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GM-CSF Expression in Pulmonary Epithelial Cells Is Regulated Negatively by Posttranscriptional Mechanisms

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Incubation of pulmonary A549 cells with D609, a phosphatidyl-choline specific phospholipase C (PC-PLC)-inhibitor, or the anti-oxidant, pyrrolidine dithiocarbamate (PTDC), markedly increased IL-1β-induced GM-CSF elaboration. This effect was observed at the mRNA level and could be partially reproduced by the protein synthesis inhibitor, cycloheximide. Following the peak in GM-CSF mRNA, the mRNA half-life ($t_{1/2}$) was 0.5–1 h. This was increased to around 3 h by cycloheximide, whilst following D609 or PDTC treatment there was essentially no degradation. These data suggest the existence of inhibitory pathways that post-transcriptionally regulate GM-CSF expression via new protein synthesis and D609- and PDTC-sensitive steps. These observations may have important clinical implications. First, drugs that target gene induction may also knock out these inhibitory pathways to lessen their effect. Second, defects in such pathways could lead to overexpression of cytokines or growth factors and contribute to the pathogenesis of inflammatory or proliferative diseases. © 2001 Academic Press

Key Words: **epithelial cell; posttranscriptional; GM-CSF; NF-kappa B; feedback; mRNA stability; mRNA superinduction.**

Bronchial epithelial cells not only act as a barrier between the internal and external environments, but also take an active role in inflammatory diseases such as asthma (1). In asthma, eosinophils accumulate in the respiratory tract via processes that are regulated by cytokines such as granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-3, and IL-5 (2). This is consistent with observations that: (i) bronchial epithelial cells enhance eosinophil survival (3), (ii) GM-CSF expression is increased in bronchial epithelial cells from asthmatics (4), and (iii) the expression of GM-CSF from these cells is increased by the proinflammatory cytokine, IL-1β (5).

The transcriptional regulation of GM-CSF is most highly characterized in T-cells. In these cells activation of the proximal promoter is substantially reduced by mutations in the NF-κB site (−85 to −76) and the conserved lymphokine element 0 (CLE0) (−54 to −31) (6). However, GM-CSF expression is also posttranscriptionally regulated via AUUUA motifs in the 3′-untranslated region (3′UTR), which regulate mRNA turnover and translational efficiency (7–9).

In this study, pulmonary A549 epithelial-like cells, which produce GM-CSF following treatment with IL-1β and are in this respect a good model of primary airway epithelial cells (10), were used to examine the regulation of GM-CSF expression.

MATERIALS AND METHODS

Cell culture and GM-CSF determination. A549 cells (ECACC) were grown to confluence in 6-well plates as described (10). Cells were incubated overnight in serum-free media and stimulated with IL-1β (R & D Systems) or phorbol 12-myristate 13-acetate (PMA) (Sigma). D609 (Alexis), pyrrolidine dithiocarbamate (PDTC) (Sigma), and cycloheximide (Sigma) were added 10 min prior to the IL-1β. Supernatants were collected at 24 h for GM-CSF ELISA (Pharmingen).

Semi-quantitative RT-PCR. RNA was extracted and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) performed using previously described protocols, conditions, and primers (10). In all cases cDNA concentration series were used to demonstrate linearity. Amplification products were quantified either by dot blot analysis or by densitometric analysis of ethidium bromide stained agarose gels. Data are expressed as a ratio of GM-CSF to GAPDH.

NF-κB activation, reporter constructs, and luciferase assay. Electrophoretic mobility shift assay (EMSA) for NF-κB was carried out as described (11). A549 cells carrying integrated luciferase reporters for the proximal GM-CSF promoter (−627/+35) and multimerized NF-κB sites were as described (10, 11). After overnight incubation in serum-free medium, confluent cells were treated and harvested 8 h later for luciferase assay (Promega).

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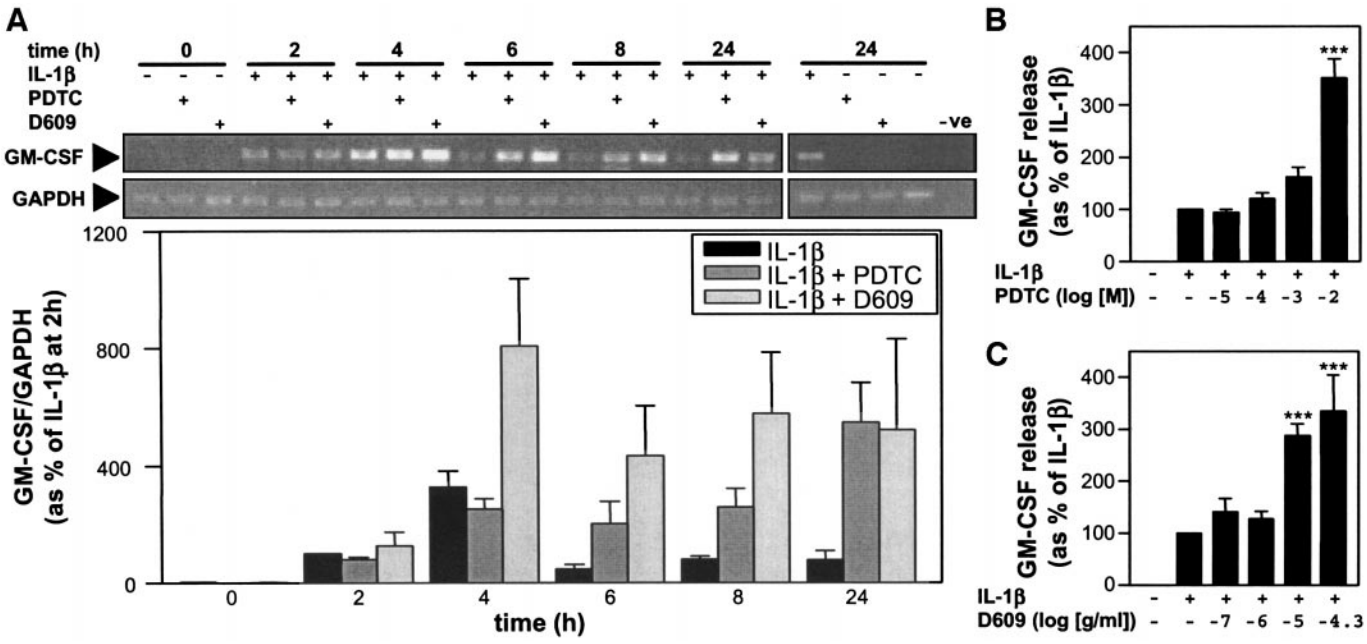

FIG. 1. Effect of PTDC and D609 on GM-CSF expression. (A) Cells were treated with IL-1β (1 ng/ml) and/or either PDTC (10 mM) or D609 (50 μg/ml) as indicated. Cells were harvested and RNA extracted for semi-quantitative RT-PCR analysis. -ve indicates a negative control for the PCR with no cDNA present. After densitometric analysis data (n = 4) were normalized to GAPDH and expressed as a percentage of IL-1β-treated at 2 h as means ± SEM. Cells were treated as above with various concentrations of either (B) PDTC or (C), D609, as indicated. After 24 h, supernatants were harvested for GM-CSF determination. Data (n = 6–8) were normalized to IL-1β treated and plotted as means ± SEM. Significance in B and C was tested by ANOVA with a Bonferroni post-test. ***P = <0.001.

RESULTS AND DISCUSSION

mRNA Expression of GM-CSF Is Transitory

Previous studies have shown that treatment of A549 cells with IL-1β results in a transcription and translation-dependent increase in GM-CSF protein (10). Following treatment with IL-1β, steady state GM-CSF mRNA rapidly increases before peaking around 4 h (see this study Fig. 1A and ref. 10). After this time, GM-CSF mRNA quickly declined towards basal levels suggesting the existence of potent negative feedback mechanisms.

PDTC and D609 Potentiate GM-CSF Expression

Addition of either the antioxidant, PDTC, or the phosphotidylcholine-specific phospholipase C (PC-PLC) inhibitor, D609, drugs that have previously been shown to inhibit NF-κB-dependent transcription (11, 12), dose-dependently elevated IL-1β-induced GM-CSF expression (Figs. 1B and 1C). Alone, PTDC, at 10 mM, and D609, at 50 μg/ml, had no effect on GM-CSF expression or cell viability as determined by trypan blue exclusion at 24 h (data not shown + Table 1). PDTC and D609 also potentiated steady state GM-CSF mRNA expression and this effect was most apparent after the peak at 4 h (Fig. 1C).

PDTC and D609 Inhibit NF-κB-Dependent Transcription

To examine whether PDTC was in fact inhibiting NF-κB, EMSA was performed (Fig. 2A). In untreated cells, NF-κB-specific DNA binding was very low. Following IL-1β treatment, two specific complexes were observed, which we have previously been shown to contain both p50 and p65 subunits of NF-κB (13). Whilst PDTC from 0.1 μM to 1 mM had no effect on NF-κB DNA binding, at 10 mM the faster migrating complex was abolished and transcriptional inhibition was observed (Figs. 2A and 2B plus data not shown). In

TABLE 1

Tolerance of A549 Cells to PDTC

Treatment	No. of viable cells as % of control n = 3 (±SEM)
Control	100
0.1 mM PDTC	142 (±49)
1 mM PDTC	149 (±75)
10 mM PDTC	81 (±19)
100 mM PDTC	0 (±0)

Note. Preconfluent A549 cells were treated with PDTC as indicated. After 24 h, cell viability was assessed using trypan blue exclusion.

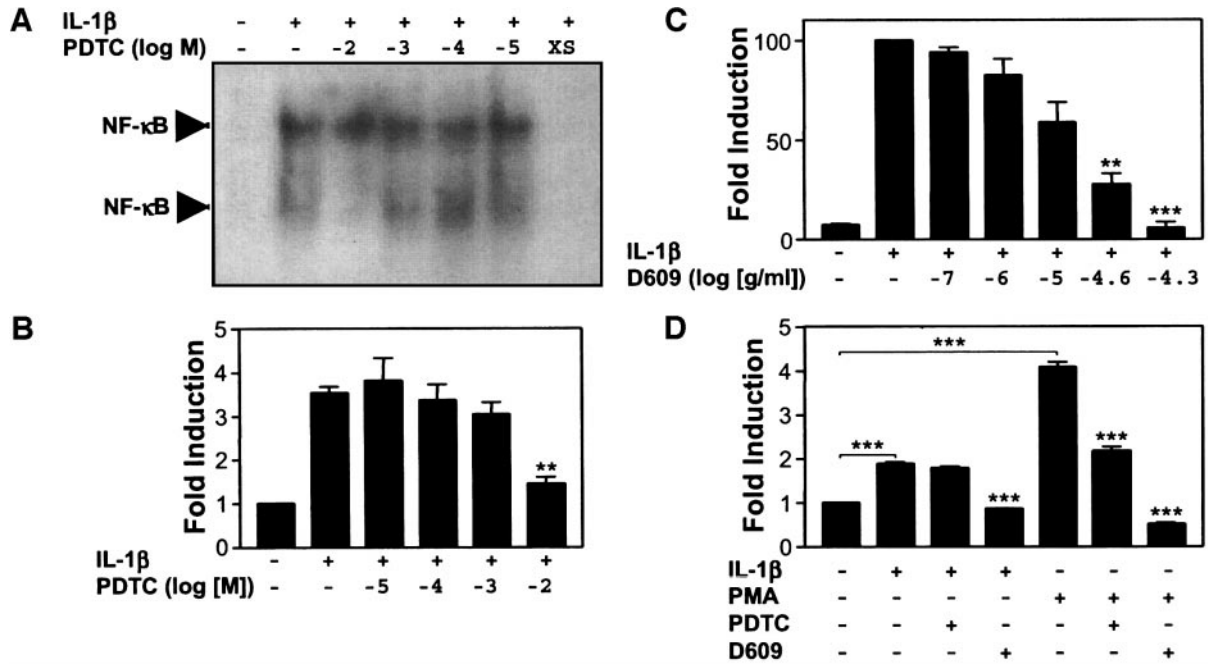

FIG. 2. PDTC and D609 inhibit NF-κB-dependent transcription. (A) Cells were treated with IL-1β (1 ng/ml) in the presence of various concentrations of PDTC. After 1 h, nuclear extracts were prepared and EMSA performed for NF-κB. An autoradiograph representative of three such experiments is shown. XS indicates the presence of a 100-fold excess of cold NF-κB probe. Specific complexes, defined by competition (XS), are indicated. (B and C) A549 cells stably transfected with the NF-κB-dependent reporter (6κB.tk.neo) were treated as indicated. Cells were harvested for luciferase determination and data ($n = 6$) plotted as fold induction as means ± SEM. (D) A549 cells stably transfected with a reporter containing the −627/+35 proximal GM-CSF promoter were treated with IL-1β (1 ng/ml) or PMA (10^{-7}M) in the presence of PDTC (10 mM) or D609 (50 μg/ml), as indicated. Cells were harvested for luciferase determination and data ($n = 3$) are plotted as fold induction as means ± SEM. Significance in B, C. and D was tested by ANOVA with a Bonferroni post-test. $^{**}P = <0.01$, $^{***}P = <0.001$.

this study the dose of PDTC required to repress NF-κB activation and NF-κB-dependent transcription was markedly higher than in other studies (12). This is consistent with the high tolerance of A549 cells to PDTC (Table 1) and may be due to the unusually high levels of anti-oxidant present in these cells (14). Similarly, D609, which inhibits NF-κB-dependent transcription, but not activation of DNA binding (11), dose-dependently inhibited NF-κB-dependent transcription (Fig. 2C).

GM-CSF Promoter Activity Is Not Enhanced by PDTC or D609

Analysis of the proximal (−627/+35) GM-CSF promoter showed that PDTC had little effect on activation by IL-1β and a partial effect on activation by phorbol ester (Fig. 2D). D609 resulted in inhibition of promoter activation by both stimuli. Likewise, analysis of a 3.3 kb GM-CSF promoter fragment revealed qualitatively similar effects to the proximal promoter suggesting that more distal promoter elements are not involved in these responses (data not shown) (10). However, on account of the low inducibility, such promoter models do not fully explain the induction of GM-CSF mRNA in A549 cells, which leads to the suggestion that posttranscriptional mechanisms may play a significant role (10). Despite this, these data indicate that the mRNA potentiation following IL-1β plus PDTC or D609 is unlikely to be due to enhanced promoter activation.

Effect of Cycloheximide on Steady State GM-CSF mRNA Levels

Numerous acute-phase and inflammatory genes demonstrate the phenomena of mRNA superinduction following translational arrest in the presence of an ongoing stimulus (13, 15). Whilst this effect is generally attributed to inhibition of labile factors that play a role in mRNA decay (15), this response may also occur transcriptionally due to enhanced activation of factors such as NF-κB or JNK (16). However, cycloheximide had no effect on steady state GM-CSF mRNA during the first 4 h of IL-1β stimulation suggesting that there was no enhancement of promoter activation (Fig. 3A). Subsequently, the decline in mRNA, following the maximum at 4 h, was inhibited by cycloheximide indicating that negative feedback inhibition is an active process that requires protein synthesis.

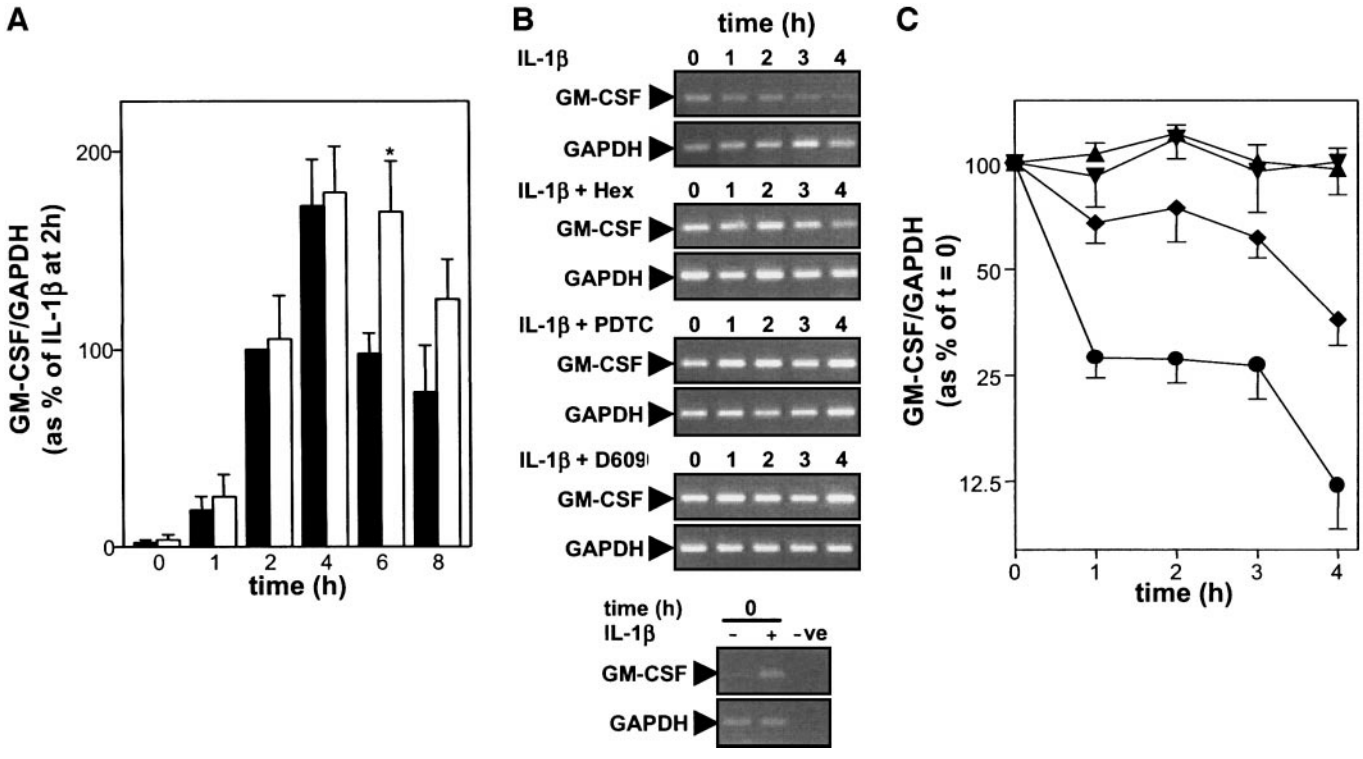

FIG. 3. Cycloheximide, PDTC, and D609 stabilize GM-CSF mRNA. (A) Cells were treated with IL-1β (1 ng/ml) (solid bars) or IL-1β plus cycloheximide (Hex) (10 μg/ml) (open bars). Cells were harvested and RNA extracted for semi-quantitative RT-PCR analysis. After dot blot analysis, data ($n = 6$) were normalized to GAPDH and expressed as a percentage of IL-1β-treated at 2 h as means ± SEM. Cells were treated with IL-1β (●) (1 ng/ml) in the presence of cycloheximide (Hex) (10 μg/ml) (◆), PDTC (10 mM) (▲), or D609 (50 μg/ml) (▼). After 4 h actinomycin D (10 μg/ml) was added ($t = 0$). Cells were then harvested as indicated and RNA extracted for semi-quantitative RT-PCR analysis of GM-CSF and GAPDH. (B 0 Representative gels are shown. Unstimulated and IL-1β-stimulated controls harvested after 4 h at the time of Actinomycin D addition (i.e., $t = 0$) and a negative PCR control (-ve) with no cDNA present are shown below. (C 0 After densitometric analysis, data ($n = 7$) were normalized to GAPDH and expressed as a percentage of $t = 0$ as means ± SEM. Significance in A was tested by Students t-test. *$P = <0.05$.

PDTC, D609, and Cycloheximide Stabilize GM-CSF mRNA

To investigate the role of posttranscriptional mechanisms, the effects of cycloheximide, PDTC, and D609 were examined on GM-CSF mRNA half-life. Cells were stimulated with IL-1β for 4 h in the presence of each drug. Transcription was arrested by the addition of actinomycin D and mRNA levels were analyzed over the subsequent 4 h (Figs. 3B and 3C). IL-1β-stimulated GM-CSF mRNA showed a $t_{1/2}$ of between 0.5–1 h, whereas in the presence of cycloheximide this value increased to around 3 h. Thus, the inhibition of translation blocks posttranscriptional negative feedback of GM-CSF. In the presence of either PDTC or D609 there was essentially no degradation of GM-CSF mRNA indicating that these drugs must additionally inhibit processes that do not require new gene synthesis.

Posttranscriptional Repression of Gene Expression

In summary, we provide evidence for posttranscriptional destabilization as a major mechanism for negative feedback inhibition of IL-1β-induced GM-CSF in pulmonary epithelial cells. These observations extend the previously characterized phenomenum of mRNA superinduction following translational blockage by inhibitors such as cycloheximide by demonstrating that inhibitors of signalling pathways may also result in superinduction. Based on the selectivity of the inhibitors used these mechanisms may involve new gene synthesis, PC-PLC and a redox step. However, further studies will be necessary to examine the true role of these and/or other signalling pathways. As both D609 and PDTC are known inhibitors of NF-κB, it is possible, but not proven, that this factor may play a role in the posttranscriptional negative feedback mechanism.

As similar posttranscriptional negative feedback processes are likely to occur with other acute phase genes, these findings may have wide reaching implications to the drug discovery process. Thus drugs that are designed to target gene transcription, for example in anti-inflammatory therapies or anti-cancer treatments, may also repress endogenous negative feedback processes. Therefore, to exploit fully the therapeutic

potential of new drugs, it may be necessary to examine both transcriptional and posttranscriptional effects. Furthermore, errors in such feedback mechanisms could represent primary molecular defects, giving rise to aberrantly high expression of inflammatory, or cancer causing genes (17). Finally, the identification of putative genes and pathways that are induced by the primary stimulus, in this case IL-1β, and act to repress primary response genes, such as GM-CSF, could be exploited therapeutically.

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