Enhanced AP-1 and NF-κB activities and stability of interleukin 8 (IL-8) transcripts are implicated in IL-8 mRNA superinduction in lung epithelial H292 cells

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Inhibition of protein synthesis may result in superinduction of short-lived transcripts and has been attributed variably to stabilization of transcripts and/or increased gene transcription. Little is known about the kinetics of these processes and relevant transcriptional elements have not been identified. In this study, we describe superinduction of interleukin 8 (IL-8) mRNA, an important inflammatory mediator, in lung epithelial-like H292 cells and identify the underlying molecular mechanisms and their kinetics. Cycloheximide (CHI, 10 μg/ml), an inhibitor of protein synthesis, maximally increased IL-8 mRNA levels 30-fold in H292 cells. Tumour necrosis factor α (TNF-α), which induced IL-8 mRNA 3-fold, synergized with CHI causing a 150-fold increase at 6 h. CHI early on increased the stability of IL-8 mRNA (from 40 min in cells cultured with medium to more than 4 h with CHI). CHI also increased transcription as shown by transfection with IL-8 promoter constructs. Truncated and mutated constructs identified NF-κB and AP-1 binding sites as primary *cis*-acting elements in IL-8 gene transcription and IL-8 mRNA superinduction. Electrophoretic mobility shift assays indicated that CHI increased NF-κB and prolonged AP-1 DNA-binding activities and that the synergism of TNF-α and CHI on IL-8 mRNA expression was paralleled by a further increase of AP-1 DNA-binding activity. This synergism was still noticed when 4 h elapsed between the addition of CHI and that of TNF-α. Taken together, our results indicate that CHI interferes with both post-transcriptional and transcriptional repressive mechanisms of IL-8 mRNA expression.

INTRODUCTION

Interleukin 8 (IL-8) belongs to the C-X-C subfamily of chemokines and is a potent chemoattractant and stimulus of neutrophils. IL-8 has also been shown to affect other inflammatory and immunocompetent cells (reviewed in [1]) and is therefore believed to play a pivotal role in inflammatory disease. IL-8 was originally purified from the medium of stimulated human blood monocytes, but its production has now been characterized in many different cell types, including fibroblasts, keratinocytes, bone marrow stromal cells, T cells and endothelial and epithelial cells. Basal production of IL-8 is usually low, but can be induced by different stimuli such as granulocyte-colony-stimulating factor, tumour necrosis factor α (TNF-α), lipopolysaccharide (LPS), IL-1β, PMA, viruses or double-stranded RNA. The 5′-regulatory region of the IL-8 gene contains several potential transcription regulatory elements. According to the cell type analysed, different sets of these transcription regulatory elements, most likely combinations of activator protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP) and nuclear factor-κB (NF-κB) DNA-binding sites, are implicated in stimulus-induced IL-8 gene transcription [2–7]. Besides regulation at the transcriptional level, IL-8 mRNA steady-state levels may be affected by post-transcriptional processes. Specific AU-rich elements (AREs) in the 3′-untranslated region (3′-UTR) of some mRNAs encoding for proto-oncogenes, transcription factors and cytokines, have been implicated in an active degradation of these mRNAs, thus shortening their half-life [8]. Similar AREs are found in the 3′-UTR of IL-8 transcripts, suggesting that their half-life may be modulated by similar mechanisms.

Coordinated regulation of gene transcription and mRNA stability may ensure transient gene expression. However, under conditions that reduce protein synthesis, mRNAs bearing AREs are expressed at increased levels and over a prolonged period, a phenomenon known as superinduction. Various inhibitors of protein synthesis, that act via different mechanisms, all give rise to superinduction [9–11], indicating that restriction of protein synthesis *per se* results in superinduction. Superinduction has been attributed variably to a stabilization of transcripts and/or an increased gene transcription [12–17]. However, little is known on the kinetics of these processes and transcriptional elements have not been identified precisely. In the present study, we assessed the contribution of transcriptional and post-transcriptional processes to IL-8 mRNA superinduction, including the kinetics of these events. Because of our interest in IL-8 expression in lung epithelial cells due to pathophysiological conditions, we used a lung adenocarcinoma-derived cell line, H292, and TNF-α as a relevant stimulus. Transcriptional processes were studied and identified using transient transfection with whole, truncated and mutated IL-8 promoter constructs and electrophoretic mobility shift assays (EMSA). Superinduction of IL-8 mRNA by the protein synthesis inhibitor cycloheximide (CHI), in the absence or presence of TNF-α, resulted in a marked stabilization of IL-8 mRNA. CHI also

Abbreviations used: IL, interleukin; TNF, tumour necrosis factor; LPS, lipopolysaccharide; CAT, chloramphenicol acetyltransferase; CHI, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AP-1, activator protein-1; C/EBP, CCAAT/enhancer binding protein; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assay; 3′-UTR, 3′-untranslated region; AREs, AU-rich elements.

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enhanced transcription, coinciding with an increased NF-κB activity and a prolonged (and further increased, only in the presence of TNF-α) AP-1 activity, as compared to the transient activation of NF-κB and AP-1 activities by TNF-α. These findings indicate that CHI interferes with both transcriptional and post-transcriptional repression of IL-8 mRNA expression in H292 cells.

MATERIALS AND METHODS

Cytokines and reagents

Unless specified otherwise, TNF-α (R & D Systems, Minneapolis, MN, U.S.A.), LPS (Sigma, St. Louis, MO, U.S.A.), IL-1β (Genzyme, Cambridge, MA, U.S.A.) and CHI (Calbiochem, Bierges, Belgium) were used at a final 10 ng/ml, 10 μg/ml, 0.5 ng/ml and 10 μg/ml, respectively. Actinomycin D (Boehringer Mannheim, Mannheim, Germany) was used in mRNA stability experiments at 10 μg/ml.

Cell culture

The human lung-derived mucoepidermoid adenocarcinoma cell line H292 (CRL 1848; American Type Culture Collection, Rockville, MD, U.S.A.) was maintained as described [18]. For experiments, H292 cells were grown in 6-well plates in RPMI-1640 medium (Gibco–BRL, Gaithersburg, MD, U.S.A.) supplemented with 10 % fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Transfection

H292 cells at 70 % confluence were transfected with 5 μg of chloramphenicol acetyltransferase (CAT) reporter vectors using the calcium/phosphate precipitation method [19]. Stimuli were added to the cultures 40 h after transfection and 7 h before RNA was isolated for analysis by Northern blot. After blotting and hybridization, CAT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were quantified using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). CAT levels were expressed relative to GAPDH levels in arbitrary units. The generation of the truncated and mutated IL-8 promoter CAT constructs was described previously [2,4]. The following mutations were introduced in -133-CAT: AP-1 site (-126/-120) from TGACTCA to TatCTCA, C/EBP site (-94/-81) from CAGTTGCAAATCGT to agcTTGCAAATCGT and NF-κB site (-80/-71) from GGAATTTCCT to taAcTTTCCT. The C/EBP and NF-κB sites from $-94(\Delta-70/-51)$-CAT were mutated to agcTTGCAAATCGT and GGAATTTaaa, respectively. Synthesized trimerized Igκ-κB (GGGACTTTCC) and trimerized IL-8 NF-κB and IL-8 AP-1 site were cloned into a -50 CAT construct [7].

RNA analysis

Total RNA was extracted from H292 cells using TRIzol (Gibco-BRL). Twenty to forty μg of total RNA were separated on 1 % agarose-formaldehyde gels using standard protocols [19] and transferred to nylon membranes (Hybond N; Amersham, Arlington Heights, IL, U.S.A.). Blots were hybridized overnight at 65 °C in a solution containing 0.5 M sodium phosphate buffer pH 7.2, 7 % (w/v) SDS, 1 mM EDTA and ^{32}P-labelled probes. After hybridization, blots were washed three times for 40 min at 65 °C with 0.5 × SSC/0.5 % (w/v) SDS and exposed to image plates (Molecular Dynamics). Subsequent hybridizations were performed after stripping probes with three washes in boiling 0.1 × SSC/0.1 % (w/v) SDS. The GAPDH probe (encoding region 488–570) and the IL-8 probe (encoding region 257–966) were derived by PCR. The CAT probe was isolated from a CAT plasmid used in this study.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to Schreiber et al. [20]. Protein recovery was determined with the Bio-Rad Protein Assay kit (Hercules, CA, U.S.A.). Four μg of protein of the nuclear extracts (adjusted to 10 μl) were incubated 15 min at room temperature with 1 μl of radiolabelled oligonucleotide probes ($\sim$ 0.25 ng, $\sim$ 25000 c.p.m.), 7 μl of a buffer composed of 20 mM Hepes, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 % (v/v) NP40, 0.5 mM PMSF, 1 mg/ml BSA, 5 % (v/v) glycerol and 2 μl of 1 mg/ml poly(dI-dC)-poly(dI-dC). Reaction mixtures were separated on 4 % non-denaturing polyacrylamide gels at 230 V at room temperature in 0.5 × TBE buffer. After drying, gels were exposed to X-ray films. Signals were quantified using an Eagle Eye (Stratagene, La Jolla, CA, U.S.A.) Oligonucleotides used in EMSA were the following: NF-κB: 5′-ATCGTGGAATTTCCTCTGAC-3′; AP-1: 5′-GTGTGATGACTCAGGTTTG-3′.

RESULTS

Superinduction of IL-8 mRNA in H292 cells

The lung-derived adenocarcinoma cell line H292 cultured in medium expressed low levels of IL-8 mRNA (Figure 1A). This level increased 1- to 3-fold after 7 h of incubation with LPS, IL-1β or TNF-α. When cells were stimulated in the presence of 10 μg/ml of CHI, levels of IL-8 mRNA increased 50–100 times. At 10 μg/ml of CHI, [^{14}C]leucine incorporation during 4 h in trichloroacetic-acid precipitable proteins, as a measure of protein synthesis, was reduced by 80 % (data not shown). Superinduction of IL-8 mRNA was also observed when cells were treated with CHI alone.

We then explored superinduction over time and in the presence of TNF-α and LPS (Figure 1B). TNF-α increased IL-8 mRNA 2-fold at 1 h, followed by a decrease at 2 h. Subsequently, IL-8 mRNA levels increased again 2- to 3-fold and remained at that level until at least 32 h. Although CHI alone appeared less potent than TNF-α after 0.5 and 1 h, it induced a constant increase of IL-8 mRNA which reached a plateau at 6 h (30-fold increase) until at least 24 h. Exposure to TNF-α plus CHI led to a synergistic effect on IL-8 mRNA expression; shortly after treatment, there was a fast increase of the IL-8 mRNA level resulting in a 150-fold increase at 6 h. LPS was a poor inducer of IL-8 mRNA. Exposure to LPS plus CHI also showed a synergistic effect on IL-8 mRNA expression, but to a lesser extent than exposure to TNF-α plus CHI. Thus, the level of IL-8 mRNA expression provided by the stimulus influenced the level of superinduction. The level of superinduction was also dependent on the concentration of CHI, both in the absence or in the presence of TNF-α (Figure 2). Interestingly, the synergistic effect of TNF-α on CHI-dependent superinduction increased with increasing concentrations of CHI.

Stability of IL-8 mRNA in H292 cells

We compared the half-life of IL-8 mRNA in H292 cells exposed for 5 h to medium, TNF-α, CHI, and TNF-α plus CHI using actinomycin D (Figure 3). IL-8 mRNA was unstable in cells exposed to medium or TNF-α, with an approximate half-life of 40 min. CHI alone or CHI plus TNF-α, considerably increased stability ($>$ 4 h), indicating that stabilization of IL-8 mRNA

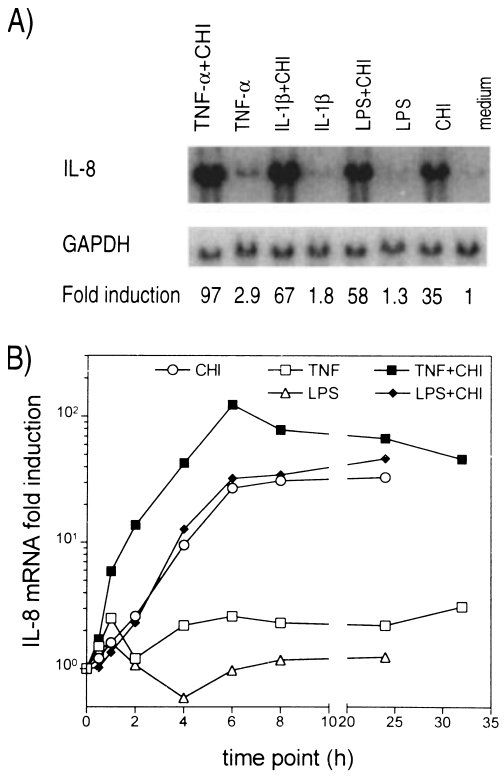

Figure 1 Superinduction of IL-8 mRNA in H292 cells

(**A**) H292 cells were maintained in medium or activated by LPS, IL-1β or TNF-α in the absence or in the presence of CHI. After 7 h of incubation, total RNA was isolated and subjected to Northern blot analysis. Signals obtained after hybridization with an IL-8 probe were quantified using a PhosphoImager and normalized to GAPDH mRNA levels. IL-8 mRNA levels were expressed relative to the level in resting cells (fold induction). (**B**) IL-8 mRNA induction by CHI, LPS, LPS + CHI, TNF-α and TNF-α + CHI was determined and analysed as described in (**A**). Similar results were obtained in four separate experiments.

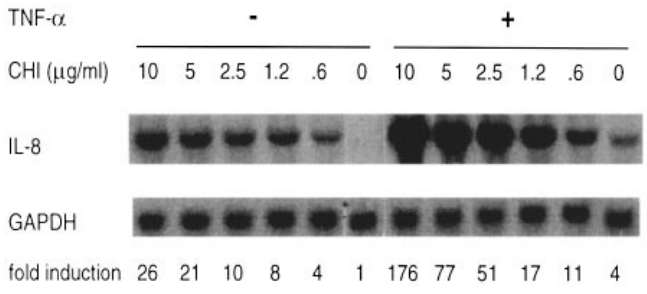

Figure 2 Dose–response of CHI on IL-8 mRNA superinduction

H292 cells were treated for 5 h with different concentrations of CHI in the presence or absence of TNF-α (10 ng/ml). Total RNA was collected and IL-8 and GAPDH levels were determined by Northern blot. IL-8 mRNA-fold induction was calculated as indicated in Figure 1. This experiment is representative of three separate experiments.

by CHI contributed to superinduction. A similar increased stability (half-life > 4 h) was detected in cells exposed for 1 h to CHI and to TNF-α plus CHI (data not shown), indicating that this process was an early event in superinduction. However, the finding that the IL-8 mRNA level increased with time in the presence of CHI and that a synergistic increase was obtained

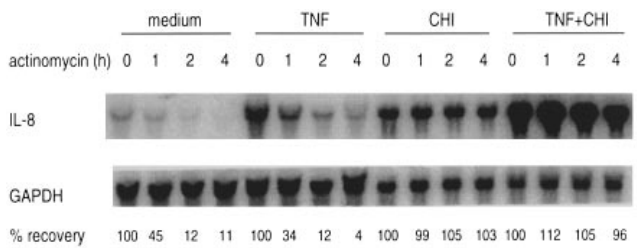

Figure 3 Stabilization of IL-8 transcripts is involved in superinduction

H292 cells were treated 5 h with medium, TNF-α, CHI or TNF-α + CHI. Actinomycin (10 μg/ml) was added to the cultures and RNA recovered after an additional 0, 1, 2 or 4 h of incubation. The extracted RNA was subjected to Northern blot analysis (40 μg RNA for cells exposed to medium and TNF-α, 20 μg RNA for cells exposed to CHI and TNF-α + CHI) and hybridized with IL-8 and GAPDH probes. IL-8 signals normalized to GAPDH were expressed relative to the corresponding level before adding actinomycin (% remaining). These results are representative of three separate experiments.

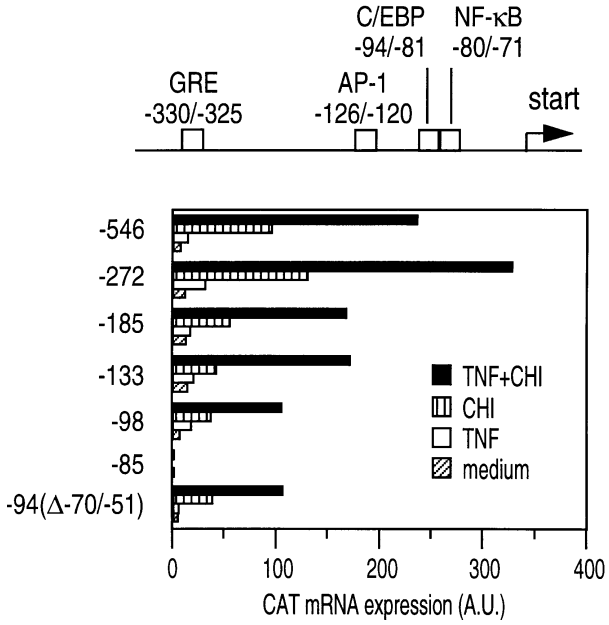

Figure 4 The IL-8 promoter can support superinduction of a CAT reporter gene

On top, schematic representation of the IL-8 promoter indicating the localization of the AP-1, C/EBP and NF-κB DNA-binding sites. H292 cells were transfected with CAT expression vectors in which various 5′-deleted fragments of the IL-8 promoter were cloned. CAT mRNA expression in cells cultured for 7 h in medium, TNF-α, CHI or TNF-α + CHI was determined as indicated in Materials and methods. Similar results were obtained in a second separate experiment.

upon TNF-α plus CHI treatment also implicated a transcriptional process in superinduction.

Role of the IL-8 promoter in superinduction of IL-8 mRNA

We then tested whether the promoter of the IL-8 gene mediated superinduction. H292 cells were transfected transiently with a CAT plasmid containing region -546 to $+44$ of the human IL-8 gene in which all elements implicated in IL-8 gene transcription regulation are located. The CAT mRNA steady-state level in H292 cells exposed for 7 h to different conditions was determined by Northern blot. As shown in Figure 4, TNF-α increased CAT mRNA expression 2-fold, whereas CHI induced a 10-fold

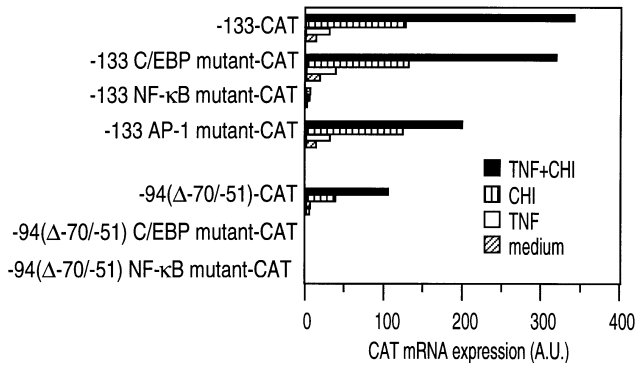

Figure 5 Requirement for an intact NF-κB site for superinduction

Mutations disrupting the C/EBP, NF-κB or AP-1 sites were introduced in −133-CAT. NF-κB and C/EBP sites from −94(Δ−70/−51)-CAT were also mutated. H292 cells transiently transfected with wild-type and mutated CAT vectors were exposed to medium, TNF-α, CHI or TNF-α + CHI during 7 h. CAT mRNA expression was determined as indicated in Materials and methods. Similar results were obtained in two separate experiments.

enhancement of CAT mRNA recovery. Exposure to TNF-α plus CHI led to a synergistic effect on the CAT mRNA level (more than 20-fold increase), as observed for IL-8 mRNA. The increased CAT mRNA expression upon CHI or TNF-α plus CHI incubation may result either from an increased transcriptional activity or from an increased half-life of CAT transcripts. To distinguish between these two possibilities, CAT mRNA stability was assessed in H292 cells exposed for 5 h to medium, TNF-α and CHI. CAT transcripts were stable and CHI did not affect their half-life (data not shown). Taken together, this demonstrated that superinduction of CAT mRNA was due to the IL-8 promoter and that CHI by itself induced transcriptional activity.

Subsequently, we determined the minimal elements of the IL-8 promoter required for transcription and superinduction. As a first approach, we used a series of CAT vectors containing truncated portions of the IL-8 promoter (Figure 4). All constructs with deletions from −546 to −98 and −94(Δ−70/−51)-CAT, but not −85-CAT, allowed superinduction of CAT mRNA albeit to variable extents. Thus, when the NF-κB site was separated from the AP-1 and the C/EBP sites (−85-CAT), H292 cells were unable to express and to superinduce CAT mRNA. In the absence of the AP-1 site, a minimal element containing the NF-κB and the C/EBP sites was required for transcription and superinduction (compare −85-CAT with −98-CAT and −94(Δ−70/−51)-CAT.

We then investigated the contribution of the different DNA-binding sites using mutated IL-8 promoter CAT constructs (Figure 5). CAT transcription was conferred with −94(Δ−70/−51)-CAT which contains both C/EBP and NF-κB sites but no AP-1 site. Mutation of −94(Δ−70/−51)-CAT at either the C/EBP site or the NF-κB site abolished CAT expression, confirming the necessity of both the NF-κB and the C/EBP sites in the absence of an AP-1 site. Mutation of the NF-κB site in −133-CAT revealed that an intact NF-κB site was essential for transcription. By contrast, mutation in the C/EBP site of −133-CAT had no effect, whereas disruption of the AP-1 site reduced CAT mRNA superinduction. Altogether, these results indicated that the NF-κB and AP-1 sites, but not the C/EBP site, were the prime target of the transcriptional regulation involved in superinduction. In agreement, CHI superinduced CAT mRNA from constructs in which a dimerized IL-8 AP-1 site or trimerized IL-8 NF-κB or Igκ NF-κB sites were cloned (data not shown). These data also indicated that AP-1 and NF-κB sites independently, albeit in multimerized constructs, can give rise to superinduction.

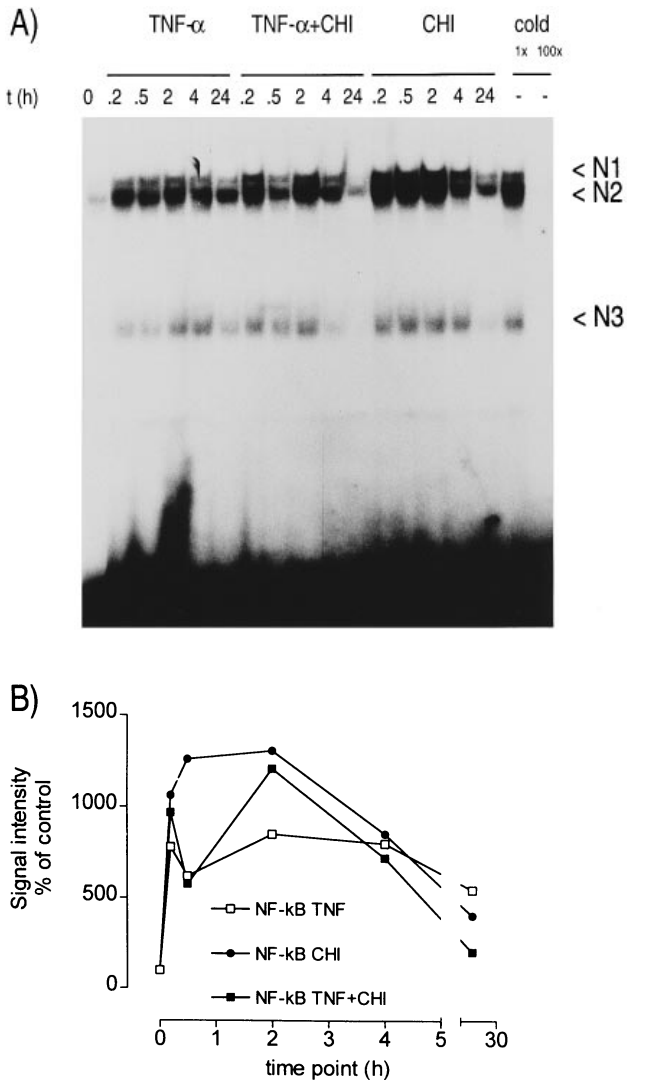

Figure 6 Modulation of the nuclear activity of NF-κB transcription factor by TNF-α and CHI

(**A**) H292 cells were treated for different periods with TNF-α, CHI or TNF-α + CHI. Nuclear proteins were extracted and used in EMSAs with an oligonucleotide specific for the IL-8 NF-κB site. Specific retarded complexes (N1, N2 and N3) were identified by competition with unlabelled specific oligonucleotide (1× and 100× excess, last two right-hand lanes respectively), but not with an irrelevant oligonucleotide (not shown). (**B**) The intensity of specific signals was expressed relative to signals obtained for resting cells and plotted as a function of time. Similar results were obtained in four separate experiments.

Nuclear activity of NF-κB and AP-1 transcriptional factors during superinduction of IL-8 mRNA

To determine whether NF-κB and AP-1 site-dependent superinduction resulted from an increased quantity in the nucleus of transcription factors, we performed EMSA (Figure 6). Nuclear extracts were isolated from H292 cells treated for different periods with TNF-α, CHI or TNF-α plus CHI. When a double-

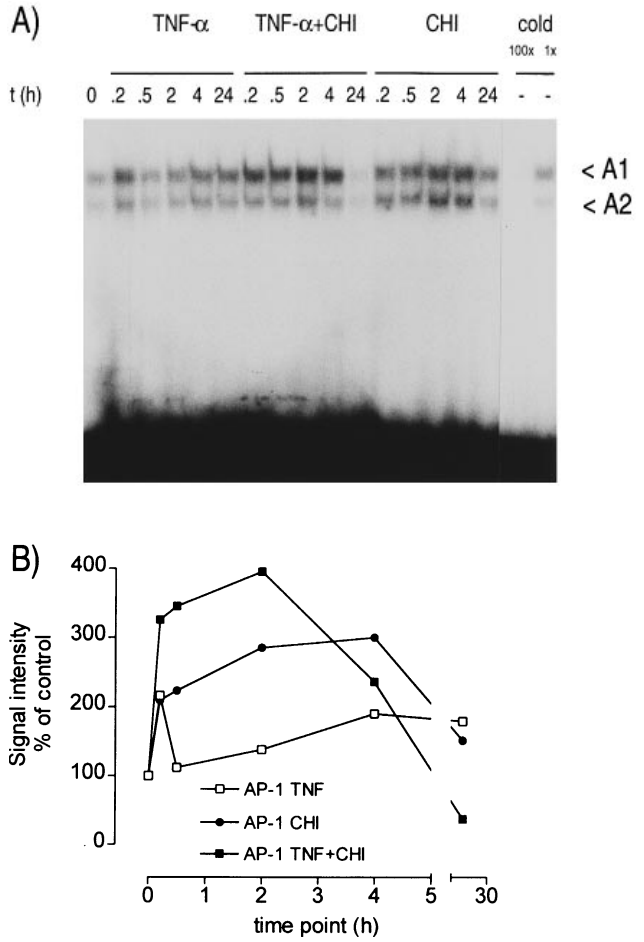

Figure 7 Synergistic effect of TNF-α and CHI on the nuclear activity of AP-1 transcription factor

(**A**) EMSAs were performed as described in Figure 6 using an oligonucleotide specific for the IL-8 AP-1 site. A1 and A2 represent specific retarded complexes. (**B**) The intensity of specific signals were expressed relative to the intensity obtained for resting cells. Similar results were obtained in three separate experiments.

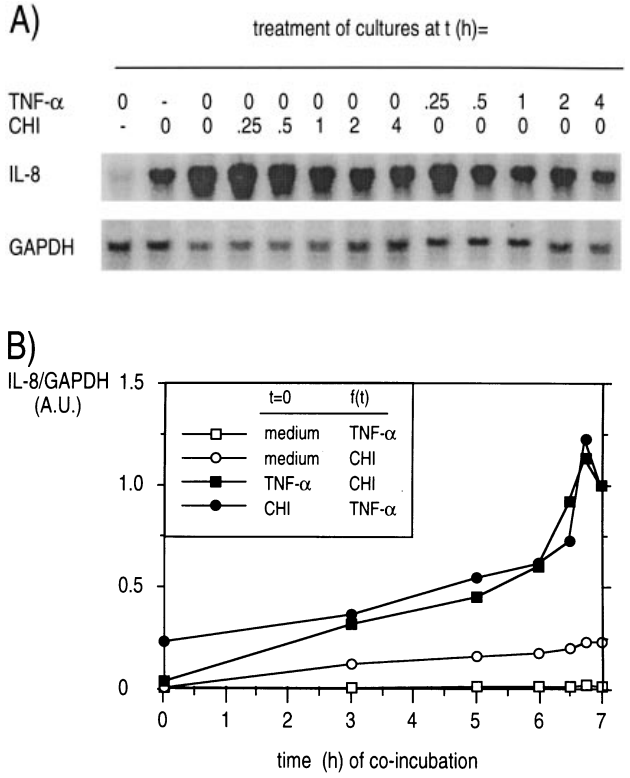

Figure 8 Effect of delayed CHI or TNF-α treatment on CHI-induced IL-8 mRNA superinduction

(**A**) H292 cells were incubated with TNF-α or CHI at the beginning of the experiment ($t = 0$). As indicated, after different times, TNF-α was added to cells cultured with CHI, whereas CHI was added to cells cultured with TNF-α. After an overall incubation of 7 h, IL-8 and GAPDH mRNA levels were determined by Northern blot. Not shown are controls in which TNF-α or CHI were added at different times to cells cultured in medium. -, No addition of TNF-α or CHI. (**B**) Normalized IL-8 mRNA signals were reported to the value obtained from cells exposed to TNF-α plus CHI for 7 h (arbitrarily set at 1). The ratios, expressed in arbitrary units (A.U.), were plotted as a function of the time of co-exposure over 7 h. These results represent the average of two separate experiments.

stranded oligonucleotide encoding the IL-8 NF-κB site was incubated with nuclear proteins from unstimulated cells, three specific DNA–protein complexes of different mobilities were distinguished. TNF-α was a potent inducer of all three complexes. In the presence of CHI alone or of CHI plus TNF-α, NF-κB-binding activity was increased more than upon TNF-α stimulation during the first 2 h of treatment. In fact, CHI alone was the most potent inducer of NF-κB-binding activity.

Analysis of the IL-8 promoter AP-1 site with extracts from unstimulated H292 cells revealed two DNA–protein complexes (Figure 7). These complexes were transiently up-regulated by TNF-α, showing a 2-fold increase after 12 min of stimulation. This was followed by a decrease to the basal level at 0.5 h. Subsequently, AP-1-binding activity increased again slightly over time. CHI alone or CHI plus TNF-α, up-regulated the two complexes at least as fast as TNF-α. However, this increase was markedly sustained during the first 2 h (TNF-α + CHI) or 4 h (CHI) of treatment. A marked additional effect on AP-1-binding activity was observed with TNF-α plus CHI compared to TNF-α or CHI alone. On the whole, this suggested that the sustained and increased binding activity of nuclear proteins interacting with NF-κB and AP-1 sites were responsible for the transcriptional process involved in IL-8 mRNA superinduction.

Time dependence on CHI vs. TNF-α on the synergistic effect of IL-8 mRNA superinduction

To provide more insight into the synergistic effect on CHI-dependent superinduction by TNF-α, we conducted the following experiment. Cells were exposed to TNF-α at different times before or after addition of CHI, and IL-8 mRNA levels were determined after an overall incubation of 7 h (Figure 8). Compared to IL-8 mRNA levels obtained with TNF-α or CHI alone, a synergistic effect on superinduction was observed with TNF-α plus CHI irrespective of the time in between the addition of TNF-α and CHI. This indicated that the contribution of TNF-α to synergism was not sensitive to reduction of protein synthesis. However, a maximal level of synergism was obtained when CHI and TNF-α were added sequentially within 30 min.

DISCUSSION

IL-8 is an important inflammatory mediator implicated in several pathophysiological conditions and is produced among others by epithelial cells. Previously, it was shown that IL-8 transcripts can

be superinduced by restriction of protein synthesis [21,22], but not in all cell lines [4]. Our primary interest is to assess whether IL-8 mRNA expression in epithelial cells exposed to viral or bacterial products, that can inhibit eukaryotic protein synthesis [23,24], is due to superinduction. As a first approach, we aimed to identify the mechanisms and the kinetics implicated in IL-8 mRNA superinduction in a lung adenocarcinoma-derived cell line, H292. Initial experiments indicated that the protein synthesis inhibitor CHI superinduced IL-8 mRNA in H292 cells as evidenced by an increase and prolonged IL-8 mRNA expression, as was shown for superinduction of IL-6 and c-fos mRNA in other cell types [12,14,16].

CHI, in the absence or presence of TNF-α markedly increased IL-8 mRNA half-life in H292 cells, indicating that, similar to reports for other superinducible mRNAs, stabilization of IL-8 transcripts contributed to superinduction [11,25]. In some studies, actinomycin, which was used here to assess IL-8 mRNA half-life, was found to cause mRNA superinduction and thus to affect the half-life measurements [26]. In H292 cells, however, actinomycin did not superinduce IL-8 mRNA, suggesting that the increase in IL-8 mRNA half-life was solely due to CHI. Stabilization of IL-8 mRNA was an early event, detectable as soon as 1 h after exposure to CHI, in support of the role of a very labile protein in degradation of IL-8 mRNA.

Next to the effect on IL-8 mRNA stability, CHI superinduced CAT transcripts of constructs containing the IL-8 promoter. We excluded that this was due to an effect of CHI on the half-life of CAT transcripts and, by extrapolation, suggest that superinduction of IL-8 mRNA also depends on transcriptional processes. Subramaniam et al. [17] showed that a synthetic copy of the serum responsive element from the c-fos promoter, cloned upstream of a thymidine kinase promoter in a CAT reporter plasmid, conferred inducibility by CHI. Ringold et al. [14], employing transfection of the human IL-6 gene under the control of its own promoter, showed a similar dependence on transcription for superinduction. However, because the construct contained the entire IL-6 gene, CHI also may have affected mRNA stability. Thus, to the best of our knowledge, the present report is the first using transient transfection with constructs controlled strictly by the relevant promoter to assess its role of transcription in superinduction. The synergism between a stimulus and CHI on mRNA expression has been reported before, but was not attributed to transcriptional or post-transcriptional processes [14]. In the present study, the synergism between TNF-α and CHI on IL-8 mRNA expression was also observed using IL-8 promoter constructs, indicating that this synergism was mediated via a transcriptional process.

Subsequent experiments employing truncated and mutated constructs were aimed to identify the transcriptional elements required for superinduction. Whereas a functional NF-κB site was indispensable, AP-1 was the second most important site involved, although C/EBP could complement NF-κB when AP-1 was absent. The potential of NF-κB and AP-1 sites to promote superinduction was further confirmed using constructs in which multimerized sites were cloned. These findings on IL-8 gene regulation are in accordance with previous reports which showed that two *cis*-acting elements were needed for IL-8 gene transcription. For example, both NF-κB and C/EBP were required for IL-8 induction by IL1-β in the glioblastoma cell line T98G [7], by TNF-α, IL1-β, PMA and hepatitis B virus X protein in the fibrosarcoma cell line 8387 [2,3], and for the IL-8 response of Jurkat T cells to PMA [6]. The involvement of NF-κB and AP-1 in H292 cells is unusual but has also been implicated in the induction of IL-8 production by TNF-α and interferon-γ in the gastric carcinoma cell line MKN45 [4].

The transcriptional process implicated in IL-8 mRNA superinduction in H292 cells was coincident with increased levels of nuclear transcription factors binding to the NF-κB and AP-1 sites present in the IL-8 promoter. TNF-α induced a rapid increase of both AP-1 and NF-κB complexes, as shown by EMSA. Subsequently, AP-1 complexes were quickly reduced to basal levels followed by a gradual increase which resembles the kinetics of AP-1 activity observed after PMA stimulation of human lung tissues [27]. In the presence of CHI there was no early decrease of AP-1 complexes, suggesting that this down-regulation depends on a labile protein. In fact, CHI is also known to superinduce c-jun and c-fos mRNA, the encoded proteins of which form AP-1 [11]. Indeed, preliminary results indicate that c-fos and c-jun mRNA are superinduced in H292 cells (results not shown), and when these transcripts are translated this may underlie the prolonged AP-1 activity.

In contrast to AP-1 complexes, NF-κB complexes remained elevated after the initial rise induced by TNF-α. In the presence of CHI, particularly in the absence of TNF-α, NF-κB complexes increased further before levelling off, suggesting that the increased NF-κB-binding activity also depends on a labile protein. Activation of NF-κB by CHI is in line with reports showing that CHI activates NF-κB by inhibiting the replenishment of the labile inhibitory protein I-κB [28].

The synergism between TNF-α and CHI on IL-8 mRNA expression may be explained by the further increased AP-1 activity as compared with that with CHI alone. CHI activates the protein kinase pathways implicated in early AP-1 activation [29,30]. On the other hand, it cannot be excluded that the synergism is dependent on superinduction of jun and fos mRNA, although preliminary results showed similar superinduction of jun and fos mRNA both in the absence or presence of TNF-α.

Newton and co-workers [31] recently reported studies on the effect of CHI on NF-κB and AP-1 activity in the alveolar type II-like cell line A549. They showed that co-exposure of this cell line to IL-1β and CHI induced a marked increase of NF-κB-binding activity compared to IL-1β alone. In contrast to NF-κB, AP-1-binding activity was not up-regulated by IL-1β and CHI. These apparent discrepancies may be related to intrinsic differences between A549 and H292 cells and/or to differences due to TNF-α and IL-1β. It should also be noted that AP-1 and NF-κB activities in A549 cells were measured using consensus oligonucleotides in which the putative DNA-binding site differed from that found in the IL-8 promoter, whereas we used IL-8 promoter-derived oligonucleotides.

In most experiments, TNF-α and CHI were added simultaneously. We anticipated that the contribution of transcriptional processes to the observed synergism was more obvious when TNF-α and CHI were added simultaneously or added separately within a short time-span. In fact, the results shown in Figure 8 illustrate just that. However, even when CHI was added 4 h after TNF-α, there still was a synergistic effect detectable. Furthermore, the effect of TNF-α on superinduction was not inhibited by 4 h of preincubation with CHI, in agreement with the fact that NF-κB and AP-1 activation do not require *de novo* protein synthesis.

In conclusion, IL-8 mRNA superinduction in H292 cells results from an increased mRNA half-life and from prolonged and increased activation of relevant transcription factors. It appears that inhibition of protein synthesis affects both transcriptional and post-transcriptional processes that are implicated in repression of IL-8 mRNA expression. The identification of the molecular mechanisms described here should facilitate further studies into IL-8 mRNA expression due to superinduction by pathophysiological conditions that may occur in the airways.

This work was supported in part by a grant from the European Community to T. R. (contract ERBCHBGCT940673) and by a grant from The Netherlands Asthma Foundation (grant 95.56).

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Received 27 August 1997/3 November 1997; accepted 5 November 1997