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Superinduction of IL-6 by Cycloheximide Is Associated With mRNA Stabilization and Sustained Activation of p38 Map Kinase and NF-κB in Cultured Caco-2 Cells

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Abstract Protein synthesis inhibitors paradoxically increase the expression of early-gene products, including various cytokines, through a process known as superinduction. Superinduction is cell-specific and the mechanisms involved are not fully understood but are usually attributed to decreased mRNA degradation. There is, however, increasing evidence that activation of signaling cascades and increased transcriptional activation may be involved as well. Recent studies suggest that IL-6 production in the intestinal mucosa is particularly important due to its anti-inflammatory and protective effects. The effect of protein synthesis inhibitors on IL-6 production in enterocytes, however, is unknown. Treatment of Caco-2 cells with cycloheximide (10 μg/ml) increased IL-6 mRNA and protein levels in IL-1β-treated cells and this was associated with increased mRNA stabilization. In addition, cycloheximide suppressed IκBα resynthesis and prolonged p38MAP kinase activation and these changes were associated with sustained activation of the transcription factor NF-κB. NF-κB activation, in turn, was prevented by the specific p38MAP kinase inhibitor SB208350. Our results suggest that superinduction of IL-6 by cycloheximide in enterocytes results from both increased mRNA stabilization and upregulated transcriptional activity mediated by prolonged activation of the p38 MAP kinase and NF-κB. J. Cell. Biochem. 91: 951–961, 2004. © 2004 Wiley-Liss, Inc.

Key words: enterocytes; cytokines; protein synthesis inhibitors; cell signaling; transcription factors

There is increasing evidence that the intestinal mucosa is an active participant in the inflammatory and metabolic responses to injury, shock, and sepsis and that some of these responses may contribute to the development of multiple organ failure in critically ill patients [Deitch, 1992; Pritts et al., 2002b]. Studies in our and other laboratories have shown that various cytokines, including interleukin-1 (IL-1) and IL-6, are produced in the intestinal mucosa during sepsis and endotoxemia [Mester et al., 1993; Jung et al., 1995; Meyer et al., 1995; Wang et al., 1998b]. Among those cytokines, IL-6 is particularly important because of its pleiotropic role in local and systemic inflammation and in the regulation of metabolic changes seen during sepsis and other inflammatory conditions [Akira et al., 1993; Stouthard et al., 1995; Wang et al., 1997].

Recent studies suggest that IL-6 may exert both pro- and anti-inflammatory effects [Papanicolaou et al., 1998]. Thus, under certain circumstances, IL-6 may contribute to morbidity and mortality in critical illness and may impair mucosal integrity, resulting in increased intestinal permeability [Calandra et al., 1991; Damas et al., 1992; Janu et al., 1996; Wang et al., 1998a]. In other situations, however, IL-6 may be protective and have anti-inflammatory properties [Barton and Jackson, 1993; Xing et al., 1998]. Interestingly, we recently found that IL-6 may induce

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thermotolerance in cultured Caco-2 cells, a human intestinal epithelial cell line [Hershko et al., 2003]. Although, it is not fully understood at present why IL-6 exerts proinflammatory and potentially harmful effects in some situations and protective effects under other circumstances, it is obvious that an increased understanding of mechanisms that regulate IL-6 production in the enterocyte may have important clinical implications.

In recent studies, we used cultured Caco-2 cells to examine the regulation of enterocyte IL-6 production. Results from those experiments suggest that enterocyte IL-6 production is upregulated at the transcriptional level by IL-1β [Parikh et al., 1997a] and that among different transcription factors that regulate the IL-6 gene, NF-κB may be particularly important for the IL-1β-induced IL-6 production in Caco-2 cells [Parikh et al., 1997b]. In other studies, we found evidence that enterocyte IL-6 production can be blocked by sodium arsenite, mainly reflecting inhibition of NF-κB activity [Hershko et al., 2002a]. In contrast, IL-6 production in IL-1β-treated Caco-2 cells was potentiated by the heat-shock response induced either by hyperthermia [Parikh et al., 1998] or treatment with a proteasome inhibitor [Pritts et al., 2002a].

As part of our ongoing efforts to examine mechanisms that regulate enterocyte IL-6 production, the present study focused on the effects of cycloheximide. In recent studies, certain protein synthesis inhibitors increased the expression of various gene products, including IL-6, a phenomenon called superinduction [Edwards and Mahadevan, 1992; Faggioli et al., 1997; Newton et al., 1997; Roger et al., 1998; Lutter et al., 2000]. Although the mechanisms of superinduction are not fully understood, various explanations that have been proposed include stabilization of mRNA, stimulation of intracellular signaling, and increased gene transcription, possibly by altered activity of transcription factors. Recent studies suggest that the mechanisms by which protein synthesis inhibitors induce superinduction may vary between different cell types and may also be different depending upon which gene is being superinduced. The influence of cycloheximide on enterocyte IL-6 production has not been reported. In the present study, we tested the hypothesis that treatment of cultured Caco-2 cells with cycloheximide results in superinduction of IL-6 and that increased mRNA stability, MAP kinase signaling, and NF-κB activation are involved in this response to cycloheximide.

MATERIALS AND METHODS

Materials

Caco-2 cells were from American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids, low-endotoxin fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, and TRIZOL were from Invitrogen (Carlsbad, NY). Human recombinant IL-1β was from Endogen (Woburn, MA). All other chemicals, unless otherwise stated, were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Caco-2 cells were grown at 37°C in 5% CO_2 in DMEM supplemented with 10% FBS, nonessential amino acids, 6 mM glutamine, 10 mM HEPES, 10 μg/ml apo-transferrin, 1 mM pyruvate, 24 mM $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen). Cells, between passages 5 and 25, were seeded at a density of 100,000 cells/cm^2 onto 6-well culture plates for ELISA and luciferase assays or onto 10-cm tissue culture plates (both from Falcon-Becton Dickinson, Franklin Lakes, NJ) for electrophoretic mobility shift assay (EMSA), Western blot analysis, reverse transcriptase-polymerase chain reaction (RT-PCR), and real-time PCR. The cells were grown for 72 h to 90% confluence before use.

Experimental Conditions

Prior to experiments, cells were washed three times with serum-free DMEM and then treated at 37°C with serum-free medium containing different concentrations of cycloheximide with and without IL-1β (0.5 ng/ml) for 0–24 h as indicated in Results. Treatment of cultured enterocytes with 0.5 ng/ml of IL-1β resulted in maximal IL-6 production in previous experiments in this laboratory [Parikh et al., 1997a]. After treatment of the cells, measurements of IL-6 protein and mRNA levels, IκBα, p38 MAP kinase protein levels, and NF-κB activity were performed as described below.

Determination of Cell Viability

Cell viability was determined by measuring mitochondrial respiration, assessed by the

mitochondrial-dependent reduction of 3-(4.5 dimehtylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan as described previously [Salzman et al., 1996]. Cell viability was not influenced by any of the experimental conditions in the present study (data not shown).

Preparation of Cytoplasmic and Nuclear Extracts

For preparation of cytoplasmic and nuclear fractions, cells were harvested by scraping into ice-cold PBS and pelleted by centrifugation at 3,800*g* for 5 min. Cells were then suspended in one packed-cell volume of lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% (v/v) Nonidet P-40, 1 mM DTT, 0.5 mM PMSF, 100 μM 4-(2-aminoehtyl)-benzenesulfonyl fluoride, 1.5 μM pepstatin A, 1.4 μM transepoxysuccinyl-L-leucylamidol, 4 μM bestatin, 2.2 μM leupeptin, 0.08 μM aprotinin, 0.0045 μM microcystin LR, 0.46 μM cantharidin, and 0.2 μM (−)-p-bromotetramisole. After incubation on ice for 5 min with intermittent vortexing, the nuclear pellet was isolated by centrifugation at 3,800*g* for 5 min. The supernatant was removed and saved as the cytoplasmic fraction. The pellet was resuspended in 1 cell volume of extract buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol (v/v), 1 mM DTT, 0.5 mM PMSF, 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1.5 μM pepstatin A, 1.4 μM transepoxysuccinyl-L-leucylamidol, 4 μM bestatin, 2.2 μM leupeptin, 0.08 μM aprotinin, 0.0045 μM microcystin LR, 0.46 μM cantharidin, and 0.2 μM (−)-p-bromotetramisole and incubated on ice for 30 min with intermittent vortexing. The nuclear debris was pelleted by centrifugation at 16,000*g* for 20 min. Protein concentrations in the nuclear and cytoplasmic extracts were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

EMSA

Aliquots of the nuclear fractions (7.5 μg protein) were incubated in buffer containing 12% glycerol (v/v), 12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 25 mM KCl, 5 mM $MgCl_2$, 0.04 μg/μl poly [d(I-C)] (Boehringer Mannheim, Indianapolis, IN), and Tris-EDTA buffer, pH 7.4. NF-κB gel shift oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The probe was end-labeled with (^{32}P) γATP using polynucleotide kinase T4 (Gibco BRL, Grand Island, NY). End-labeled probe was purified from unincorporated (^{32}P) γATP using a purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA buffer, pH 7.4. Labeled probe was added to nuclear extracts and the samples were incubated for 30 min on ice. Samples were subjected to electrophoretic separation on a nondenaturing 5% poly-acrylamide gel at 30 V using Tris borate EDTA buffer (0.45 M Tris-borate, 0.001 M EDTA, pH 8.3). Blots were dried at 80°C for 3 h and analyzed by exposure to PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis

Aliquots of nuclear or cytoplasmic fractions containing 25 μg of protein were boiled in equal amounts of loading buffer (125 mmol/l Tris-hydrochloride, pH 6.8, 4% sodium dodecyl sulphate, 20% glycerol and 10% 2-mercaptoethanol) for 3 min, then separated by electrophoresis on 8–16% Tris-glycine gradient gel (Invitrogen, San Diego, CA). A protein ladder (see, Blue; Invitrogen, San Diego, CA) was included as a molecular weight marker. The proteins were transferred to nitrocellulose membranes (Xcell II Blot Module; Novex), which were blocked in 5% nonfat dried milk in Tris-buffered saline (pH 7.6), containing 0.05% Tween 20 for 1 h. The membranes were then incubated with antibodies against IκBα, p38 MAP kinase, or phosphorylated p38 MAP kinase (Cell signaling Technology, Beverly, MA) for 1 h and then washed three times with TTBS before incubation with peroxidase-conjugated secondary antibody for 45 min. Following successive washes, the membranes were incubated in enhanced chemiluminescence reagents and exposed on radiographic film (Eastman-Kodak, Rochester, NY).

Determination of IL-6

IL-6 levels were determined by ELISA (Endogen, Cambridge, MA) according to the manufacturer's guidelines. The limit of detection was 1 pg/ml.

RT-PCR

Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method

[Chomczynski and Sacchi, 1987] using TRIZOL (Gibco-BRC). Total RNA was reverse transcribed to cDNA by adding 2 μg of the RNA samples to a mixture containing 25 mM $MgCl_2$, 10× PCR buffer, nucleotide triphosphates, RNase inhibitor, oligo d(T), and reverse transcriptase. The samples were incubated in a Perkin-Elmer Thermal Cycler (Norwalk, CT) at 65°C for 5 min, 25°C for 1 min, 42°C for 90 min, and at 99°C for 5 min. The resulting cDNA was stored at −20°C until used. PCR primer pairs for human IL-6 were from Clontech Laboratories (Palo Alto, CA). The 5′-sense (5′-ATG AAC TCC TTC CCA CCA GCG C-3′) and 3′-anti-sense (5′-GAA GAG CCC TCA GGC TGG ACT G-3′) amplified a 628 bp fragment of the published IL-6 mRNA extending from bp 34 to 661. Primers for the house-keeping gene GAPDH were used in all samples for evidence of even amplification. Aliquots (2 μg) of cDNA were added to Taq and anti-Taq polymerase (Life Technologies, Gaithensburg, MD), PCR buffer, and the primers listed above. PCR was performed using a Perkin-Elmer Thermal Cycler by heating the samples (40 cycles) to 94°C for 45 s, 60°C for 30 s, 72°C for 5 min and cooled to 4°C. Ten microliters of PCR products were then separated by electrophoresis at 80 V in a 1% agarose gel stained with ethidium bromide (0.02%) for 30 min.

Real-Time PCR

Real-time PCR was performed as described in detail recently [Wray et al., 2003]. In short, RNA was extracted as described above. First-strand cDNA synthesis was performed using the Superscript first strand system for real-time PCR (Life Technologies, Rockville, MD) with oligo dT as the primer according to manufacturer's protocol. As an additional quality control, *Arabidopsis thaliana* mRNA was added to each RNA sample prior to cDNA synthesis. Real-time PCR was performed in a Smart Cycler (Cepheid, Sunnyvale, CA) using the Pre-Developed Taqman® Assay Reagents (Applied Biosystems, Langen, Germany) for human IL-6 according to the manufacturer's directions. In addition, real-time PCR was performed for the house-keeping gene GAPDH and results were normalized for GAPDH mRNA levels. Measurements were taken at the end of the 72°C extension step in each cycle, and the second-derivative method was used to calculate the threshold cycle. Melt curve analysis showed a single sharp peak for all samples. Real-time PCR was also performed with primers specific for *Arabidopsis thaliana*. Fluorescence growth curves and threshold cycle for *Arabidopsis thaliana* mRNA were equal for all samples ensuring equal cDNA loading.

Statistical Analysis

Results are expressed as means ± SEM. Student's test or ANOVA followed by Tukey's test was used for statistical analysis. Experiments were performed at least three times to provide evidence of reproducibility.

RESULTS

Treatment of cultured Caco-2 cells with 0.5 ng/ml of IL-1β for 24 h resulted in increased IL-6 production (Fig. 1A). The amount of IL-6 produced by the IL-1β-stimulated cells was similar to that found in previous reports from our laboratory [Parikh et al., 1997a,b, 1998; Hershko et al., 2002a; Pritts et al., 2002a]. The effect of IL-1β on IL-6 production was substantially potentiated by cycloheximide at concentrations ranging from 10 to 100 μg/ml with the most pronounced effect of cycloheximide noticed at the lowest concentration tested. At the highest concentration tested (1,000 μg/ml), IL-6 production was actually completely blocked. In separate experiments, overall protein synthesis measured as incorporation of ^{14}C-labeled leucine into protein was inhibited by approximately 80% by 10 μg/ml of cycloheximide and was completely blocked by higher concentrations of the drug (data not shown). Addition of cycloheximide alone (10 μg/ml) to the culture medium resulted in only a minimal increase in IL-6 production, suggesting that treatment with cycloheximide resulted in IL-6 superinduction by potentiating the effects of IL-1β.

The concentration of IL-1β used here (0.5 ng/ml) was based on previous reports in which we found that IL-6 production was maximally stimulated by this concentration of IL-1β [Parikh et al., 1997a]. In order to test whether cycloheximide would potentiate the effect of a lower concentration of IL-1β, cells were treated with 0.1 ng/ml of the cytokine. This concentration of IL-1β did not in itself result in significantly increased production of IL-6, whereas, in the presence of cycloheximide (10 μg/ml), IL-6 production was significantly upregulated (Fig. 1B). This result suggests that cycloheximide increases both the response and sensitivity to

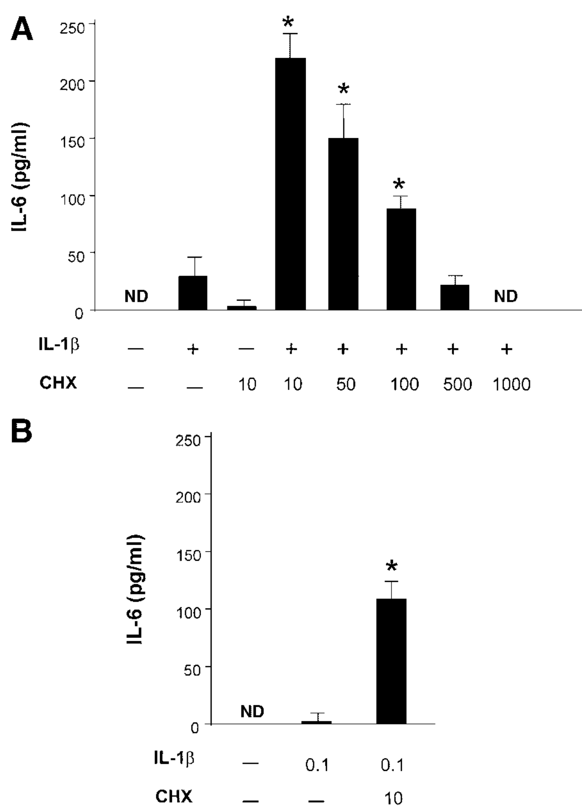

Fig. 1. **A**: The effect of different concentrations of cycloheximide (CHX) on IL-6 production in IL-1β-stimulated cultured Caco-2 cells. Cells were treated with IL-1β (0.5 ng/ml) and different concentrations of CHX (0–1,000 μg/ml) for 24 h. IL-6 levels were determined in the culture medium by ELISA. Results are means ± SEM with n = 6 for each treatment. ND, nondetectable. *$P < 0.05$ versus cells incubated with IL-1β alone. **B**: The effect of CHX (10 μg/ml) on IL-6 production in Caco-2 cells stimulated with 0.1 ng/ml of IL-1β. IL-6 levels were determined in the culture medium by ELISA after 24 h of treatment. Results are for means ± SEM with n = 6 for each condition. ND, nondetectable. *$P < 0.05$ versus IL-1β alone.

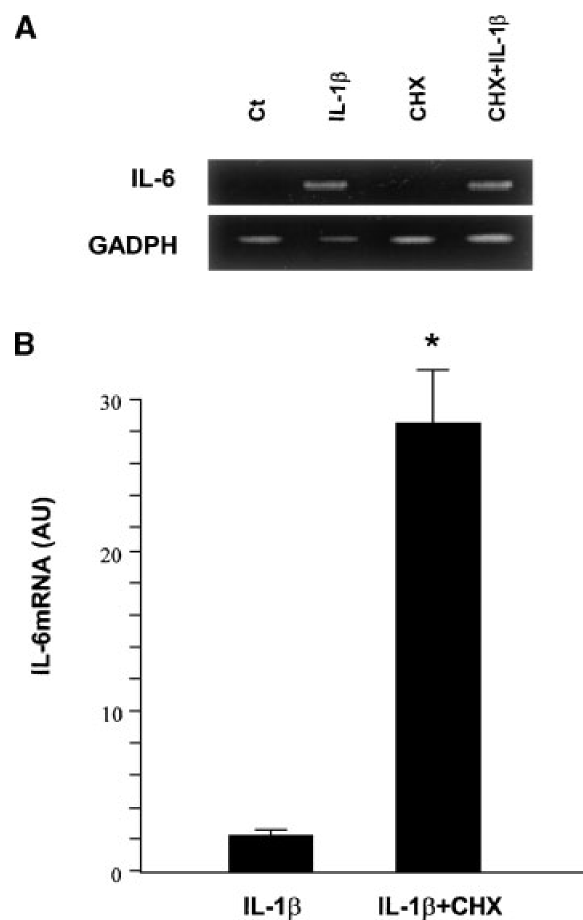

Fig. 2. **A**: The effects of IL-1β (0.5 ng/ml) and cycloheximide (10 μg/ml) on the expression of IL-6 mRNA determined by reverse transcriptase-PCR in cultured Caco-2 cells. Cells were treated for 4 h with IL-1β, cycloheximide, or both as indicated above the blots. Ctr, untreated cells. **B**: The effects of IL-1β and cycloheximide on the expression of IL-6 mRNA determined by real-time PCR in cultured Caco-2 cells. Cells were treated for 4 h with IL-1β (0.5 ng/ml) or IL-1β + cycloheximide (10 μg/ml). Results are expressed as arbitrary units (AU) after normalization for GAPDH mRNA and are means ± SEM with n = 3 for each treatment. *$P < 0.05$.

IL-1β with regards to IL-6 production in Caco-2 cells.

In previous studies, treatment of cells with cycloheximide resulted in accumulation of gene transcripts [Faggioli et al., 1997; Lutter et al., 2000]. To examine if a similar mechanism may be involved in enterocytes, Caco-2 cells were treated with 0.5 ng/ml of IL-1β and/or 10 μg/ml of cycloheximide for 4 h followed by determination of IL-6 mRNA expression by RT-PCR. Similar to previous observations [Parikh et al., 1997a], IL-6 mRNA was not detectable in untreated control cells, whereas treatment with IL-1β resulted in upregulated IL-6 mRNA levels (Fig. 2A). Cycloheximide alone did not induce expression of IL-6 mRNA. In order to test whether the combined treatment with IL-1β and cycloheximide resulted in increased IL-6 mRNA levels compared with IL-1β treatment alone, quantification was performed by real-time PCR. The addition of cycloheximide to IL-1β-treated cells resulted in a 14–15-fold increase in IL-6 mRNA levels (Fig. 2B).

Increased IL-6 mRNA levels, as observed here, may reflect increased stability (reduced degradation) of the transcript, increased gene transcription, or a combination of these changes. In order to examine the influence of cycloheximide on IL-6 mRNA degradation, the decline in IL-6 mRNA levels in cultured Caco-2 cells

was monitored in the presence of actinomycin D (Act D). Because Act D blocks mRNA production, the decrease in mRNA levels under these conditions reflects degradation of mRNA. Cells were treated with IL-1β (0.5 ng/ml) and cycloheximide (10 μg/ml) for 4 h to induce high levels of IL-6 mRNA. The cells were then washed three times and incubated for an additional 4 h in the presence of Act D (10 μg/ml) and IL-1β (0.5 ng/ml) with or without cycloheximide (10 μg/ml). The rate of decline of IL-6 mRNA levels was reduced in the presence of cycloheximide (Fig. 3A), suggesting that the degradation of IL-6 mRNA was inhibited by cycloheximide. Thus, stabilization of mRNA may be a mechanism by which cycloheximide induces superinduction of IL-6 in IL-1β-treated Caco-2 cells.

In order to test whether the superinduction of IL-6 under the present experimental conditions requires active gene transcription, we next measured IL-6 production in IL-1β-treated Caco-2 cells in the absence or presence of cycloheximide or Act D. Results from this experiment showed that Act D completely blocked IL-6 production in cells treated with IL-1β alone or IL-1β together with cycloheximide (Fig. 3B). Although, this result does not allow us to conclude that cycloheximide results in increased IL-6 gene transcription in IL-1β treated cells, the data clearly show that ongoing gene transcription is needed for the superinduction to occur.

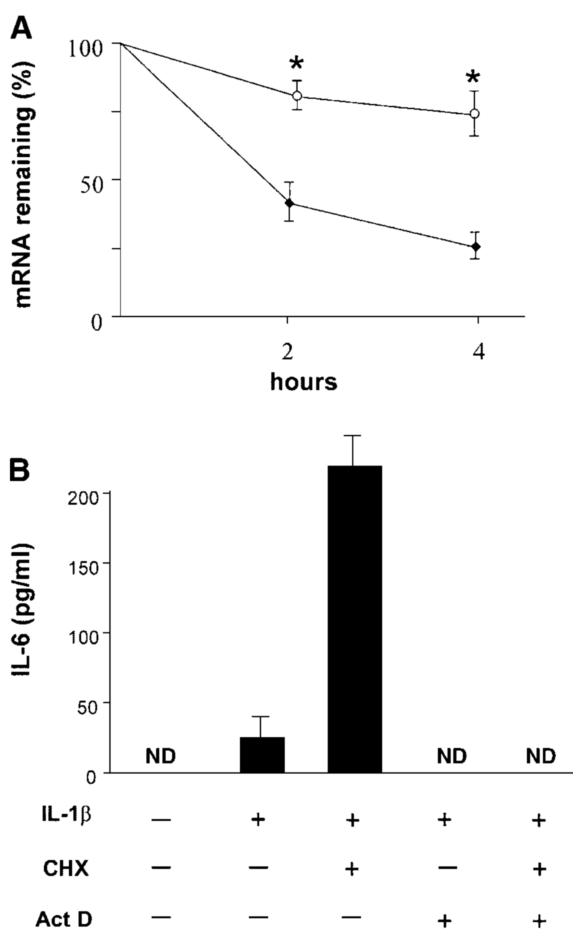

Fig. 3. A: The effect of cycloheximide on the degradation of IL-6 mRNA determined by real-time PCR. Cultured Caco-2 cells were treated for 4 h with IL-1β (0.5 ng/ml) and cycloheximide (10 μg/ml). Cells were then washed and incubated for an additional 4 h in medium containing 10 μg/ml of actinomycin D (Act D) with (○) or without (◆) 10 μg/ml of cycloheximide. The expression of IL-6 mRNA at 0, 2, and 4 h was determined by real-time PCR and results are expressed as percent (%) of initial (0 h) mRNA levels. $^{*}P < 0.05$ between the two conditions. B: The effect of actinomycin D (Act D) on IL-6 production in cultured Caco-2 cells. Cells were pretreated for 30 min with Act D (10 μg/ml) followed by treatment with IL-1β (0.5 ng/ml) with or without cycloheximide (10 μg/ml) for 24 h. IL-6 levels were determined in the culture medium by ELISA. Results are means ± SEM with n = 6 for each condition. ND, nondetectable.

Previous studies from our and other laboratories suggest that among the different transcription factors that regulate the IL-6 gene (i.e., NF-κB, AP-1, CREB, and C/EBP), NF-κB activation is particularly important for IL-6 production in IL-1β-treated Caco-2 cells [Parikh et al., 1997b; Vanden Berghe et al., 1999; Pritts et al., 2002a]. To examine whether NF-κB may be involved in the cycloheximide-induced IL-6 superinduction, NF-κB DNA binding activity was determined by EMSA. Treatment of Caco-2 cells with IL-1β increased NF-κB DNA binding activity (Fig. 4A). Treatment of the cells with cycloheximide alone did not activate DNA binding. The increase in NF-κB activity caused by IL-1β was transient and was no longer present at 6 h. Interestingly, when cycloheximide was added to the medium, the activation of NF-κB caused by IL-1β was extended in time and persisted for at least 6 h (Fig. 4A, sixth lane).

NF-κB is normally sequestered in the cytoplasm in an inactive form by the inhibitory proteins IκB. Activation of NF-κB is mediated by phosphorylation, ubiquitination, and degradation of IκB by the 26S proteasome [Karin and Ben-Neriah, 2000]. This allows the active transcription factor to translocate to the nucleus where it binds to its DNA recognition sites in responsive genes. To examine whether the prolonged activation of NF-κB, noticed in cells treated with IL-1β and cycloheximide, was associated with altered IκBα levels, cells were

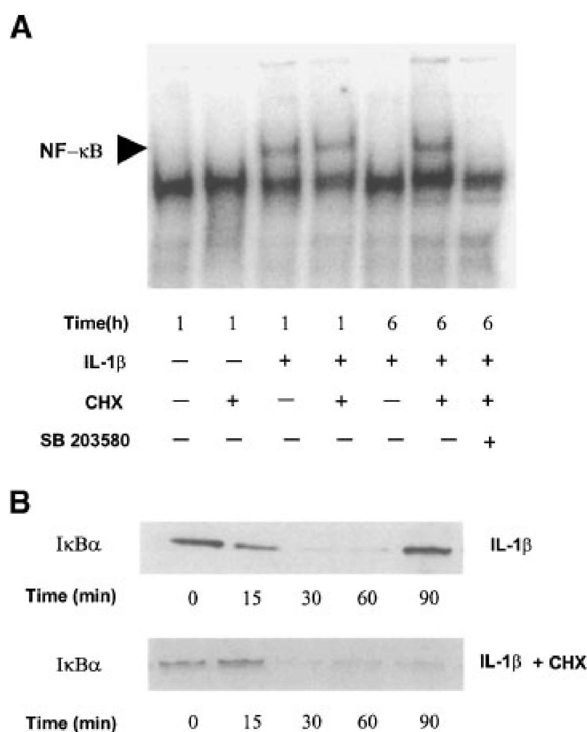

Fig. 4. **A**: The effect of IL-1β and cycloheximide on NF-κB DNA binding activity in cultured Caco-2 cells. Cells were treated with IL-1β (0.5 ng/ml) and cycloheximide (10 μg/ml) for 1 or 6 h as indicated. Some cells were pretreated with SB 203580 (10 μM) for 30 min and then treated with IL-1β and cycloheximide for 6 h in the continued presence of SB 203580 (**last lane**). Nuclear extracts were then subjected to EMSA. **B**: The effect of cycloheximide on IκBα levels in IL-1β-stimulated cultured Caco-2 cells. Cells were treated with IL-1β (0.5 ng/ml) with (**lower pannel**) or without (**upper pannel**) cycloheximide (10 μg/ml) for 0–90 min as indicated. IκBα levels in cytoplasmic extracts were determined by Western blot analysis.

treated with IL-1β alone or together with cycloheximide. Cytoplasmic extracts were then subjected to Western blot analysis to determine IκBα levels. In IL-1β treated cells, IκBα rapidly decreased and was undetectable after 30 min (Fig. 4B, upper panel). This decline in IκBα levels was followed by reaccumulation of IκBα after 90 min, consistent with NF-κB regulated resynthesis of IκBα [Henkel et al., 1993]. Treatment of cells with cycloheximide did not alter the rate of IL-1β-induced IκBα degradation, but reaccumulation of IκBα was prevented at least up to 90 min (Fig. 4B, lower panel). Thus, the prolonged activation of NF-κB noticed in cycloheximide-treated cells (see Fig. 4A, sixth lane) may at least in part reflects inhibited resynthesis of IκBα.

In previous studies, NF-κB activation was found to reflect activation of the MAP kinase signaling pathway, in particular p38 MAP kinase [Zechner et al., 1998; Craig et al., 2000]. We next examined the potential involvement of p38 MAP kinase in IL-1β- and cycloheximide-treated Caco-2 cells. Treatment of the cells with IL-1β alone or in combination with cycloheximide rapidly (15 min) increased cellular levels of phosphorylated (activated) p38 (p-p38) (Fig. 5A). The increase in p-p38 MAP kinase levels caused by IL-1β was transient and p-p38 was no longer detectable after 60 min in cells treated with IL-1β alone. This decline in p-p38 levels was blocked by cycloheximide; thus, in cells treated with both IL-1β and cycloheximide, cellular levels of p-p38 MAP kinase remained increased at 60 min (Fig. 5A, last lane in upper panel).

We next tested whether activation of the p38 MAP kinase pathway contributed to IL-6 production in Caco-2 cells treated with IL-1β and cycloheximide. This was done by treating cells with the p38 MAP kinase inhibitor SB203580 [Cuenda et al., 1995]. This treatment reduced IL-6 production in a dose-dependent fashion to approximately the same level as noticed in cells treated with IL-1β alone (Fig. 5B and compare with Fig. 1A). In contrast, treatment of the cells with the p42/44 MAP kinase inhibitor PD98059 resulted in only marginal inhibition of IL-6 production in cells treated with IL-1β and cycloheximide. These observations suggest that p38 MAP kinase is particularly important for the superinduction of IL-6 in IL-1β-treated Caco-2 cells.

In order to test whether activation of p38 MAP kinase is important for the sustained NF-κB DNA binding activity noticed in cells treated with IL-1β and cycloheximide, the influence of SB203580 on NF-κB activity was determined by EMSA. The sustained activation of NF-κB noticed at 6 h in cells treated with IL-1β and cycloheximide was prevented by SB203580 (Fig. 4, last lane) suggesting that p38 MAP kinase signaling regulates NF-κB activation in cycloheximide-treated cells.

DISCUSSION

In the present study, treatment of IL-1β-stimulated Caco-2 cells with cycloheximide resulted in increased IL-6 mRNA levels and IL-6 protein production. This response to cycloheximide was associated with increased IL-6 mRNA stability and activation of p38 MAP kinase and NF-κB, suggesting that multiple

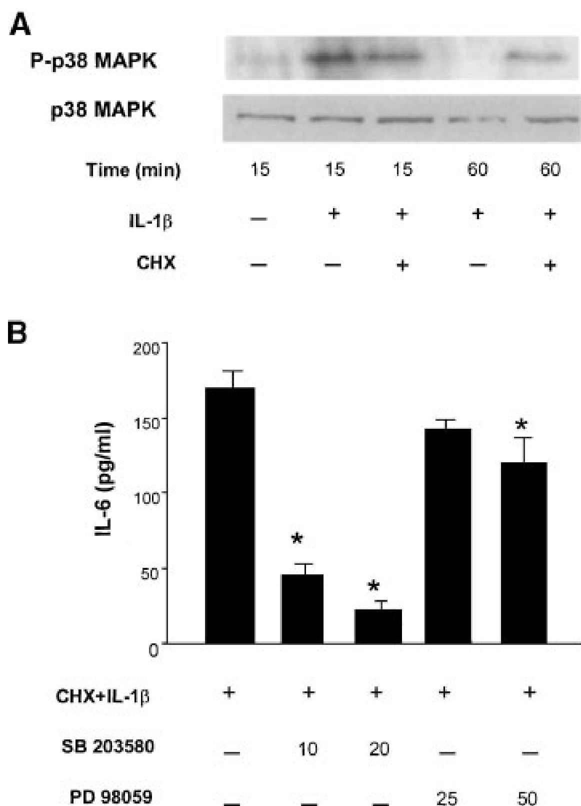

Fig. 5. **A**: The effect of IL-1β and cylcoheximide on p38 MAP kinase levels in cultured Caco-2 cells. Cells were treated with IL-1β (0.5 ng/ml) and cycloheximide (10 μg/ml) for 15 or 60 min as indicated. Phosporylated p38 MAP kinase (p-p38 MAPK) and total p38 MAP kinase levels from cytoplasmic extracts were determined by Western blot analysis. **B**: The effects of the p38 MAP kinase inhibitor SB203580 and the p42/44 MAP kinase inhibitor PD98059 on IL-6 production in IL-1β- and cyclohex-imide-treated cells. Cells were treated for 30 min with the inhibitors followed by IL-1β (0.5 ng/ml) and cycloheximide (10 μg/ml) for 24 h (the MAP kinase inhibitors were present in the culture medium also during the 24 h treatment period). The concentrations of SB 203580 and PD98059 used in the experiments are given as (μM) below the graph. IL-6 levels were determined in the culture medium by ELISA. Results are means ± SEM with n = 6 for each condition. *$P < 0.05$ versus cells incubated with IL-1β and cycloheximide.

mechanisms were involved in the superinduction of IL-6. To our knowledge, this is the first report of IL-6 superinduction in intestinal epithelial cells. This is significant considering the fact that enterocyte and mucosal IL-6 production is influenced by conditions of both systemic and local inflammation [Mitsuyama et al., 1991; Mester et al., 1993; Jung et al., 1995; Meyer et al., 1995; Pritts et al., 2002b] and that IL-6 has multiple biological effects in gut mucosa where it can exert both pro- and anti-inflammatory effects [Calandra et al., 1991; Damas et al., 1992; Barton and Jackson, 1993; Janu et al., 1996; Papanicolaou et al., 1998; Wang et al., 1998a; Xing et al., 1998; Hershko et al., 2003]. Thus, an increased understanding of treatments and mechanisms that regulate enterocyte IL-6 production may have both biological and clinical implications.

Superinduction of IL-6 with cycloheximide was reported previously in other cell types, including fibroblasts, lung epithelial cells, HeLa cells, and a breast carcinoma cell line [Faggioli et al., 1997; Vanden Berghe et al., 1999; Lutter et al., 2000]. It should be noted, however, that mechanisms involved in IL-6 superinduction seems to be cell specific. For example, superinduction of IL-6 in fibroblasts and airway epithelial cells was mainly caused by mRNA stabilization [Lutter et al., 2000], whereas in HeLa and breast cancer cells, superinduction depended mainly on increased transcriptional activity, secondary to NF-κB activation [Faggioli et al., 1997]. Thus, it is important to specifically examine the influence of cycloheximide on IL-6 production and its mechanisms in different cell types in which IL-6 production has significant biological effects.

It should be noted that although cycloheximide is a protein synthesis inhibitor, at least some of the mechanisms of cycloheximide-induced IL-6 superinduction do not necessarily reflect inhibited protein synthesis. The observation in the present study that there was an inverse relationship between cycloheximide concentrations and IL-6 superinduction would support that conclusion. A similar observation was reported recently by Itani et al. [2002]. They found that the alpha-epithelial Na^+ channel (α-ENaC) gene was superinduced in dexamethasone-treated canine kidney cells by cycloheximide at a concentration that did not inhibit protein synthesis. Furthermore, the protein synthesis inhibitor puromycin did not influence α-ENaC gene expression and anisomycin (an additional protein synthesis inhibitor) actually reduced α-ENaC mRNA levels in dexamethasone-treated cells. Thus, protein synthesis inhibition does not seem to be sufficient or necessary, at least for some of the mechanisms that result in superinduction. In the present study, one of the potential mechanisms by which cycloheximide induced superinduction was a prolonged NF-κB activation, possibly reflecting inhibited synthesis of IκBα. It is possible that the inhibition of IκBα

synthesis reflected the protein synthesis inhibitory effect of cycloheximide, although other mechanisms can not be ruled out from the present data.

At least from a theoretical standpoint, it may be argued that the increased IL-6 expression observed in Caco-2 cells treated with IL-1β and cycloheximide may reflect inhibited turnover of IL-1β resulting in increased IL-1β concentrations. This is highly unlikely, however, because in previous studies [Parikh et al., 1997a] we found that IL-6 production was maximally stimulated by IL-1β at a concentration of 0.5 ng/ml (in fact, a small reduction of IL-6 production was noticed at higher IL-1β concentrations). Thus, it is not likely that the increased IL-6 expression seen in cells treated with IL-1β and cycloheximide was the result of higher IL-1β concentrations.

We found here that cycloheximide alone induced only a minor production of IL-6 in cultured Caco-2 cells and did not influence IL-6 mRNA levels or NF-κB activity. This contrasts to previous reports in which cycloheximide alone induced IL-6 superinduction in lung epithelial cells, HeLa cells, and a breast carcinoma cell line [Faggioli et al., 1997; Lutter et al., 2000]. Similar to the present study, however, cycloheximide alone did not influence the gene expression in cultured canine kidney cells [Itani et al., 2002]. Thus, the influence of cycloheximide on gene activation is probably cell specific and at least under certain circumstances, cycloheximide acts mainly by potentiating the effect of another stimulus, such as IL-1β (present report) or dexamethasome [Itani et al., 2002].

Superinduction of IL-6 mRNA levels may reflect changes in gene transcription, mRNA stability, or a combination of both. Results reported here suggest that both mechanisms may be involved in cycloheximide-induced IL-6 superinduction in cultured Caco-2 cells. The increased (sustained) NF-κB activation, together with the observation that Act D completely blocked the IL-6 superinduction, suggest that increased IL-6 gene transcription may, at least in part, account for the superinduction observed under the present experimental conditions. Also, in certain other [Faggioli et al., 1997], but not all [Lutter et al., 2000] cell lines, increased mRNA levels after treatment with protein synthesis inhibitors reflected enhanced transcriptional activity.

When the decline of IL-6 mRNA levels was followed in cells in the presence of Act D, the results suggested that IL-6 mRNA was stabilized by cycloheximide, providing an additional mechanism by which IL-6 mRNA levels were increased. Similar to this observation, cycloheximide was found to stabilize IL-6 transcripts in fibroblasts and lung epithelial cells [Lutter et al., 2000]. In contrast, in breast cancer cells, HeLa cells, and canine kidney cells, cycloheximide induced superinduction without affecting mRNA stability [Faggioli et al., 1997; Newton et al., 1997; Itani et al., 2002], supporting the concept that the response to cycloheximide with regards to mechanisms of gene superinduction is cell specific.

The present findings that SB203580 inhibited the increase in IL-6 production and blocked the sustained NF-κB activation in cells treated with IL-1β and cycloheximide suggest that p38 MAP kinase signaling was involved in the superinduction of IL-6 noticed here. In other studies as well, evidence for a role of p38 MAP kinase was found in cycloheximide-induced superinduction [Itani et al., 2002]. The mechanisms by which p38 MAP kinase signaling mediates superinduction are not completely understood at present but may be multiple. For example, recent studies suggest that activation of NF-κB is p38 MAP kinase-dependent [Zechner et al., 1998; Craig et al., 2000]. This may, at least in part, explain why in the present study, SB203580 inhibited NF-κB DNA binding activity in Caco-2 cells treated with IL-1β and cycloheximide. Stabilization of mRNA caused by p38 MAP kinase-mediated binding of AUF1 and AUBF to AU-rich segments of the IL-6 mRNA [Sirenko et al., 1997; Winzen et al., 1999] offers another potential mechanism by which p38 MAP kinase may mediate cycloheximide-induced superinduction.

In addition to NF-κB, the IL-6 gene is regulated by the transcription factors AP-1, C/EBP, and CREB [Vanden Berghe et al., 2000]. There is evidence that the importance of different transcription factors for the regulation of IL-6 varies with cell type and with stimulus [Vanden Berghe et al., 1999]. Although, we have found evidence in recent studies that the IL-6 gene is regulated by both AP-1, C/EBP and CREB in IL-1β-treated Caco-2 cells [Hungness et al., 2000, 2002; Hershko et al., 2002b]; in other experiments, we found that NF-κB is particularly important for IL-6 expression in

these cells [Parikh et al., 1997b; Hershko et al., 2002a, Pritts et al., 2002a]. In the present study, therefore, we focused on the potential involvement of NF-κB in the cycloheximide-induced superinduction of IL-6. Although, our results support a role of NF-κB in the superinduction of IL-6 noticed here, the experiments do not rule out involvement of other transcription factors as well. Indeed, in a recent study in lung epithelial cells, superinduction of IL-6 mRNA was dependent on increased C/EBP activity [Roger et al., 1998]. It will be important in future studies to test the role of other transcription factors, in addition to NF-κB, in cycloheximide-induced superinduction of IL-6 in intestinal epithelial cells.

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