Importance of post-transcriptional regulation of chemokine genes by oxidative stress

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The transcription factor, nuclear factor κB (NF-κB), is activated by various stimuli including cytokines, radiation, viruses and oxidative stress. Here we show that, although induction with H_2O_2 gives rise to NF-κB nuclear translocation in both lymphocyte (CEM) and monocyte (U937) cells, it leads only to the production of mRNA species encoding interleukin-8 (IL-8) and macrophage inflammatory protein 1α in U937 cells. Under similar conditions these mRNA species are not observed in CEM cells. With the use of a transient transfection assay of U937 cells transfected with reporter constructs of the IL-8 promoter and subsequently treated with H_2O_2, we show that (1) IL-8-promoter-driven transcription is stimulated in both U937 and CEM cells and (2) the NF-κB site is crucial for activation because its deletion abolishes activation by H_2O_2. The production of IL-8 mRNA in U937 cells is inhibited by the NF-κB inhibitors clasto-lactacystin-β-lactone and E-64D (L-3-trans-ethoxycarbonyloxirane-2-carbonyl-L-leucine-3-methyl amide) but requires protein synthesis *de novo*. Moreover, inhibition of the p38 mitogen-activated protein kinase also decreases the IL-8 mRNA up-regulation mediated by H_2O_2. Taken together, these results show the importance of post-transcriptional events controlled by a p38-dependent pathway in the production of IL-8 mRNA in U937. The much lower activation of p38 in CEM cells in response to H_2O_2 could explain the lack of stabilization of IL-8 mRNA in these cells.

Key words: free radicals, monocytes/macrophages, mRNA, transcription factors.

INTRODUCTION

During an inflammatory response, cells are exposed to reactive oxygen species (ROS) such as H_2O_2, superoxide anion ($O_2^{-\bullet}$) and hydroxyl radicals ($OH^{\bullet}$) derived from activated macrophages and neutrophils [1]. ROS, in addition to being efficient antimicrobial effector molecules, are also key mediators of inflammation [1–3] and have profound effects on gene transcription [4]. One of the downstream targets of ROS is nuclear factor κB (NF-κB), which is the first eukaryotic transcription factor that has been shown to respond directly to oxidative stress [5].

The NF-κB transcription factor binds specific DNA sequences as dimers. These consist of proteins belonging to the Rel/NF-κB family. In mammals, this family contains p50, p52, p65 (RelA), RelB and c-Rel (Rel) [6,7]. These five proteins harbour a related, but non-identical, 300-residue-long Rel homology domain (RelHD), which is responsible for dimerization, nuclear translocation and specific DNA binding. In addition, RelA, RelB and c-Rel, but not p50 or p52, contain one or two transactivating domains. NF-κB complexes are sequestered in the cytoplasm of most resting cells by inhibitory proteins belonging to the inhibitor κB (IκB) family [8–10], comprising IκBα, IκBβ, IκBε, p100 and p105. When cells are stimulated by proinflammatory cytokines such as interleukin-1β (IL-1β) or tumour necrosis factor α (TNF-α), the signal transduction pathway leading to the phosphorylation and degradation of IκB proteins has recently been clarified in HeLa and L293 cells ([11–13]; reviewed in [14]). The pathway includes a 700–900 kDa complex called signalosome involving the IκBα kinases (IKK-α and IKK-β) and the NF-κB essential modulator ('NEMO'). Important partners are proteins associated with the TNF-α or IL-1 receptors, the NF-κB-inducing kinase ('NIK'), and mitogen-activated protein kinase kinase 1 ('MEKK-1'). IκB kinase β phosphorylates IκBα on Ser-32 and Ser-36, which targets IκBα for ubiquitination and rapid degradation by the 26 S proteasome. These reactions are extremely rapid, the IκBα protein being completely degraded within minutes of cell stimulation, before being resynthesized to replenish the IκBα cytoplasmic pool. This rapid and transient degradation of IκBα contrasts with its slow and sustained disappearance observed by several authors when cells are induced by an extracellular generation of ROS, as occurs during inflammation [5,15,16]. These different kinetics of IκBα degradation very probably reflect the existence of different signalling pathways leading to IκBα phosphorylation. We showed recently that NF-κB activation by H_2O_2 does not involve the phosphorylation of Ser-32 and Ser-36 of IκBα but requires the phosphorylation of Tyr-42 and serine/threonine residues within the C-terminal region of IκBα [16].

Many genes are controlled by NF-κB (reviewed in [17]). Among them, genes encoding chemokines are important because these small proteins have a crucial role in both the immune and inflammatory responses (reviewed in [18]). Although several genes encoding chemokines are controlled by NF-κB and by other transcription factors such as NF-IL6, activator protein 1 (AP-1) and Sp1, it is not yet clear which subset of chemokine genes is activated by H_2O_2 and in which cell type this activation

Abbreviations used: ActD, actinomycin D; AP-1, activator protein 1; DTT, dithiothreitol; E-64D, L-3-trans-ethoxycarbonyloxirane-2-carbonyl-L-leucine-3-methyl amide; EMSA, electrophoretic mobility-shift assay; ERK, extracellular signal-regulated protein kinase; IκB, inhibitor κB; IL, interleukin; IP-10, interferon-γ-inducible protein 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; lactacystin, clasto-lactacystin-β-lactone; LPS, lipopolysaccharide; Ltn, lymphotactin; MAPK, mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase 2; MCP-1, monocyte chemotactic protein 1; MIP-1, macrophage inflammatory protein 1; NF-κB, nuclear factor κB; ROS, reactive oxygen species; RPA, ribonuclease protection assay; RT–PCR, reverse-transcriptase-mediated PCR; TNF-α, tumour necrosis factor α; UTR, untranslated region.

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can be observed. Here we show that two chemokine genes (*IL-8* and *MIP-1α*, the latter coding for macrophage inflammatory protein 1α) are up-regulated by H_2O_2 in U937 cells, whereas their mRNA species do not accumulate in CEM cells. The differential regulation between these two cell types is not related to the activation of transcription factors or to the transcription of chemokine genes but is more probably due to a cell-specific post-transcriptional stabilization requiring protein synthesis *de novo* and p38 mitogen-activated protein kinase activation.

MATERIALS AND METHODS

Cell culture and treatment

The human promyeloid cell line U937 and the human T-lymphoid cell line CEM were grown in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 2 mM glutamine (BioWhittaker) and 10% (v/v) fetal bovine serum (BioWhittaker). HeLa cells were grown in EMEM medium (BioWhittaker) containing 2 mM glutamine and 10% (v/v) fetal calf serum. Cells were seeded in fresh medium 12–24 h before any experiment and adjusted to a concentration of 10^6 cells/ml just before the experiment. For treatment with cycloheximide, the reagent was prepared at 10 mg/ml just before use and added at 50 μg/ml in culture medium 1 h before stimulation of the cells with H_2O_2 or lipopolysaccharide (LPS). Cycloheximide (50 μg/ml) was added again after 4 h of incubation. For treatment with clasto-lactacystin-β-lactone (lactacystin) and L-3-trans-ethoxycarbonyl-oxirane-2-carbonyl-L-leucine-3-methyl amide (E-64D), the reagents were added at 50 μM and 25 μg/ml respectively in culture medium 1 h before stimulation of the cells with H_2O_2 or LPS. For treatment with SB203580, this inhibitor was added at 10 μM 60 min before stimulation with H_2O_2 or LPS. Actinomycin D (ActD) was added at 5 μg/ml 3 h after stimulation with H_2O_2.

Chemicals

H_2O_2 (Merck, Germany) was diluted in RPMI 1640 immediately before use. LPS from *Escherichia coli* serotype 0111:B4, PMA, lactacystin, cycloheximide and ActD were purchased from Sigma (St Louis, MO, U.S.A.). LPS was dissolved in sterile water; PMA and lactacystin were dissolved in DMSO. Cycloheximide was diluted in RPMI medium immediately before use. TNF-α (Roche, Mannheim, Germany) was used at 100 units/ml in RPMI 1640. IL-1β (Roche) was used at 200 units/ml in RPMI 1640. The calpain inhibitor E-64D was purchased from Peptide International (Osaka, Japan) and resuspended in DMSO. The Ca^{2+} ionophore ionomycin from *Streptomyces conglobatus* was purchased from Calbiochem–Novachem (San Diego, CA, U.S.A.) and dissolved in DMSO. The inhibitor of the p38 kinase SB203580 was purchased from Alexis (Lausen, Switzerland) and resuspended at 20 mM in DMSO.

Extraction of nuclear proteins

Cells (5×10^6) were harvested and washed in 1 ml of cold PBS [137 mM NaCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4/2.7 mM KCl (pH 7.4)]. Cells were centrifuged at 15000 ***g*** for 15 s and re-suspended in 400 μl of cold hypotonic buffer [10 mM Hepes/KOH (pH 7.9)/10 mM KCl/2 mM $MgCl_2$/0.1 mM EDTA/0.1 mM PMSF/1 mM dithiothreitol (DTT) for CEM cells and 10 mM Hepes/KOH (pH 7.9)/10 mM KCl/2 mM $MgCl_2$/0.1 mM EDTA/0.1 mM PMSF/1 mM DTT/protease inhibitor cocktail Complete™ (Roche)/0.1% (v/v) Nonidet P40 for U937 cells]. Cells were left to swell on ice for 10 min and then vortex-mixed for 2 or 3 s. The suspension was then centrifuged at 15000 ***g*** for 30 s and the pellets containing nuclei were re-suspended in 25 μl of cold hypertonic buffer [50 mM Hepes-KOH (pH 7.9)/2 mM $MgCl_2$/0.1 mM EDTA/300 mM NaCl/0.1 mM PMSF/1 mM DTT/protease inhibitor cocktail Complete™] and left on ice for 20 min. After centrifugation (15000 ***g*** for 15 min at 4 °C), aliquots of supernatant, containing the nuclear proteins, were stored at −80 °C. Protein concentrations were measured by the Bradford method (Bio-Rad, Hercules, CA, U.S.A.).

Electrophoretic mobility shift assay (EMSA)

EMSA and supershifting analysis were performed as described by Ryseck et al. [19]. In brief, 5 μg of nuclear proteins were incubated for 30 min at room temperature in a final volume of 10 μl with 0.2 nM of ^{32}P-labelled oligonucleotide probe in binding buffer [20 mM Hepes/KOH (pH 7.9)/75 mM NaCl/1 mM EDTA/5% (v/v) glycerol/0.5 mM $MgCl_2$/1 mM DTT] containing 2 μg of BSA and 1.25 μg of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech, Roosendael, The Netherlands) for the NF-κB site of the MIP-1α promoter and of the monocyte chemotactic protein 1 (MCP-1) promoter, and 0.5 μg of BSA and 0.25 μg of poly(dI-dC)·poly (dI-dC) for the NF-κB site of the IL-8 promoter. For competition experiments, excess unlabelled probe was added to the binding buffer. For supershift assays, specific polyclonal antibodies against p50, p65, c-Rel, RelB and p52 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were used in the binding buffer for 20 min before addition of the probe. DNA–protein complexes were then resolved on a non-denaturing 6% (w/v) polyacrylamide gel run for 2 h at 300 V in TBE [10 mM Tris/10 mM H_3PO_4/7 mM EDTA, (pH 8.5)] at 4 °C. The gels were then dried and autoradiographed on a Fuji X-ray film (Fujifilm, Sint Niklaas, Belgium). They were also analysed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The probes (Eurogentec, Liège, Belgium) contained the sequence of the NF-κB site of the MCP-1 promoter [20] (5′-GGTTCATGGAA-GATCCCTCCT-3′ and 5′-TTGGAGGAGGGATCTTCCAT-G-3′), the MIP-1α promoter [21] (5′-GGTTCTTAAAAATT-TCCCTCCT-3′ and 5′-TTGGAGGAGGGAAATTTTTAAG-3′) and the IL-8 promoter [22,23] (5′-GGTTATCGTGGAA-TTTCCTCTG-3′ and 5′-TTGGCAGAGGAAATTCCACG-AT-3′). The oligonucleotide probes were labelled by filling in with the Klenow DNA polymerase (Roche). The probes (100 ng) were labelled with 3 μCi of [α-^{32}P]dATP and [α-^{32}P]dCTP (3000 Ci/mmol; Du Pont de Nemours International, Zaventem, Belgium) and unlabelled dTTP and dGTP (Roche), then purified on a Sephadex G-25 (Amersham Pharmacia Biotech) column and stored at −20 °C until use. Specific radioactivity was always more than 10^8 c.p.m./μg.

IL-8 detection

The release of IL-8 in cell supernatants taken from U937 or CEM cells treated with H_2O_2, LPS or PMA was quantitatively assayed by a double-antibody ELISA kit with recombinant IL-8 as standard (ELISA, CYTOScreen; Biosource Europe, Nivelles, Belgium) in accordance with the manufacturer's protocol. This assay has a sensitivity of detection of 5 pg/ml. Results are expressed as means for 10 separate experiments.

Ribonuclease protection assays (RPAs)

RPAs were performed with an RNase protection assay kit purchased from PharMingen (San Diego, CA, U.S.A.). In brief, total RNA was isolated from 7×10^6 stimulated U937 or CEM

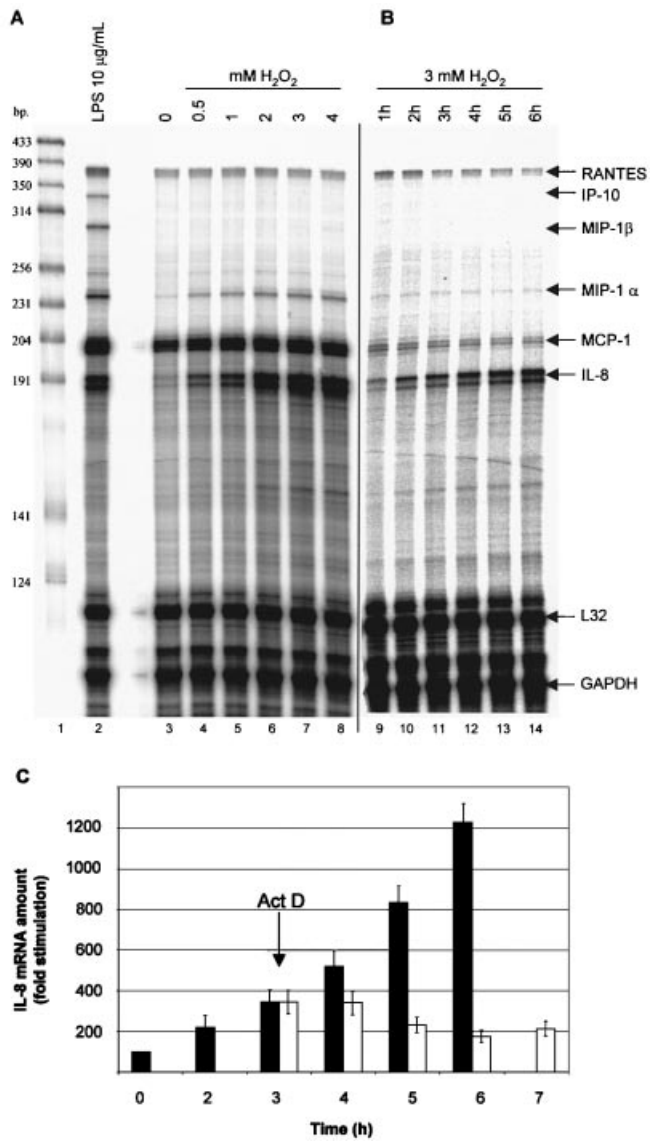

Figure 1 Treatment with H_2O_2 leads to the expression of chemokine mRNA in U937 cells

Total RNA (10 μg) from U937 cells treated with H_2O_2 was analysed by RPA with radiolabelled multiprobes containing RNA species complementary to those for the chemokines Ltn, RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8 and I-309 and also for the two housekeeping genes L32 and GAPDH. (**A**) U937 cells treated with increasing H_2O_2 concentrations and total RNA species extracted after 6 h. (**B**) U937 cells treated with 3 mM H_2O_2 and total RNA species extracted at various times after the treatment. The non-digested probes were used as molecular mass markers. Quantification was performed on a PhosphorImager. (**C**) Relative amounts of IL-8 mRNA quantified by RPA in U937 cells treated with 3 mM H_2O_2 for 3 h and then incubated in the presence of 5 μg/ml ActD (open columns) for the indicated periods; filled columns show stimulation in the absence of ActD. The band intensities were measured on a PhosphorImager and are reported relative to the internal standards L32 and GAPDH. The half-life was determined by plotting the relative amount of IL-8 mRNA to GAPDH-L32, as a percentage of the maximum value at the time of ActD addition, against time.

cells with Tripur (Roche). The multiprobe hCK5, containing templates for the chemokines lymphotactin (Ltn), RANTES, interferon-γ-inducible protein (IP-10), MIP-1β, MIP-1α, MCP-1, IL-8, I-309 and the housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was labelled with [α-^{32}P]UTP (3000 Ci/mmol; Du Pont de Nemours International) with T7 RNA polymerase. Labelled probe (7×10^5 c.p.m.) was hybridized to 10 μg of total RNA for 16 h at 56 °C. mRNA probe hybrids were treated with RNase and proteinase K and then extracted with phenol/chloroform (1:1, v/v). Protected hybrids were resolved on a denaturing 5 % (w/v) polyacrylamide sequencing gel. The gel was then dried and autoradiographed on a Fuji X-ray film (Fujifilm). Quantifications were made on a PhosphorImager (Molecular Dynamics).

IL-8 mRNA detection by reverse-transcriptase-mediated PCR (RT–PCR)

For RT–PCR, total RNA was isolated from 5×10^6 CEM cells with the Tripure reagent (Roche). Total RNA was treated with DNAse I (RNAse free enzyme; Roche) and 1 μg was used for reverse transcription in a 15 μl reaction mixture containing 2.5 mM random primers (Amersham Pharmacia Biotech), 1.6 mM dNTPs, 0.35 μl RNA guard (Amersham Pharmacia Biotech), 16 mM DTT (Life Technologies, Carlsbad, CA, U.S.A.), 250 units of Moloney-murine-leukaemia virus reverse transcriptase (Life Technologies) and first-strand buffer (Life Technologies). PCR was performed with the primers 5′-ATG-ACTTCCAAGCTGGCCGTGGCT-3′ and 5′-TCTCAGGC-CCTCTTCAAAAACTTCTC-3′. In brief, 2.5 μl of cDNA was amplified in a 50 μl mix containing PCR buffer, 1.75 mM $MgCl_2$, 0.25 μl of AmpliTaq (PerkinElmer, Norwalk, NJ, U.S.A.), 0.2 mM dNTP (Amersham Pharmacia Biotech) and 0.5 μM of each primer. Amplified cDNA were separated on a Nusieve 4 % (w/v) agarose gel, stained with ethidium bromide and photographed under UV radiation.

Plasmids

Plasmid −1481-IL8-luc contained a human IL-8 promoter fragment of 1481 bp, plasmid −133-IL8-luc a human IL-8 promoter fragment of 133 bp and plasmid −133-IL8-NF-κB-mut-luc a human IL-8 promoter fragment of 133 bp with the NF-κB site GGAATTTCCT (−80 to −71 bp) mutated to TAACTTTCCT. Plasmid −133-IL-8-AP-1-mut-luc contained a human IL-8 promoter fragment of 133 bp with the AP-1 site TGACTCA (−126 to −120 bp) mutated to TATCTCA. Plasmid −133-IL-8-NF-IL6-mut-luc [containing a human IL-8 promoter fragment of 133 bp with the NF-IL6 site CAGTTGCAAATCGT (−94 to −81 bp) mutated to AGCTTGCAAATCGT] and plasmid AP-1-LUC (containing the AP-1 site of the promoter of the collagenase) were kindly provided by Dr W. Vanden Berghe and Dr G. Haegeman (Ghent University, Ghent, Belgium).

Cell transfection and luciferase assay

Transient transfection was performed in 22.5×10^6 exponentially growing U937 or CEM cells with a mixture of 400 μg of DEAE-dextran and 10 μg of hIL-8–LUC reporter (in which LUC represents luciferase) in 1 ml of sterile STBS medium [25 mM Tris/HCl (pH 7.4)/137 mM NaCl/5 mM KCl/0.6 mM Na_2HPO_4/0.7 mM $CaCl_2$/0.5 mM $MgCl_2$]. After 30 min at 37 °C the cells were treated for 3 min with 10 % (v/v) DMSO, then washed twice with STBS and once with RPMI 1640. Cells were then resuspended in 28 ml of fresh culture medium and returned to the incubator. At 24 h after transfection, the 28 ml of transfected cells was divided into three aliquots of 9.3 ml, then treated or not with H_2O_2, 10 μg/ml LPS or PMA-ionomycin. After 8 h of treatment, cells were harvested, cell extracts were prepared and luciferase activity was measured with the chemiluminescent assay kit Luciferase Reporter Gene Assay (Roche)

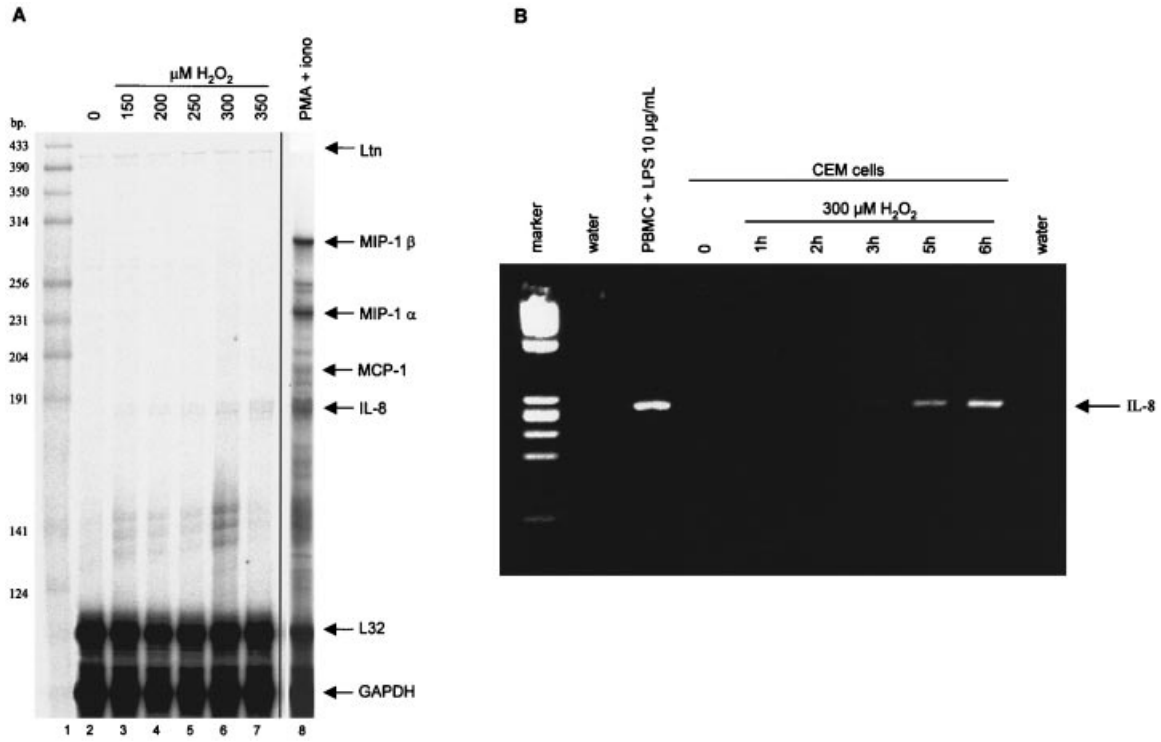

Figure 2 H_2O_2 leads to very low IL-8 mRNA expression in CEM cells

(**A**) Total RNA (10 μg) from CEM cells treated with H_2O_2 was analysed by RPA with radiolabelled multiprobes containing complementary RNAs to those for the chemokines Ltn, RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8 and I-309 and also for the two housekeeping genes L32 and GAPDH. CEM cells were treated with increasing H_2O_2 concentrations and total RNA was extracted after 6 h. A positive control was performed for 6 h with 0.2 μM PMA and 2 μM ionomycin (iono). The non-digested probes were used as molecular mass markers. Quantification was performed on a PhosphorImager. (**B**) RT–PCR detection of IL-8 mRNA in CEM cells treated with H_2O_2. Non-quantitative RT–PCR analysis of IL-8 mRNA produced in CEM cells treated with 300 μM H_2O_2. Total RNA was extracted from 0 to 6 h after treatement and subjected to RT–PCR with primers specific for IL-8 mRNA. A positive control was performed with total RNA extracted from PBMC and treated for 6 h with 10 μg/ml LPS. RT–PCR performed with the same primers but without RNA was used as a negative control (water). The marker was φX174 DNA digested with *Hae*III.

in accordance with the manufacturer's instructions. The luciferase activity of the samples was normalized with the protein concentration measured by the Bradford method (Bio-Rad). Results are expressed as means for four independent experiments.

Western blotting

Washed U937 cells (10^6) were lysed in 100 μl of sample buffer [62.5 mM Tris/HCl (pH 6.8)/2% (w/v) SDS/10% (v/v) glycerol/50 mM DTT/0.1% Bromophenol Blue] for SDS/PAGE and immediately boiled for 4 min. DNA was sheared by sonication. A 15 μl aliquot was loaded in each lane. The phospho-p38 protein was detected by Western blot analysis with specific polyclonal antibody against phospho-p38 mitogen-activated protein kinase (MAPK) (Thr-180/Tyr-182) (New England Biolabs, Beverly, MA, U.S.A.). The non-activated p38 protein was detected by Western blot analysis with specific polyclonal antibody against p38 MAPK (New England Biolabs). Activated extracellular signal-regulated protein kinase (ERK) was detected with E10 monoclonal antibody against phospho-ERK-1/-2 MAPK (Thr-202/Tyr-204) (New England Biolabs). Samples were subjected to SDS/PAGE [12% (w/v) gel]. After transfer to a PVDF membrane (Roche) and blocking with PBS-Tween [3.78 mM NaH_2PO_4/16 mM K_2HPO_4/0.15 mM NaCl/0.1% (v/v) Tween 20] plus 5% (w/v) dried milk, the membrane was incubated overnight at 4 °C under agitation with the first antibody (1:1000 dilution), washed and then incubated with the second peroxidase-conjugated antibody. The reaction was revealed by the enhanced chemoluminescence detection method (ECL® kit; Amersham, Little Chalfont, Bucks., U.K.).

Immunoprecipitation and assay of p38 MAPK

After treatment with H_2O_2, the cells were lysed for 10 min on ice in lysis buffer [20 mM Hepes (pH 7.4)/50 mM sodium β-glycerophosphate/2 mM EGTA/1% (v/v) Triton X-100/10% (v/v) glycerol/150 mM NaCl/10 mM NaF/1 mM Na_3VO_4/2 mM DTT/1 mM PMSF/3 μg/ml aprotinin/10 μM E-64D/2 μg/ml pepstatin/5 μg/ml leupeptin]. Samples were clarified by centrifugation at 4 °C for 10 min at 16000 ***g***. p38 MAPK was immunoprecipitated by the addition of 5 μl of polyclonal antibody against p38 MAPK (New England Biolabs). Samples were incubated at 4 °C for 1 h with mixing before the addition of 30 μl of a 50% slurry of Protein A-coated beads in lysis buffer and incubation for an additional 2 h. The beads were washed four times in 1 ml of wash buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM EDTA/25 mM sodium β-glycerophosphate/10 mM sodium tetrapyrophosphate/1 mM Na_3VO_4/2 mM DTT/1% (v/v) Triton X-100/0.5% sodium deoxycholate/0.1% SDS] and twice in 1 ml of kinase assay buffer [20 mM Hepes (pH 7.5)/20 mM sodium β-glycerophosphate/200 mM NaCl/2 mM DTT/10 mM $MgCl_2$/10 mM NaF/0.1 mM Na_3VO_4/0.5 mM EDTA/0.5 mM EGTA/0.05% Brij 35], containing a cocktail of anti-proteases. p38 MAPK was assayed with 1 μg of recombinant MAPK-activated protein

kinase 2 (MAPKAPK-2) (a gift from Dr A. Clark, Kennedy Institute of Rheumatology, Imperial College, London, U.K.) as the substrate in kinase assay buffer containing 20 μM ATP and 4 μCi of [γ-^{32}P]ATP in an assay volume of 30 μl. Samples were incubated at 20 °C for 20 min with agitation, and the reaction was stopped by the addition of 4 × SDS/PAGE sample buffer. The samples were separated by SDS/PAGE [10 % (w/v) gel]. Gels were stained with Coomassie Brilliant Blue R-250; ^{32}P incorporation into MAPKAPK-2 was quantified on a PhosphorImager (Molecular Dynamics).

RESULTS

H_2O_2 activates chemokine mRNA expression in U937 cells

To determine which genes encoding chemokines could be activated in response to oxidative stress in a T-lymphocytic cell line (CEM cells) and in a promonocytic cell line (U937 cells), these cells were treated with increasing concentrations of H_2O_2 and total RNA was extracted 6 h after treatment and hybridized with several probes detecting chemokine mRNA species. The inductions with H_2O_2 were performed in conditions leading to NF-κB activation in both cells (results not shown) and to similar cytotoxic effects (results not shown). Figure 1(A) shows that untreated U937 cells expressed basal levels of RANTES and MCP-1 mRNA species. After treatment with increasing H_2O_2 concentrations, only IL-8 and MIP-1α mRNA species were up-regulated, whereas the quantities of Ltn, RANTES, IP-10, MCP-1, MIP-1β and I-309 mRNA species were not significantly modified. A positive control performed with LPS permitted us to visualize an up-regulation of six out of eight chemokine mRNA species analysed (Figure 1A). The extent of activation after H_2O_2 treatment, determined with internal controls (L32 and GAPDH), was more than 10-fold for IL-8 and 2.4-fold for MIP-1α. mRNA synthesis was also followed as a function of time after stimulation with H_2O_2. As shown in Figure 1(B), only the level of IL-8 mRNA increased from 3 to 6 h after treatment, the increase being sustained up to 9 h (results not shown). In contrast, the synthesis of MIP-1α, RANTES and MCP-1 mRNA species did not markedly increase with time (Figure 1B).

The half-life of the IL-8 mRNA was also measured in U937 cells after treatment of cells with H_2O_2 for 3 h before the addition of ActD, which blocks transcription. As shown in Figure 1(C), the addition of ActD significantly decreased the amount of IL-8 mRNA with a half-life of approx. 270 min. The half-life of the IL-8 mRNA in untreated U937 cells was less than 90 min (results not shown). These results showed unambiguously that oxidative stress led to a significant stabilization of the IL-8 mRNA in U937 cells.

When CEM cells were treated with H_2O_2 under conditions in which NF-κB activation was clearly detectable, almost no chemokine mRNA was detected on the gel (Figure 2A). This absence of activation was not due to a general failure in chemokine gene regulation, because treatment with PMA-ionomycin led to the transcription of four out of eight genes analysed (Figure 2A). To ensure that the lack of chemokine mRNA production in CEM cells was not due to suboptimal oxidative stress conditions, CEM cells were treated with a much larger range of H_2O_2 concentrations. However, owing to an important cytotoxic effect, no chemokine mRNA species could be detected on the gel (results not shown). This lack of chemokine mRNA in CEM cells was not due to a deficiency in NF-κB activation. EMSA showed that stimulation of CEM cells with H_2O_2 at micromolar concentrations (100–300 μM) induced a strong

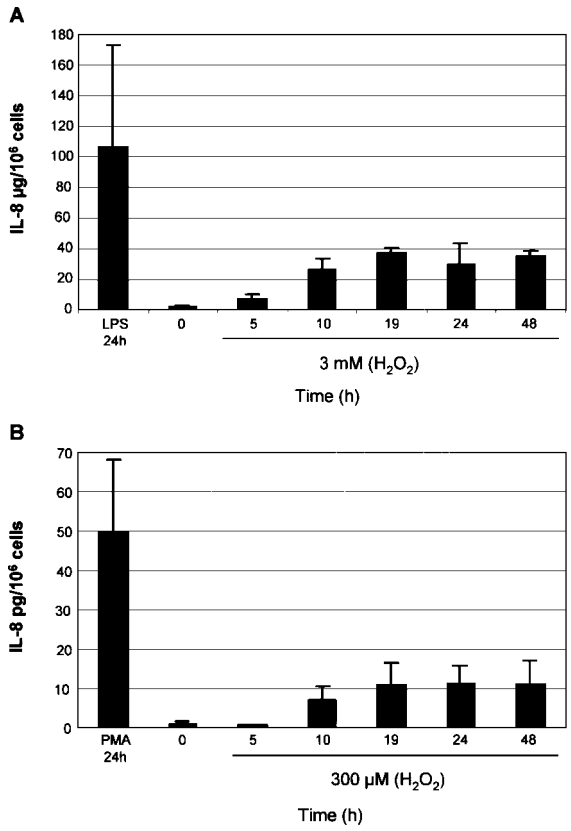

Figure 3 IL-8 is released in cell supernatant after treatment with H_2O_2

IL-8 release into the supernatants of H_2O_2-treated U937 cells (**A**) or CEM cells (**B**). U937 cells were treated with 3 mM H_2O_2 and CEM cells with 300 μM H_2O_2. The positive control for IL-8 production in U937 cells was with LPS (10 μg/ml) for 24 h and in CEM cells with 0.2 μM PMA for 24 h. Results are means ± S.D. for 10 independent experiments.

NF-κB DNA-binding activity, reaching its maximum between 120 and 240 min of treatment and sustained for up to 300 min (results not shown). Supershifting experiments performed with antibodies directed against NF-κB proteins demonstrated that the NF-κB complex induced after 120 min was displaced by antibodies against both p50 and p65 and also against c-Rel (results not shown).

Experiments were also performed with THP-1 (a monocytic cell line) and Jurkat JR (a lymphocytic cell line). Treatment of these cells with H_2O_2 gave rise to the same behaviour in terms of chemokine mRNA production as observed with U937 and CEM cells respectively (results not shown). From these results we conclude that H_2O_2, although activating NF-κB in both monocytes and lymphocytes, only led to a RPA-detectable accumulation of a subset of chemokine mRNA species in monocytic cells but not in lymphocytic cells.

Because the quantities of chemokine mRNA species produced in CEM cells treated by H_2O_2 could well have been less than the detection limit of the RPA technique, we followed the production of IL-8 mRNA by RT–PCR in CEM cells treated with 300 μM H_2O_2. RT–PCR, a non-quantitative technique, permitted the detection of IL-8 mRNA in CEM cells at 5 and 6 h after the treatment with H_2O_2 (Figure 2B), demonstrating that the IL-8 mRNA was produced in CEM cells but at a concentration too low for detection by RPA.

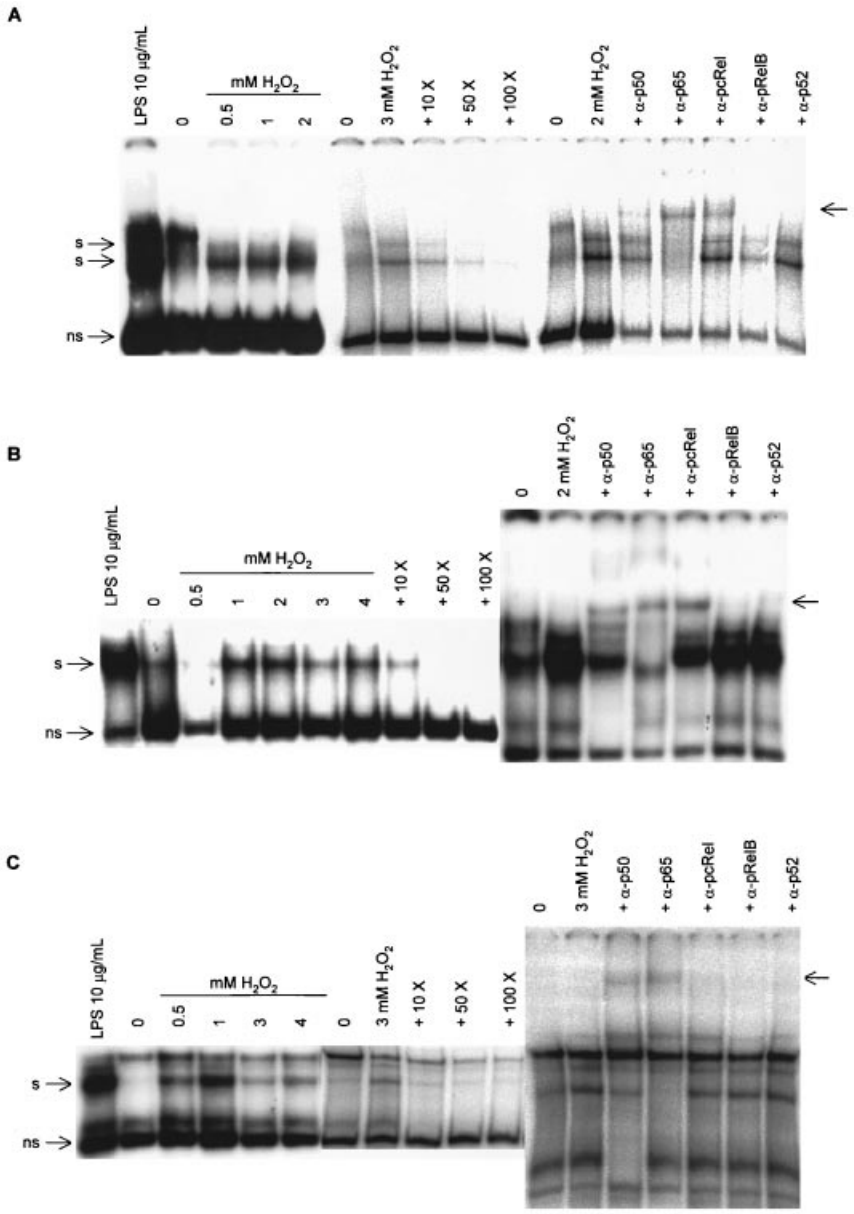

Figure 4 NF-κB binding on the IL-8, MCP-1 and MIP-1α promoters

EMSA analysis of NF-κB DNA-binding activities in U937 cells after stimulation with increasing concentrations of H_2O_2. The NF-κB binding sites were from the IL-8 (**A**), MCP-1 (**B**) and MIP-1α (**C**) promoters. The non-specific (ns) and specific (s) bindings are indicated by arrows. Competition experiments were performed with 10-fold, 50-fold or 100-fold excesses of unlabelled probe. Supershift experiments were performed with antibodies against p50, p65, c-Rel, Rel B and p52. Supershifted complexes are indicated by arrows at the right.

IL-8 is released into the cell supernatant after treatment with H_2O_2

Because IL-8 mRNA was up-regulated by H_2O_2 in U937 cells, we measured the level of IL-8 released in cell supernatants at various times after activation. Treatment of U937 cells with 3 mM H_2O_2 led to a substantial release of IL-8 (Figure 3A). The level of IL-8 released 19 h after treatment (approx. 40 μg of IL-8/10^6 cells) was one-third of that achieved by LPS treatment. IL-8 synthesis remained similar up to 48 h after treatment. In contrast, when IL-8 was measured in the supernatants from CEM cells, the level of IL-8 was very low and was close to the ELISA kit detection limit. IL-8 levels were approx. 10 pg/10^6 cells when measured from 19 to 48 h after H_2O_2 (300 μM) treatment and approx. 50 pg/10^6 cells after 24 h in the presence of PMA (Figure 3B). Although very low, the amount of IL-8 released by H_2O_2-treated CEM cells was just above the detection limit and was in agreement with the small amount of mRNA measured in these cells under identical conditions.

NF-κB binding on the IL-8, MCP-1 and MIP-1α promoters

Because several chemokine-encoding genes contain NF-κB elements in their promoter, we analysed nuclear extracts from H_2O_2-treated U937 or CEM cells for their capacity to bind to these various NF-κB sites. EMSA analysis of nuclear extracts

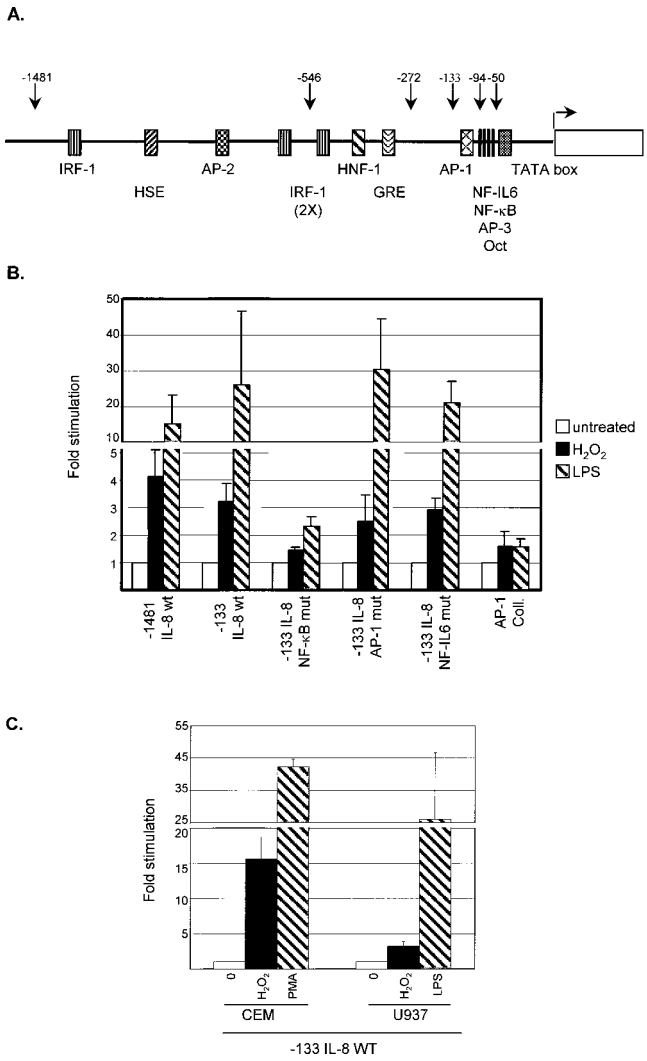

Figure 5 IL-8 promoter transactivation induced by H_2O_2 in U937 cells requires NF-κB

(**A**) DNA-responsive elements borne by the IL-8 promoter. (**B**) Transient transfection assays of either H_2O_2-treated or LPS-treated U937 cells with various constructs derived from the human IL-8 promoter. Full-length (−1481 bp) and shorter (−133 bp) versions were used for transfection. An IL-8 promoter mutated at various sites was also used: at the NF-κB site (from −80 to −71 bp; GGAATTTCCT), the AP-1 site (from −126 to 120 bp; TGACTCA) and the NF-IL6 site (from −94 to −81 bp; CAGTTGCAAATCGT), which were mutated into TAACTTTCCT, TATCTCA and AGCTTGCAAATCGT respectively. The transfected cells were treated with 3 mM H_2O_2 or with LPS (10 μg/ml) for 8 h, or left untreated. Luciferase activities were measured and are shown relative to protein concentrations. Results are means ± S.E.M. for four separate experiments. (**C**) Transient transfection assays of H_2O_2-treated U937 or CEM cells with the −133 bp version of the IL-8 promoter controlling the luciferase gene. The experimental conditions were the same as in (**B**).

from U937 treated with increasing concentrations of H_2O_2 revealed binding to the IL-8 NF-κB probe with a maximal induction at 2 mM H_2O_2 (Figure 4A), the band intensity being decreased at higher H_2O_2 concentrations (results not shown). Two retarded bands were resolved on the gel, corresponding to p50/p65 and p65/c-Rel dimers respectively. The two bands were specific because they were competed for by an excess of unlabelled probe (Figure 4A). Similar results were obtained with CEM cells stimulated by a micromolar range of H_2O_2 concentrations. Maximal binding of NF-κB on the IL-8 probe was observed at 300 μM H_2O_2. The proteins involved in the complex were p50/p65 (results not shown). Binding to the NF-κB site of the MCP-1 promoter was also observed in U937 cells (Figure 4B). This binding was maximal at 1 mM H_2O_2 and decreased slightly at higher concentrations. Competition experiments demonstrated that this band was specific and contained mainly p65 and p50. Part of this complex also contained c-Rel. Experiments on CEM cells showed similar results, with a maximal binding of NF-κB at 300 μM H_2O_2. The NF-κB complex was made from the same NF-κB members (results not shown). A complex inducible at 0.5 and 1 mM H_2O_2 was observed with the MIP-1α probe (Figure 4C). Under these conditions a constitutive complex was also observed on the gel and was identified as non-specific by competition analysis. The inducible complex was specific and made of p50 and p65 (Figure 4C). CEM cells stimulated with 300 μM H_2O_2 exhibited the same pattern of NF-κB activation (results not shown). These results allow us to conclude that treatment of U937 and CEM cells with H_2O_2 induces various NF-κB complexes that bind to the NF-κB-responsive elements contained in the promoters of the genes encoding IL-8, MCP-1 and MIP-1α.

IL-8 gene transcription induced by H_2O_2 in U937 cells requires NF-κB binding

To determine whether or not transcription factors other than NF-κB may be required for the transcriptional activation of the IL-8 gene by H_2O_2 in U937 cells, transient transfection experiments were performed with various constructs of the IL-8 gene promoter, cloned upstream of the luciferase gene. These constructs were used because the IL-8 promoter contained several elements shown to bind transcription factors that were potentially regulated by oxidative stress (Figure 5A). When a fragment bearing the whole IL-8 promoter was used for transient transfection experiments, a 4-fold stimulation of the luciferase activity was observed after treatment with 3 mM H_2O_2 (Figure 5B). A positive control performed with LPS yielded a 15-fold increase in luciferase activity (Figure 5B). A similar stimulation of the luciferase activity was obtained after the treatment of U937 cells with H_2O_2 or LPS using a shorter version of the IL-8 promoter (−133 bp), demonstrating that all the elements responsive to oxidative stress were situated in the proximal promoter (Figure 5B). Mutation of the NF-κB responsive elements borne by this construct (−133 IL-8 NF-κB mut) strongly decreased the inducibility by both H_2O_2 and LPS, indicating that the NF-κB sites were required for the proper induction of gene transcription (Figure 5B). In the case of induction with H_2O_2, mutation of the AP-1 site decreased the luciferase activity to a smaller extent than mutation of the NF-κB site (a 22% decrease compared with a 55% decrease respectively), demonstrating that the AP-1 transcription factor was involved in the activation of the IL-8 promoter. Mutation of the NF-IL6 site did not significantly decrease the luciferase activity induced by H_2O_2 or LPS, showing that binding of the transcription factor to this site was not important for the transcription induced by these two stimuli (Figure 5B). In addition, we showed that the luciferase gene driven by the AP-1 sites of the collagenase gene promoter was not activated in U937 cells treated with H_2O_2 or with LPS (Figure 5B).

With the use of a transient transfection assay with a luciferase reporter construct driven by the IL-8 promoter, we also demonstrated in CEM cells an increase in luciferase activity after treatment with H_2O_2 (Figure 5C), showing that the NF-κB complexes translocated after activation with H_2O_2 were transcriptionally active.

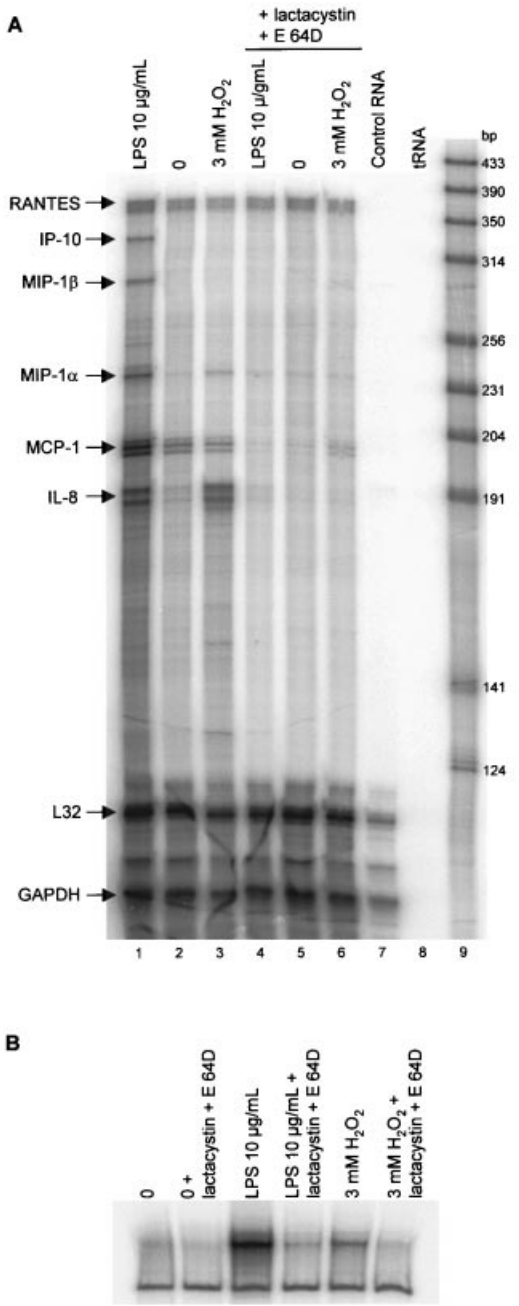

Figure 6 IL-8 mRNA accumulation induced by H_2O_2 in U937 cells requires the activation and binding of NF-κB

(**A**) Total RNA (10 μg) from U937 cells treated with 10 μg/ml LPS or 3 mM H_2O_2 in the presence or the absence of lactacystin (50 μM) and E-64D (25 μg/ml) was analysed by RPA with a radiolabelled multiprobe containing RNA species complementary to the mRNA species for Ltn, RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8 and I-309 and for two housekeeping genes (L32 and GAPDH). The non-digested probes were used as molecular mass markers. Quantification was performed on a PhosphorImager. (**B**) NF-κB activation in U937 cells treated with either LPS (10 μg/ml) or H_2O_2 (3 mM) in the presence or the absence of lactacystin (50 μM) and E-64D (25 μg/ml). Nuclear extracts from these cells were analysed by EMSA with a labelled probe containing the NF-κB site of the HIV-1 long terminal repeat.

In RPA experiments we confirmed the results described above and showed that NF-κB induction by H_2O_2 was necessary for the proper transcriptional activation of the genes encoding IL-8 and MIP-1α. This was demonstrated by the use of two specific

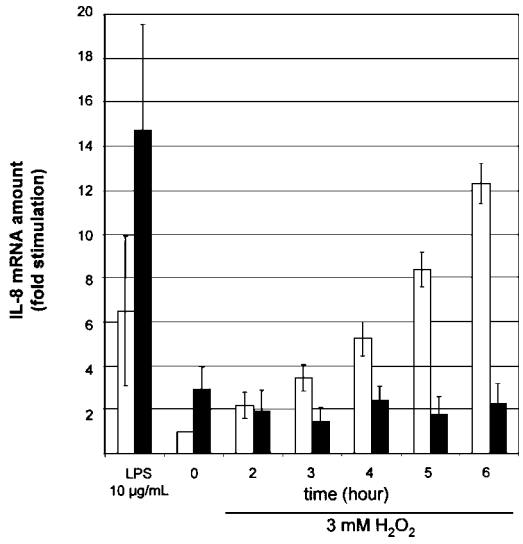

Figure 7 Efficient accumulation of IL-8 mRNA after activation by H_2O_2 requires protein synthesis *de novo*

Relative amount of IL-8 mRNA detected by RPA in U937 cells treated with LPS (10 μg/ml) or H_2O_2 (3 mM) for increasing periods in the presence (filled columns) or absence (open columns) of 50 μg/ml cycloheximide. Quantification was performed on a PhosphorImager. Results are means $\pm$ S.D. for three independent experiments.

inhibitors of NF-κB activation by H_2O_2: lactacystin, which inhibits the 26 S proteasome, and E-64D, which inhibiting the calpain proteases [22]. As shown in Figure 6(A), addition of these two inhibitors to U937 cells before treatment with H_2O_2 completely abolished the detection of the IL-8 and MIP-1α mRNA species. A similar inhibition was recorded with LPS-treated U937 cells. The NF-κB-containing complexes observed with EMSA were strongly decreased when the two inhibitors were added to U937 cells before treatment with LPS or with H_2O_2 (Figure 6B).

Efficient accumulation of IL-8 mRNA after activation by H_2O_2 requires *de novo* protein synthesis

Cytokine and chemokine mRNA species are also controlled at the post-transcriptional level; for some of them, stabilization requires protein synthesis [24–26]. To evaluate the importance of this regulation in U937 cells treated with H_2O_2, we followed the accumulation of these mRNA species in the presence of cycloheximide, which inhibits *de novo* protein synthesis.

Treatment of U937 cells with 3 mM H_2O_2 led to an accumulation of the IL-8 mRNA up to 10-fold after 6 h (Figure 7). MCP-1 and MIP-1α mRNA species did not accumulate over time (results not shown). When U937 cells were treated with cycloheximide and then incubated in the presence of H_2O_2, the accumulation of the IL-8 mRNA was significantly decreased (Figure 7). This demonstrated that H_2O_2 induced two important factors for proper IL-8 mRNA accumulation: NF-κB, required for transcription of the gene encoding IL-8, and unknown factor(s) that were probably implicated in the stabilization of IL-8 mRNA.

Efficient accumulation of IL-8 mRNA after activation by H_2O_2 is dependent on p38 activation

Because several previous studies have emphasized the role of the MAPK cascade, especially p38, in the post-transcriptional

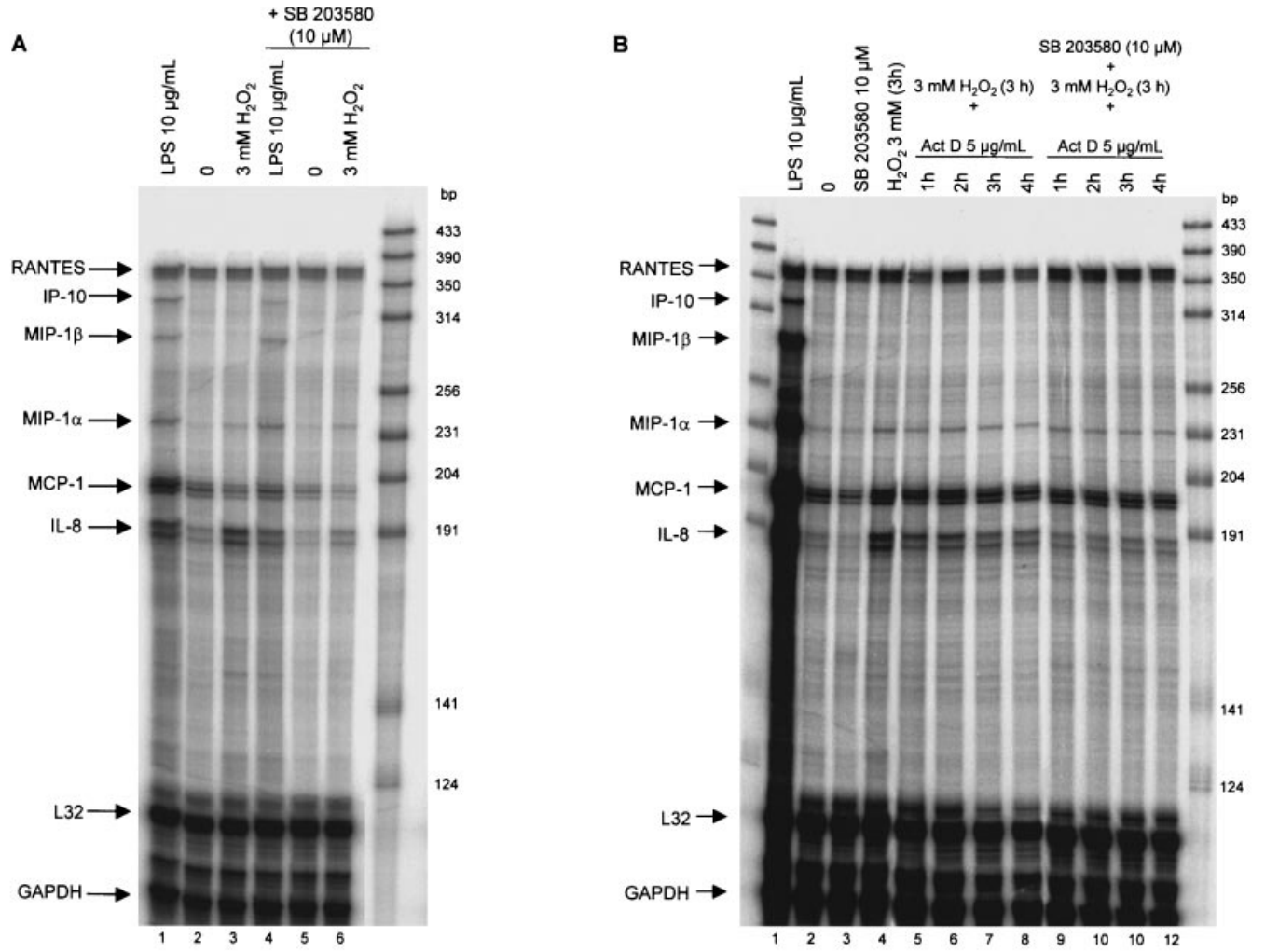

Figure 8 Efficient accumulation of IL-8 mRNA after activation of U937 cells by H_2O_2 is dependent on p38 activation

(**A**) Total RNA (10 μg) from U937 cells pretreated or not with 10 μM SB203580 for 1 h and treated with 3 mM H_2O_2 or 10 μg/ml LPS for 6 h was analysed by RPA with radiolabelled multiprobes containing RNA species complementary to those for the chemokines Ltn, RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8 and I-309 and for the two housekeeping genes L32 and GAPDH. (**B**) Total RNA (10 μg) from U937 cells pretreated or not with 10 μM SB203580 for 1 h and induced with 3 mM H_2O_2 for 3 h before being treated with ActD (5 μg/ml) for the indicated periods. RNA was analysed by RPA with radiolabelled multiprobes as described for (**A**).

regulation of several mRNA species [27–29], we studied the effects of the p38 specific inhibitor SB203580 on the H_2O_2-induced accumulation of IL-8 mRNA. Figure 8(A) shows RPA performed with total RNA from U937 cells pretreated for 1 h with the p38 inhibitor SB203580 at 10 μM and incubated for 6 h with 3 mM H_2O_2. This experiment showed a 4.25-fold decrease in the accumulation of the IL-8 mRNA after treatment with H_2O_2. In contrast, the LPS-mediated accumulation of the IL-8 mRNA was decreased less in the presence of SB203580 (Figure 8A). These results demonstrate that the p38 MAPK was implicated in the post-transcriptional stabilization of the IL-8 mRNA. To confirm that SB203580 exerted its effect solely at a post-transcriptional level, the amount of IL-8 mRNA was determined in the presence or absence of this inhibitor with or without ActD to measure the effect of the inhibitor on post-transcriptional regulation only. As shown in Figure 8(B), the addition of ActD to H_2O_2-treated U937 cells decreased the amount of IL-8 mRNA with a half-life of 270 min. Pretreatment of U937 cells with SB203580 led to a further decrease in IL-8 mRNA, demonstrating that the p38 inhibitor exerted its effect only at the post-transcriptional level (Figure 8B).

To confirm the involvement of p38, we performed a Western blot analysis with anti-(phospho-p38) antibody to determine whether p38 activation could be achieved by oxidative stress in these cells. Figure 9(A) shows that H_2O_2 clearly led to p38 phosphorylation in U937 cells. Maximal activation of p38 in U937 cells was observed 10 min after stimulation with H_2O_2 (approx. 2.4-fold compared with untreated cells) and it was sustained for up to 30 min (Figure 9A). SB203580 (10 μM) significantly inhibited p38 activation when U937 cells were treated under identical conditions (Figure 9A). Unexpectedly, LPS did not activate p38 under these conditions but repressed its activation compared with the basal activity in untreated U937 cells (Figure 9A). The addition of SB203580 further decreased the amount of phospho-p38 in cells treated by LPS or left

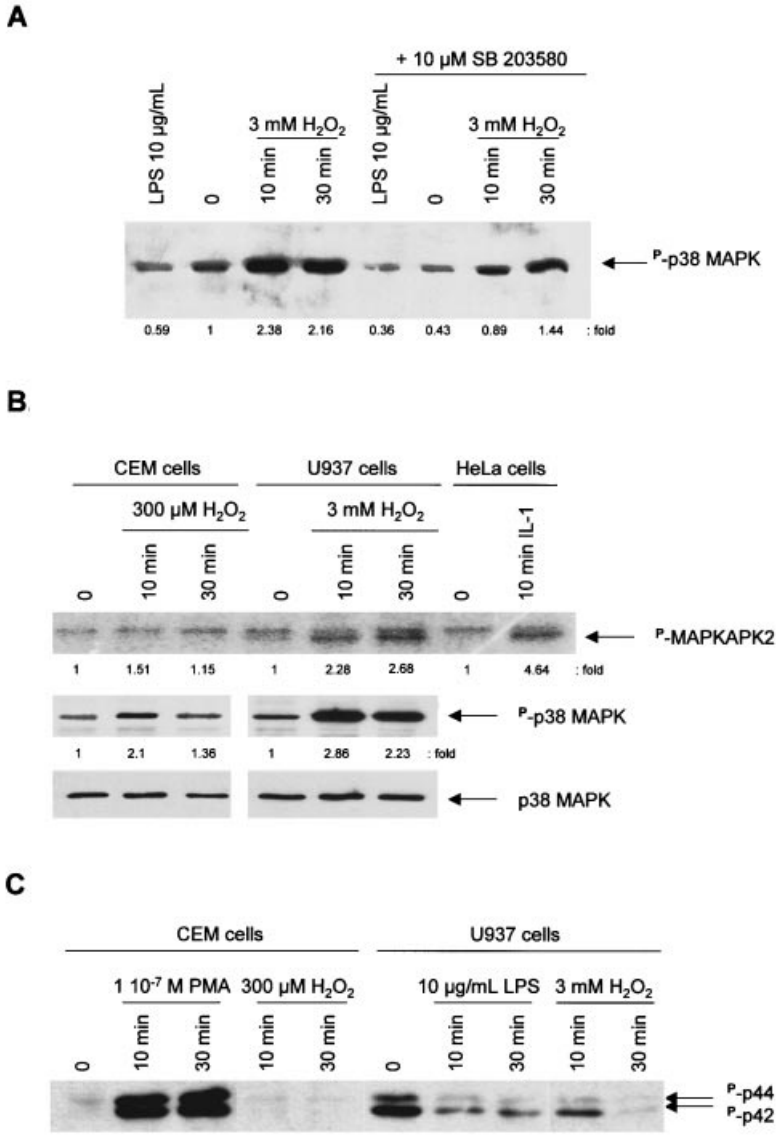

Figure 9 Activation of MAPK by H_2O_2 in U937 cells and in CEM cells

(**A**) Total extracts (15 μl) from U937 cells pretreated or not with 10 μM SB203580 for 1 h and treated with 3 mM H_2O_2 for 10 or 30 min or 10 μg/ml LPS for 10 min were analysed by Western blotting with an antibody specific for the activated phosphorylated form of p38. (**B**) p38 activation in CEM cells treated with 300 μM H_2O_2 and U937 cells treated with 3 mM H_2O_2 for similar periods. p38 activation was measured either by Western blot analysis with an anti-(phospho-p38) antibody or by a kinase assay with MAPKAPK-2 as substrate. (**C**) ERK-1/-2 activation in either U937 or CEM cells by H_2O_2. Positive controls were performed with PMA in CEM cells and with LPS in U937 cells.

untreated. Because the p38 inhibitor also decreased the amount of IL-8 mRNA in both untreated and LPS-induced U937 cells (Figure 8A), it could be suggested that the constitutive amount of phospho-p38 present in U937 cells might participate in IL-8 mRNA stabilization. In contrast, when CEM and U937 cells were compared in terms of p38 activation, it turned out that H_2O_2 only very weakly and transiently activated p38 in CEM cells, although the overall amount of p38 was not very different in the two cell lines (Figure 9B). To confirm these results, p38 kinase assays were also performed with MAPKAPK-2 as substrate. As shown in Figure 9(B), a rather weak and transient MAPKAPK-2 phosphorylation was observed in CEM cells in comparison with U937 cells, confirming the observations made by Western blotting. These results suggest that the barely detectable increase of IL-8 mRNA stabilization observed in CEM cells was related to the low and transient increase in activated p38.

It was recently shown that the ERK pathway might also have a role in post-transcriptional stabilization of several transcripts [30]. To assess the importance of this pathway in IL-8 mRNA stabilization by oxidative stress, U937 and CEM cells were activated by H_2O_2 under the same conditions as those described above and were analysed by Western blotting with an anti-(phospho ERK-1/-2) (p42 and p44). As shown in Figure 9(C), H_2O_2 did not activate ERK-1/-2 in CEM cells, whereas PMA yielded significant ERK activation. In U937 cells, the constitutive level of activated ERK was substantially greater than in CEM cells but this level was decreased by treatment with H_2O_2, demonstrating that the stabilization of IL-8 mRNA depended only on the p38 pathway, not on the ERK pathway.

DISCUSSION

The results presented here show for the first time that cells of the monocyte/macrophage lineage (U937) subjected to an oxidative stress accumulate several chemokine mRNA species with a strong preference for IL-8 mRNA. The main pathways leading to IL-8 mRNA production involve (1) the nuclear translocation of transcription factor NF-κB and binding to its responsive elements within the IL-8 gene promoter and (2) post-transcriptional stabilization of the IL-8 mRNA involving both protein synthesis *de novo* and p38 activation. Although lymphocytes (CEM) are also responsive to oxidative stress in terms of NF-κB activation, the level of IL-8 mRNA accumulation is much lower than in U937, probably owing to the absence of a post-transcriptional stabilization of IL-8 mRNA in these cells.

Chemokines are a group of small (8–14 kDa), mostly basic, structurally related proteins that regulate the trafficking of various types of leucocyte through interactions with a subset of seven-transmembrane G-protein-coupled receptors [18,31,32]. Approximately 40 chemokines have now been identified in humans. They act mainly on neutrophils, monocytes, lymphocytes and eosinophils and have a pivotal role in host defence mechanisms. They have been divided into two major subfamilies on the basis of the arrangement of the two N-terminal cysteine residues, CXC and CC, depending on whether the first two cysteine residues have a residue between them (CXC) or are adjacent (CC).

The genes for these families are currently designed *SCY* (small secreted cytokine), with *SCYa* corresponding to the CC subfamily and *SCYb* to the CXC subfamily. Two other classes of chemokine have been described: Ltn (C, *SCYc*) and fractalkine (CX3C, *SCYd*). Many of the genes encoding chemokines have been mapped and they cluster at specific loci: CC chemokine genes are grouped at 17q11.2–12 and CXC chemokine genes at 4q13. Because chemokines are produced by different cell types in both immunological and inflammatory reactions [33], it was to be expected that the genes that encode chemokines are regulated by transcription factors activated under these conditions. NF-κB is a central element of the transcription factors important for the immune system and for inflammation. Indeed, many genes encoding chemokines bear NF-κB-responsive elements in their promoter and their deletion usually abolishes transcription [34,35]. When NF-κB is activated, for instance by pro-inflammatory cytokines or LPS, chemokine gene transcription can be detected in many cell types [36]. In the present study, we demonstrate that six out of eight chemokine genes analysed are activated in U937 cells on treatment with LPS, whereas four out of eight tested are up-regulated in CEM on treatment with PMA-ionomycin. Because both LPS and PMA-ionomycin are strong NF-κB inducers, it is very likely that seven out of the eight chemokine genes analysed are regulated by NF-κB in lymphocytes and monocytes. Among them, transcription of the genes encoding IL-8 and MIP-1α is activated after treatment with H_2O_2. In contrast, the other chemokine genes studied (those encoding Ltn, RANTES, IP-10, MCP-1 and MIP-1β) are not activated by H_2O_2. Because NF-κB is the main redox-controlled transcription factor involved in the regulation of chemokine genes, NF-κB is the candidate main regulator of the transcription of IL-8 and MIP-1α genes but is less important for Ltn, RANTES, IP-10, MCP-1 and MIP-1β and is irrelevant for I-309 gene transcription.

Another important observation is the higher responsiveness to H_2O_2 of the gene encoding IL-8 compared with the gene encoding MIP-1α. Whereas the latter gene is induced at low level after treatment with H_2O_2, only the IL-8 mRNA accumulates with time in U937 cells. This accumulation is less when protein synthesis is inhibited, indicating that one or more proteins synthesized *de novo* are involved in post-transcriptional regulation. This post-transcriptional stabilization was not seen with MIP-1α in U937 cells. This contrasts with the report of post-transcriptional stabilization of the MIP-1α mRNA observed after oxidative stress in rat alveolar macrophages [37]. Similarly, this stabilization does not take place for the MCP-1 mRNA, the corresponding band of which seems to decrease in intensity with time after treatment, which might be due to a destabilization of the MCP-1 mRNA by treatment with H_2O_2. Transcriptional activation of the gene encoding IL-8 through NF-κB activation has previously been reported in non-monocytic cells, such as hepatoma cells, fibroblasts or epithelial cells [22,23,38]. In the present study we have also demonstrated the crucial role of NF-κB and the unexpected importance of post-transcriptional regulation of the gene encoding IL-8 by oxidative stress. This regulation turned out to be cell-specific, being observed in U937 cells but not in CEM cells. Indeed, in T lymphocytes, NF-κB is both strongly activated by H_2O_2 and transcriptionally active on the IL-8 promoter, as measured by EMSA and transient transfection assays, but no IL-8 mRNA could be detected by RPA in these cells. Regulation of mRNA stability is often mediated by AUUUA sequences (ARE) within the 3′ untranslated region (UTR) [39,40]. Four AU-rich elements have been identified in the 3′ UTR of the IL-8 mRNA [34]. Several factors that specifically interact with AU-rich elements have been implicated in the positive or negative regulation of mRNA stability [41,42]. Our results might be explained by postulating that H_2O_2 promotes the synthesis of one or more factors that bind either to the AUUUA or the GA-rich sequences of the 3′ UTR of the IL-8 mRNA, the factor(s) being inducible in U937 cells but absent or non-inducible in CEM cells. In addition, these unknown stabilizing factors would require phosphorylation mediated by p38. In this hypothesis, H_2O_2 would exert a direct positive effect on a post-transcriptional regulator. Among the various proteins shown to bind to the AU-rich element, a physiological function was demonstrated only for AUF1, TTP and HuR, which destabilize [43–45] and stabilize [46–49] AU-rich mRNA species respectively. HuR might therefore be a candidate for H_2O_2-mediated IL-8 mRNA stabilization. However, HuR was recently found to exert its stabilizing effect independently of the p38 MAPK pathway when overexpressed in a macrophage cell line [50]. Alternatively, H_2O_2 might lead to the removal of a destabilizing protein such as AUF1, present in a cytosolic fraction, which was shown to accelerate the decay of c-Myc mRNA *in vitro* [51,52]. In that case, H_2O_2 would inhibit a negative regulator. It should be pointed out that either of these pathways requires protein synthesis *de novo* because the addition of cycloheximide abolishes the stabilization of IL-8 mRNA.

Future tasks will be (1) to characterize the redox-controlled proteins mediating the stabilization of IL-8 mRNA and to determine whether H_2O_2 functions as a positive or negative regulator, and (2) to characterize the transduction pathways controlling the post-transcriptional regulation, with special attention to the potential target of p38. Recently, several reports emphasized the role of the MAPK cascade, especially p38, in the regulation of the stability of several mRNA species such as those encoding cyclo-oxygenase 2 [27] and vascular endothelial growth factor [28]. For IL-8 mRNA, MAPK kinase 7 (MKK7), an activator of the p38/JNK pathways, induced IL-8 synthesis and transcription from a minimal IL-8 promoter [29]. Furthermore, MKK7 synergized in both effects with the NF-κB-inducing kinase [29], underscoring the importance of NF-κB not only in the transcriptional regulation but also in the post-transcriptional

regulation of the gene encoding IL-8. Because the p38 signalling cascade is activated by H_2O_2 in U937 cells, p38 might be a key intermediate in IL-8 mRNA accumulation in U937 cells. It can be postulated that correct binding to the 3′ UTR elements requires the p38-mediated phosphorylation of the proteins synthesized *de novo*. ERK-1/-2 was also recently shown to be involved in the post-transcriptional stabilization of mRNA [30]. However, ERK-1/-2 was not activated by H_2O_2 in U937 and CEM cells, showing that p38 could very well be the only important factor for the stabilization of IL-8 mRNA by H_2O_2. The lack of stabilization of IL-8 mRNA in CEM cells was not due to a general deficiency in p38 inducibility because TNF-α strongly activated p38 within 10 min of treatment (results not shown). Other inducers such as IL-1β and PMA did not clearly activate p38 in CEM cells (results not shown). It should be pointed out that although PMA did not activate p38 in CEM cells, it led to an important activation of ERK-1/-2 (Figure 9C) and to a strong up-regulation of several chemokine genes (Figure 2A) in these cells, emphasizing the importance of ERK-1/-2 in this process. In U937 cells, H_2O_2 was the strongest p38 activator in comparison with TNF-α, IL-1β and PMA (results not shown), suggesting that the stabilization of chemokine mRNA species after treatment with these agents would require the activation of other MAPKs than p38.

In summary, H_2O_2 leads to an efficient transcription of the gene encoding IL-8 in monocytic cells; protein synthesis is required for the increase in IL-8 mRNA. Significantly, oxidative stress controls the stabilization of IL-8 mRNA through a pathway requiring SB203580-sensitive p38 activity. The targets of this p38 pathway are now under investigation.

This work was supported by grants from the National Fund for Scientific Research (Belgium), Télévie (Belgium) and the centre anticancereux (University of Liège). J.P. is a Research Director at the National Fund for Scientific Research (Belgium); C.J. is supported by a FRIA fellowship.

REFERENCES

1 Winyard, P. G. and Blake, D. R. (1997) Antioxidants, redox-regulated transcription factors, and inflammation. Adv. Pharmacol. **38**, 403–421
2 Janssen, Y. M., Van Houten, B., Borm, P. J. and Mossman, B. T. (1993) Cell and tissue responses to oxidative damage. Lab. Invest. **69**, 261–274
3 Weiss, S. J. (1989) Tissue destruction by neutrophils. N. Engl. J. Med. **320**, 365–376
4 Sun, Y. and Oberley, L. W. (1996) Redox regulation of transcriptional activators. Free Radical Biol. Med. **21**, 335–348
5 Schreck, R., Rieber, P. and Baeuerle, P. A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J. **10**, 2247–2258
6 Karin, M. and Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu. Rev. Immunol. **18**, 621–663
7 Hatada, E. N., Krappmann, D. and Scheidereit, C. (2000) NF-κB and the innate immune response. Curr. Opin. Immunol. **12**, 52–58
8 Lentsch, A. B. and Ward, P. A. (2000) The NF-κB/IκB system in acute inflammation. Arch. Immunol. Ther. Exp. **48**, 59–63
9 Verma, I. M. and Stevenson, J. (1997) IκB kinase: beginning, not the end. Proc. Natl. Acad. Sci. U.S.A. **94**, 11758–11760
10 Huxford, T., Huang, D. B., Malek, S. and Ghosh, G. (1998) The crystal structure of the IκBα/NF-κB complex reveals mechanisms of NF-κB inactivation. Cell **95**, 759–770
11 Karin, M. and Delhase, M. (2000) The IκB kinase (IKK) and NF-κB: key elements of proinflammatory signalling. Semin. Immunol. **12**, 85–98
12 DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. and Karin, M. (1997) A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature (London) **388**, 548–554
13 Woronicz, J. D., Gao, X., Cao, Z., Rothe, M. and Goeddel, D. V. (1997) IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. Science **278**, 866–869
14 Israel, A. (2000) The IKK complex: an integrator of all signals that activate NF-κB? Trends Cell Biol. **10**, 129–133
15 Schoonbroodt, S., Legrand-Poels, S., Best-Belpomme, M. and Piette, J. (1997) Activation of the NF-κB transcription factor in a T-lymphocytic cell line by hypochlorous acid. Biochem. J. **321**, 777–785
16 Schoonbroodt, S., Ferreira, V., Best-Belpomme, M., Boelaert, J. R., Legrand-Poels, S., Korner, M. and Piette, J. (2000) Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of IκBα in NF-κB activation by an oxidative stress. J. Immunol. **164**, 4292–4300
17 Pahl, H. L. (1999) Activators and target genes of Rel/NF-κB transcription factors. Oncogene **18**, 6853–6866
18 Rossi, D. and Zlotnik, A. (2000) The biology of chemokines and their receptors. Annu. Rev. Immunol. **18**, 217–242
19 Ryseck, R. P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzanski, P. and Bravo, R. (1992) RelB, a new Rel family transcription activator that can interact with p50-NF-κB. Mol. Cell. Biol. **12**, 674–684
20 Martin, T., Cardarelli, P. M., Parry, G. C., Felts, K. A. and Cobb, R. R. (1997) Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF-κB and AP-1. Eur. J. Immunol. **27**, 1091–1097
21 Grove, M. and Plumb, M. (1993) C/EBP, NF-κB, and c-Ets family members and transcriptional regulation of the cell-specific and inducible macrophage inflammatory protein 1α immediate-early gene. Mol. Cell. Biol. **13**, 5276–5289
22 Lakshminarayanan, V., Drab-Weiss, E. A. and Roebuck, K. A. (1998) H_2O_2 and tumor necrosis factor-α induce differential binding of the redox-responsive transcription factors AP-1 and NF-κB to the interleukin-8 promoter in endothelial and epithelial cells. J. Biol. Chem. **273**, 32670–32677
23 Lakshminarayanan, V., Beno, D. W., Costa, R. H. and Roebuck, K. A. (1997) Differential regulation of interleukin-8 and intercellular adhesion molecule-1 by H_2O_2 and tumor necrosis factor-α in endothelial and epithelial cells. J. Biol. Chem. **272**, 32910–32918
24 Winzen, R., Kracht, K., Ritter, B., Wilhelm, A., Chen, C. Y., Shyu, A. B., Muller, M., Gaestel, M., Resch, K. and Holtmann, H. (1999) The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. EMBO J. **18**, 4969–4980
25 Stoeckle, M. Y. (1991) Post-transcriptional regulation of gro-α, -β, -γ, and IL-8 mRNAs by IL-1 β. Nucleic Acids Res. **19**, 917–920
26 Shi, M. M., Godleski, J. J. and Paulauskis, J. D. (1995) Molecular cloning and posttranscriptional regulation of macrophage inflammatory protein-1α in alveolar macrophages. Biochem. Biophys. Res. Commun. **115**, 289–295
27 Lasa, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J. and Clark, A. R. (2000) Regulation of cyclooxygenase 2 mRNA stability by the mitogen-activated protein kinase p38 signaling cascade. Mol. Cell. Biol. **20**, 4265–4274
28 Pages, G., Berra, E., Milanini, J., Levy, A. P. and Pouysségur, J. (2000) Stress-activated protein kinases (JNK and p38/HOG) are essential for vascular endothelial growth factor mRNA stability. J. Biol. Chem. **275**, 26484–26491
29 Holtmann, H., Winzen, R., Holland, P., Eickemeier, S., Hoffmann, E., Wallach, D., Malinin, N. L., Cooper, J. A., Resch, K. and Kracht, M. (1999) Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. Mol. Cell. Biol. **10**, 6742–6753
30 Rutault, K., Hazzalin, C. A. and Mahadevan, L. C. (2001) Combinations of ERK and p38 MAPK inhibitors ablate tumor necrosis factor-α (TNF-α) mRNA induction. J. Biol. Chem. **276**, 6666–6674
31 Zlotnik, A. and Yoshie, O. (2000) Chemokines: a new classification system and their role in immunity. Immunity **12**, 121–127
32 Rollins, B. J. (1997) Chemokines. Blood **90**, 909–928
33 Baggiolini, M. and Loetscher, P. (2000) Chemokines in inflammation and immunity. Immunol. Today **21**, 418–420
34 Villarete, L. H. and Remick, D. G. (1996) Transcriptional and post-transcriptional regulation of interleukin-8. Am. J. Pathol. **149**, 1685–1693
35 Roebuck, K. A., Carpenter, L. R., Lakshminarayanan, V., Page, S. M., Moy, J. N. and Thomas, L. N. (1999) Stimulus-specific regulation of chemokine expression involves differential activation of the redox-responsive transcription factors AP-1 and NF-κB. J. Leukoc. Biol. **65**, 291–298
36 Kaufmann, A., Gemsa, D. and Sprenger, H. (2000) Differential desensitization of lipopolysaccharide-inducible chemokine gene expression in human monocytes and macrophages. Eur. J. Immunol. **30**, 1562–1567
37 Shi, M. M., Godleski, J. J. and Paulauskis, J. D. (1996) Regulation of macrophage inflammatory protein-1α mRNA by oxidative stress. J. Biol. Chem. **271**, 5878–5883

38 DeForge, L. E., Preston, A. M., Takeuchi, E., Kenney, J., Boxer, L. A. and Remick, D. G. (1993) Regulation of interleukin 8 gene expression by oxidant stress. J. Biol. Chem. **268**, 25568–25576
39 Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C. Y., Shyu, A. B., Muller, M., Gaestel, M., Resch, K. and Holtmann, H. (1999) The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. EMBO J. **18**, 4969–4980
40 Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S. and Cerami, A. (1986) Identification of a common nucleotide sequence in the 3′-untranslated region of mRNA molecules specifying inflammatory mediators. Proc. Natl. Acad. Sci. U.S.A. **83**, 1670–1674
41 Chen, C. Y., Gherzi, R., Andersen, J., Gaietta, G., Jurchott, K., Royer, H. D., Mann, M. and Karin, M. (2000) Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation. Genes Dev. **14**, 1236–1248
42 Hew, Y., Lau, C., Grzelczak, Z. and Keeley, F. W. (2000) Identification of a GA-rich sequence as a protein-binding site in the 3′-untranslated region of chicken elastin mRNA with a potential role in the developmental regulation of elastin mRNA stability. J. Biol. Chem. **275**, 24857–24864
43 Buzby, J. S., Brewer, G. and Nugent, D. J. (1999) Developmental regulation of RNA transcript destabilisation by A + U-rich elements is AUF1-dependent. J. Biol. Chem. **274**, 33973–33978
44 Lieberman, A. P., Pitha, P. M. and Shin, M. L. (1990) Protein kinase regulates tumor necrosis factor mRNA stability in virus-stimulated astrocytes. J. Exp. Med. **172**, 989–992
45 Lai, W. S., Carballo, E., Thorn, J. M., Kennington, E. A. and Blackshear, P. J. (2000) Interactions of CCCH zinc-finger proteins with mRNA. Binding of tristetraproline-related zinc finger proteins to AU rich elements and destabilization of mRNA. J. Biol. Chem. **275**, 17827–17837
46 Fan, X. C. and Steitz, J. A. (1998) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. EMBO J. **17**, 3448–3460
47 Levy, N. S., Chung, S., Furneaux, H. and Levy, A. P. (1998) Hypoxic stabilisation of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J. Biol. Chem. **273**, 6417–6423
48 Peng, S. S., Chen, C. Y., Xu, N. and Shyu, A. B. (1998) RNA stabilisation by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J. **17**, 3461–3470
49 Wang, W. S., Caldwell, M. C., Lin, S., Furneaux, H. and Gorospe, M. (2000) HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation. EMBO J. **19**, 2340–2350
50 Dean, J. L. E., Wait, R., Mathani, K. R., Sully, G. A., Clark, A. R. and Saklavtvala, J. (2001) The 3′ untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR. Mol. Cell. Biol. **21**, 721–730
51 Brewer, G. (1991) An A + U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro. Mol. Cell. Biol. **11**, 2460–2466
52 Zhang, W., Wagner, B. J., Ehrenman, K., Schaefer, A. W., DeMaria, C. T., Crater, D., DeHaven, K., Long, L. and Brewer, G. (1993) Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. Mol. Cell. Biol. **13**, 7652–7665

Received 3 April 2001/9 August 2001; accepted 19 September 2001