Immunology 1999 **97** 309–315

Regulation of macrophage inflammatory protein-2 gene expression by oxidative stress in rat alveolar macrophages

M. M. SHI,* I.-W. CHONG,† J. J. GODLESKI‡ & J. D. PAULAUSKIS‡ **Genomic Pathology Laboratory, Pathology and Experimental Toxicology, Parke-Davis Pharmaceutical Research, Warner-Lambert Company and Department of Pathology, the University of Michigan Medical School, Ann Arbor, MI, USA,* †*Department of Internal Medicine, Kaohsiung Medical College, Kaohsiung, Taiwan, and* ‡*Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA*

SUMMARY

Chemokines are important mediators in the development of inflammation. Our previous work demonstrated that an oxidative stress can up-regulate mRNA expression of a CC chemokine macrophage inflammatory protein (MIP)-1α in rat alveolar macrophages. In the present study, we further investigate whether an oxidative stress can regulate the gene expression of a related CXC chemokine MIP-2, involved in both neutrophil chemotaxis and activation. A rat alveolar macrophage cell line (NR8383) was exposed to 10 μg/ml bacterial lipopolysaccharide (LPS) and MIP-2 mRNA levels dramatically increased after 4 hr of stimulation. This increase by LPS was attenuated by co-treatment with the antioxidants *N*-acetylcysteine and dimethylsulphoxide, suggesting that the induction of MIP-2 mRNA is mediated via the generation of reactive oxygen species. To assess directly the role of oxidative stress on regulation of MIP-2 mRNA expression, macrophages were exposed to H_2O_2. MIP-2 mRNA levels had significantly increased after 1 hr exposure to 0·5 mM H_2O_2, were maximally increased after 4 hr and decreased after 6 hr. Co-treatment of macrophages with the transcriptional inhibitor actinomycin D eliminated the H_2O_2-induction of MIP-2 mRNA, implicating a role for transcriptional activation in increased expression of MIP-2. Genomic cloning of the rat MIP-2 gene 5′-flanking region has identified a consensus nuclear factor-κB (NF-κB) binding site. Gel-mobility shift assays revealed NF-κB binding to the MIP-2 promoter/enhancer sequence was induced by H_2O_2. LPS treatment for 4 hr also significantly activated NF-κB binding, which could also be attenuated by pretreatment with *N*-acetylcysteine at the doses that reduced MIP-2 mRNA expression. The half-life of MIP-2 mRNA transcripts was also increased by H_2O_2 treatment. These observations indicate that MIP-2 gene expression is subject to both transcriptional and post-transcriptional control in response to an H_2O_2 oxidative stress.

INTRODUCTION

Inflammation, which includes leucocyte migration and activation, is a classic response to pathogen invasion or tissue damage. The recent literature has established a group of structurally related peptides, termed chemokines, which are responsible for recruitment of specific leucocyte subsets to the inflammatory sites.[1–3] The chemokine superfamily can be divided into two subsets based on the position of the two cysteine residues at the N terminus: CXC chemokines contain a single amino acid between the first two cysteines; or CC chemokines which have two adjacent cysteines.[1] Interleukin-8 (IL-8), a CXC chemokine, appears to be the prominent neutrophil chemotactant in humans.[4] The rodent counterpart of human IL-8 has not been identified, even after vigorous cloning efforts from different groups. Previous work from our group and others has established that two rodent CXC chemokines, macrophage inflammatory protein-2 (MIP-2) and KC, act in rodents as functional homologues for human IL-8.[5–8] MIP-2 is a potent neutrophil chemoattractant both *in vitro* and *in vivo*.[5,6,8] It can also increase the oxidative metabolism in these cells and appears to be a mitogen for epithelial cells.[6,8] Increased MIP-2 expression has been implicated in the development of pulmonary inflammation induced by a variety of stimuli, including bacterial endotoxin, reovirus, ozone, sulphur dioxide and the transition metals vanadium and manganese.[9–14]

Received 10 December 1998; revised 25 February 1999; accepted 25 February 1999.

Abbreviations: AM, alveolar macrophage; AP-1, activator protein-1; AUBF, adenosine–uridine-binding factor; DMSO, dimethyl sulphoxide; EMSA, electrophoresis mobility shift assay; IL, interleukin; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; NAC, N-acetylcysteine; NF-κB, nuclear factor-κB; nt, nucleotide; UTR, untranslated region.

Correspondence: Dr M. M. Shi, Pathology and Experimental Toxicology, Parke-Davis Pharmaceutical Research, Warner-Lambert Company, Ann Arbor, MI 48105, USA.

© 1999 Blackwell Science Ltd

Lipopolysaccharide (LPS)-induced IL-8 production in human peripheral blood mononuclear cells can be blocked by antioxidants, suggesting a role for reactive oxygen species (ROS) in the regulation of chemokine expression.[15,16] We have also found that LPS caused a time-dependent increase of MIP-2 mRNA and protein expression in rat alveolar macrophages (M. M. Shi *et al.* submitted for publication). Other recent work from our laboratory provided direct evidence that oxidative stress generated by H_2O_2 and menadione could induce mRNA expression of a CC chemokine, MIP-1α, in a rat alveolar macrophage cell line NR8383.[17] The objective of the study reported here was to test the hypothesis that an oxidative stress can serve as a common signal to regulate both CC and CXC chemokines in alveolar macrophages. We utilized a rat alveolar macrophage (AM) cell line to investigate the regulation of MIP-2 gene expression in response to oxidative stress. LPS-induction of MIP-2 mRNA expression was attenuated by antioxidants, suggesting that the induction of MIP-2 mRNA expression by LPS is mediated through the generation of ROS. H_2O_2 treatment directly caused a transient induction of MIP-2 mRNA expression, which involves transcriptional regulation. We recently cloned a full-length rat MIP-2 cDNA (M. M. Shi *et al.* GenBank accession number U45965) and the 5′-flanking region of the MIP-2 gene (M. M. Shi *et al.* GenBank accession number U83656). A search of this rat MIP-2 gene 5′-flanking region revealed a consensus nuclear factor-κB (NF-κB) and an activator protein-1 (AP-1) binding site. We found that the up-regulation of MIP-2 mRNA involves both transcriptional activation of gene expression and post-transcriptional regulation of mRNA half-life. Gel-mobility shift assays suggest that transcriptional activation of MIP-2 gene expression involves NF-κB activation.

MATERIALS AND METHODS

Materials

Polyclonal antibodies to p65 (Rel A) and p50 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The radiolabels [α-^{32}P]dATP (6000 Ci/mmol) and [γ-^{32}P]dATP (3000 Ci/mmol) were from DuPont-NEN (Boston, MA). If not specified, other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and culture conditions

The rat AM cell line, NR8383, was developed from normal rat AMs and provided by Dr R. Helmke.[18] These cells behave similarly to primary AMs relative to phagocytosis, the respiratory burst, and cytokine release.[18–22] Cells were cultured in RPMI-1640 supplemented with 5% equine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown in a humidified incubator at 37° with 5% CO_2.

Cell treatment

Tissue culture plates (100 mm) were precoated with polyhydroxymethylacrylate to prevent cell adherence as previously described.[23] Cells were plated at a density of 1×10^6 cells/ml in serum-free RPMI-1640 for at least 4 hr prior to addition of LPS or H_2O_2. For inhibitor studies, AMs were treated with H_2O_2 along with 5 μg/ml actinomycin D to inhibit transcription. For antioxidant treatment, cells were incubated with 1, 10, or 20 mM *N*-acetylcysteine (NAC) or 1% dimethyl sulphoxide (DMSO) for 1 hr, followed by the addition of 10 μg/ml LPS for 4 hr.

RNA extraction

Total cellular RNA was isolated from AMs using a guanidinium method as previously described.[17,24] RNA was dissolved in diethyl pyrocarbonate-treated TE buffer [10 mM Tris/1 mM ethylenediamine tetraacetic acid (EDTA), pH 7·4] and stored at −70° before gel electrophoresis.

Northern analysis

Total cellular RNA (10 μg/lane) was denatured in 50% formamide/7% formaldehyde, resolved in a 1% agarose/7% formaldehyde gel and transferred and ultraviolet (UV) cross-linked to nylon membranes (Schleicher & Schuell, Keene, NH). A 0·33-kilobase rat MIP-2 cDNA was labelled with [α-^{32}P]dATP (6000 Ci/mmol) by random primer labelling (Life Technologies, Inc., Gaithersburg, MD).[9] Prehybridization (3 hr) and hybridization with ^{32}P-labelled MIP-2 cDNA (overnight) were carried out in 0·5 M $NaPO_4$/1 mM EDTA/7% sodium dodecyl sulphate (SDS)/150 μg/ml tRNA at 65°.[25] Blots were washed once in 0·1 × SSC (20 × = 3 M NaCl, 0·3 M sodium citrate)/0·1% SDS at room temperature and twice at 52° before autoradiography and densitometry. The same blots were stripped of hybridized MIP-2 probe and rehybridized with a radiolabelled cDNA fragment of mouse β-actin cDNA (American Type Culture Collection, Rockville, MD) as an internal control. Several blots used in this study had been previously probed with MIP-1α cDNA and stripped.[17]

Determination of mRNA half-life

Actinomycin D (final concentration of 5 μg/ml) was added to the media of control cells or AMs treated with 0·5 mM H_2O_2 for 1 hr. Cells were sampled at times indicated through 6 hr and levels of MIP-2 mRNA were determined by Northern analysis. The integrated band values, as determined by densitometry, were normalized to β-actin RNA and 100% mRNA was set at time 0 after actinomycin D treatment.

Nuclear protein extraction and electrophoresis mobility shift assay (EMSA)

After treatment with 0·5 or 1 mM H_2O_2 or LPS in the presence or absence of antioxidant NAC for different times, nuclear protein was extracted from 10 million cells/treatment using a modified Dignam protocol and frozen at −70°.[26] Total nuclear protein concentrations were determined before EMSA using a bicinchoninic acid protein assay (Pierce, Rockford, IL).

Oligonucleotides including the putative rat NF-κB-binding sequence: 5′-GAGCTGCGGGAATTTCCCAGCC-3′ (−100 to −78) and AP-1-binding sequence: 5′-GGGCACTTGAGTCAGAACTCA-3′ (−244 to −224) were synthesized according to the actual sequences determined from the rat MIP-2 gene promoter region (M. M. Shi *et al.* GenBank accession number U83656). Double-stranded DNA probes containing the AP-1- or NF-κB-binding regions were 5′ end-labelled with [γ-^{32}P]dATP and T4 polynucleotide kinase (Promega, Madison, WI), and purified by G-50 Sephadex columns (Pharmacia Biotech, Pittscatway, NJ). Five micrograms of the protein/nuclear extract from control or H_2O_2-treated cells was added to the ^{32}P-labelled DNA fragments (50 000 c.p.m.) in a reaction buffer to a final volume of 10 μl containing 2 μg of

poly(dI-dC), 10 μg of bovine serum albumin, 10 mM Tris–HCl (pH 7·5), 50 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA and 5% glycerol. DNA–protein complexes were resolved on 5% non-denaturing polyacrylamide gels electrophoresed in low ionic strength buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA) and visualized by autoradiography. For supershift assays, the antibodies to p65 or p50 were added to the above mixture at a concentration of 4 μg per 10 μl and the samples were allowed to incubate at room temperature for 1 hr prior to gel loading. The presence of an additional gel shift band or the disappearance of the original band were recognized by comparison with antibody-free parallel samples.

RESULTS

Antioxidants attenuate the MIP-2 mRNA production in response to LPS

Steady-state mRNA levels encoding MIP-2 in AMs were dramatically induced in response to 10 μg/ml LPS treatment for 4 hr (Fig. 1). To test the potential roles of reactive oxygen intermediates in LPS-induced MIP-2 mRNA expression, AMs were exposed to LPS in the presence of varying doses of the antioxidants NAC or DMSO. NAC is a synthetic antioxidant which can replenish intracellular glutathione levels.[27] DMSO has been widely used as an antioxidant and has been proposed to inhibit oxygen free radical production.[28] AMs were pretreated with 0, 1, 10, or 20 mM NAC or 1% DMSO for 1 hr before challenging with 10 μg/ml LPS for 4 hr. The low dose of NAC (1 mM) did not influence the LPS-induction of MIP-2 mRNA expression, while both NAC at 10 or 20 mM and DMSO at 1% significantly decreased this induction (Fig. 1).

Induction of MIP-2 mRNA by H_2O_2

To assess directly the role of oxidants on MIP-2 mRNA expression, AMs were exposed to H_2O_2 for up to 6 hr and MIP-2 mRNA was measured by Northern analysis. Levels of MIP-2 mRNA were rapidly induced as early as 1 hr following exposure to 0·5 mM H_2O_2, maximally induced after 4 hr and returned to the control level by 6 hr (Fig. 2a). H_2O_2 at 0·1 mM did not induce a change in MIP-2 mRNA levels (data not shown). The induction of MIP-2 mRNA expression at 4 hr following 0·5 mM H_2O_2 exposure could be partially attenuated by pretreatment with 1 mM NAC for 1 hr (Fig. 2b).

Effect of actinomycin D on the elevation of MIP-2 mRNA levels by H_2O_2

Increased mRNA expression is commonly controlled by transcriptional or post-transcriptional mechanisms. To determine whether H_2O_2 elevates MIP-2 mRNA levels through transcrip-

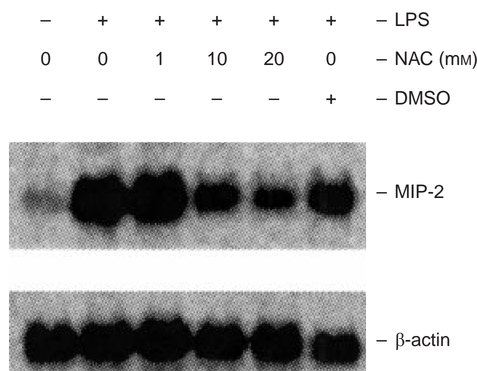

Figure 1. Effect of NAC and DMSO on MIP-2 mRNA levels in response to LPS in the rat alveolar macrophage cell line, NR8383. Cells were incubated with 10 μg/ml LPS for 4 hr and MIP-2 mRNA expression was determined by Northern analysis as described in the Materials and Methods. For antioxidant treatment, cells were incubated with 1, 10, or 20 mm NAC or 1% DMSO for 1 hr, followed by the addition of 10 μg/ml LPS for 4 hr. Upper panel, radio image of Northern blot hybridized with a radiolabelled MIP-2 cDNA. Lower panel, the same membrane hybridized with a mouse β-actin cDNA. Results are representative of two independent experiments.

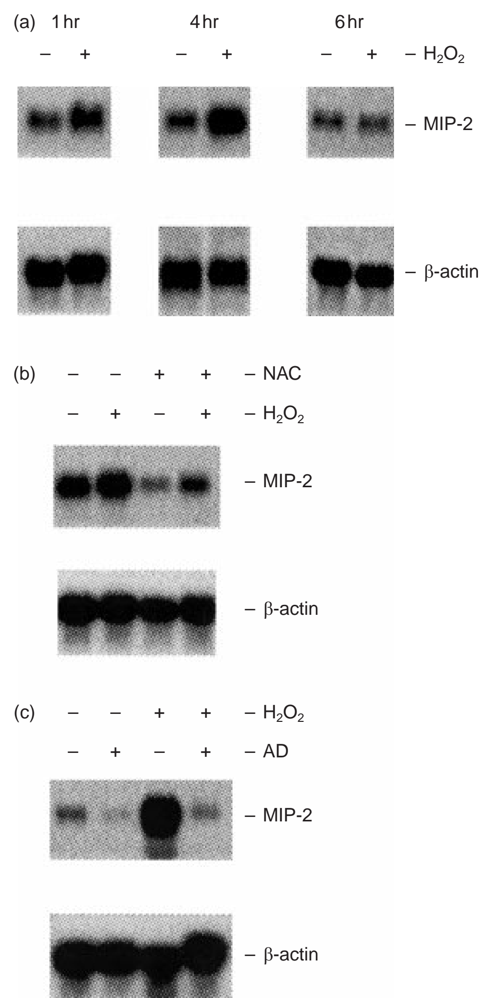

Figure 2. Induction of MIP-2 mRNA levels by H_2O_2 in a rat alveolar macrophage cell line, NR8383. (a) Cells were incubated with 0 or 0·5 mM H_2O_2 in serum-free RPMI for 1, 4, or 6 hr. (b) Cells were incubated with 1 mM NAC for 1 hr, followed by the addition of 0·5 mM H_2O_2 for 4 hr. (c) Cells were treated with 0 or 0·5 mM H_2O_2 in the presence or absence of 5 μg/ml actinomycin D for 1 hr. Total cellular RNA was extracted and Northern analysis was performed as described in the Materials and Methods. Upper panel, autoradiogram of Northern blot hybridized with radiolabelled MIP-2 cDNA. Lower panel, the same membrane hybridized with a mouse β-actin cDNA. Results are representative of two independent experiments.

tional activation of MIP-2 gene, we utilized a commonly used transcriptional inhibitor actinomycin D as previously described.[17,29] AMs were exposed to 0·5 mM H_2O_2 in the presence or absence of 5 μg/ml actinomycin D for 1 hr and the MIP-2 mRNA levels were quantified by Northern analysis. The elevation of MIP-2 mRNA by H_2O_2 was blocked by co-incubation with actinomycin D, suggesting that the increase, at least at this early time-point, involves transcriptional regulation (Fig. 2c).

Activation of NF-κB binding to the MIP-2 promoter by H_2O_2

To investigate further the transcriptional regulation of MIP-2 gene expression, we recently cloned the MIP-2 gene 5′-flanking region and identified consensus transcription factor NF-κB (−92 to −83) and AP-1 binding sites (−236 to −229) (M. M. Shi *et al.* GenBank accession number U83656). In this study, we performed EMSA to determine whether either of these two putative sites are bound during H_2O_2 treatment. Oligonucleotides containing NF-κB- or AP-1-binding sites identified in the MIP-2 promoter region were synthesized and used to probe nuclear extracts from control and H_2O_2-treated AMs. Binding of the NF-κB sequence was markedly induced after 1 mM H_2O_2 treatment for 1 hr and was still much higher than control levels after 6 hr of H_2O_2 treatment (Fig. 3a). H_2O_2-induced nuclear protein–DNA binding was further characterized immunochemically using antibodies against known members of the NF-κB family. The protein–DNA complex was supershifted by anti-p50 antibody and abrogated by anti-p65 antibody (Fig. 3a). These results indicate that H_2O_2 activates the DNA binding for both the p50 and p65 subunits of the NF-κB transcription factor. Binding to the AP-1 sequence was weak and was not influenced by 0·5 mM H_2O_2 treatment (data not shown).

Activation of NF-κB binding to the MIP-2 promoter by LPS is attenuated by NAC

Binding of the NF-κB sequence was also markedly induced after 10 μg/ml LPS treatment for 4 hr (Fig. 3b). AMs were also pretreated with 0, 1, 10, or 20 mM NAC for 1 hr before challenging with 10 μg/ml LPS for 4 hr and then, nuclear extracts were prepared and EMSA was performed. NAC at 10 or 20 mM significantly reduced the activation of NF-κB-binding by LPS, while 1 mM NAC was ineffective. Again, the protein–DNA complex was supershifted by anti-p50 antibody and abrogated by anti-p65 antibody. These results indicate that LPS activates the DNA binding for both the p50 and p65 subunits of the NF-κB transcription factor and this activation of NF-κB could be attenuated by relatively high doses of the antioxidant NAC.

Effect of H_2O_2 on MIP-2 mRNA half-life

The possible contribution of changes in MIP-2 mRNA stability to its increased expression in response to H_2O_2 was also evaluated by measuring MIP-2 mRNA half-life ($t_{\frac{1}{2}}$). Control and AMs exposed to 0·5 mM H_2O_2 for 1 hr were treated with 5 μg/ml actinomycin D to stop transcription. Cells were sampled for 6 hr post-actinomycin D treatment and levels of MIP-2 mRNA were quantified by Northern analysis (Fig. 4a). The integrated band values, as determined by densitometry, were normalized to β-actin RNA (Fig. 4b). In the presence of

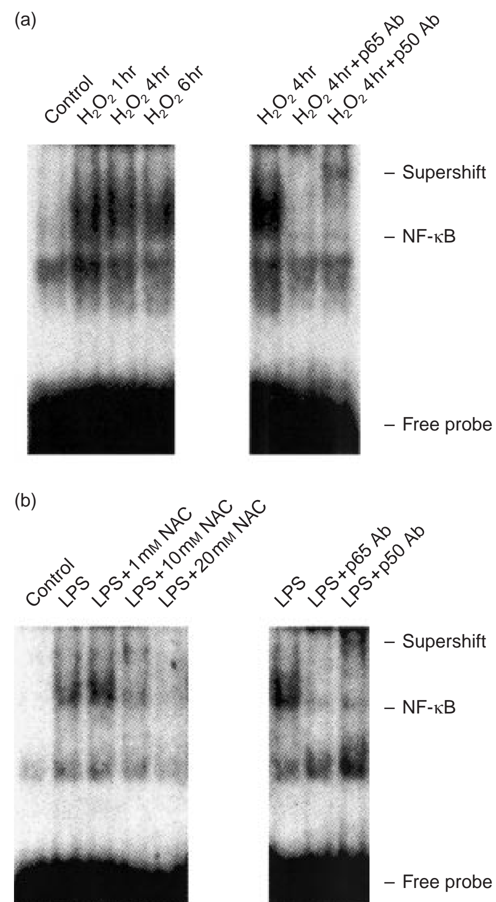

Figure 3. Effects of H_2O_2 and LPS on NF-κB binding to an oligonucleotide probe containing a putative consensus sequence within the MIP-2 promoter region. (a) Cells were treated without or with 1 mM H_2O_2 for the times indicated. (b) Cells were incubated with 1, 10, or 20 mM NAC for 1 hr, followed by the addition of 10 μg/ml LPS for 4 hr. Then, nuclear extracts were prepared and electrophoretic mobility shift assays were performed using ^{32}P-labelled oligonucleotide containing the NF-κB-binding site. For supershift assays, nuclear extracts from cells treated with 1 mM H_2O_2 (a) or 10 μg/ml LPS (b) for 4 hr were preincubated with 4 μg of antibodies to p65 or p50 before gel loading. Binding of specific antibodies to the NF-κB subunits either eliminates the formation of radiolabel–NF-κB complex or results in a higher molecular weight complex that migrates more slowly on the gel (supershift). The autoradiogram is a representative of three separate experiments.

actinomycin D, MIP-2 mRNAs from both control and H_2O_2-treated macrophages display biphasic degradation kinetics, with an initial rapid first-order decay followed by a plateau after 3 hr. The initial phase of mRNA decay in control cells was very quick, with a $t_{\frac{1}{2}}$ of ≈2 hr. H_2O_2 treatment significantly increased the half-life of MIP-2 mRNA, with an initial phase $t_{\frac{1}{2}}$ greater than 3 hr, suggesting that the induction of MIP-2 mRNA by H_2O_2 also involves post-transcriptional regulation of the MIP-2 mRNA transcript.

DISCUSSION

MIP-2 is a primary CXC chemokine that can cause neutrophil chemotaxis and activation. Elevation of MIP-2 mRNA

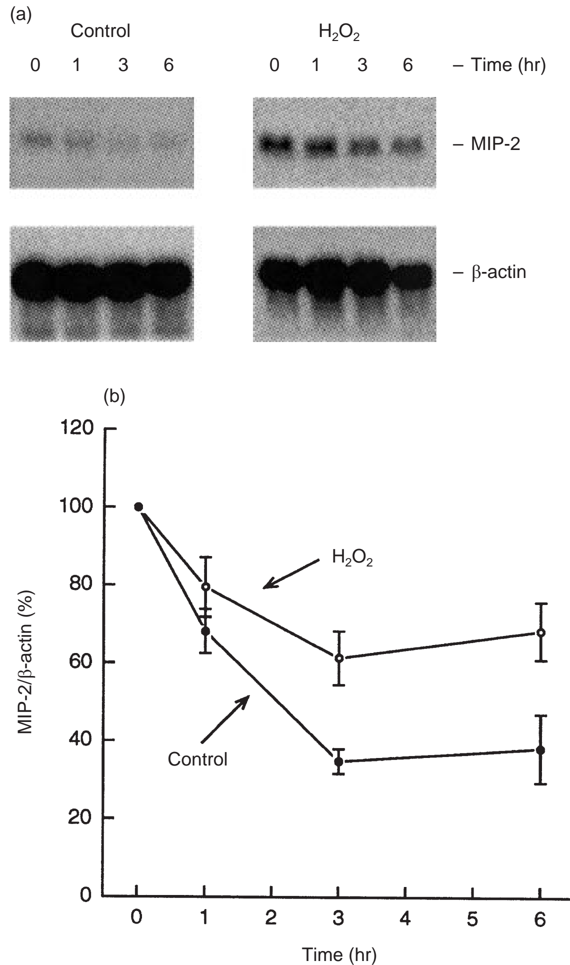

Figure 4. Effect of H_2O_2 on the half-life of MIP-2 mRNA. (a) Cells were untreated (control), or treated with 0·5 mM H_2O_2 for 1 hr. Actinomycin D was then added to a final concentration of 5 μg/ml, and at the times indicated, total RNA was isolated and Northern analysis was performed as described in the Materials and Methods. Upper panel, autoradiogram of Northern blot hybridized with radio-labelled-MIP-2 cDNA. Lower panel, the same membrane hybridized with a mouse β-actin cDNA. This is a representative blot from three independent experiments. (b) Densitometric quantification of the decay of MIP-2 mRNA normalized to β-actin RNA from control cells and from cells treated with H_2O_2. Results are mean ± SE of three independent experiments.

expression has been implicated in the development of inflammatory diseases in several animal models, such as pulmonary inflammation induced by ozone, SO_2 and the transitional metals vanadium and manganese.[11–14] While ozone and SO_2 are direct oxidants, transitional metals have been shown to induce cellular oxidative stress.[14,30] In the current study, we investigated whether induction of MIP-2 mRNA parallels the generation of ROS. AMs treated with LPS for 4 hr showed significant increases in MIP-2 mRNA levels (Fig. 1). In addition, LPS-induction of MIP-2 mRNA expression was attenuated by pretreatment with the antioxidants NAC and DMSO, further suggesting a role for ROS in the regulation of this gene. There is increasing evidence that increased expression of other cytokines by LPS also involves reactive oxygen intermediates. For example, the induction of a human CXC chemokine IL-8 by LPS can also be blocked by a variety of antioxidants, including DMSO.[15,16] The primary cellular source of ROS production in response to LPS has been suggested to be mitochondrial.[31] The molecular mechanism of ROS production in response to LPS treatment is not clear.

Other recent work from our laboratory provides direct evidence that oxidative stress in the form of H_2O_2 and menadione can directly induce MIP-1α mRNA expression in an AM cell line, NR8383.[17] In combination with this study, we suggest that the induction of both CXC and CC chemokines involves ROS production. We hypothesize that ROS may serve as a common signal to regulate the gene expression of most, if not all, chemokines, thereby initiating and propagating the inflammatory process. To study the regulation of a CXC chemokine MIP-2 gene expression, we exposed AMs directly to H_2O_2, a commonly used *in vitro* system to generate oxidative stress. H_2O_2 exposure at 0·5 mM caused a transient induction of MIP-2 mRNA levels at 1 hr and 4 hr post-treatment (Fig. 2a). Co-incubation with actinomycin D blocked the induction of MIP-2 mRNA by H_2O_2 (Fig. 2c), suggesting that the transcriptional regulation of the MIP-2 gene is involved.

To characterize the transcriptional regulation of the MIP-2 gene, we recently cloned the rat MIP-2 gene 5′-flanking region. Analysis of the 5′-flanking region of rat MIP-2 gene revealed only putative AP-1 and NF-κB consensus sequences (M. M. Shi *et al.* GenBank accession number U83656). NF-κB motifs have been found in the 5′-regulatory regions of an increasing number of cytokine genes, including human IL-8 gene.[32] Mammalian NF-κB is a heterodimeric transcription factor which can induce a variety of genes involved in inflammation and acute-phase immune responses.[33,34] AP-1 is a transcription factor composed of various *fos* and *jun* family gene products, which form homodimers or heterodimers and bind to a common *cis*-acting element. The expression of *fos* family and *jun* family genes can be induced by a variety of extracellular stimuli, such as serum, growth factors, phorbol esters, calcium ionophore and ionizing radiation.[35] ROS have been implicated in the activation of both transcription factors NF-κB and AP-1,[36–38] but oxidative induction of NF-κB or AP-1 is not a universal phenomenon.[39,40] In the current study, H_2O_2 treatment induced the formation of NF-κB complexes in a time-dependent fashion. In addition, the NF-κB complexes could be supershifted by specific antisera against p50 and abrogated by antisera against the p65 subunit of the NF-κB, indicating the induction of a p65–p50 dimer (Fig. 3a). The AP-1 binding to the DNA probes from MIP-2 promoter was not influenced by H_2O_2 treatment. AMs treated with LPS for 4 hr also showed significant activation of NF-κB (Fig. 3b). Similar to the Northern results (Fig. 1), LPS-induction of NF-κB activation was attenuated by pretreatment with the antioxidant NAC (Fig. 3b), further suggesting a role for ROS in the regulation of gene transcription. Taken together, these results suggest that the induction of MIP-2 gene expression in AMs is most likely to be through the activation of NF-κB.

Oxidative regulation of mRNA stability is another potentially important regulatory mechanism.[41,42] We previously reported that LPS- and H_2O_2-induction of mRNA for another chemokine, MIP-1α, also involves post-transcriptional stabilization of MIP-1α mRNA.[17,43] MIP-1α belongs to the CC chemokine subfamily, rather than the CXC group to which MIP-2 belongs. The two chemokine subfamilies represent two

sets of genes clustered on two different chromosomes, suggesting their regulation might be controlled differently.[1] Quantification of control MIP-2 mRNA half-life revealed a biphasic pattern, with initial rapid first-order kinetics and a $t_{\frac{1}{2}}$ of around 2 hr, followed by a plateau at around 30% of the initial message levels (Fig. 4). As shown by others, this arrest of degradation is most likely a general effect attributed to the transcription inhibitors.[44] H_2O_2 treatment significantly increased MIP-2 mRNA half-life at the initial phase with a $t_{\frac{1}{2}} > 3$ hr and reached a plateau at around 60% of initial mRNA levels. The instability of mRNA in many cytokines is determined by a specific *cis*-element (AU-rich element) in the 3′-untranslated regions (UTRs) of their mRNAs.[45] It was previously postulated that MIP-1α mRNA stabilization may be attributed to the presence of multiple copies of the reiterated AUUUA motifs within the 3′-untranslated region of the mRNA. These sequences are typically conserved in these regions of cytokine and growth factor mRNAs and areimplicated in mRNA stability and translational control.[46,47] Adenosine–uridine-binding factor (AUBF) is a 33 000 MW cytoplasmic protein found in many tumour cell lines or activated normal lymphocytes. It binds to multiple AUUUA elements in the 3′-UTRs of many cytokine and growth factor mRNAs and stabilizes them.[48] The binding of AUBF to RNA templates was reported to be redox sensitive.[49] Sequence analysis of the MIP-2 cDNA 3′-UTR also revealed multiple copies of the ATTTA motifs (M. M. Shi *et al.* GenBank accession number U45965). H_2O_2 treatment increased the mRNA half-lives of both MIP-2 and MIP-1α, suggesting that an oxidative stress can influence mRNA stability through a common oxidative control mechanism. The possible role of AUBF in the regulation of chemokine gene mRNA stabilization deserves further investigation.

In conclusion, the present study demonstrates that the induction of MIP-2 mRNA expression by LPS is attenuated by antioxidants, suggesting ROS as a mediator for chemokine expression. H_2O_2 treatment increases MIP-2 mRNA expression through both transcriptional control, most likely involving NF-κB activation, and post-transcriptional control via increased mRNA transcript stability.

ACKNOWLEDGMENTS

This research was supported by NIH Grant ES05703, HL54958, Giles F. Filley Memorial Award from the American Physiological Society and Parker B. Francis Award to M. M. Shi. M. M. Shi is an Edward Livingston Trudeau Scholar of the American Lung Association.

REFERENCES

1. Oppenheim J.J., Zachariae C.O.C., Mukaida N. & Matsushima K. (1991) Properties of the novel proinflammatory supergene 'intercrine' cytokine family. *Annu Rev Immunol* **9,** 617.
2. Miller M.D. & Krangel M.S. (1992) Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. *Crit Rev Immunol* **12,** 17.
3. Baggiolini M., Dewald B. & Moser B. (1994) Interleukin-8 and related chemotactic cytokines – CXC and CC chemokines. *Adv Immunol* **55,** 97.
4. Leonard E. & Yoshimura T. (1990). Neutrophil attractant/activation protein-1 (NAP-1[Interleukin-8]). *Am J Respir Cell Mol Biol* **2,** 479.
5. Wolpe S.D., Sherry B., Juers D., Davatelis G., Yurt R.W. & Cerami A. (1989) Identification and characterization of macrophage inflammatory protein 2. *Proc Natl Acad Sci USA* **86,** 612.
6. Frevert C.W., Farone A., Danaee H., Paulauskis J.D. & Kobzik L. (1995) Functional characterization of rat chemokine macrophage inflammatory protein-2. *Inflammation* **19,** 133.
7. Frevert C.W., Huang S., Danaee H., Paulauskis J.D. & Kobzik L. (1995) Functional characterization of the rat chemokine KC and its importance in neutrophil recruitment in a rat model of pulmonary inflammation. *J Immunol* **154,** 335.
8. Driscoll K.E., Hassenbein D., Howard B.W. *et al.* (1995) Cloning, expression, and functional characterization of rat MIP-2: a neutrophil chemoattractant and epithelial cell mitogen. *J Leukoc Biol* **58,** 359.
9. Huang S., Paulauskis J.D., Godleski J.J. & Kobzik L. (1992) Expression of macrophage inflammatory protein-2 and KC mRNA in pulmonary inflammation. *Am J Pathol* **141,** 981.
10. Farone A.L., Frevert C.W., Farone M.B. *et al.* (1997) Serotype-dependent induction of pulmonary neutrophilia and inflammatory cytokine gene expression by reovirus. *J Virol* **70,** 7079.
11. Driscoll K.E., Simpson L., Carter J., Hassenbein D. & Leikauf G.D. (1993) Ozone inhalation stimulates expression of a neutrophil chemotactic protein, macrophage inflammatory protein 2. *Toxicol Appl Pharmacol* **119,** 306.
12. Farone A., Huang S., Paulauskis J.D. & Kobzik L. (1995) Airway neutrophilia and chemokine mRNA expression in sulfur dioxide-induced bronchitis. *Am J Respir Cell Mol Biol* **12,** 345.
13. Pierce L.M., Alessandrini F., Godleski J.J. & Paulauskis J.D. (1996) Vanadium-induced chemokine mRNA expression and pulmonary inflammation. *Toxicol Appl Pharmacol* **138,** 1.
14. Tsai C.S., Shi M.M., Godleski J.J. & Paulauskis J.D. (1996) Manganese-induced pulmonary inflammation and increased expression of pro-inflammatory cytokine mRNA in rat lung cells. *Am J Respir Critical Care Med* **153,** A614.
15. Deforge L.E., Fantone J.C., Kenney J.S. & Remick D.G. (1992) Oxygen radical scavengers selectively inhibit interleukin 8 production in human whole blood. *J Clin Invest* **90,** 2123.
16. Deforge L.E., Preston A.M., Takeuchi E., Kenney J., Boxer L.A. & Remick D.G. (1993) Regulation of interleukin-8 gene expression by oxidative stress *J Biol Chem* **268,** 25 568.
17. Shi M.M., Godleski J.J. & Paulauskis J.D. (1996) Regulation of macrophage inflammatory protein-1α by oxidative stress. *J Biol Chem* **271,** 5878.
18. Helmke R.J., Boyd R.L., German V.F. & Mangos J.A. (1987) From growth factor dependence to growth factor independence: the genesis of an alveolar macrophage cell line. *In Vitro Cell Develop Biol* **23,** 567.
19. Krieg D.P., Helmke R.J., German V.F. & Mangos J.A. (1988) Resistance of mucoid *Pseudomonas aeruginosa* to nonopsonic phagocytosis by alveolar macrophages in vitro. *Infect Immun* **56,** 3173.
20. Helmke R.J., German V.F. & Mangos J.A. (1989) A continuous alveolar macrophage cell line: comparisons with freshly derived alveolar macrophages. *In Vitro Cell Dev Biol* **25,** 44.
21. Hidalgo H.A., Helmke R.J., German V.F. & Mangos J.A. (1991) Role of the zymolyase-sensitive cyst wall of *Pneumocystis carinii* in the oxidative burst of macrophages. *J Protozoo* **38,** 30S.
22. Hidalgo H.A., Helmke R.J., German V.F. & Mangos J.A. (1992) *Pneumocystis carinii* induces an oxidative burst in alveolar macrophages. *Infect Immun* **60,** 1.
23. Folkman J. & Moscona A. (1987) Role of cell shape in growth control. *Nature* **273,** 345.
24. Chirgwin J., Przbyla A., MacDonald R. & Rutter W. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18,** 5294.
25. Church G.M. & Gilbert W. (1984) Genomic sequencing. *Proc Natl Acad Sci USA* **74,** 5463.
26. Dignam J.D., Lebovitz R.M. & Roeder R.G. (1983) Accurate

transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucl Acid Res* **11,** 1475.
27. Meister A. & Anderson M.E. (1983) Glutathione. *Annu Rev Biochem* **52,** 711.
28. Repine J.E., Eaton J.W., Anders M.W., Hoidal J.R. & Fox R.B. (1979) Generation of hydroxyl radical by enzymes, chemicals, and human phagocytes in vitro. *J Clin Invest* **64,** 1642.
29. Shi M.M., Kugelman A., Iwamoto T., Tian L. & Forman H.J. (1994) Quinone-induced oxidative stress elevates γ-glutamylcysteine synthetase activity in rat lung epithelial L2 cells. *J Biol Chem* **269,** 26 512.
30. Grabowski G., Paulauskis J. & Godleski J.J. (1995) Tyrosine phosphorylation events in the respiratory burst of rat lung macrophages induced by vanadium. *FASEB J* **9,** A947.
31. Remick D.G. & Villarete L. (1996) Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates. *J Leukoc Biol* **59,** 471.
32. Yasumoto K., Okamoto S., Mukaida N., Murakami S., Mai M. & Matsushima K. (1992) Tumor necrosis factor α and interferon γ synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-κB-like binding sites of the interleukin 8 gene. *J Biol Chem* **267,** 22506.
33. Baeuerle P.A. & Henkel T. (1994) Function and activation of NF-κB in the immune system. *Annu Rev Immunol* **12,** 141.
34. Thanos D. & Maniatis T. (1995) NF-κB: a lesson in family values. *Cell* **80,** 529.
35. Karin M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270,** 16483.
36. Sen C.K. & Packer L. (1996) Antioxidant and redox regulation of gene transcription. *FASEB J* **10,** 709.
37. Schreck R., Rieber P. & Baeuerle P.A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NFκB transcription factor and HIV-1. *EMBO J* **10,** 2247.
38. Abate C., Patel L., Rauscher I. & Curran T. (1990) Redox regulation of fos and jun DNA-binding activity in vitro. *Science* **249,** 1157.
39. Das K.C., Lewis-Molock Y. & White C.W. (1995) Activation of NF-kappa B and elevation of MnSOD gene expression by thiol reducing agents in lung adenocarcinoma (A549) cells. *Am J Physiol* **269,** L588.
40. Choi H.S. & Moore D.D. (1993) Induction of c-fos and c-jun gene expression by phenolic antioxidants. *Mol Endocrinol* **7,** 1596.
41. Clerch L.B., Iqbal J. & Massaro D. (1991) Perinatal rat lung catalase gene expression: influence of corticosteroid and hyperoxia. *Am J Physiol* **260,** L428.
42. Clerch L.B. & Massaro D. (1992) Oxidation-reduction-sensitive binding of lung protein to rat catalase mRNA. *J Biol Chem* **267,** 2853.
43. Shi M.M., Godleski J.J. & Paulauskis J.D. (1995) Molecular cloning and posttranscriptional regulation of macrophage inflammatory protein-1α in alveolar macrophages. *Biochem Biophys Res Commun* **211,** 289.
44. Stoecklin C., Hahn S. & Moroni C. (1994) Functional hierarchy of AUUUA motifs in mediating rapid interleukin-3 mRNA decay. *J Biol Chem* **269,** 28 591.
45. Chen F.Y., Amara F.M. & Wright J.A. (1993) Mammalian ribonucleotide reductase R1 mRNA stability under normal and phorbol ester stimulating conditions: involvement of a cis–trans interaction at the 3′ untranslated region. *EMBO J* **12,** 3977.
46. Shaw G. & Kamen R. (1986) A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46,** 659.
47. Brewer G. (1991) An A+U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro. *Mol Cell Biol* **11,** 2460.
48. Malter J.S. (1989) Identification of an AUUUA-specific messenger RNA binding protein. *Science* **246,** 664.
49. Malter J.S. & Hong Y. (1991) A redox switch and phosphorylation are involved in the post-translational up-regulation of the adenosine-uridine binding factor by phorbol ester and ionophore. *J Biol Chem* **266,** 3167.