

Upregulation of expression of the chemokine receptor CCR5 by hydrogen peroxide in human monocytes

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THE objective of this study was to test the hypothesis that an oxidative stress can serve as a signal to regulate the expression of CCR5. When human monocytes were exposed to graded concentration of hydrogen peroxide (H₂O₂), CCR5 mRNA levels increased maximally at 4 h of exposure to 200 μM of H₂O₂ and decreased by 24 h of treatment. Pretreatment of monocytes with the NF-κB inhibitor BAY 11-8072 blocked the H₂O₂-induced augmentation of CCR5 mRNA expression, suggesting a role for this transcription factor in the regulation of CCR5 expression. CCR5 protein expression on the plasma membrane was also increased by treatment with H₂O₂, as assessed by flow cytometry. This was accompanied by enhanced responsiveness of H₂O₂-pretreated monocytes to the CCR5 ligand MIP-1β in terms of chemotaxis and *c-fos* gene activation. Our results suggest that oxidative stress may indeed modulate the expression of chemokine receptors and thus contribute to regulation of the inflammatory process.

Key words: Reactive oxygen species, Monocyte, Macrophage, *c-fos*, Chemotaxis

Introduction

Recruitment of inflammatory cells to a tissue site is a complex process that depends in part on the local expression of appropriate chemoattractant proteins termed chemokines. The interaction of chemotactic factors with their corresponding receptors on leukocytes constitutes the basis for chemotaxis to inflammatory sites and cell activation. In addition to the production of chemotactic factors, the level of expression of their receptors on target cells will determine the magnitude of the response. CCR5 is a G-protein-coupled receptor that binds the chemokines monocyte inflammatory protein (MIP)-1α, MIP-1β and RANTES.^{1,2} CCR5 is expressed on monocytes/macrophages, activated T cells and dendritic cells in lymphoid tissues, the ovaries and the lungs.³ It has been proposed to be implicated in several inflammatory diseases, such as rheumatoid arthritis, asthma and glomerulonephritis.^{4–7}

Inflammatory cells, such as macrophages and neutrophils, play an important role in host defense, but may also contribute to tissue injury through the release of reactive oxygen species (ROS).^{8–10} Increased production of ROS is now believed to be implicated in the pathogenesis of inflammatory disease such as acute respiratory distress syndrome, asthma, rheumatoid arthritis and AIDS.^{11–14} In addition, ROS are recognized as mediators of cell apoptosis.^{15–19} The role of the second messenger in

signal transduction requires small, non-toxic concentrations of ROS that can be produced by every cell type. ROS have been shown to affect the production of the chemokine interleukin (IL)-8 in dendritic cells and monocytes,^{20,21} monocyte chemotactic protein (MCP)-1 in monocytes and MIP-1α in the alveolar macrophages cell line NR8383.²² These effects may be mediated in part through the action of ROS on redox-sensitive proteins, such as the transcription factor nuclear factor (NF)-κB,^{23–29} or some upstream protein kinases.³⁰ NF-κB regulates many of the genes involved in the immune response, including IL-2, IL-6, IL-8, interferon-γ, tumour necrosis factor (TNF)-α, vascular cell adhesion molecule-1 and intracellular adhesion molecule-1.³¹ It is a heterodimer of 50 kDa (p50) and 65 kDa (p65) subunits and is found in the cytoplasm in an inactive form, bound to the inhibitory protein IκB through the p65 moiety.^{32,33} In response to various stimuli, including proinflammatory cytokines, IκB-α is first phosphorylated by IKK and then rapidly degraded by the proteasome, allowing translocation of NF-κB to the nucleus and subsequent gene activation.^{34,35}

The CCR5 gene promoter contains consensus binding sequences for the transcriptional activating factor NF-κB.³⁶ In this study, we investigated the hypothesis that ROS can increase the expression of the chemokine receptor CCR5 in human monocytes and thus contribute in the inflammatory process. We show here that hydrogen peroxide (H₂O₂), a cell membrane-permeable ROS, can indeed upregulate

CCR5 expression in monocytes and increase their responsiveness to the CCR5 ligand MIP-1 β .

Materials and methods

Chemical reagents

H₂O₂ and dimethyl-sulfoxide were from Fisher Scientific (Fair Lawn, NJ, USA). MIP-1 β was obtained from Pepro Tech (Rocky Hill, NJ, USA) and actinomycin D was from Merck Sharp & Dohme International (Rahway, NJ, USA). BAY 11-7082 was from Calbiochem (San Diego, CA, USA) and catalase was from Sigma-Aldrich Chemie (Steinheim, Germany).

Preparation of monocytes

Human venous blood from healthy medication-free volunteers was collected on citrate/dextrose/adrenaline. The peripheral blood mononuclear leukocytes were enriched by dextran sedimentation, layered over a Ficoll–Hypaque cushion and centrifuged at 400 \times g for 20 min. Mononuclear leukocytes were collected at the interface and washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS). Monocytes were then purified by adherence (60 min, 37°C) to the surface of plastic petri dishes coated with defibrinated autologous serum and removed with ethylenediamine tetraacetic acid (EDTA) (0.01 M) in RPMI 1640, 10% FBS. This was effective in enriching the cell population to greater than 90% monocytes, with a viability greater than 98% as assessed by Wright–Giemsa staining and trypan blue exclusion, respectively. Cells were resuspended in RPMI 1640, 10% FBS, at a final concentration of 2 \times 10⁶ cells/ml. Monocytes were left to rest overnight in polypropylene tubes to allow them to return to baseline status following initial activation by adherence. The medium was then removed by pipetting and replaced by fresh RPMI 1640 without FBS 1 h before stimulation with hydrogen peroxide. The 30% H₂O₂ stock was diluted in RPMI immediately before treatment. Cell viability after treatment was measured by trypan blue exclusion.

mRNA studies

After appropriate treatment, cells were pelleted in 15 ml polypropylene tubes and the total cellular RNA was isolated by acid guanidium thiocyanate–phenol–chloroform extraction according to Chomczynski and Sacchi.³⁷ RNA (10 μ g) was separated by electrophoresis on 1% agarose and transferred onto a Hybond-N (Amersham, Arlington Heights, IL, USA) membrane for northern analysis. The cDNA corre-

sponding to the whole coding sequence of human CCR5 was obtained as previously described³⁸ from genomic DNA of Raji cells using the primers 5'-GCTCTAGAGATTATCAAGTGTC-3' (sense), and 5'-GGGGTACCTCACAAGCCCACAGATATTTCTGCTCCC-3' (antisense). The *c-fos* cDNA probe was obtained from the American Type Culture Collection (Rockville, MD, USA). Control hybridizations were performed with the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe obtained from the American Type Culture Collection. The probes were labelled with a multiprime DNA labeling system (Amersham) using [³²P]dCTP (specific activity > 3000 Ci/mmol; Amersham). Membranes were prehybridized for 4 h in a mixture containing 120 mM of Tris, 600 mM of NaCl, 8 mM of EDTA, 0.1% sodium pyrophosphate, 0.2% sodium dodecyl sulphate, and 100 μ g/ml of heparin. Hybridization was performed overnight at 68°C in the same mixture in which the concentration of heparin was increased to 625 μ g/ml and dextran sulfate at 10% was added. The membranes were then washed once at room temperature for 20 min in 2 \times SSC (1 \times SSC: 0.15 M of NaCl, 0.15 M of sodium citrate, pH 7); once with 0.1 \times SSC, 0.1% sodium dodecyl sulphate at 68°C for 60 min, and then rinsed at room temperature with 0.1 \times SSC. The membranes were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA) with intensifying screens for 24 h at -80°C. Signal intensity was quantitated by densitometry using a UMAX PowerLook II scanner with the UMAX Magic Scan DA3.1 software and analysed with the NIH Image 1.61 software (Bethesda, MD, USA) on a Macintosh 7300/200 computer. Densitometric values are expressed as ratios of receptor/GAPDH or receptor/28s RNA densitometry quantification and normalized for control = 1.

Flow cytometry

The expression of CCR5 on the surface of monocytes was assessed with the monoclonal anti-CCR5 antibody (2D7) (PharMingen, Mississauga, Ontario, Canada) and anti-IgG2a antibody (PharMingen) at 2.5 μ g/ml, as previously described.³⁸ In brief, 3 \times 10⁵ treated cells were washed twice with PBS and labelled for 30 min at 4°C with anti-CCR5, washed with cold PBS and incubated for 30 min at 4°C with FITC-conjugated goat anti-mouse IgG (Bio/Can Scientific, Mississauga, Ontario, Canada). Finally, cells were washed and resuspended in PBS before flow cytometry analysis with a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). Results are expressed as the mean peak channel fluorescence for each treatment.

Chemotaxis assay

Monocyte chemotactic activity was performed in modified single-well Boyden chambers separated by 5 μ m pore size polycarbonate filters (Neuroprobe, Cabin John, MD, USA), as previously described.³⁸ MIP-1 β or medium was added to the lower chamber and 200 μ l of monocytes (6×10^5) in Gey's BSS (Gibco-BRL, Burlington, ON, Canada), supplemented with 2% bovine serum albumin, were added to the upper chamber. In chemokinesis assays, both chambers contained an equal concentration of MIP-1 β . After incubation for 2 h at 37°C, the filters were disassembled and the upper side of the filters were scraped free of cells. Cells on the lower side were removed with 5 mM of EDTA, centrifuged and counted.

Statistical analysis

Data were analysed for statistical significance using analysis of variance or Student's *t*-test for paired values, as appropriate. Differences were considered significant at $p < 0.05$. Results are expressed, when relevant, as means \pm SEM.

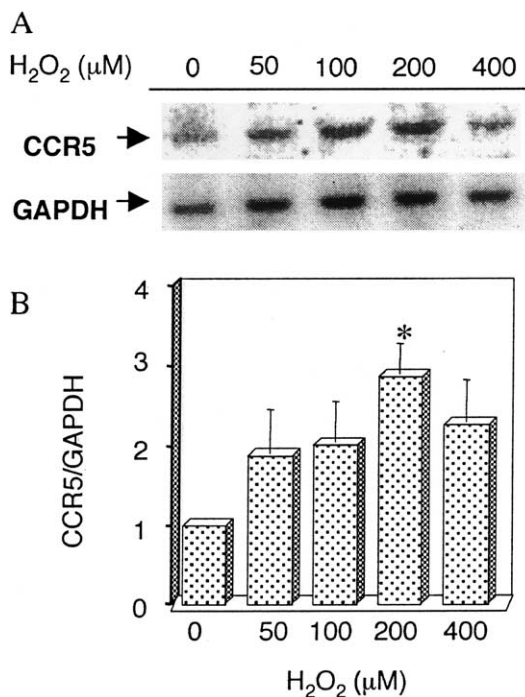


FIG. 1. Induction of CCR5 mRNA expression by H₂O₂ in human monocytes. Human monocytes were incubated for 4 h with graded concentrations of H₂O₂ in serum-free RPMI 1640 medium and total RNA was extracted and analysed by northern blotting for CCR5 mRNA expression. (A) Autoradiogram of one representative experiment. (B) Densitometric quantification of CCR5 mRNA normalized to GAPDH mRNA. The results represent the means \pm SEM of nine independent experiments. * $p < 0.05$, H₂O₂-treated versus control cells.

Results

Monocytes isolated from different blood donors were treated with graded concentrations of H₂O₂ for 4 h and mRNA levels encoding CCR5 were measured by northern blot analysis. As illustrated in Fig. 1, H₂O₂ induced a concentration-dependent augmentation of CCR5 mRNA expression in human monocytes with a maximum induction at 200 μ M of H₂O₂. Parallel experiments were performed to evaluate the effect of H₂O₂ on the expression of the chemokine receptor CXCR4 and the receptor for the lipid mediator, platelet-activating factor (PAFR). As illustrated in Fig. 2A, H₂O₂ also induced an increase in CXCR4 mRNA expression, albeit of a lesser magnitude, but had no effect on PAFR mRNA expression (Fig. 2B). In a time-course study, illustrated in Fig. 3, the effect of H₂O₂ on CCR5 mRNA levels was detectable as early as at 2 h of treatment, was maximal by 4 h and decreased by 24 h.

Increased mRNA expression is commonly controlled by a transcriptional or post-transcriptional mechanism. To determine whether H₂O₂ elevated CCR5 mRNA levels through transcriptional activation, monocytes were pretreated with Actinomycin D, a transcriptional inhibitor, for 15 min. The cells were then treated with medium or 200 μ M of H₂O₂ for 3 h. Total cellular RNA was then extracted and northern blot analysis was performed. As illustrated in Fig. 4, this treatment blocked the H₂O₂-induced accumulation of CCR5 mRNA, suggesting that H₂O₂-regulated CCR5 expression involves a transcriptional mechanism.

Since the transcription factor NF- κ B has been shown to be affected by H₂O₂ in some cell lines and in T lymphocytes,^{29,39} and because the CCR5 gene promoter contains consensus binding sequences for this transcription factor,³⁶ we examined

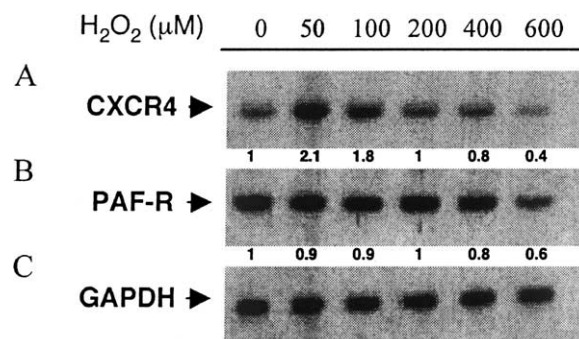


FIG. 2. Effect of H₂O₂ on the expression of CXCR4 and PAFR mRNA in human monocytes. Human monocytes were incubated for 4 h with graded concentrations of H₂O₂ in serum-free RPMI 1640 medium and total RNA was extracted and analysed by northern blotting for CXCR4 and PAFR mRNA expression. (A) Autoradiogram of northern blot hybridized with radiolabelled CXCR4 cDNA. The same membrane was rehybridized with radiolabelled PAFR (B) or GAPDH (C) cDNA.

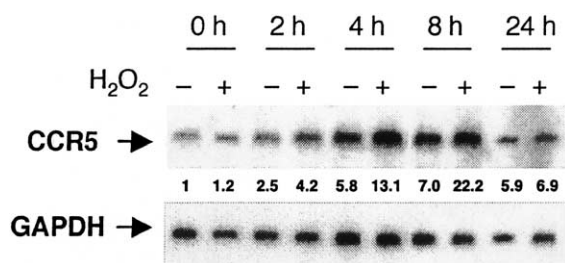


FIG. 3. Time course of H₂O₂-induced upregulation of CCR5 gene expression. Monocytes were incubated in the absence or presence of H₂O₂ (200 μM) for the indicated time periods followed by northern blot analysis of CCR5 gene expression. Total RNA was then extracted and analysed by northern blot for CCR5 and GAPDH gene expression. The results are representative of two independent experiments.

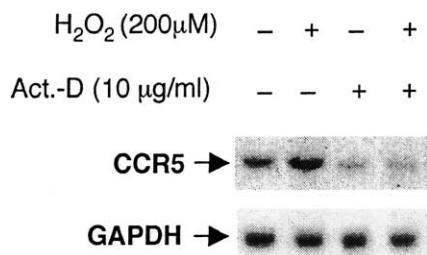


FIG. 4. Effect of Actinomycin D (Act-D) on CCR5 mRNA expression in response to H₂O₂. Monocytes were incubated in the absence or presence of Actinomycin D (10 μg/ml) for 15 min before addition of either medium or H₂O₂ for 3 h. Total RNA was then extracted and analysed by northern blot for CCR5 and GAPDH gene expression. The results are representative of two independent experiments.

the effect of an inhibitor of NF-κB on H₂O₂-induced expression of CCR5 mRNA. As shown in Fig. 5, CCR5 mRNA induction by H₂O₂ was largely inhibited following pretreatment with 20 μM of BAY 11-7082, an inhibitor of IκB-α phosphorylation. Thus, the stimulatory effects of H₂O₂ on CCR5 expression appears to be mediated, at least in part, through the activation of the NF-κB signalling pathway.

We then investigated whether H₂O₂-induced accumulation of CCR5 mRNA was associated with an increased expression of CCR5 protein on the cell

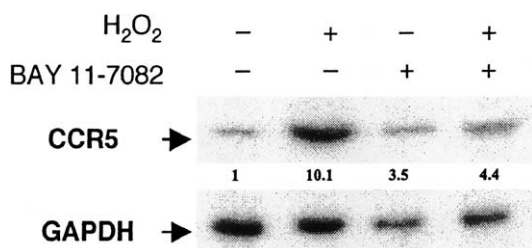


FIG. 5. Effect of BAY 11-7082 pretreatment on H₂O₂-induced CCR5 mRNA expression. Monocytes were incubated in the absence or presence of Bay 11-7082 (20 μM) for 30 min before addition of either medium or H₂O₂ (200 μM). Total RNA was extracted and analysed by northern blot for the expression of CCR5 and GAPDH mRNA. The results are representative of three independent experiments.

surface. Monocytes were treated for 4 h with either medium or 200 μM H₂O₂. Supernatants were then replaced with new medium and cells were incubated for 16 h. Flow cytometry studies were then performed using the 2D7 monoclonal anti-CCR5 antibody. As illustrated in Fig. 6, H₂O₂ treatment of monocytes induced a marked increase in membrane expression of CCR5, as compared with untreated cells (H₂O₂-treated monocytes, 6.11 ± 1.7 peak channel fluorescence versus control cells, 2.44 ± 0.6; *p* < 0.05).

To assess the specificity of action of H₂O₂, monocytes were treated with H₂O₂ in the presence or absence of catalase, which degrades H₂O₂ to H₂O and O₂. As expected, the H₂O₂-induced upregulation of CCR5 expression was prevented by catalase both at the mRNA and protein levels (Fig. 7A,B). Interestingly, however, catalase itself was found to increase the expression of CCR5.

We next investigated whether the H₂O₂-induced upregulation of CCR5 expression was associated with augmented responsiveness of treated monocytes to the CCR5 ligand MIP-1β. Receptor function was first evaluated for MIP-1β-induced chemotaxis; as shown in Fig. 8, monocytes pretreated with H₂O₂ showed enhanced responsiveness to the chemotactic action of MIP-1β. Chemokinetic activity in response to MIP-1β was not affected, however (data not shown). CCR5 function was also evaluated in terms of MIP-1β-induced *c-fos* expression. After a 4 h pretreatment with 200 μM of H₂O₂, followed by an incubation of 16 h in new medium, cells were treated for 30 min with 50 or 100 ng/ml of MIP-1β and the total RNA was

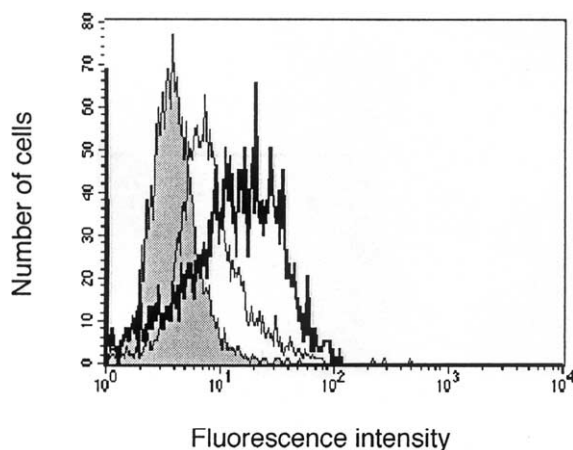


FIG. 6. Effect of H₂O₂ on CCR5 protein expression. Monocytes were treated with 200μM of H₂O₂ for 4 h in serum-free medium and centrifuged for 5 min at 1000 rpm. The medium was then replaced by new medium and the cells were incubated for 16 h. The medium was then removed by aspiration without centrifugation. Cells were labelled with either an irrelevant antibody (shaded) or anti-CCR-5 (2D7) antibody, followed by FITC-conjugated goat anti-mouse antibody. Cells were analysed by flow cytometry (untreated cells, thin line; H₂O₂-treated cells, thick line). A representative experiment of nine independent experiments is illustrated.

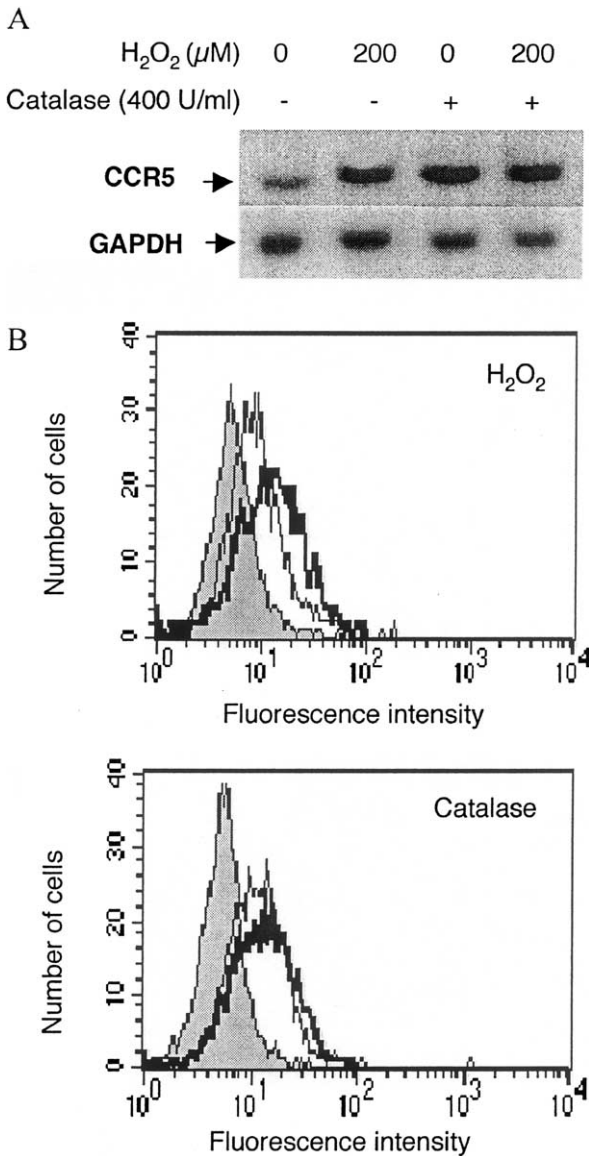


FIG. 7. Effect of catalase on CCR5 expression. (A) Monocytes were treated with 200 μ M of H₂O₂ in the absence or presence of catalase (400 U/ml) for 4 h in serum-free medium and total RNA was extracted and analysed by northern blotting for CCR5 mRNA expression. Autoradiogram of one representative experiment. (B) Monocytes were treated with 200 μ M of H₂O₂ in the absence or presence of catalase (400 U/ml) for 4 h in serum-free medium. The medium was then replaced following centrifugation by new medium and the cells were incubated for 16 h at 37°C. The medium was then removed by aspiration without centrifugation. Cells were labelled with either an irrelevant antibody (shaded) or anti-CCR-5 (2D7) antibody, followed by FITC-conjugated goat anti-mouse antibody. Cells were analysed by flow cytometry (untreated cells, thin line; H₂O₂-treated cells, thick line).

extracted for northern blot analysis. As illustrated in Fig. 9, *c-fos* mRNA expression was markedly increased in H₂O₂-pretreated monocytes following exposure with MIP-1 β , compared with untreated cells. These observations suggest that the increased expression of CCR5 following H₂O₂ treatment is associated with enhanced functional activities of the receptor.

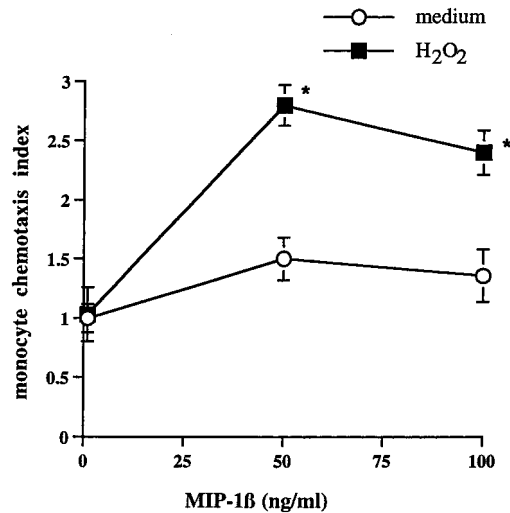


FIG. 8. Effect of H₂O₂ on monocyte chemotaxis. Monocytes were treated with either medium or 200 μ M of H₂O₂ for 4 h in serum-free medium. The medium was then replaced by new medium and the cells were incubated for 16 h at 37°C. Chemotactic activity was measured in response to MIP-1 β using a modified Boyden chamber chemotaxis assay. The figure illustrates the means \pm SEM of triplicate determinations of one representative experiment.

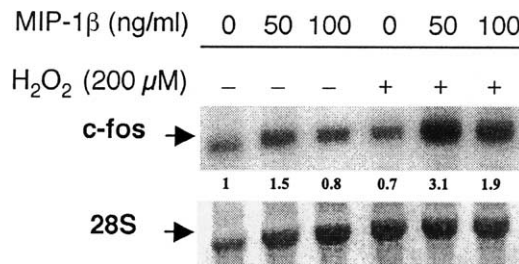


FIG. 9. Effect of H₂O₂ on MIP-1 β -induced *c-fos* expression. Monocytes were treated with either medium or 200 μ M of H₂O₂ for 4 h in serum-free medium. The medium was then replaced by new medium and the cells were incubated for 16 h at 37°C. MIP-1 β or medium were then added for 20 min and total RNA was extracted for northern blot analysis. The autoradiogram of one experiment representative of two independent experiments is shown.

Discussion

It is now accepted that a wide variety of cell types can respond to oxidative stress by the induction of multiples genes controlled by redox-sensitive proteins.⁴⁰ Several investigators have demonstrated that ROS can modulate gene expression of chemokines such as IL-8,⁴¹ MCP-1^{21,42} and MIP-1 α ,²² suggesting a role for ROS in the inflammation process. In this report, we demonstrated that externally applied H₂O₂, a cell-permeable oxidant, significantly increased mRNA expression of the chemokine receptors CCR5 and, to a lesser extent, CXCR4 in human monocytes. Similar findings were recently reported by Sacconi *et al.*,⁴³ who also found that the antioxidant pyrrolidinedithiocarbamate (PDTC) downregulated the expression of CCR5 and CXCR4.

We further extended our studies by showing that the upregulation of CCR5 by H₂O₂ was dependent on transcriptional activity and could be prevented by inhibition of NF- κ B activation. Moreover, the H₂O₂-induced augmentation of CCR5 mRNA levels was associated with enhanced CCR5 expression at the cell surface. We further demonstrated that these newly induced receptors were functional since pretreatment with H₂O₂ was associated with an augmented responsiveness of monocytes to MIP-1 β in terms of cell migration as well as *c-fos* gene activation.

The transcription factor NF- κ B is involved in the expression of a wide variety of genes controlling immune and inflammatory responses. Several lines of evidence suggest a role for ROS as a common and critical intermediate in NF- κ B activating signals. For example, the antioxidant PDTC or the overexpression of superoxide dismutase have been shown to be inhibitory for TNF-induced activation of NF- κ B in Jurkat T cells.^{27,44–47} It has also been shown that addition of H₂O₂ to specific cell types results in the appearance of the phosphorylated form of I κ B- α , whereas treatment with reducing agents such as dithiothreitol and β -mercaptoethanol enhances DNA-binding activity of NF- κ B itself.⁴⁸ Consistent with these reports, treatment with antioxidants or overexpression of antioxidant enzymes can block I κ B- α phosphorylation and degradation induced by lipopolysaccharide, TNF- α and phorbol-myristate acetate (PMA).³⁵ These observations suggest that redox regulation may affect more than one step in the NF- κ B activating pathway. Although the molecular mechanism of the redox regulation of NF- κ B is still unclear, signalling molecules such as protein kinases, which are involved in the dissociation of I κ B from the NF- κ B complex, may be targets of the redox regulation. Our study also suggests a role for the NF- κ B pathway in the effect mediated by H₂O₂ on CCR5 expression in monocytes. Since the pretreatment of cells with an I κ B- α phosphorylation inhibitor resulted in a partial inhibition of the H₂O₂-mediated induction of CCR5 mRNA, we can suppose that I κ B- α itself or some upstream factors, such as one or more protein kinases, could be the target for the oxidant. Moreover, the CCR5 gene promoter also contains consensus binding sequences for the transcriptional activating factor AP-1 and for such regulatory elements as STAT,³⁶ which have recently been shown to be redox sensitive.³⁰ These findings present the possibility that additional transcription factors might be implicated in the regulation of CCR5 expression by H₂O₂.

Regulation of chemokine receptors by reactive oxygen species may be important in the pathogenesis of several inflammatory diseases. ROS have been shown to be produced by immune cells after activation by proinflammatory cytokines, such as IL-1 and TNF- α .⁴⁹ ROS are also implicated in a number of

inflammatory diseases where expression of CCR5 ligands is enhanced, such as in rheumatoid arthritis, sepsis and autoimmune encephalomyelitis.^{50–53} Understanding the effect of ROS in the direct environment of inflammatory cells could lead to a better understanding of the mechanisms implicated in the progression of these diseases.

In conclusion, our results demonstrate that ROS in the extracellular environment of human monocytes can increase the expression of the chemokine receptor CCR5 through redox-sensitive transcription factors, such as NF- κ B. In an inflammatory disease context, activated phagocytic cells would release inflammatory mediators and ROS, such as H₂O₂. H₂O₂ accumulation in the environment of immune cells would then promote an increase in CCR5 expression on the cell surface of monocytes, leading to a greater response to the chemokines MIP-1 α , MIP-1 β and RANTES. This increased response to chemokines would extend the inflammatory process and possibly facilitate a greater accumulation of activated monocytes at the inflammatory site.

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