Dexamethasone inhibits ozone-induced gene expression of macrophage inflammatory protein-2 in rat lung

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Abstract To address the potential role of the chemokine macrophage inflammatory protein-2 (MIP-2) in airway inflammation, we examined whether MIP-2 may play a role in ozone-induced neutrophilic inflammation of airways and its modulation by dexamethasone in rat lung. Following ozone exposure, MIP-2 mRNA expression in the lung peaked at 2 h after exposure and slowly declined thereafter. Dexamethasone suppressed ozone-induced MIP-2 mRNA expression and neutrophil accumulation in the lung. We suggest that the MIP-2 mRNA induction may switch on the neutrophilic influx observed in this model of lung inflammation. Furthermore, the MIP-2 expression is regulated by dexamethasone which may represent one of the mechanisms by which glucocorticoids exert their potent anti-inflammatory properties.

Key words: Ozone; Rat lung; Inflammation; Northern blot; MIP-2 mRNA; Dexamethasone

1. Introduction

Acute ozone exposure of humans results in a significant decrease in lung function parameters and an increase in airway reactivity [1,2]. This effect has also been observed in dogs [3], guinea pigs [4] and rats [5]. Airway hyper-reactivity induced by ozone exposure is associated with epithelial injury [5–8] and an influx of inflammatory cells, mainly neutrophils [6,9,10]. Several studies have shown that neutrophils play a role in ozone-induced impairment of pulmonary function. In perfused rat lung, neutrophils potentiate the ozone-induced decrease in lung function and airway epithelial injury [5]. Furthermore, ozone-induced airway hyper-reactivity in dogs was prevented by neutrophil depletion with hydroxyurea [3]. Indeed, activated neutrophils are known to release reactive oxygen radicals and proteolytic enzymes, both of which are capable of producing airway reactivity [11] and tissue damage [12]. However, the mechanisms by which neutrophils are recruited in the lung are poorly understood.

Chemokines are a large group of chemotactic and proinflammatory cytokines [13,14]. They represents a family of polypeptides, containing four conserved cysteine residues, which can be subdivided into two large groups depending on whether or not there is an intervening amino acid between the two cysteines yielding the C-X-C (or the α) or C-C (or the β) families. Macrophage inflammatory protein (MIP-2) is a 6 kDa basic protein purified from the endotoxin-stimulated macrophage RAW 264.7 cell line [15]. The gene encoding MIP-2 has been cloned and the sequence shows the chemokine α subfamily of this

cytokine [16]. MIP-2 is a potent chemotactic agent for human polymorphonuclear leukocytes, and its in vivo injection results in neutrophil infiltration [15].

In this study, we investigate the effect of ozone exposure on the gene expression of MIP-2 mRNA in Brown–Norway rat lung and its modulation by glucocorticoids. We report that ozone exposure induces the expression of the chemokine MIP-2 mRNA, and that this expression may underlie the neutrophilic influx observed after ozone exposure. Furthermore, we show that the expression of MIP-2 mRNA is regulated by dexamethasone.

2. Materials and methods

2.1. Ozone exposure

Ozone was generated by passing laboratory air (1 l/min) through a Sander Ozoniser (Model IV; Sander GmbH, Germany). The output was diluted with compressed air (10 l/min) controlled by a gas flowmeter (Platon Flow Control, Basingstoke, UK) and fed into a 32 l box. The concentration of ozone was determined by using specific gas sampling tubes (Dragerwerk, AG, Germany) and was maintained at 1 or 3 ppm by regular measurement at the output port of the box. Pathogen-free Brown–Norway rats (250–350 g) were exposed to ozone for 2 or 6 h, and as a control, rats inhaled filtered air only.

2.2. Cell retrieval and counting

Rats were administered an overdose of pentobarbitone (100 mg/kg intravenously), and lungs were lavaged with 20 ml of saline through a polyethylene tube introduced through the tracheotomy. Bronchoalveolar lavage (BAL) fluid was centrifuged and the cell pellet was resuspended in 1 ml of Hanks' balanced salt solution. Using Kimura stain (dilution 1:10), total cell counts were made in a Neubauer chamber using light microscopy. Differential cell counts were performed on cytospin preparations stained by May–Grünwald stain. Cells were identified as macrophages, neutrophils, eosinophils, lymphocytes, basophils, and epithelial cells by standard morphology. After BAL, lung tissue was removed and stored at −70°C.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) and sequencing

For the preparation of MIP-2 cDNA, a Brown–Norway rat was administered lipopolysaccharide (*Escherichia coli*; Sigma; 100 μg intratracheally). Total cellular RNA from rat BAL cells recovered 4 h later was isolated according to the method of Chomczynski and Sacchi [17]. Total cellular RNA from BAL cells recovered from LPS-stimulated rat was reverse transcribed in a 20 μl reaction volume with 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂, 2 mM dNTP, 1 U RNasin (Promega, Madison), 17-base oligo-dT as primer and 7.5 U reverse transcriptase at 42°C for 60 min. PCR was performed on 1 μl of reverse transcriptase product as a final concentration of 1 × PCR buffer, 250 μM dNTP, 0.5 μM each of sense and antisense primers and 2.5 U *Taq* polymerase (Promega, Southampton, UK) in a total volume of 40 μl using a thermal cycler (Techne, Cambridge, UK). The primers were designed from the published sequences for murine MIP-2 cDNA [16] as follows: sense-5′GGCACAATCGGTACGATCCAG and anti-sense-5′ACCCTGCCAAGGGTTGACTTC. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: sense 5′TCCCTCAAGATTGTCAGCAA and antisense-5′AGATCCA-

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CAACGGATACATT [18]. The PCR reagents were overlaid with mineral oil and amplification was carried out through 25–30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extending at 72°C for 2 min. The PCR products were electrophoresed in 2% agarose gels to visualize the MIP-2 and GAPDH bands. The sizes of the PCR products generated were 287 bp for MIP-2 and 309 bp for GAPDH. The PCR product was excised and purified using Geneclean II (Stratech, Luton, UK). The purified products were used as cDNA probes for Northern blot analysis. The MIP-2 PCR product was also analysed by sequencing. Cycle sequence was performed on 100 ng of PCR product and 1 pmol of each primer. Cycling conditions were 30 s at 95°C, 30 s at 55°C and 30 s at 72°C using exo pfu– (Stratagene, Cambridge, UK). The purified product was used as cDNA probes for Northern blot analysis. Sequence analysis of MIP-2 cDNA was homologous to rat MIP-2 but not to other chemokines.

2.4. Northern blot analysis

The 287 bp MIP-2 PCR product and the 1272-bp PstI fragment from rat GAPDH cDNAs were labelled by random priming using [α-^{32}P]dCTP (3000 Ci/mmol; Amersham, UK). Total cellular or messenger RNA from rat lung or bronchoalveolar lavage cells were subjected to electrophoresis on a 1% agarose/formaldehyde gel and blotted onto Hybond-N membranes (Amersham, UK). The prehybridization and hybridisation were carried out at 42°C in buffer containing 5× Denhardt's solution, 5× standard saline citrate (SSC), 50 mM Na$_2$HPO$_4$, 0.1% sodium dodecyl sulfate (SDS), 250 μl/ml sonicated denatured salmon sperm DNA and 50% formamide. Each blot was washed to a stringency of 0.1 × SSC, 0.1% SDS for 20 min at 60°C and exposed at – 80°C for 1–7 days to Kodak X-OMAT S film. Autoradiographic bands were quantified by densitometric scanning (Quantity One software; PDI, New York, NY).

2.5. Statistics

Data are presented as mean ± S.E.M. Statistical analysis of results was performed by Mann–Whitney U-test for stepwise comparison. P values less than 0.05 were considered to be significant.

3. Results and discussion

Recruitment of inflammatory cells in the lungs may contribute to tissue injury as a result of mediators released from these cells. Ozone exposure induces airway hyper-reactivity which is associated with a flux of inflammatory cells, mainly neutrophils, into the lung and airways of human and animals [2,6,9,10]. The molecular mechanisms underlying these responses are poorly understood. The principal biological property of chemokines is their ability to attract and activate leukocytes [13,14]. In this study we investigated the gene expression of the chemokine MIP-2 following ozone exposure of BN rats and its modulation by dexamethasone.

Fig. 1. MIP-2 gene expression in lungs of Brown–Norway rats inhaling filtered air (CTRL) or exposed for 6 h to 3 ppm ozone (lanes 3–11). The lungs were removed at 2, 8 and 24 h after ozone exposure and assayed for MIP-2 mRNA expression. The top panel shows the Northern blot data with lung mRNA using the MIP-2 and the GAPDH cDNA probes. The estimated size for the MIP-2 mRNA transcript is 1.3 kb. The bottom panel represents the densitometric measurement of the Northern blot analyses. The level of MIP-2 mRNA expression was normalised to that of the GAPDH signal to account for differences in loading or transfer of the mRNA. Data are the mean ± S.E.M. of 3–5 observations.

MIP-2 has been identified as a secretory product of mouse macrophages [15,19]. MIP-2 is chemotactic for neutrophils in vitro and can elicit a localised neutrophilic inflammation when injected subcutaneously in mice or when given intracisternally in rabbits [15,20]. MIP-2 has been shown to be increased in rat
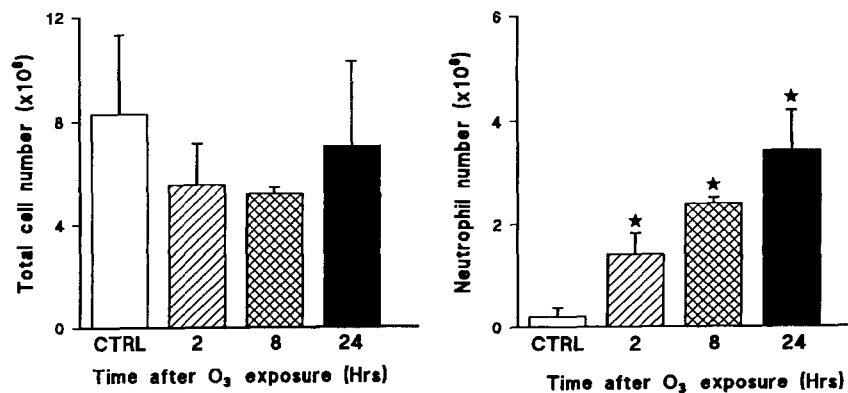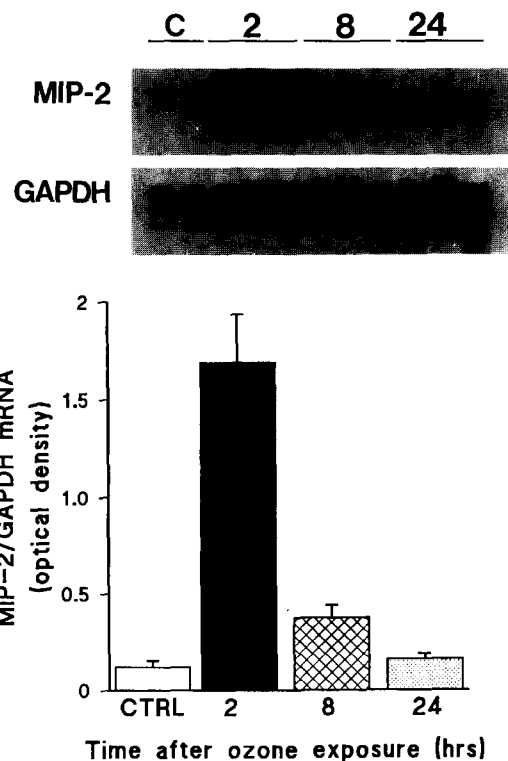

Fig. 2. Effect of ozone on total cell number (right panel) and neutrophils (left panel) recovered by bronchoalveolar lavage in Brown–Norway rats. After ozone exposure (3 ppm for 6 h), animals were lavaged at 2, 8 and 24 h after exposure. Control rats inhaled filtered air only (CTRL). Data are the mean ± S.E.M. of 3–5 animals. *P < 0.05 compared to controls.
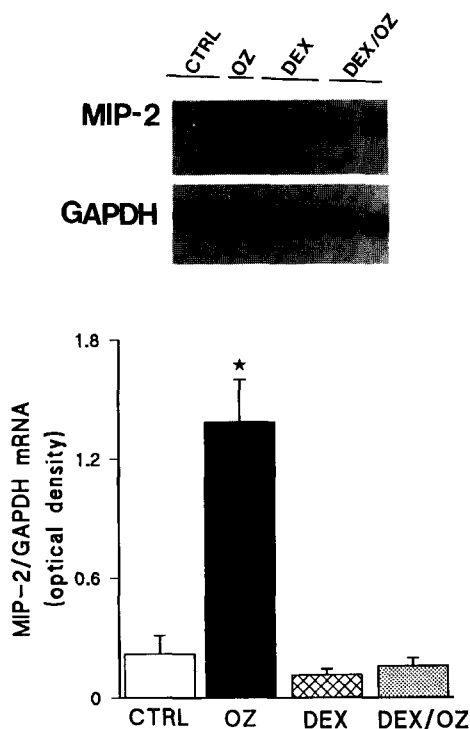
Fig. 3. Effect of dexamethasone on ozone-induced MIP-2 gene expression. Rats were exposed to filtered air (CTRL), to 3 ppm ozone for 6 h (OZ), or treated with dexamethasone (3 mg/kg i.p. 1 h prior to ozone (DEX/OZ) or filtered air exposure (DEX)). Lung mRNA was extracted 2 h after air or ozone exposure and assayed for MIP-2 mRNA expression. The top panel shows the Northern blot data using the MIP-2 and the GAPDH cDNA probes. The lower panel shows the mean densitometric measurements of the autoradiograms. Data are the mean ± S.E.M. of 5–8 observations. *$P < 0.05$ compared to controls.

Ozone-exposed rats also demonstrated a significant increase in neutrophil counts in BAL fluid without a significant change in total cell counts in BAL fluid (Fig. 2). The neutrophil recruitment was significantly increased at 2, 8 and 24 h post-ozone exposure. There was no significant difference in the number of macrophages, eosinophils, lymphocytes or epithelial cells recovered in BAL fluid in animals exposed to ozone or inhaling filtered air. The MIP-2 mRNA expression preceded the increase in neutrophils. The delay between the increase in MIP-2 mRNA and neutrophil recruitment may reflect the rate of MIP-2 protein turnover within the cells. These results suggest that MIP-2 may be responsible, at least in part, for neutrophil influx observed after ozone exposure. In support of this conclusion, it has been shown in a mouse model of inflammation induced by mycobacterial antigen that antiserum against MIP-2 was effective in reducing peritoneal neutrophilia [23]. The increase of MIP-2 mRNA at a time when there was a significant increase in neutrophil number might suggest the presence of additional stimuli for neutrophil recruitment. There are a number of factors, including MIP-1α and cytokine-induced neutrophil chemoattractant (CINC), which are thought to play a role in inflammatory cell recruitment [24,25]. In this respect, preliminary data from our laboratory suggest the induction by ozone of CINC mRNA in rat lung. Thus the contribution of other factors in ozone-induced neutrophil accumulation is not excluded.

There are several potential sources of MIP-2 in the lung. The most probable source is macrophages. Driscoll and Schlesinger [26] have shown that ozone stimulates alveolar macrophages to release a chemotactic activity for neutrophils. Furthermore, macrophages obtained from BAL fluid have been previously shown to express MIP-2 mRNA following LPS stimulation [27,28]. MIP-2 mRNA has been recently shown to be induced by tumour necrosis factor in rat alveolar macrophages and in rat fibroblasts and epithelial cell lines [21]. Using in-situ hybridization, Xu et al. [22] have demonstrated that following topical administration of IL-1β, MIP-2 mRNA is detected in mononuclear cells within the airway wall, with some contribution by epithelial cells. Thus, the macrophage and the airway epithelium are the most likely sources of increased expression we observed after in vivo exposure to ozone.

Glucocorticoids are the most potent and effective agents in controlling chronic inflammatory disease [29]. Glucocorticoids have been shown to modulate the transcription of several genes by binding to a specific DNA sequence termed glucocorticoid response elements (GREs) located in the promoter region of
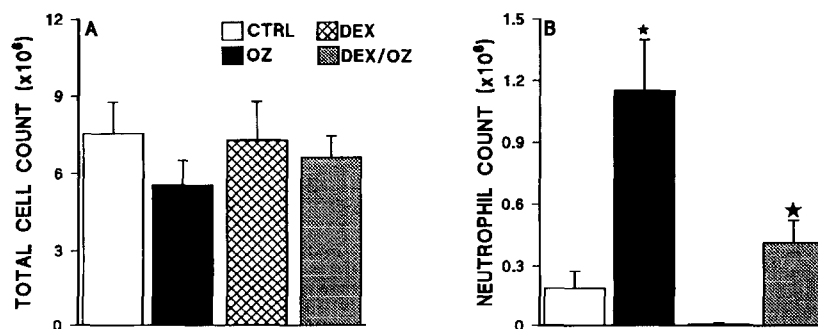
lung after mineral dust exposure [21] or interleukin 1β instillation [22].

In this study, we showed that ozone exposure of BN rats to 3 ppm for 6 h results in increased expression of MIP-2 mRNA (Fig. 1). The MIP-2 mRNA signal was barely detectable in RNA obtained from animals exposed to filtered air only. The expression peaked at 2 h after exposure and rapidly declined thereafter. A similar profile of MIP-2 expression was found in bronchoalveolar lavage cells (data not shown). The MIP-2 induction was also seen after a shorter exposure time (2 h) and lower (1 ppm) ozone concentration (data not shown).

Fig. 4. Effect of ozone and/or dexamethasone on the total cell number (A), and neutrophils (B) recovered by bronchoalveolar lavage in Brown–Norway rats. After ozone exposure (3 ppm for 6 h), animals were lavaged at 2 h after exposure. Control rats inhaled filtered air only (CTRL). There was a significant increase in neutrophils (compared to control) which was significantly inhibited by dexamethasone pre-treatment (compared to ozone). Data are the mean ± S.E.M. of 3–5 animals.

steroid-responsive target genes [29]. We have investigated whether the synthetic glucocorticoid, dexamethasone, could modulate the expression of MIP-2 mRNA. Dexamethasone alone had no effect on the level of MIP-2 mRNA expression. However, it significantly reduces the ozone-induced MIP-2 mRNA expression to the control level (Fig. 3). This inhibition was also reflected at the level of neutrophil influx into the lung (Fig. 4). Indeed, dexamethasone significantly reduced ozone-induced neutrophilia in bronchoalveolar lavage fluid without having any effect on the total cell number, eosinophils or macrophages recovered in BAL fluid. (Fig. 4).

In conclusion, we have shown that MIP-2 mRNA appears to be an important mediator in ozone-induced rat lung inflammation. We therefore suggest that the release of MIP-2 may be responsible, at least in part, for the recruitment of neutrophils observed in this model of ozone-induced lung inflammation. We have also demonstrated that the ozone-induced MIP-2 mRNA expression is regulated by steroids in vivo and this could represent one of the mechanisms by which glucocorticoids exert their potent anti-inflammatory properties.

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