EXPRESSION AND REGULATION OF MACROPHAGE INFLAMMATORY PROTEIN-2 GENE BY VANADIUM IN MOUSE MACROPHAGES

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Abstract—Environmental and occupational exposure to vanadium (V) dusts results in inflammation mainly confined to the respiratory tract. Macrophages apparently play an important role in mediating the inflammation via the production of many chemokines. In the current study, we investigated whether vanadium can regulate the gene expression of a CXC chemokine macrophage inflammatory protein-2 (MIP-2), and to determine the molecular mechanisms controlling MIP-2 gene expression. A mouse macrophage cell line RAW 264.7 was treated with sodium metavanadate (NaVO$_3$) at the dose of 0.5, 5, or 10 μg/ml V. Northern blot analysis showed that induction of MIP-2 mRNA expression was in a dose-dependent manner. To define the time course of the inflammatory response, RAW 264.7 cells were exposed to 5 μg/ml V, MIP-2 mRNA in macrophages increased markedly as early as 1 h after treatment, maximally induced at 4 h and reduced to 2-fold above control levels by 6 and 8 h. The protein levels of MIP-2 in conditioned media, measured by enzyme-linked immunosorbent assay (ELISA), was well correlated with the levels of MIP-2 mRNA following all of the treatments in the study. In addition, the increase in MIP-2 mRNA expression by vanadium was attenuated by co-treatment with the antioxidant N-acetylcysteine (NAC), at the doses of 10 and 20 mM, suggesting that the induction of MIP-2 mRNA is mediated via the generation of reactive oxygen species (ROS). To further investigate transcriptional regulation of the MIP-2 gene expression by vanadium, we performed RNA decay assay by measuring the half-life of MIP-2 mRNA. Co-treatment of macrophages with the transcriptional inhibitor actinomycin D at 5 μg/ml following exposure to 5 μg/ml V for 4 h revealed complete stabilization of vanadium-induced MIP-2 mRNA and no sign of mRNA degradation, at least, for 6 h, in comparison to the half-life of MIP-2 mRNA was approximately 2.5 h by bacterial lipopolysaccharide (LPS) treatment, supporting post-transcriptional stabilization as the predominant role of MIP-2 gene expression. In conclusion, these observations demonstrate that *in vitro* vanadium can induce MIP-2 mRNA expression, mediating, at least in part, via the production of ROS. In addition, the increase in

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MIP-2 mRNA level involves, most likely, post-transcriptional control via increased mRNA stability.

INTRODUCTION

Vanadium, a widely distributed and fairly abundant element in the Earth's crust, is a constituent of many ores, coals, and oils. Occupational exposure to vanadium is common in mining, petrochemical, steel and utilities industries (1, 2). The term "boilermakers' bronchitis" refers to the respiratory tract irritation in workers associated with vanadium exposure (3). Increased mining of vanadium-bearing ores and combustion of fossil fuels for energy production resulted in high levels of vanadium-containing particles or fumes in the environment (1–4). Therefore, it recently has received considerable attention in the context of occupational and environmental exposure. The lung is a primary site of contact with inhaled particles and microbes. Exposure to vanadium dusts has been well known to cause inflammation mainly confined to the airway and lung (1–4), but little is known about the mechanisms of vanadium-induced pulmonary inflammation.

Inflammation, which includes migration and activation of various immune cells such as neutrophils and mononuclear phagocytes, is a classic response to air pollutants, microbial pathogens or tissue injury. Recruitment of inflammatory leukocytes from blood to a site of injury depends on the expression of appropriate chemotactic cytokines termed "chemokines" (5–7). Because of their roles in lung host defense, alveolar macrophages (AMs) are thought to play a critical role in mediating many inflammatory responses via production of chemokines.

Interleukin-8 (IL-8), a CXC chemokine, appears to be the prominent neutrophil chemoattractant in humans. Previous work by our group and others have demonstrated that, macrophage inflammatory protein-2 (MIP-2), a member of CXC chemokine (6–10), acting as functional homologues in rodents for human, is a primary neutrophil chemotactic factor in the initiation and propagation of inflammatory process in rodent models (7, 8, 11, 12). To date, a clear correlation between MIP-2 mRNA expression and transition metal-induced pulmonary inflammation has been established for manganese (13). In order to identify the cellular and molecular control mechanisms underlying vanadium-induced lung inflammation, animal models have recently been developed to demonstrate that significant neutrophil influx into the lung was observed after vanadium exposure, preceding by increased MIP-2 mRNA levels in AMs (14). In addition, reactive oxygen species (ROS) synthesis by AMs was increased in response to vanadium exposure, which was mediated through activation of NADPH oxidase, suggesting ROS may play an important role in regulation of MIP-2 gene expression (15). However, increased MIP-2 mRNA levels in macrophages after vanadium

exposure *in vitro* have not been shown yet, and the mechanisms of regulation of MIP-2 gene expression in response to vanadium still remain poorly understood.

In the present study, because it is apparent that vanadium exist in well oxygenated blood, as well as body fluids in +5 oxidation state as VO_3^- (1, 16), we chose sodium metavanadate ($NaVO_3$) to investigate the regulation of MIP-2 gene expression by utilizing a mouse macrophage cell line RAW 264.7. Since ROS have been implicated in the control of MIP-2 gene expression, we used N-acetylcysteine (NAC), a synthetic antioxidant which can replenish intracellular glutathione levels (17), to deteremine if the induction of MIP-2 mRNA expression by vanadium was attenuated by antioxidants. Furthermore, we examined the protein levels of MIP-2 in supernatants obtained from all of the treatments in the study by using enzyme-linked immunosorbent assay (ELISA) to clarify if induction of MIP-2 mRNA expression subsequently resulted in secretion of MIP-2 protein. To extend these studies of MIP-2 gene regulation, we applied a commonly used RNA decay assay as previous described (18, 19) by measuring the half-life of MIP-2 mRNA to evaluate if the elevation of MIP-2 mRNA was modulated by transcriptional or post-transcriptional mechanisms.

MATERIALS AND METHODS

Cells and Culture Condition. The mouse macrophage cell line, RAW 264.7 was purchased from American Type Culture Collection (ATCC, Manassas, VA). The cell line has the properties of normal macrophages relative to phagocytosis (20, 21). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine and 10% fetal bovine serum. Cells were grown in a humidified incubator at 37°C with 5% CO_2.

Cell Treatment. Cells were plated in tissue culture plates (100 mm) at a density of 1×10^6 cells/ml in serum free DMEM for at least 4 h prior to addition of $NaVO_3$. For dose response study, cells were treated with $NaVO_3$ at the dose of 0.5, 5, or 10 μg/ml V, while in time course study, cells were exposed to 5 μg/ml V for 1, 4, 6, and 8 h. In addition, cells were treated with 10 μg/ml LPS for 4 h as a positive control. For antioxidant treatment, cells were incubated with 1, 10 or 20 mM NAC for 1 h, followed by the addition of 5 μg/ml vanadium (V).

RNA Extraction and Northern Blot Analysis. Total cellular RNA was prepared using a modified acid guanidinium thiocyanate-phenol/chloroform method (22) and quantified by absorbance at 260 nm. Total RNA (25 μg/lane) was denatured in 6.5% formaldehyde/50% formamide, fractionated on a 1.1% formaldehyde/1.2% agarose gels and transferred and UV cross-linked to a nylon membrane (Schleicher & Schuell, GmbH, Dassel, Germany). The filters were hybridized with a random-primed, Digoxigenin-labeled, 0.33 kb rat MIP-2 cDNA for 18 h at 65°C. After hybridization, blots were washed twice in 2× sodium chloride and sodium citrate buffer (SSC)/0.1% sodium dodecyl sulfate (SDS) at room temperature (RT), then in 0.1× SSC/0.1% SDS twice at 68°C. After 1× blocking solution was added to block reaction for more than 30 min at RT, the blots were incubated for 30 min with an anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer Mannheim GmbH, Germany). For signal detection, the blots were incubated in CSPD-ready-to-use solution (Boehringer Mannheim, GmbH, Germany) at 37°C for 15 min and then exposed the blots to X-ray film at RT. The same blots were stripped and subsequently rehybridized with a mouse β-actin cDNA (ATCC, Manassas, VA) as an internal control.
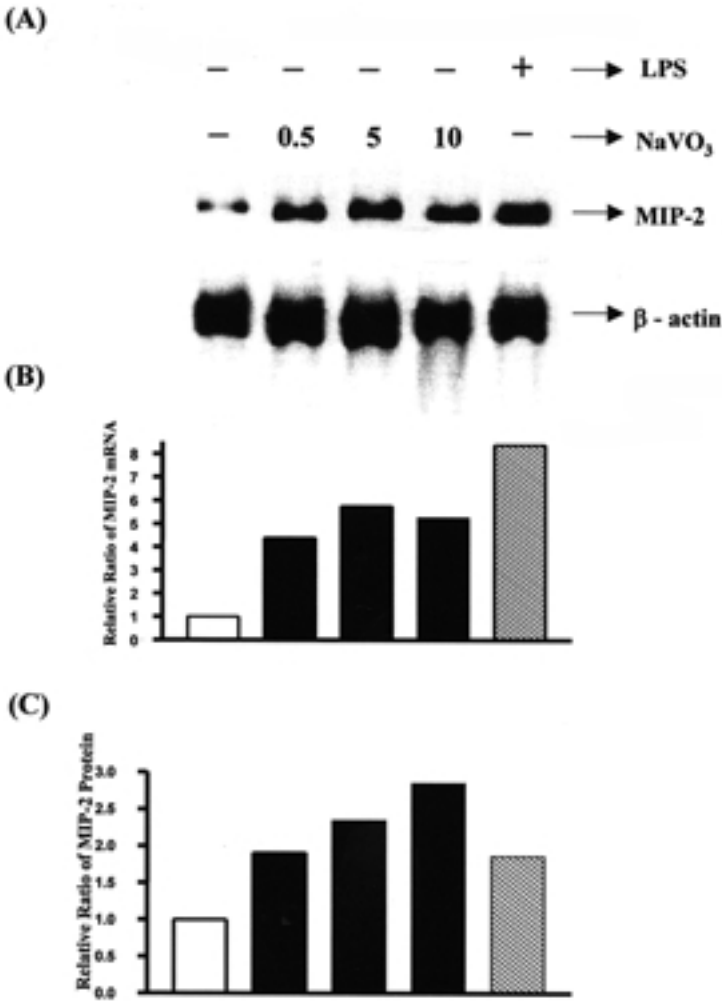
MIP-2-Specific Enzyme-Linked Immunosorbent Assay (ELISA). A double ligand rat MIP-2 specific ELISA (Biosource International, Camarillo, CA) was used to quantify extracellular immuno-reactive MIP-2. Briefly, neat and a 1/10 dilution of conditioned culture media (50 μl) obtained from all of the treatments in this study were added to flat-bottomed 96-well microtiter plates coated with MIP-2 antibody. After a 2 h incubation at 37°C, plates were washed four times with wash buffer followed by the addition of 50 μl/well of biotinylated rabbit anti-MIP-2 antibody and again incubated for 1 h at 37°C. Plates were washed four times, streptavidin peroxidase conjugate added and incubated an additional 30 min. The plates were washed again and chromogen substrates added for 30 min before stop solution was added. Plates were read at 450 nm in a 96-well plate reader (Millipore, Bedford, MA).

mRNA Half-Life Determination. Following treatment of RAW 264.7 cells with 10 μg/ml LPS (as a control) or 5 μg/ml V for 4 h, actinomycin D was added to the media to a final concentration of 5 μg/ml. Cells were sampled at times indicated through 6 h and levels of MIP-2 mRNA determined by Northern blot analysis. The integrated band values, as determined by densitometry, were normalized to β-actin mRNA and 100% mRNA was set at time 0 after actinomycin D treatment.

RESULTS

NaVO$_3$ Can Induce MIP-2 mRNA Expression In Vitro. To directly assess the role of vanadium on MIP-2 mRNA expression, RAW 264.7 cells were exposed to NaVO$_3$ at 0.5, 5, or 10 μg/ml V for 4 h under cell culture conditions as described in Methods. Northern blot analysis demonstrated that NaVO$_3$ could directly induce MIP-2 mRNA in RAW 264.7 cells in a dose-dependent manner (Fig. 1A, 1B), with no significant decrease in cell viability (>90%, data not shown). The MIP-2 mRNA induction was 4.4-fold over control levels at 0.5 μg/ml V and as high as 5.3-fold over control at 10 μg/ml V (Fig. 1A, 1B). Whereas, to evaluate the time course of vanadium effect on MIP-2 mRNA expression. RAW 264.7 cells were exposed to NaVO$_3$ for up to 8 h. Levels of MIP-2 mRNA were rapidly reduced as early as 1 h following exposure to 5 μg/ml V, maximally induced after 4 h and reduced but still 2-fold above control levels by 6 and 8 h (Fig. 2A, 2B). The induction of MIP-2 mRNA expression at 4 h following 5 μg/ml V exposure could be markedly attenuated by pretreatment with 10 mM NAC for 1 h (Fig. 2A, 2B).

NAC Attenuates the MIP-2 mRNA Expression in Response to NaVO$_3$. Steady-state mRNA levels encoding MIP-2 in RAW 264.7 cells were dramatically induced in response to 5 μg/ml V treatment for 4 h (Fig. 3A, 3B). To test the potential roles of ROS in NaVO$_3$-induced MIP-2 mRNA expression, RAW 264.7 cells were pretreated with 0, 1, 10 or 20 mM NAC for 1 h prior to exposure with 5 μg/ml V for 4 h. The low dose of NAC (1 mM) did not influence the expression of MIP-2 mRNA induced by NaVO$_3$, while both NAC at 10 or 20 mM significantly attenuated the NaVO$_3$-induced MIP-2 mRNA expression (Fig. 3A, 3B). The MIP-2 mRNA levels was approximately 5.7-fold higher than

(A)

| − | − | − | − | + | ⟶ LPS |

| − | 0.5 | 5 | 10 | − | ⟶ NaVO₃ |

⟶ MIP-2

⟶ β - actin

(B)

*Relative Ratio of MIP-2 mRNA*

(C)

Relative Ratio of MIP-2 Protein

Fig. 1. Induction of MIP-2 mRNA and secretion of MIP-2 protein levels in a mouse macrophage cell line, RAW 264.7, by increasing doses of NaVO₃. Cells were treated with 0.5, 5 or 10 μg/ml V for 4 h, while 10 μg/ml LPS was used as a positive control. (A) Northern blot analysis for MIP-2 and β-actin mRNA was performed. (B) Densitometric quantification of MIP-2 mRNA was normalized to β-actin and the corrected density from each dose point was further divided by that of normal control and presented as a relative ratio. (C) Protein levels of MIP-2 in conditioned media were divided by that of normal control and presented as a relative ratio. Results are representative of two independent experiments.

(A)

(B)

(C)
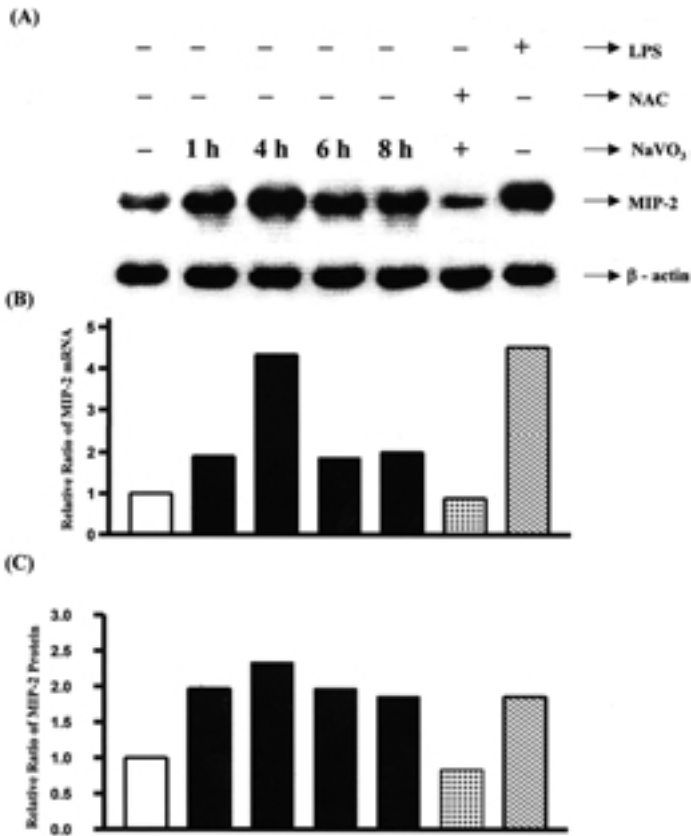
Fig. 2. Time course of MIP-2 mRNA induction and protein secretion in a mouse macrophage cell line, RAW 264.7, by NaVO₃. Cells were treated with control vehicle or 5 μg/ml V for 1, 4, 6, or 8 h, while 10 μg/ml LPS was used as a positive control. For antioxidant treatment, cells were incubated with 10 mM NAC for 1 h, followed by the addition of 5 μg/ml V for 4 h. (A) Northern blot analysis for MIP-2 and β-actin mRNA was performed. (B) Densitometric quantification of MIP-2 mRNA was normalized to β-actin and the corrected density from each time point was further divided by that of normal control and presented as a relative ratio. (C) Protein levels of MIP-2 in conditioned media were divided by that of normal control and presented as a relative ratio. Results are representative of two independent experiments.

the control levels following NaVO₃ treatment alone, but decreased to 0.9-fold over control with NAC pretreatment and NaVO₃ exposure (Fig. 3A, 3B).

Secretion of MIP-2 Protein Correlates with Expression of MIP-2 mRNA. RAW 264.7 cells were treated with 5 μg/ml V for up to 8 h and MIP-2 in conditioned culture media was measured by ELISA. NaVO₃ caused a dose-dependent induction of MIP-2 protein secretion (Fig. 1C), while mean MIP-2 protein levels

(A)

(B)

(C)
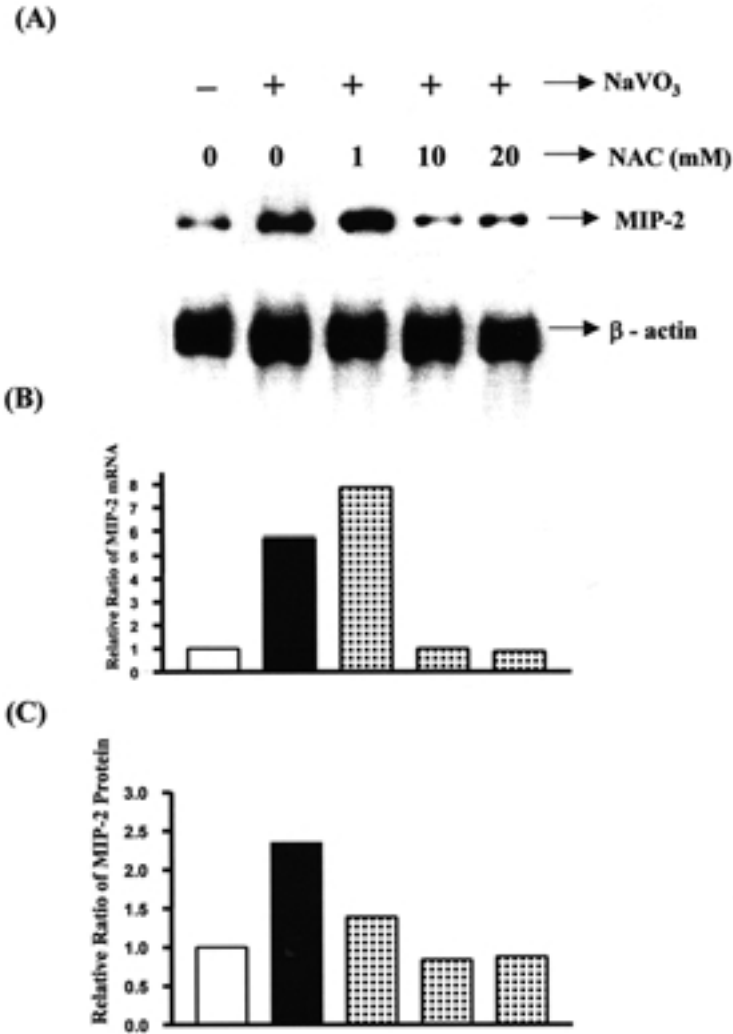
Fig. 3. Effect of NAC on suppressing the induction and protein secretion of MIP-2 mRNA by NaVO$_3$. Cells were pretreated with 0, 1, 10 or 20 mM NAC for 1 h prior to exposure with 5 μg/ml V for 4 h. (A) Northern blot analysis for MIP-2 and β-actin mRNA was performed. (B) Densitometric quantification of MIP-2 mRNA was normalized to β-actin and the corrected density from each dose point was further divided by that of normal control and presented as a relative control. (C) Protein levels of MIP-2 in conditioned media were divided by that of normal control and presented as a relative ratio. Results are representative of two independent experiments.

in the media were higher after 1 h treatment, significantly increased after 4 h, and diminished but still nearly 2-fold above control levels by 8 h (Fig. 2C). A clear correlation between MIP-2 mRNA expression and protein secretion after $NaVO_3$ exposure has been established very well both in dose response and time course studies (Fig. 1C, 2C). In fact, a very good correlation was also found between MIP-2 mRNA expression and protein secretion after all of the treatments. (Fig. 1C, 2C, 3C)

Increase of MIP-2 mRNA Expression by $NaVO_3$ Is Mediated Through Post-Transcriptional Stabilization. Increased mRNA expression may be modulated by transcriptional or post-transcriptional mechanisms. The possible contribution of changes in MIP-2 mRNA post-transcriptional stability to its increased expression was evaluated by measuring the half-life ($t_{1/2}$) of MIP-2 mRNA. We applied a commonly used RNA decay assay to determine the mechanisms through which $NaVO_3$ elevates MIP-2 mRNA expression. For better signal detection, RAW 264.7 cells were exposed to 10 μg/ml LPS (as a control) or 5 μg/ml V for 4 h, followed by 5 μg/ml actinomycin D to stop transcription. Cells were sampled through 6 h post-actinomycin D treatment and levels of MIP-2 mRNA quantified by Northern blot analysis (Fig. 4A). The integrated band values as determined by densitometry, were normalized to β-actin mRNA. In the presence of actinomycin D, MIP-2 mRNAs from both LPS control and $NaVO_3$-treated macrophages displayed divergent degradation kinetics. MIP-2 mRNA from LPS control cells decayed quickly with a $t_{1/2}$ of approximately 2.5 h, however, $NaVO_3$ treatment significantly stabilized the MIP-2 mRNA degradation, at least, for 6 h (Fig. 4B), indicating that the induction of MIP-2 mRNA by $NaVO_3$ involved, most likely, in post-transcriptional stability of MIP-2 mRNA transcripts.

DISCUSSION

Inflammatory changes in the upper and lower airways as well as pronounced reversible reductions in lung function are associated with environmental and occupational exposure to vanadium-containing particles. MIP-2, a primary CXC chemokine, can cause neutrophil chemotaxis and activation. Elevated MIP-2 mRNA expression has been implicated in the development of lung inflammation in a number of animals models, such as lung inflammation induced by bacterial endotoxin, virus, ozone, SO_2 and transition metal manganese (13, 23–27). Our observations in this study clearly demonstrate that sodium metavanadate is capable of inducing MIP-2 mRNA expression in a macrophage cell line. The levels of MIP-2 mRNA observed in response to vanadium exposure were dose-dependent, whereas in time course studies, rapid expression of MIP-2 mRNA was induced as early as 1 h after vanadium exposure, maximally to be expressed at
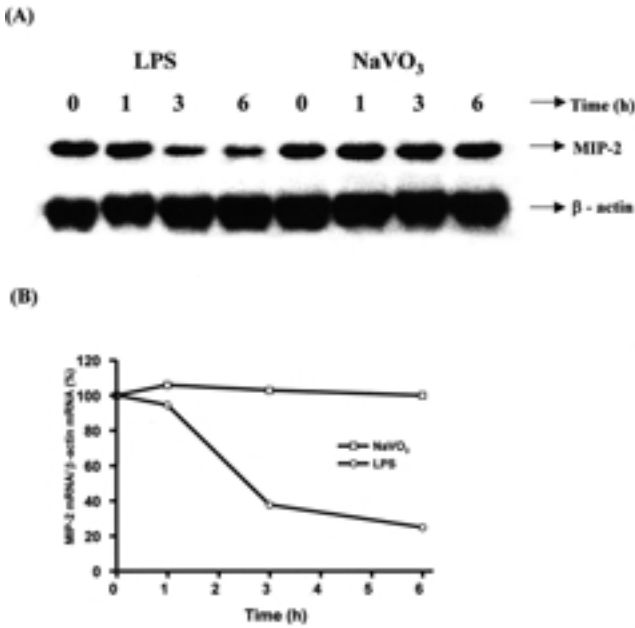
Fig. 4. Effect of NaVO₃ on the half-life of MIP-2 mRNA. (A) cells were treated with 10 μg/ml LPS (control), or 5 μg/ml V for 4 h. Actinomycin D was then added to a final concentration of 5 μg/ml, and at times indicated, total RNA was isolated and Northern blot analysis was performed. β-actin was used as an internal control. (B) Densitometric quantification of the decay of MIP-2 mRNA was normalized to β-actin mRNA from cells treated with LPS (——○——) or NaVO₃ (——□——). 100% mRNA was set at time 0 following actinomycin D treatment. Results are representative of two independent experiments.

4 h, and was low, but still 2-fold above control levels at 8 h, indicating MIP-2 may be an important mediator in vanadium-induced lung inflammation. In addition, the levels of MIP-2 protein in conditioned media from all these treatments correlated with MIP-2 mRNA expression very well, suggesting that expression of MIP-2 mRNA can reflect in MIP-2 protein translation and secretion.

Previous work from our lab provided evidence that intratracheal instillation of NaVO₃ resulted in a significant neutrophilia, whereas expression of MIP-2 mRNA was induced in bronchoalveolar lavage cells preceding and correlating with the extent of inflammation (14). To our knowledge, this is the first report demonstrating that vanadium can induce expression of MIP-2 mRNA in macrophages *in vitro*. The relationship between the metal toxicity and the respiratory tract depends on the nature of the offending agents, its physicochemical form, the dose, exposure condition and host factors (28). For this study, we chose sodium metavanadate as a representative member of vanadium compounds. The

choice was made based on the facts that within biological systems, extracellular vanadium is predominantly in +5 oxidation state as VO_3^- (vanadate, metavanadate) (1, 16).

Macrophages, when activated by a variety of inflammatory stimuli, are well known to release substantial quantities of oxidative metabolism products including superoxide anions, hydrogen peroxide and hydroxyl radicals (29, 30). An association between ROS and the upregulation of MIP-1α, a CC chemokine, and MIP-2, a CXC chemokine, mRNA expression in macrophages has been established (18, 23, 31). Previous work from our laboratory provided evidence that expression of MIP-1α and MIP-2 mRNA in a rat alveolar macrophage cell line induced by endotoxin or oxidative stress such as H_2O_2 and menadione, a quinone compound that undergoes redox cycling and generates ROS continuously (18), was attenuated by antioxidants like NAC or dimethyl sulfoxide (DMSO) suggesting that ROS may serve as a common signalling pathway to regulate gene expression of most, if not all chemokines, thereby initiating and propagating the inflammatory responses. In addition, recent reports directly implicated ROS production, mediating through activation of NADPH oxidase complex, was significantly increased in alveolar macrophages on exposure to metavanadate (15). Taken together, these observations suggested to us that, like many other inflammatory stimuli, vanadium-induced increase in MIP-2 mRNA expression and protein secretion in macrophages may be mediated by ROS. In support of this hypothesis, pretreatment of these cells with antioxidant NAC was able to suppress the expression of MIP-2 mRNA by vanadium in a dose-dependent fashion. This implied that vanadium may regulate the expression of MIP-2 chemokines by the production of ROS in macrophages.

To further distinguish between an increase in transcriptional rate and post-transcriptional stabilization of preexisting transcripts of MIP-2 by vanadium, we performed a commonly used RNA decay assay by measuring the half-life of MIP-2 mRNA. Our initial studies have indicated that co-incubation with actinomycin D, a DNA-primed RNA synthesis inhibitor, almost completely eliminated the increase of MIP-2 mRNA by LPS, suggesting that transcriptional regulation of MIP-2 gene is involved. In addition, LPS-induced elevation of MIP-2 mRNA has a half-life, similar to that of untreated cells, further supporting the concept of transcriptional activation, not mRNA stability, of MIP-2 gene expression (unpublished data). However, both enhancement of transcription and post-transcriptional stabilization are responsible for increased MIP-2 mRNA levels induced by H_2O_2 and menadione treatment (32), indicating that their regulation might be controlled differently by different stimuli.

In the present study, the half-life of LPS-induced (as a control) MIP-2 mRNA revealed a biphasic pattern, with a $t_{1/2}$ of around 2.5 h, followed by a plateau at around 30% of the initial levels, on the contrary, vanadium treatment significantly increased MIP-2 mRNA half-life and maintained the mRNA lev-

els at around 100% of initial mRNA levels, suggesting increased MIP-2 mRNA by vanadium involves, most likely, post-transcriptional stabilization. It was previously reported that instability of many chemokines may be attributed to the presence of multiple copies of the reiterated AUUUA motifs within the 3′-untranslated region of their mRNAs (33). A cytoplasmic protein, adenosine-uridine binding factor (AUBF), binds specifically to AUUUA motifs within the 3′-untranslated region of many cytokine, chemokine, oncogene, and growth factor mRNAs, substantially stabilizing these transcripts and increasing their half-lives (34–36). The binding of AUBF to RNA transcripts appears to be redox sensitive and increases with oxidative stress, thereby increasing the half-life of mRNA transcripts (37). Thus, redox state is crucial in the control of redox-sensitive protein, and subsequently influence a spectrum of chemokine gene expression. Sequence analysis of the MIP-2 cDNA 3′-UTR also revealed multiple copies of the ATTTA motifs, which may explain why the half-life of MIP-2 mRNA increased after vanadium treatment. However, the MIP-2 mRNA expression was not subject to mRNA stability control, at least, by LPS treatment in our previous study, implicating it is unlikely that simple presence of AUUUA motifs can determine whether MIP-2 mRNA half-life will be influenced in response to an inflammatory stimulus. The mechanisms responsible for the differential regulation of MIP-2 mRNA half-lives by different stimuli deserves further investigation.

In summary, the present study demonstrates that vanadium can induce MIP-2 mRNA expression in a dose-dependent manner *in vitro*. Whereas, the levels of MIP-2 mRNA are induced maximally at 4 h in time course study and the production of MIP-2 protein are well correlated with the expression of MIP-2 mRNA after all of the treatments in this study. The induction of MIP-2mRNA expression by vanadium is attenuated by antioxidant NAC, suggesting ROS serve as a mediator for chemokine expression. Additionally, vanadium treatment completely abolishes MIP-2 mRNA degradation, at least, in the initial 6 h, indicating increased mRNA expression is modulated, most likely, by post-transcriptional control via increased mRNA transcript stability.

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