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Expression and regulation of the macrophage inflammatory protein-1alpha gene by nicotine in rat alveolar macrophages

Abstract

Cigarette smoking causes inflammation mainly confined to the airway and lung. Nicotine is one of the primary constituents in cigarette smoke. Alveolar macrophages apparently play a pivotal role in mediating pulmonary inflammation via the production of chemokines. Macrophage inflammatory protein-1alpha (MIP-1alpha), a member of CC chemokines, has been shown to contribute to

monocyte/macrophage and neutrophil chemotaxis and activation. Our previous work demonstrated that MIP-1alpha mRNA expression in macrophages is induced by a variety of stimuli. In the present study, we further investigate whether nicotine can regulate the gene expression of MIP-1alpha in macrophages and determine the mechanism leading to increased expression. A rat alveolar macrophage (RAM) cell line, NR8383, was treated with nicotine at a dose of 3.1, 31, 310 muM, or 3.1 mM. Northern blot analysis showed that the induction of MIP-1alpha mRNA expression was dose-dependent. To define the time course of the inflammatory response, RAM cells were exposed to 31 muM nicotine, MIP-1alpha mRNA was induced as early as 1 h after treatment, was maximally expressed at 4 and 6 hours, and reduced by 8 hours. Western blot analysis demonstrated a single band with an estimated molecular weight of 10 kD for MIP-1alpha which was induced after nicotine treatment, suggesting that expression of MIP-1alpha mRNA could reflect in protein synthesis. In addition. the increase in MIP-1alpha mRNA expression induced by nicotine was attenuated by co-treatment with the antioxidant N-acetylcysteine (NAC), at doses of 10 and 20 mM, suggesting that the induction of MIP-1alpha mRNA is mediated via the generation of reactive oxygen species (ROS). To further investigate transcriptional regulation of the MIP-1alpha gene expression, RAM cells were exposed to nicotine. MIP-1alpha mRNA levels were significantly increased in nuclear RNA preparations, indicating that transcriptional activation is involved in increased expression of MIP-1alpha mRNA. Moreover, we performed RNA decay assay by measuring the half-life of MIP-1alpha mRNA. Treatment of RAM cells with the transcriptional inhibitor actinomycin D following exposure to nicotine revealed that the half-life of MIP-1alpha mRNA was markedly increased by nicotine treatment, supporting a role of post-transcriptional stabilization in MIP-1alpha gene expression. These observations indicate that nicotine can induce MIP-1alpha mRNA expression and protein synthesis in RAM cells, mediating, at least in part, via the generation of ROS. In addition, the increase in MIP-1alpha mRNA level involves, both transcriptional activation and post-transcriptional stabilization.

Full text

INTRODUCTION

Cigarette smoking is the most clearly recognized factor leading to the development of chronic obstructive pulmonary disease (COPD) [1-3]. Several

studies suggest that lung injury in smokers is due to the effect of prolonged low-grade inflammation, and especially to the imbalance between proteases and antiproteases or oxidants and antioxidants in the lung [4, 5]. In bronchoalveolar lavage (BAL) studies, increased numbers of neutrophils, mononuclear phagocytes and eosinophils have been demonstrated in lavage fluid from smokers. These inflammatory cells are thought to produce large amount of proteases and oxidants, thus contributing to the subsequent lung injury [6, 7]. In addition, because of their roles in lung host defense, alveolar macrophages (AMs) are considered to be an important target for inhaled irritants and toxins.It has been proposed that the influx of these inflammatory cells may occur because of chemotactic factors generated in the AMs in response to cigarette smoke.

The chemokine superfamily can be divided into two subsets based on the position of the first two cysteines at the N-terminus: CXC chemokines contain a single amino acid between the first two cysteines, and CC chemokines which have two adjacent cysteines [8-10]. Interleukin-8 (IL-8), a CXC chemokine, appears to be a prominent neutrophil chemoattractant [11]. High concentrations of IL-8 have been demonstrated in sputum, serum and BAL fluid from patients with COPD [12-15]. Whereas macrophage inflammatory protein-1alpha (MIP-1alpha), a CC chemokine, has been suggested to be another primary mediator inducing pulmonary inflammation. Previous work from our group and others has indicated that MIP-1alpha is a potent monocyte/neutrophil chemotactic factor in the initiation and propagation of the inflammatory process both *in vitro* and *in vivo* [16]. Expression of MIP-1alpha mRNA has been reported to be upregulated in AMs in response to a variety of stimuli, including bacterial endotoxin, oxidative stress, and transition metals vanadium and manganese [17-19]. However, whether the expression of MIP-1alpha mRNA in AMs is induced by cigarette smoke has not yet been shown.

There has been extensive research on how smoking damages the lung. Most of the discussion up to this point has dealt with the cellular basis of lung inflammation on exposure to cigarette smoke. However, little is known about the molecular control mechanisms underlying this disease. A large number of components, more than 4,000, are present in cigarette smoke. Nicotine is recognized to be the major inducer of tobacco dependence and the main chemical constituent of cigarette smoke [20, 21]. Although nicotine itself is not carcinogenic, whether nicotine is sufficient to contribute to COPD in human

exposure to cigarette smoke is still unknown. To date, only a few reports have investigated whether nicotine can induce the production of inflammatory mediators, and the results have been inconclusive and contradictory [22-27]. In the present study, we chose a rat alveolar macrophage (RAM) cell line (NR8383) to investigate the regulation of MIP-1alpha gene expression in response to nicotine. Since reactive oxygen species (ROS) have been implicated to be a common signal regulating both CC and CXC chemokines, we used N-acetylcysteine (NAC), a synthetic antioxidant which can replenish intracellular glutathione levels, to determine if the induction of MIP-1alpha mRNA expression by nicotine was attenuated by antioxidants. Furthermore, we characterized MIP-1alpha protein in cell lysates using Western blot analysis to find out if induction of MIP-1alpha mRNA expression is subsequently reflected in synthesis of MIP-1alpha protein. To confirm whether the increase of in MIP-1alpha mRNA levels was mediated through transcriptional activation, we designed a new transcriptional activity assay for quantitative determination of MIP-1alpha mRNA in nuclear extracts because synthesis and maturation of mRNA occur exclusively in the nucleus. Moreover, we applied a commonly used RNA decay assay as previous described [19], measuring the half-life of MIP-1alpha mRNA to clarify whether the expression of MIP-1alpha mRNA was modulated *via* post-transcriptional control.

MATERIALS AND METHODS

Materials

Polyclonal antibody to MIP-1alpha was obtained from Biosource International (Camarillo, CA, USA). If not specified, other materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cells and culture conditions

The RAM cell line, NR8383, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line has the properties of normal macrophages relative to phagocytosis, respiratory burst and cytokine release. Cells were cultured in RPMI-1640 medium containing 5% fetal equine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mug/ml streptomycin. Cells were grown in a humidified incubator at 37° C, with 5% CO_2.

Cell treatment

Tissue culture plates (100 mm) were precoated with polyhydroxymethylacrylate to prevent cell adhesion as previously described [18]. Cells were plated at a density of 1 x 10^6 cells/ml in serum-free RPMI for at least 4 hours prior to addition of nicotine. For the dose response study, cells were treated with nicotine at the dose of 3.1, 31, 310 muM, or 3.1 mM, while in the time course study, cells were exposed to 31 muM nicotine for 1, 4, 6, and 8 hours. In addition, cells were treated with 10 mug/ml LPS for 4 hours as a positive control. For antioxidant treatment, cells were incubated with 1, 10, or 20 mM NAC for 1 hour, followed by the addition of 31 muM nicotine.

RNA extraction

Total cellular RNA was isolated from RAM cells using a modified acid guanidinium thiocyanate-phenol/chloroform method [28], and quantified by absorbance at 260 nm.

Northern blot analysis

Total RNA (25 mug/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, 0.18 Kb rat MIP-1alpha cDNA for 18 hours at 65° C. After hybridization, blots were washed twice in 2 x sodium chloride and sodium citrate buffer (SSC)/0.1% sodium dodecyl sulfate (SDS) at room temperature (RT), then in 0.1 x SSC/0.1% SDS twice at 68° C. After 1 x blocking solution was added to block the 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, Dassel, Germany). For signal detection, the blots were incubated in CSPD-ready-to-use solution (Boehringer Mannheim GmbH, Dassel, Germany) at 37° C for 15 min and then exposed to X-ray film at RT. The same blots were stripped and subsequently rehybridized with a mouse beta-actin cDNA (ATCC, Manassas, VA, USA) as an internal control.

Western blot analysis

After treatment, RAM cells were lysed in a buffer containing 1% (W/V) Triton X-100, 5 mug/ml leupeptin, 2 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, and 0.5 unit/ml aprotinin in phosphate-buffered saline (PBS). The lysed cells were scraped, rapidly frozen in liquid nitrogen, and sonicated for 30 sec at maximum potency. The extracts were then centrifuged for 10 min at 13,000 rpm at 4° C. After determining the protein concentration in supernatants, 20 mug of total protein was resolved using 10% SDS-polyacrylamide gel electrophoresis (PAGE) before being transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Blotted filters were probed with MIP-1alpha polyclonal antibody (25 mug/ml), and a secondary alkaline phosphatase-conjugated goat anti-mouse IgG (1:3000, Bio-Rad, Hercules, CA, USA). The MIP-1alpha protein was visualized using a colorimetric reaction when the substrate nitroblue-tetrazo-lium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) were added (Promega, Mandison, WI).

Transcriptional activity assay

RAM cells were treated with 31 muM nicotine for 6 hours. Cells were harvested and washed in PBS. Pelleted cells were resuspended in buffer containg 10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; and 0.5 mM dithiothreitol (DTT), maintained on ice, and lysed by being rapidly and repeatedly drawn through a 26-gauge needle [29]. After centrifugation, the nuclear and cytoplasmic extracts were separated, the nuclear pellet was washed with PBS, and the RNA was purified with Isogen (Nippon Gene Co., LTD. Toyoma, Japan) in accordance with the manufacturer's protocol. The expression of MIP-1alpha mRNA in the nuclear and cytoplasmic extracts was subsequently quantified by Northern blot analysis as described above.

Determination of mRNA half-life

Following treatment of RAM cells with 10 mug/ml LPS (as a control) or 31 muM nicotine for 4 h, actinomycin D was added to the medium to a final concentration of 5 mug/ml. Cells were sampled at times throughout the 9 hours as indicated and levels of MIP-1alpha mRNA were determined by Northern blot analysis. The integrated band values, as determined by densitometry, were normalized to beta-actin mRNA, and 100% mRNA was set at time 0 after actinomycin D treatment.

RESULTS

*Nicotine induces MIP-1*alpha *mRNA expression in a dose-dependent fashion*

To directly assess the role of nicotine in MIP-1alpha mRNA expression, RAM cells were exposed to nicotine at 3.1, 31, 310 muM, or 3.1 mM for 4 hours under the cell culture conditions described in Methods. Northern blot analysis demonstrated that nicotine could directly induce MIP-1alpha mRNA expression in RAM cells in a dose-dependent fashion (Figure 1A), with no significant decrease in cell viability (> 95%, data not shown). The MIP-1alpha mRNA induction was 2-fold greater than the control at 3.1 muM nicotine, 4-fold greater than the control at 31 muM nicotine, up to 6-fold at 310 muM nicotine, and as high as 9-fold greater than the control at 3.1 mM nicotine (Figure 1B).

*Nicotine induces MIP-1*alpha *mRNA expression in a time course manner*

To evaluate the time course of the effect of nicotine on MIP-1alpha mRNA expression, RAM cells were exposed to nicotine for up to 8 hours. Levels of MIP-1alpha mRNA were rapidly induced as early as 1 hour following exposure to 31 muM nicotine, maximally induced after 4 and 6 hours, and reduced by 8 hours (Figures 2A, 2B). The induction of MIP-1alpha mRNA expression at 4 hours following exposure to 31 muM nicotine could be partially attenuated by pretreatment with 10 mM NAC for 1 h (Figures 2A, 2B).

*Nicotine induces MIP-1*alpha *protein synthesis with a molecular weight of 10 kD*

RAM cells were treated with 31 muM nicotine for up to 6 hours, and MIP-1alpha proteins in cell lysates were identified by Western blot analysis. A single band with an estimated molecular weight of 10 kD for MIP-1alpha was induced after nicotine treatment (Figure 3). Moreover, it looked to be a trend that the protein was synthesized maximally at 6 hours after nicotine treatment, which correlated with the expression of MIP-1alpha mRNA. In addition, pretreatment with NAC could attenuate the production of MIP-1alpha protein (Figure 3).

*NAC attenuates the MIP-1*alpha *mRNA expression in response to nicotine*

Steady-state mRNA levels encoding MIP-1alpha in RAM cells were induced in response to the 31 muM nicotine treatment for 4 hours (Figure 4A). To test the potential roles of ROS as a common signal in nicotine-induced MIP-1alpha mRNA expression, RAM cells were pretreated with 0, 1, 10 or 20 mM NAC for 1 hours before challenge with 31 muM nicotine for 4 hours. The low dose of NAC (1 mM) hardly affected the expression of MIP-1alpha mRNA induced by nicotine, while both 10 and 20 mM NAC did (Figures 4A, 4B).

*Increase in MIP-1*alpha *mRNA expression induced by nicotine involves transcriptional activation*

Increased mRNA expression is commonly controlled by transcriptional or post-transcriptional mechanisms. To confirm the role of transcriptional activation in the regulation of MIP-1alpha mRNA expression, RAM cells were treated with 31 muM nicotine for 6 hours. Cell nuclei were extracted and the transcription rates for the MIP-1alpha gene were determined as described in Methods. At 6 hours, the expression of MIP-1alpha mRNA was markedly increased in whole cell and nuclear RNA preparations (Figures 5A, 5B) indicating that induction of MIP-1alpha mRNA by nicotine is, most likely, caused by transcriptional activation of the gene. Moreover, we demonstrated that the expression of MIP-1alpha mRNA in cytoplasmic RNA preparations was also increased, albeit to a lesser extent, (Figures 5A, B) suggesting that a post-transcriptional mechanism may also be responsible for this induction.

*Increase in MIP-1*alpha *mRNA expression by nicotine is mediated through post-transcriptional stabilization*

The possible contribution of the stability of MIP-1alpha mRNA transcripts to its increased expression was assessed by measuring the half-life ($t_{1/2}$) of MIP-1alpha mRNA. For better signal detection, RAM cells were treated with 10 mug/ml LPS (as a control) or 31 muM nicotine for 4 hours followed by 5 mug/ml actinomycin D, a DNA-primed RNA synthesis inhibitor, to stop transcription. Cells were sampled throughout the 9 hours, post-actinomycin D treatment and levels of MIP-1alpha mRNA were quantified by Northern blot analysis (Figure 6A). The integrated band values as determined by densitometry, were normalized to beta-actin mRNA. In the presence of actinomycin D, MIP-1alpha mRNAs from both LPS (control) and nicotine-treated RAM cells displayed divergent degradation kinetics. MIP-1alpha mRNA from LPS-treated cells decayed quickly,

with a $t_{1/2}$ of approximately 2 hours, however, nicotine treatment significantly stabilized the MIP-1alpha mRNA degradation with a $t_{1/2}$ of about 5 hours (Figure 6B), suggesting that the induction of MIP-1alpha mRNA by nicotine is involved, in part, in the post-transcriptional stability of MIP-1alpha mRNA transcripts.

DISCUSSION

MIP-1alpha, a primary CC chemokine, has been proved to be a potent chemoattractant both for neutrophils and monocytes/macrophages. The functional roles of MIP-1alpha, and its importance in neutrophil/monocyte recruitment during inflammation have been supported indirectly by the demonstration of increased expression of MIP-1alpha mRNA within injured lung [17-19] and characterized directly using recombinant MIP-1alpha protein in both *in vitro* and *in vivo* studies [16]. In the present study, we investigated whether nicotine can regulate the gene expression of MIP-1alpha in AMs, and we tried to elucidate the underlying mechanisms of the induction. Our observations clearly demonstrate that nicotine is capable of inducing MIP-1alpha mRNA expression in an alveolar macrophage cell line. The levels of MIP-1alpha mRNA observed in response to nicotine were dose-dependent (Figure 1), in addition, in the time-course study, rapid expression of MIP-1alpha mRNA was induced as early as 1 hour after nicotine treatment, maximally expressed at 4 and 6 hours and reduced by 8 hours (Figure 2), indicating MIP-1alpha may be an important mediator in nicotine-induced lung inflammation.

To our knowledge, this is the first report demonstrating that nicotine can induce expression of MIP-1alpha mRNA in AMs *in vitro*. Nicotine, a tertiary amine composed of a pyridine and a pyrrodine ring, is the most widely use addictive alkaloid in the world [20, 21]. It reaches plasma concentrations of 50-500 ng/ml (approximately 0.31 to 3.1 muM in our experiment) in cigarette smokers, and has an effect on lung inflammation *in vivo*. However, the immunoregulatory effects of nicotine have not been fully clarified and the reported data are often conflicting, especially on the effects of nicotine on cytokine production. Several studies have demonstrated that nicotine significantly inhibits the production of the proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-alpha) and IL-1 in human and murine cells [22-24, 27]. In contrast, it has been reported that nicotine can induce the expression and production of cytokines, such as IL-2, IL-6, and interferon-gamma (IFN-gamma) *in vitro* and in an animal model [25, 26]. Moreover, investigations of the effect of nicotine on inflammatory cells have

indicated that nicotine is directly chemotactic for neutrophils but not monocytes [30]. Our finding of the toxic effect of nicotine is in agreement with the observation that nicotine can cause the expression of chemokines.

To date, elevated MIP-1alpha mRNA has been implicated in the development of lung inflammation in a number of animal models, such as lung inflammation induced by bacterial endotoxin, oxidative stress, and transition metals [18, 19, 31]. While demonstration of increased MIP-1alpha mRNA expression suggests the hypothesis that this chemokine contributes to pulmonary inflammatory cell activation and influx, this hypothesis must be tested at the protein level, because changes in mRNA expression are not necessarily indicative of changes in protein production. To clarify this, we performed Western blot analysis using a recombinant rat MIP-1alpha antibody for demonstrating the protein in cell lysates. A single band with an estimated molecular weight of 10 kD for MIP-1alpha was induced after nicotine treatment (Figure 3). This denaturing PAGE-estimated size is comparable to the calculated, putative mature protein size of 10 kD for rat MIP-1alpha which has 92 amino acids [8, 17]. Furthermore, there seemed to be a trend that the synthesis of MIP-1alpha protein was induced maximally at 6 h after nicotine treatment, correlating with the change in MIP-1alpha mRNA expression. In addition, NAC could attenuate the MIP-1alpha protein synthesis.

However, the signalling pathway by which nicotine induced MIP-1alpha gene expression is still unknown. AMs, when activated by a variety of stimuli, are known to release substantial quantities of ROS, including superoxide anions, hydrogen peroxide, and hydroxyl radicals. Previous work from our lab, and others has provided evidence that expression of MIP-1alpha mRNA in a RAM cell line induced by endotoxin or oxidative stress such as H_2O_2 and menadione, a quinone compound that generates ROS continuously, was attenuated by antioxidants such as NAC and dimethyl sulfoxide (DMSO), suggesting that ROS may serve as a common signal transduction pathway to regulate gene expression of most, if not all chemokines [18]. In support of this hypothesis, pretreatment of RAM cells with the antioxidant NAC in the present study was shown to suppress the expression of MIP-1alpha mRNA by nicotine in a dose-dependent fashion (Figure 4), indicating that nicotine may regulate the expression of the MIP-1alpha chemokine by the generation of ROS in AMs. Although few studies have explored the possible modulation of MIP-1alpha expression by ROS, most of them used an endotoxin challenge or direct oxidants

as a stimulus for oxidative stress. The significance of our present study is that it directly links the ROS to increased MIP-1alpha mRNA expression in AMs in response to nicotine.

Reactive oxygen species are well known to activate gene expression *via* modulation of the intracellular reduction-oxidation (redox) state of nuclear proteins. In mammalian cells, ROS have been implicated in the activation of transcription factors nuclear factor-kappaB (NF-kappaB) and activator protein-1 (AP-1) [32, 33]. Our previous work and that of others have also suggested that the induction of MIP-2 - (a CXC chemokine, acting as functional homologue in rodents for human IL-8), gene expression by oxidative stress is most likely to be through the activation of NF-kappaB [19, 34]. These reports demonstrate that ROS is crucial in the control of either transcription factors or redox-sensitive proteins, subsequently initiating transcriptional activation. Since the level of mRNA in the nucleus can reflect new mRNA synthesis, we used a transcription activity assay. The expression of MIP-1alpha mRNA was markedly increased in nuclear RNA in response to nicotine (Figure 5), suggesting that the transcriptional activation of the MIP-1alpha gene is involved. Although details of the 5'-flanking region of rat MIP-1alpha are unavailable at present, its murine counterpart contains an NF-kappaB-like binding region [35]. Whether the transcriptional induction of MIP-1alpha gene by nicotine is through an NF-kappaB binding region, or other 5' sequences deserves further investigation.

In addition, stability of mRNA transcripts also plays an important role in the regulation of MIP-1alpha gene expression. Our previous work has demonstrated that LPS-induced elevation of MIP-2 mRNA had a half-life, similar to that of control cells, supporting the notion that it is transcriptional activation, not mRNA stability, that is primarily responsible for the elevation of MIP-2 mRNA [19], whereas, LPS-induced elevation of MIP-1alpha mRNA had a half-life, longer than that of control cells, suggesting that post-transcriptional stabilization is involved in the regulation of MIP-1alpha gene expression [17]. The discrepancy between the regulation of the two chemokine subfamilies may be because two sets of genes clustered on two different chromosomes might be controlled differently. In the present study, the half-life of LPS-induced (as a control) MIP-1alpha mRNA revealed a rapid degradation with a $t_{1/2}$ of around 2 hours, however, nicotine treatment significantly increased the half-life of MIP-1alpha mRNA to about 5 hours (Figure 6), suggesting that increased MIP-1alpha mRNA induced by nicotine was mediated, at least in past, through post-transcriptional

stabilization. It has been proposed that the instability of many chemokines may be attributed to the presence of multiple copies of the reiterated AUUUA motifs within the 3'-untranslated region (UTR) of their mRNAs [36]. A cytoplasmic protein, adenosine-uridine binding factor (AUBF), binds specifically to AUUUA motifs within the 3'-UTR of many chemokine mRNAs, substantially stabilizing these transcripts and increasing their half-lives [37-39]. 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 [40]. Sequence analysis of the MIP-1alpha cDNA 3'-UTR also revealed six copies of the ATTTA motifs, which may explain why the half-life of MIP-1alpha mRNA increased after nicotine treatment. Taken together, the fact that the redox state is crucial in the control of post-transcriptional stabilization and that ROS serve as a mediator for transcriptional activation, indicates that ROS may play the pivotal role in the regulation of MIP-1alpha gene expression by nicotine.

CONCLUSION

In conclusion, the present study demonstrates that nicotine can induce MIP-1alpha mRNA expression and protein synthesis in macrophages. The induction of MIP-1alpha mRNA expression by nicotine was attenuated by the antioxidant NAC, suggesting ROS serve as a common signalling pathway for MIP-1alpha mRNA expression. In addition, the increase in MIP-1alpha mRNA expression is mediated through two distinct mechanisms: transcriptional activation and post-transcriptional stabilization.

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Figures

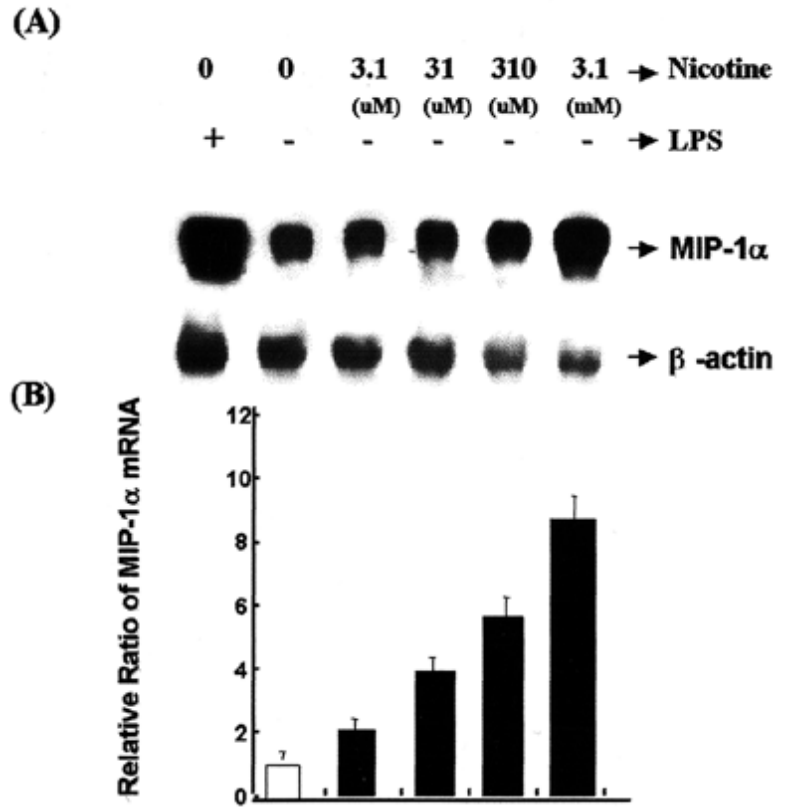


Figure 1

Induction of MIP-1alpha mRNA in rat alveolar macrophages by increasing doses of nicotine.

Cells were treated with 3.1, 31, 310 muM, or 3.1 mM nicotine for 4 hours, while 10 mug/ml LPS was used as a positive control. **A:** Northern blot analysis for MIP-1alpha and beta-actin mRNA was performed. The blot shown is representative of three independent experiments. **B:** Densitometric quantification of MIP-1alpha mRNA was normalized to beta-actin and the corrected density from each dose point was further divided by that for the of normal control and presented as a relative ratio. Results are mean ± SD of three independent experiments.

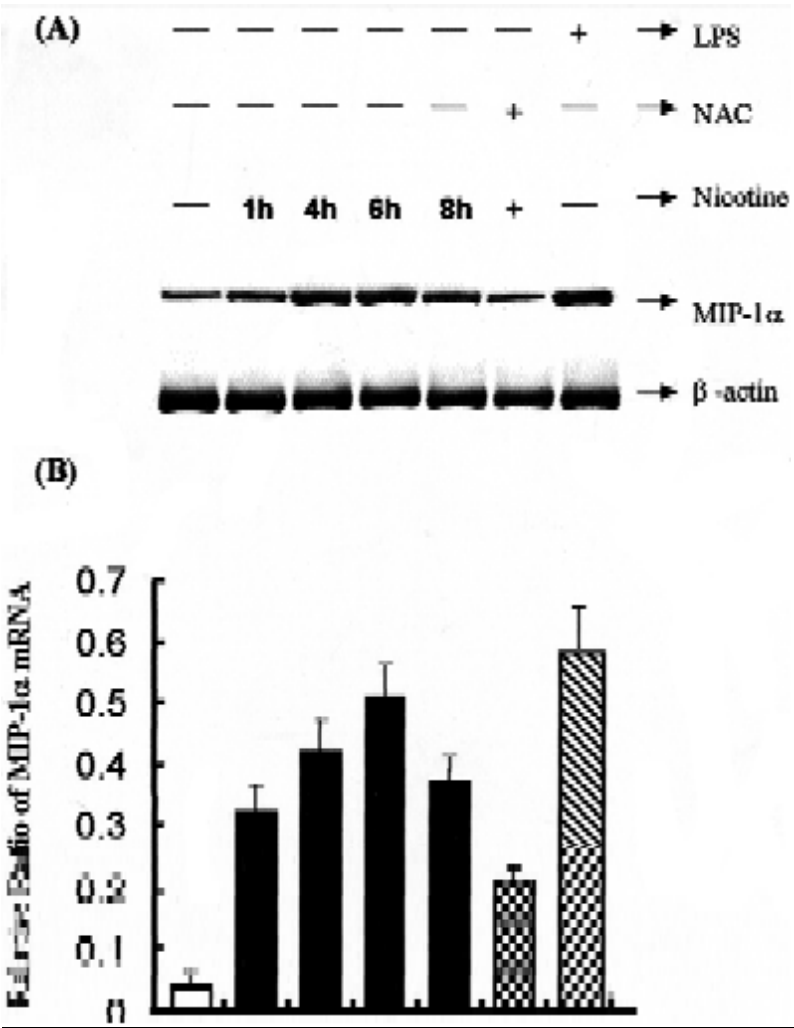


Figure 2

Time course of MIP-1alpha mRNA induction in rat alveolar macrophages by nicotine.

Cells were treated with control vehicle or 31 muM nicotine for 1, 4, 6, or 8 hours, while 10 mug/ml LPS was used as a positive control. For antioxidant treatment, cells were incubated with 10 mM NAC for 1 hours, followed by the addition of 31 muM nicotine for 4 hours. **A:** Northern blot analysis for MIP-1alpha and beta-actin mRNA was performed. The blot shown is representative of three independent experiments. **B:** Densitometric quantification of MIP-1alpha mRNA was normalized to beta-actin and the corrected density from each time point was further divided by that of normal control and

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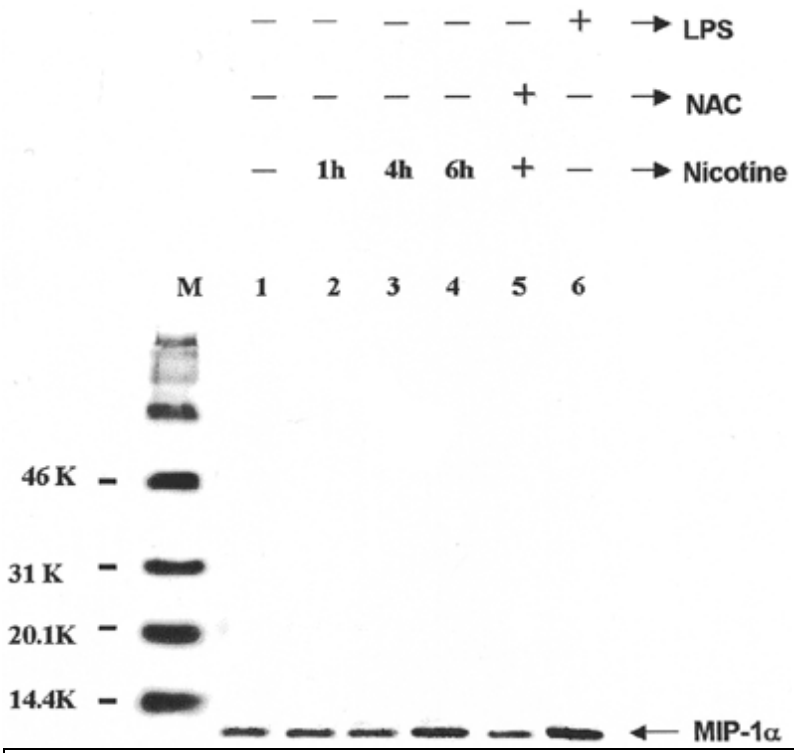


Figure 3

Western blot analysis of cell lysates from rat alveolar macrophages exposed to 31 muM nicotine for 1, 4, or 6 hours, while 10 mg/ml LPS was used as a positive control.

For antioxidant treatment, cells were incubated with 10 mM NAC for 1 hour, followed by the addition of 31 muM nicotine for 4 hours. A single band with an estimated molecular weight of 10 kD for MIP-1alpha was induced. Sizes of the molecular markers are indicated. The blot shown is representative of three independent experiments.

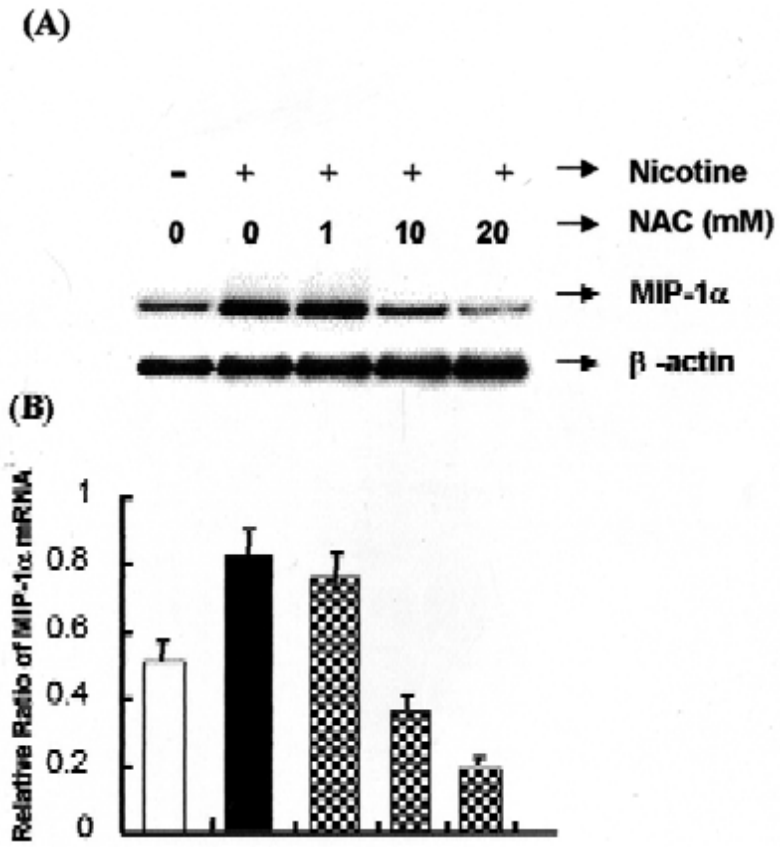


Figure 4

Effect of NAC on suppression of the induction of MIP-1alpha by nicotine.

Cells were pretreated with 0, 1, 10, or 20 mM NAC for 1 hour prior to exposure with 31 muM nicotine for 4 hours. **A:** Northern blot analysis for MIP-1alpha and beta-actin mRNA was performed. The blot shown is representative of three independent experiments. **B:** Densitometric quantification of MIP-1alpha mRNA was normalized to beta-actin and the corrected density from each dose point was further divided by that of normal control and presented as a relative ratio. Results are mean ± SD of three independent experiments.

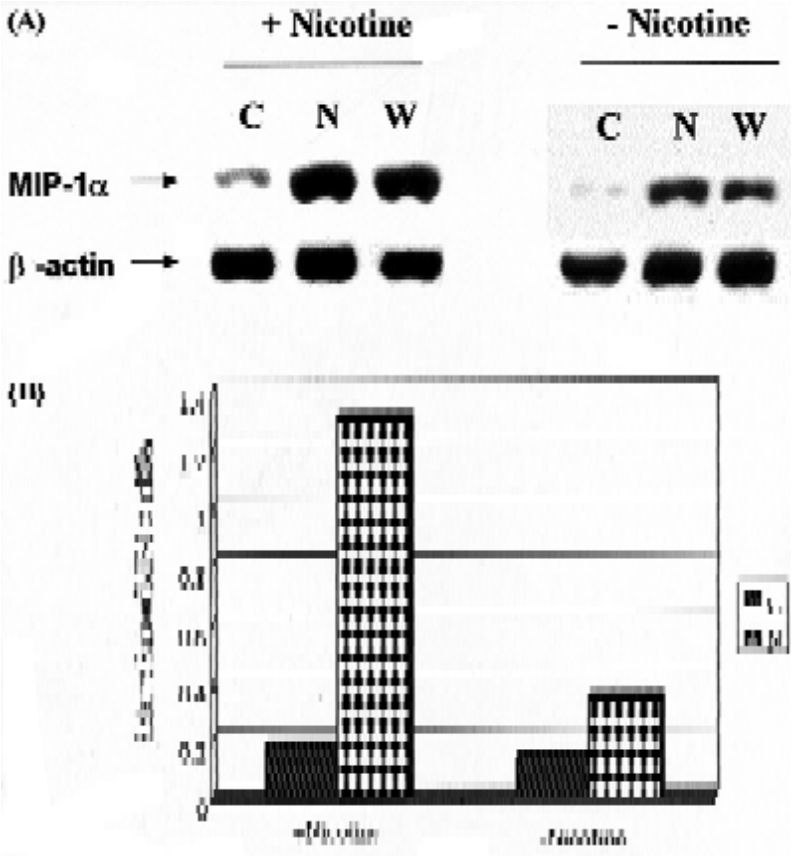
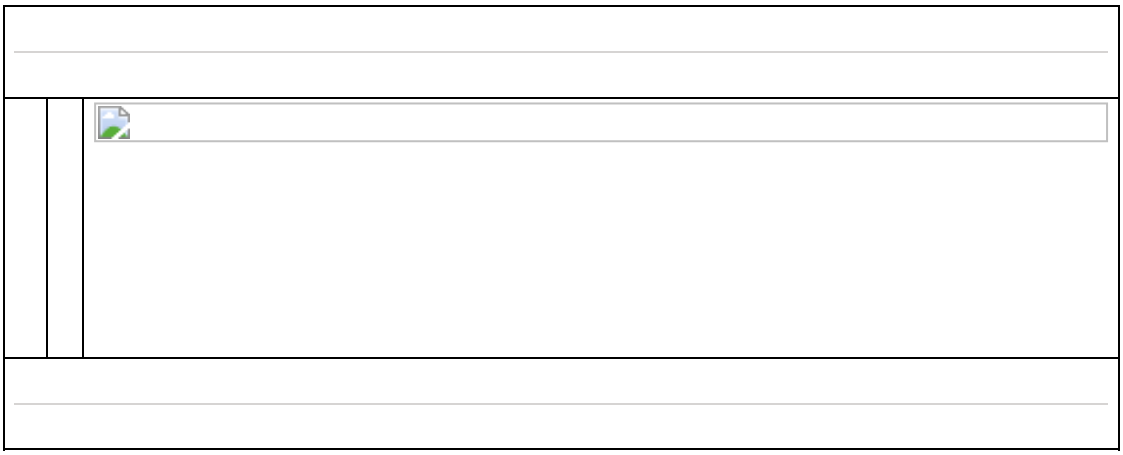


Figure 5

Transcriptional rate of the MIP-1alpha gene in rat alveolar macrophages exposed to nicotine.

Cells were exposed to 31 muM nicotine for 6 hours. After treatment, RAM cells were fractionated into nuclear (N) and cytoplasmic (C) fractions, and equivalent amounts of RNA (25 mug) prepared from each fractions, and corresponding whole-cell (W) were subjected to Northern blot analysis **A:** Northern blot analysis for MIP-1alpha and beta-actin mRNA was performed. The blot shown is representative of three independent experiments. **B:** Densitometric quantification of MIP-1alpha mRNA was normalized to beta-actin and presented as a relative ratio.





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In this issue

Osteoprotegerin and inflammation

N. Saidenberg Kermanac'h, Natacha Bessis, M. Cohen-Solal, M.C. De Vernejoul, Marie-Christophe Boissier
bone resorption, inflammation, osteoprotegerin, RANK.

Impact of glycosylation on the effect of cytokines. A special focus on oncology

A.-L. Chamorey, N. Magné, X. Pivot, G. Milano
cytokine glycosylation, oncology, erythropoietin, granulocyte-colony stimulating factors, interleukins, interferons.

Role of the pro-inflammatory cytokines tumor necrosis factor-alpha, interleukin-1beta, interleukin-6 and interleukin-8 in the pathogenesis of the otitis media with effusion

M. G. Smirnova, S. L. Kiselev, N. V. Gnuchev, J. P. Birchall, J. P. Pearson
endotoxin, cytokines, middle ear inflammation, otitis media.

Macrophages/microglial cells in patients with cerebral malaria

M. H. Deininger, P. G. Kremsner, R. Meyermann, H. Schluesener
cerebral malaria, cytokines, macrophages.

Cytokines in the generation and maturation of dendritic cells: recent advances

G. M. Zou, Y. K. Tam
dendritic cells, cytokines, generation, maturation, differentiation.

Interleukin-10 promoter polymorphism in multiple sclerosis: association with disease progression

L. Almeras, B. Meresse, J. de Seze, D. Lefranc, Sylvain Dubucquoi, I. Fajardy, P. Vermersch, L. Prin
interleukin-10, polymorphism, microsatellite, multiple sclerosis.

Effects of early dexamethasone therapy on pulmonary fibrogenic mediators and respiratory mechanics in preterm

infants

G. Vento, P. G. Matassa, F. Ameglio, E. Capoluongo, L. Tortorolo, C. Romagnoli
TGF-beta1, bFGF, VEGF, pulmonary function testing, tracheobronchial aspirate fluid, dexamethasone.

Analysis of the systemic immune response in HIV-1-infected patients suffering from opportunistic Candida infection

C. Punzón, S. Resino, J.-M. Bellón, Ma A. Muñoz-Fernández, M. Fresno
Candida, HIV, cytokine, phagocytes, polymorphonuclear, T cell.

Interferon-alpha suppresses leptin levels: studies in interferon-alpha treated patients with hepatitis C virus infection and murine adipocytes

S. Kaser, A. Kaser, W. Vogel, J. R. Patsch, H. Tilg
interferon-alpha, leptin, chronic hepatitis C virus infection, adipocytes.

The CD14-260 C => T promoter polymorphism co-segregates with the tumor necrosis factor-alpha (TNF-alpha)-308 G => A polymorphism and is associated with the interleukin-1beta (IL-1beta) synthesis capacity of human leukocytes

M. Heesen, B. Bloemeke, B. Bachmann-Mennenga, D. Kunz
CD14, tumor necrosis factor-alpha, interleukin-1beta, endotoxin, genetic polymorphism, linkage disequilibrium, polymerase chain reaction.

Extracellular matrix proteins influence phenotype and cytokine expression in human breast cancer cell lines

K. Hirtenlehne, M. Pec, E. Kubista, C. F. Singer
breast cancer, cell line, extracellular matrix (ECM), tumor cell phenotype, malignant potential, cytokine expression pattern.

Expression and regulation of the macrophage inflammatory protein-1alpha gene by nicotine in rat alveolar macrophages

I.-W. Chong, S.-R. Lin, J.-J. Hwang, M.-S. Huang, T.-H. Wang, J.-Y. Hung, J. D. Paulauskis
nicotine, macrophage inflammatory protein-1alpha (MIP-1alpha), rat alveolar macrophage (RAM).

Recombinant human interleukin (IL)-1beta-mediated regulation of hypoxia-inducible factor-1alpha (HIF-1alpha) stabilization, nuclear translocation and activation requires an antioxidant/reactive oxygen species (ROS)-sensitive mechanism

J. J. Haddad
antioxidant, airway epithelium, cytokine, HIF-1alpha, IL-1beta, mitochondria, oxygen sensing, reactive oxygen species.

Thioredoxin specifically cross-desensitizes monocytes to MCP-1

S. Pagliei, P. Ghezzi, C. Bizzarri, V. Sabbatini, G. Frascaroli, S. Sozzani, G. Caselli, R. Bertini
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