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Nitric Oxide Modulates MCP-1 Expression in Endothelial Cells: Implications for the Pathogenesis of Pulmonary Granulomatous Vasculitis

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Abstract—Monocyte chemoattractant protein-1 (MCP-1) is a pivotal mediator of angiocentric granuloma formation in glucan-induced pulmonary granulomatous vasculitis. Based on the rationale that mononuclear phagocytes retrieved from granulomas are rich sources of nitric oxide (NO) and that the recruitment of mononuclear phagocytes into lesions abates as granuloma formation slows, we tested the hypothesis that MCP-1 gene expression is regulated by a NO-sensitive mechanism. Preexposure of endothelial cell (EC) monolayers to NO donor compounds markedly reduced cytokine-induced MCP-1 expression and cytosolic-to-nuclear translocation of nuclear factor-kappa B (NF-κB), reversed fluctuations in endothelial reduced glutathione (GSH) pools but did not affect cGMP concentrations. The lungs of mice bearing targeted disruptions of the inducible nitric oxide synthase (*iNOS*) gene exhibited significantly higher concentrations of MCP-1 following glucan infusion than did those of wild-type mice. Cumulatively, these data suggest that NO suppresses MCP-1 expression by blunting the redox changes associated with cytokine-induced EC activation.

KEY WORDS: monocyte chemoattractant protein-1; pulmonary granulomatous vasculitis; nitric oxide; endothelial cells; *iNOS* knockout mice.

INTRODUCTION

Pulmonary granulomas develop in response to a variety of microbial agents (eg. *Mycobacterium tuberculosis*), inhaled foreign particulates (e.g. metal dusts such as beryllium), and etiologically unknown factors (e.g. Wegener's granulomatosis, sarcoidosis) (1). Several forms of pulmonary granulomas are characterized by monocyte/macrophage-rich collections of inflammatory cells centered around blood vessels (i.e. angiocentric). Monocyte chemoattractant protein-1 (MCP-1), a member of the C-C or beta subfamily of chemokines has been identified as a pivotal mediator of granuloma formation. Secreted by a variety of cell types, including endothelial cells (ECs), smooth muscle cells, fibroblasts, and monocytes, MCP-1 induces monocyte recruitment and activation, and can upregulate adhesion molecule expression and cytokine production by monocytes (2). Monocyte chemoattractant protein-1 can activate monocytes by inducing calcium flux and a respiratory burst (3).

Our previous studies have revealed that vascular wall MCP-1 expression is obligatory for lesion development in a rodent model of pulmonary granulomatous vasculitis (4). In this model, the intravenous infusion of particulate yeast cell wall glucan results in the rapid, synchronous development of foreign-body-type granulomas that, when fully mature, are composed primarily of monocytes and macrophages. Recent studies indicate that the upregulation of MCP-1 is, in part, regulated by the cytokines TNF-α and IL-1β (5). MCP-1 expression during the

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pathogenesis of glucan-induced granuloma formation is biphasic with an early (1 h) blood-vessel wall associated rise in MCP-1 and a later (6–24 h) rise in MCP-1 associated with granuloma cells *per se* (6). Experiments in which neutrophils have been either selectively depleted or their adherence to vascular wall endothelium inhibited by means of infused sialyl-Lewis glycomimetic P-selectin antagonists have revealed that neutrophils are obligatory for full granuloma development even though they are not present in definitive lesions (48–96 h) (7, 8). These observations, coupled with evidence that the infusion of catalase inhibits granuloma formation and that pharmacologic modulation of intracellular glutathione redox status modulates MCP-1 expression in granulomatous vasculitis, support the hypothesis that neutrophils and locally produced H_2O_2 are important mediators of monocyte recruitment (9).

While there is extensive evidence suggesting that the expression of proinflammatory mediators including MCP-1, is redox sensitive, the intracellular mediators and transduction pathway(s) are complex and incompletely understood (10). In this study, we tested the hypothesis that locally produced NO regulates the expression of endothelial MCP-1. The rationale for this hypothesis in the context of glucan-induced granulomatous vasculitis rests on evidence that mononuclear phagocytes in pulmonary lesions release NO discretely at sites of granuloma formation. In this scenario, NO may temporally and anatomically delimit the extent of an evolving granulomatous lesion by either down-regulating further MCP-1 elaboration and/or by preventing MCP-1 expression in vessel wall cells adjacent to the lesion. In the present context, "delimitation" indicates the cessation of net "growth" of a granuloma. While there is likely to be an overlap in processes, development of an individual granuloma must reach a maximum size before it begins to then "resolve". The *in vitro* data presented suggest that locally produced NO may regulate endothelial MCP-1 expression by blunting cytokine- and/or reactive oxygen intermediate-induced changes in cellular redox status.

MATERIALS AND METHODS

Unless otherwise specified, materials were purchased from Sigma Chemical Company (St. Louis, MO). NONOates were purchased from Cayman Chemicals (Ann Arbor, MI). Mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords as previously described (11).

Treatment of HUVECs for Enzyme Immunoassay (EIA) Experiments

Aqueous solutions of NO were generated using NONOate compounds. NONOates are nucleophilic NO adducts that spontaneously decompose at neutral pH to release NO (12). As indicated in the results, we employed combinations of two short half-life NO donors, Methylamine hexamethylene methylamine (MAHMA) NONOate ($t_{1/2} = 1$ min at 37°C) and Diethylamine (DEA) NONOate ($t_{1/2} = 2$ min at 37°C), or Dipropylenetriamine (DPTA) NONOate ($t_{1/2} = 3$ h at 37°C) for longer sustained release. NONOate stock solutions were prepared in 0.01 N NaOH and then diluted into M199 medium to initiate NO release. After a 30-min exposure to NO, HUVECs were stimulated with either TNF-α or IL-1β(1 ng/mL) for 8 h. The conditioned media collected at the end of this 8-h incubation period were subjected to an MCP-1 enzyme immunoassay (EIA). Cell viability was assessed by using a fraction of the conditioned media from the EIA experiment in a cytotoxicity detection (LDH) assay (Boehringer, Mannheim, Germany). Cell viability was always $\geq$95%.

MCP-1 Enzyme Immunoassay (EIA)

MCP-1 EIAs were carried out as previously decribed (13).

Ribonuclease (RNase) Protection Assay

Total RNA was extracted from endothelial cells using Tri Reagent according to manufacturer's instructions. The RNase protection assays were performed using the RiboQuant Multi-probe kit from Pharmingen (San Diego, CA) and carried out as previously described (13).

Extraction of Nuclear Protein

Nuclear extracts were prepared and protein concentrations determined as previously decribed (9, 14).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSAs) were carried out as previously described using a gel shift assay system kit (Promega, Madison, WI) (11).

Glutathione Assay

The glutathione assay was performed using a kit from Oxis International (Portland, OR) following the manufacturer's instructions. Briefly, cells were washed with phosphate buffered saline (PBS) and homogenized in ice-cold 5% metaphosphoric acid. The homogenate was centrifuged and the resulting aqueous layer was utilized for the assay. Reduced glutathione (GSH) was utilized as a standard. Spectrophotometric determinatons were performed in triplicate using a El_x808 plate reader set to a wavelength of 405 nm.

Induction of Pulmonary Granulomatosis

Glucan-induced pulmonary granulomatous vasculitis was induced in BL6 mice or *iNOS* knockout mice as previously decribed (4). Briefly, glucan from Bakers yeast (Sigma) was suspended in phosphate bufferered saline (5 mg glucan in 1 mL of PBS). The suspension was sonicated, vortexed, and 100 μL were infused slowly via the heat-dilated tail vein. Control animals received vehicle (PBS) only. At specified intervals following glucan administration, animals were euthanized and their lungs and hearts removed *en bloc*. One lung was instilled with 10% buffered formalin phosphate (Fisher Scientific, Pittsburgh, PA), followed by processing for light and electron microscopy. The other lung was immediately snap-frozen in liquid nitrogen and subsequently processed for use in EIAs of tissue extracts.

cGMP Assay

cGMP concentrations were measured with a kit from Assay Designs (Ann Arbor, MI) according to manufacturer's instructions. Briefly, HUVECs that had been grown in 24-multiwell plates were washed with HBSS to remove traces of serum. The cells were incubated with the indicated test compounds for 15 min and subsequently 1 mM isobutylmethylxanthine (IBMX) was added to inhibit the phosphodiesterases (5-min incubation). Hank's balance salt solution (HBSS) was then aspirated and 250 μL of 0.1 N hydrochloric acid (HCl) were added to each well to stop enzymatic reactions and to extract cGMP. After 30 min, the HCl extract was collected and stored until analyzed. To normalize cGMP values, the protein content in each well was measured by the Bradford method after solubilization with 1 N sodium hydroxide (NaOH).

Densitometric Scanning

Autoradiographs from RNase protection assays and EMSAs were scanned using Polaroid PhotoMAX Pro and integrated densities were determined using Scion Image for windows (Scion Corporation, Frederick, MD). To adjust for differences in sample loading between wells in the RNase protection assay, MCP-1 mRNA levels were expressed as ratios of integrated densities for MCP-1 to GAPDH products.

Statistical Analysis

Statistical analysis was performed using SAS, version 8.2 (SAS Institute). All values are expressed as the means $\pm$ standard error. Data were analyzed by analysis of variance (ANOVA).(15) Probability (p) values of <0.05 were considered significant.

RESULTS

Exogenous NO Suppresses Cytokine-Induced MCP-1 in HUVECS

Exposure of HUVECs to either TNF-α or IL-1β resulted in dose-dependent increases in MCP-1 production (Fig. 1). When cytokine-activated cells were pretreated with a combination of the short half-life donors MAHMA-NONOate ($t_{1/2} = 1$ min at 37°C) (50 μM) and DEA NONOate ($t_{1/2} = 2$ min) (50 μM), significant reductions in secreted MCP-1 concentrations were observed. These effects were not observed when the NO donor compound and the cytokine were added simultaneously or when NO exposure followed cytokine stimulation (data not shown). Similar results were observed when the NO donor, DPTA-NO ($t_{1/2} = 3$ h at 37°C) (50 μM) was substituted for DEA-NO (data not shown). These data indicate that exposure of HUVECs to chemically generated NO prior to stimulation with TNF-α or IL-1β results in marked reductions in MCP-1 production.

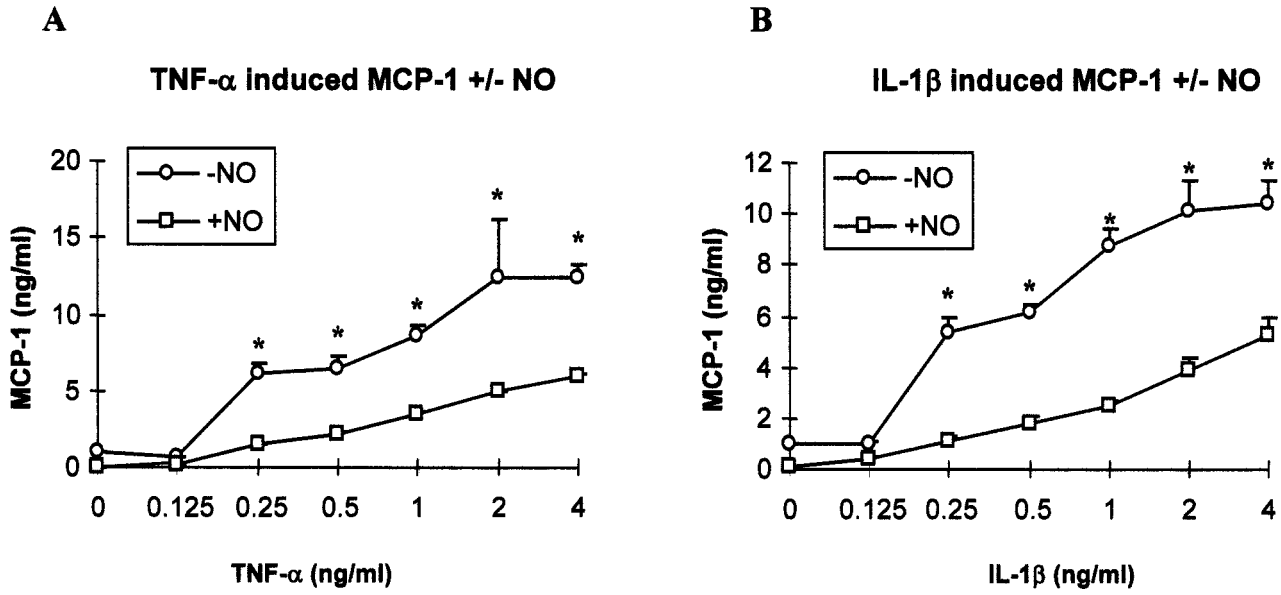

Fig. 1. Suppression of cytokine-induced MCP-1 by exogenous NO. HUVECs were treated for 30 min with medium alone (○) or NONOate (□), respectively, followed by a 12-h incubation period with either a) TNF-α or b) IL-1β. These data represent means ± SE of a single experiment that is representative of four separate experiments. In each experiment, conditions were examined in triplicate. Asterisk* denotes significant difference in MCP-1 concentrations in presence and absence of NO donor.

NO Donors Suppress MCP-1 mRNA

In order to determine whether the observed decreases in cytokine-induced MCP-1 protein secretion in response to NO pretreatment were also associated with changes in MCP-1 mRNA concentrations, we utilized a multi-probe RNase protection assay. No MCP-1 mRNA was detectable in either medium-treated HUVECs or HUVECs that had been treated with a combination of MAHMA and DEA NONOates (Fig. 2). As expected, exposure of HUVECs to TNF-α or IL-1β (4 h) resulted in marked increases in MCP-1 mRNA concentrations (Fig. 2). When cells were treated with MAHMA and DEA NONOates prior to cytokine stimulation significant reductions in MCP-1 mRNA were observed when compared to HUVECs exposed to cytokines alone (Fig. 2). These observations indicate that the NO-mediated reductions in MCP-1 protein production in HUVECs measured by EIA (Fig. 1) are accompanied by reduced MCP-1 mRNA levels.

Exogenous NO Suppresses Cytokine-Induced NF-κB Translocation but Does Not Affect AP-1 Activation

Cytokine-induced MCP-1 gene expression in endothelial cells depends on the cooperative interaction of the redox-sensitive transcription factors NF-κB and AP-1 (16). We examined the activation of NF-κB and AP-1 by EMSA (Fig. 3). Low concentrations of NF-κB were detected in nuclear extracts derived from media-treated cells (lane 1, Fig. 3A). As expected, cytokine stimulation of HUVECs resulted in a marked increase in the cytosolic-to-nuclear translocation of NF-κB (lanes 2 and 3, Fig. 3A). The activation of NF-κB was suppressed by pretreatment with MAHMA and DEA NONOates (lanes 5 and 6, Fig. 3A). No changes in the nuclear concentrations of AP-1 were observed in endothelial cells upon activation by cytokines (lanes 2 and 3 compared to lane 1; Fig. 3B). Moreover, NO donors had no effect on activation of AP-1 (lanes 4, 5, and 6; Fig. 3B). These data indicate that cytokine-induced activation of NF-κB but not AP-1, is sensitive to NO exposure. Since NF-κB is a redox-sensitive transcription factor, these data suggest that alterations of cellular redox status may be mechanistically important in NO-mediated suppression of endothelial MCP-1 production.

Exogenous NO Blunts Cytokine-Induced Fluctuations in Endothelial Reduced Glutathione (GSH) Concentrations

In order to further address the potential role of changes in cellular redox status as a mechanism for

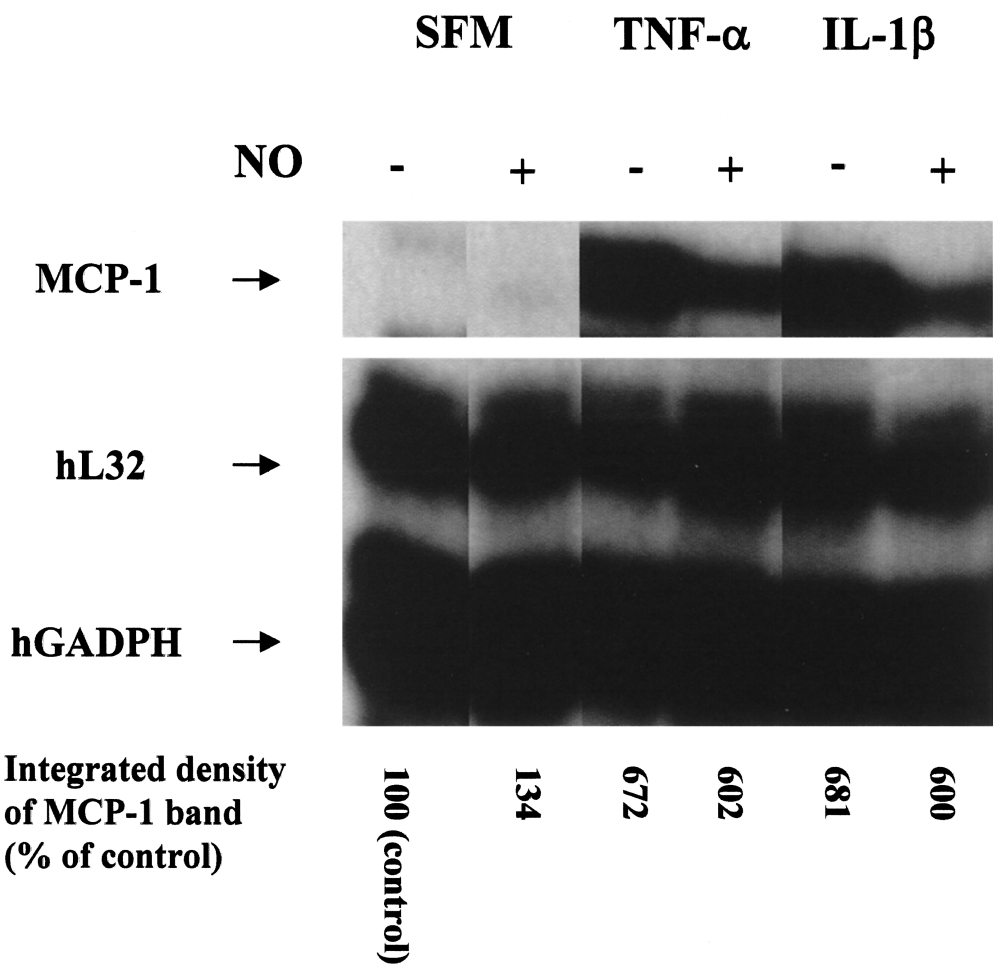

Fig. 2. Suppression of cytokine-induced MCP-1 mRNA concentrations by exogenous NO. HUVECs were treated for 30 min with medium (SFM) or a combination of MAHMA NONOate and DEA NONOate (50 μM each), followed by a 4-h incubation period with TNF-α or IL-1β. RNase protection assay was utilized in order to detect specific transcripts for MCP-1 and for the "housekeeping" genes, *L32* and *GAPDH*, as controls. The data shown are representative of three separate experiments.

NO-mediated suppression of MCP-1 expression, we examined the effects of cytokines and NO on cellular GSH concentrations. TNF-α treatment of HUVECs resulted in a 30% decrease in intracellular GSH concentration when compared to cells treated with serum-free medium (SFM) alone (Fig. 4A). Preexposure of HUVECs to NO partially reversed this effect, restoring GSH concentrations to 93% of the resting level (Fig. 4A). However, a more than 20% increase in intracellular GSH pools from baseline was observed in IL-1β-stimulated HUVECs (Fig. 4A). Incubation with NO prior to activation with IL-1β resulted in a decrease in GSH concentrations to the basal level (Fig. 4A). Hence, cytokine-induced alterations in cellular GSH pools were prevented by preexposure of HUVECs to NO donors.

Exogenous NO Does Not Modulate Cytokine-Induced Endothelial cGMP Production

Nitric oxide exerts its relaxation effect on smooth muscle cells by activating soluble guanylate cyclase (sGC) that in turn leads to increased intracellular concentrations of cyclic GMP (cGMP).(17) To ascertain whether the observed effect of NO on MCP-1 suppression is accompanied by increased cGMP levels, we treated HUVECs with NO donor compounds and measured cellular cGMP

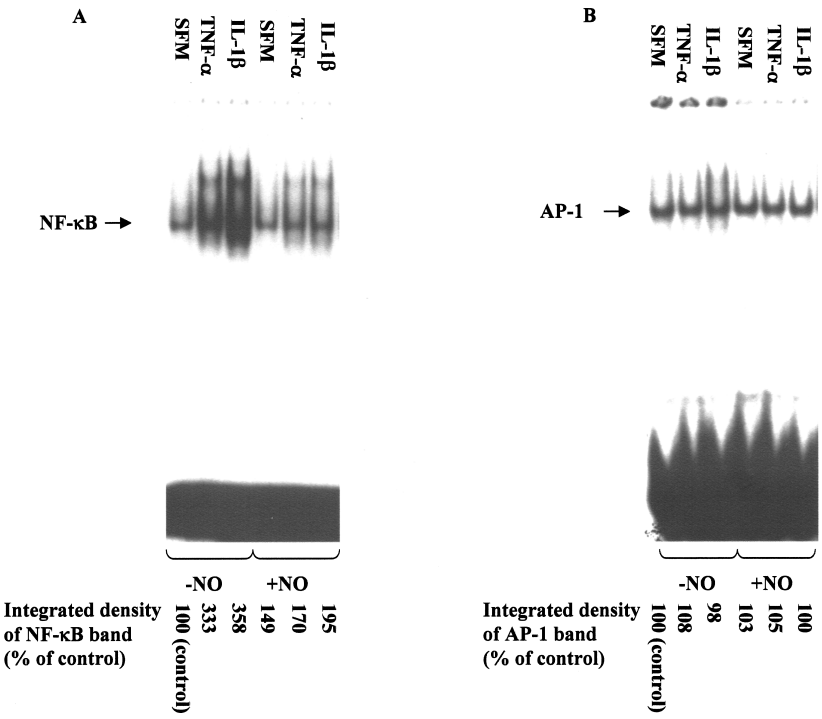

Fig. 3. Effect of exogenous NO on cytokine-mediated NF-κB translocation and on activation of AP-1. HUVECs were treated for 30 min with medium (SFM) or a combination of MAHMA NONOate and DEA NONOate (50 μM each), followed by a 45-min incubation period with either TNF-α or IL-1β respectively. Nuclear extracts were prepared as described in "METHODS" and were incubated in presence of a radiolabeled NF-κB or AP-1 consensus oligonucleotide probe for 30 min at room temperature. Samples were run on a nondenaturing 4% polyacrylamide gel. Competition with a 50-fold excess of unlabeled (cold) NF-κB or AP-1, respectively, abrogated the gel shift (data not shown).

levels. In HUVECs, NO treatment did not significantly alter the intracellular concentration of cGMP measured at 15 min after exposure (Fig. 4B, HUV). This is in contrast to smooth muscle cells in which cGMP levels began to rise by 15 min (Fig. 4B, SMC). These data suggest that NO-mediated suppression of TNF-α and IL-1β-induced MCP-1 expression in endothelial cells is not mediated by sGC. This observation, in conjunction with the above data, is consistent with the hypothesis that NO-mediated suppression of cytokine-induced MCP-1 expression is mediated by a redox-sensitive mechanism.

Inhibition of Endothelial NO Synthesis Increases Cytokine-Induced MCP-1 Secretion

The effect of inhibition of endothelial NO synthesis on TNF-α-mediated MCP-1 expression was studied using L-N^G-nitroarginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase. HUVECs treated with medium alone secreted low concentrations of MCP-1 (Fig. 5). A significant rise in MCP-1 concentration was detected in cells exposed to either 1ng/mL or 10 ng/mL, respectively, of TNF-α (Fig. 5). Pretreatment of HUVECs with 4 mM, 1 mM, and 250 μM L-NAME, respectively, resulted in significant increases in TNF-α-mediated MCP-1 secretion (Fig. 5). Concentrations of L-NAME less than or equal to 62.5 μM were ineffective in modulating MCP-1 secretion (Fig. 5). These data suggest that inhibition of cellular nitric oxide production results in an augmentation of MCP-1 concentration and that endogenously synthesized NO plays an important role in regulating cytokine-induced MCP-1 expression.

iNOS Knockout Exhibit Increased Pulmonary MCP-1 Concentrations

We have previously decribed the time course for pulmonary MCP-1 expression following glucan infusion (9).

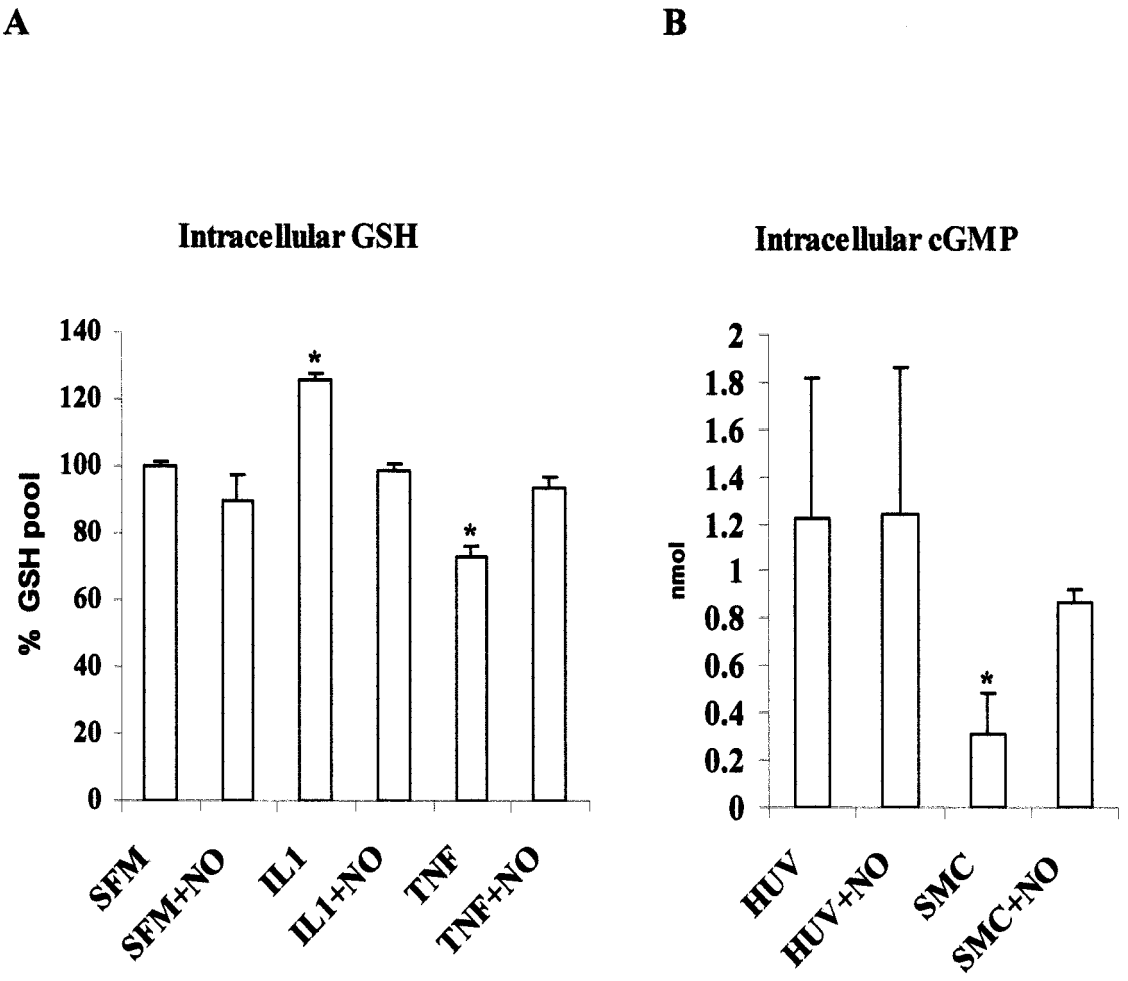

Fig. 4. Effect of NO donors on intracellular GSH pools in HUVECs (A) and cGMP pools in HUVECs and SMCs (B). Endothelial and smooth muscle cells were treated with a combination of MAHMA and DEA NONOate (50 μM each). GSH and cGMP determinations were performed as described in "METHODS." The data shown is representative of three separate experiments. Asterisk* denotes significant difference in GSH and cGMP concentrations in presence and absence of NO donor. HUV = human umbilical vein endothelial cells; SMC = human vascular smooth muscle cells.

Here, MCP-1 concentrations following intravenous glucan administration were measured in the lungs of BL6 wild type and strain-mutated *iNOS* knockout mice. As shown in Fig. 6, immediately following glucan-infusion to BL6 wild-type mice, virtually no MCP-1 was detected in the lungs (time 0). At 1.5 h after glucan infusion, MCP-1 concentrations were significantly elevated. Pulmonary MCP-1 concentrations peaked at 24 h. At 96 h after glucan infusion, a return towards baseline MCP-1 concentration was observed. In *iNOS* knockout mice, pulmonary MCP-1 concentrations followed a similar temporal pattern with a peak at 24 h and a decline towards baseline concentrations at 96 h post glucan infusion. However, when compared to wild-type controls, pulmonary MCP-1 concentrations in *iNOS* knockout mice were significantly elevated at 0 and 24 h following glucan infusion. These data suggest that in mice, the absence of inducible nitric oxide synthase results in augmented pulmonary MCP-1 concentrations at baseline and following glucan infusion.

DISCUSSION

The present data indicate that exposure of ECs to NO prior to TNF-α or IL-1β-induced activation significantly

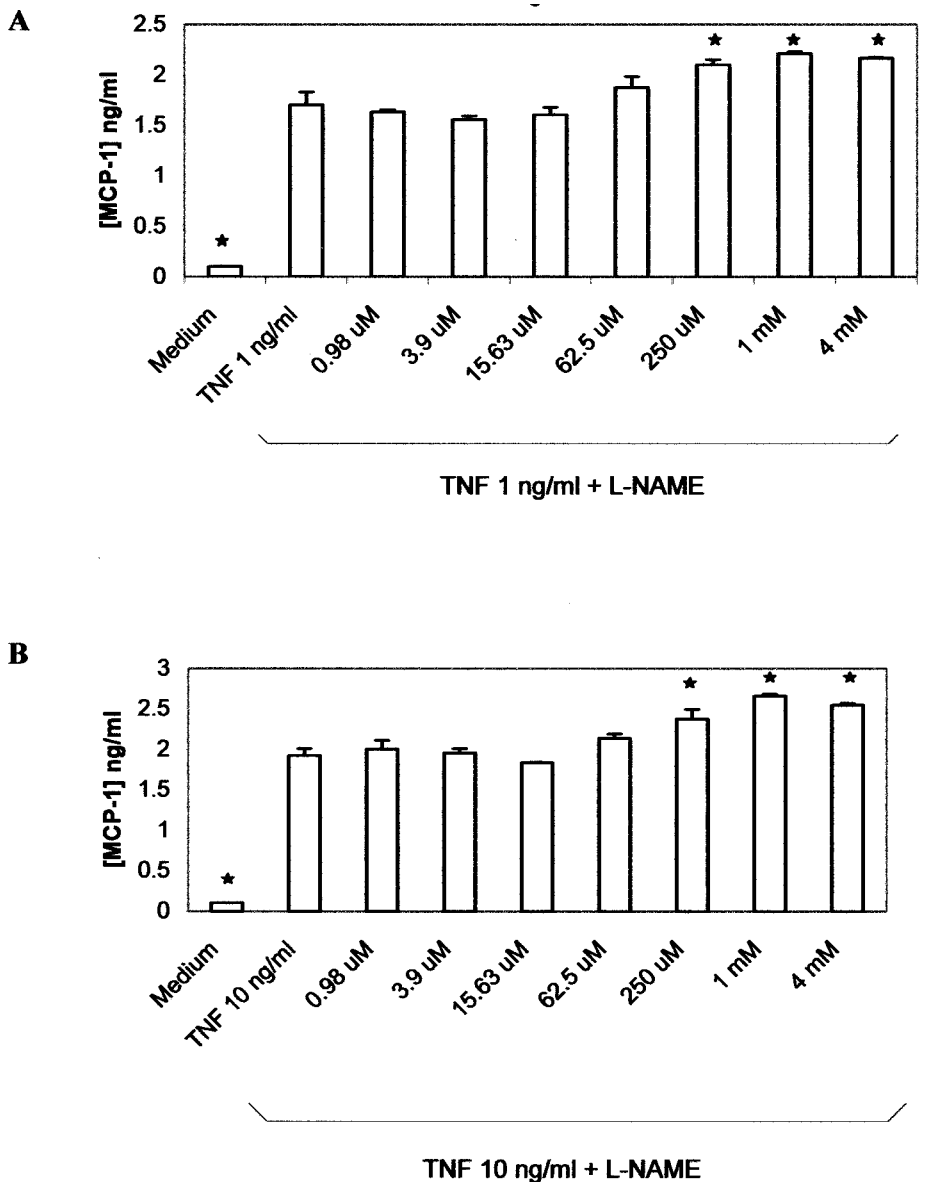

Fig. 5. Effect of endogenous NOS inhibition on cytokine-mediated MCP-1 expression. HUVECs were treated with medium or the indicated concentrations of L-NAME, respectively, for 2 h. The cells were subsequently incubated in presence of medium alone or TNF-α at either 1ng/mL or 10 ng/mL, respectively for 8 h. At the end of this incubation period, conditioned media were removed and subjected to MCP-1 EIAs. The data shown is representative of three separate experiments, each performed in triplicate. Asterisk* denotes significant (at $\alpha = 0.05$) difference from MCP-1 concentrations in TNF-α-stimulated endothelial cells as determined by Bonferroni comparison of means.

suppresses expression of MCP-1. Conversely, inhibition of endogenous nitric oxide synthesis by L-NAME results in signficant increases in cytokine-induced MCP-1 concentrations. Consistent results were also observed in a rodent model of pulmonary granulomatous vasculitis. The lungs of *iNOS* knockout mice exhibited substantially greater concentrations of MCP-1 following intravenous glucan administration than did those of wild-type mice suggesting that NO is an important regulator of MCP-1 expression during granuloma formation.

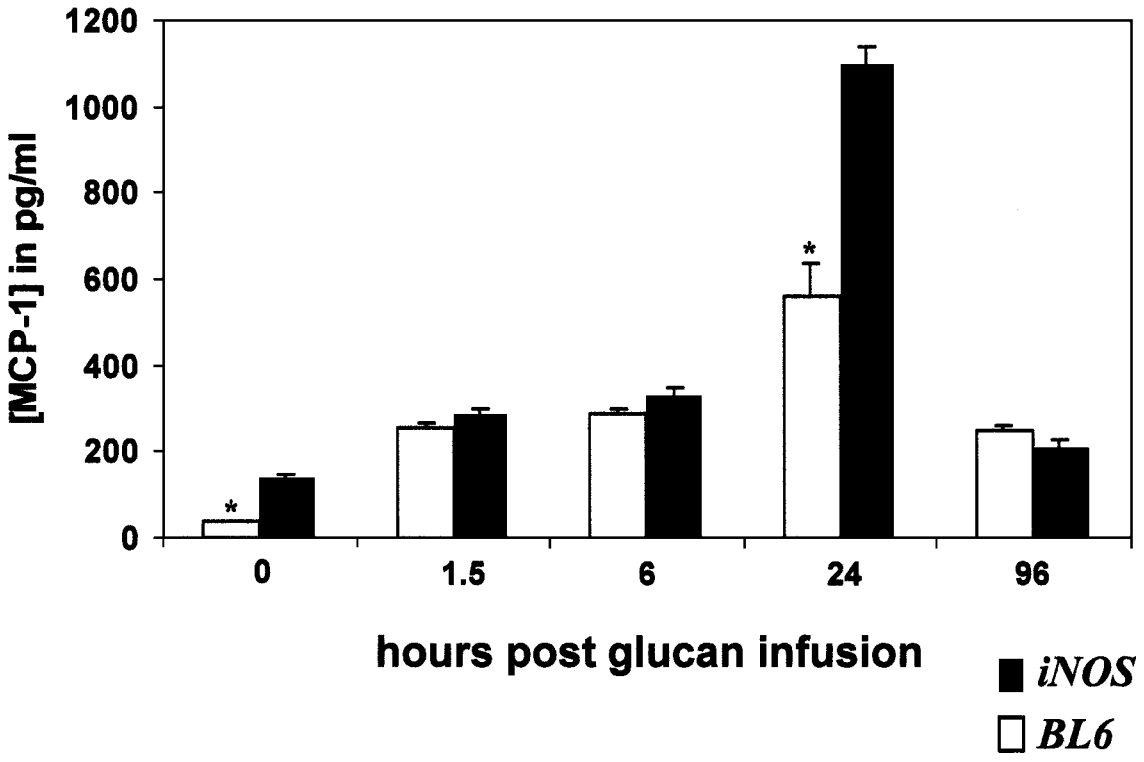

Fig. 6. Pulmonary MCP-1 concentrations in wild type (□) and iNOS knockout mice (■) at varying time points following intravenous glucan administration. Mice were sacrificed at the indicated times after glucan infusion and lung extracts were subjected to MCP-1 EIAs. Three animals were used per time point and each sample was analyzed in triplicate. Asterisk* denotes significant difference ($p < 0.05$) in pulmonary MCP-1 concentrations between iNOS and BL6 mice at the indicated time points following glucan infusion.

This study also suggests that the redox-sensitive transcription factor NF-κB, but not AP-1, is associated with the NO-mediated suppression of cytokine-induced MCP-1 expression. Futhermore, treatment of HUVECs with NO donors did not significantly alter the intracellular concentration of cGMP, suggesting that the NO-mediated suppression of TNF-α and IL-1β-induced MCP-1 expression in endothelial cells is not mediated by soluble guanylate cyclase (sGC). Exogenously supplied NO, however, reversed cytokine-induced alterations in intracellular GSH concentrations lending further support to the hypothesis that the NO-mediated suppression of cytokine-induced MCP-1 expression is mediated by a redox-sensitive mechanism. Our data agree with recent reports from several other laboratories. Zeiher *et al*. (18) reported that inhibition of basal NO production in ECs by the NOS inhibitor N^G-nitro-L-arginine (L-NAG) increased MCP-1 mRNA levels to $250 \pm 20\%$ of controls and resulted in a marked increase in monocyte chemotactic activity. As in this study, generation of exogenous NO resulted in a decrease in MCP-1 at both the protein and mRNA levels (18). Tsao *et al*. (19) obtained similar results in rabbit aortic smooth muscle cells that were stimulated with lipopolysaccharide (LPS) or oxidized LDL (oxLDL) (19). Khan *et al*. (20) reported that NO donors also reduce VCAM-1 and ICAM-1 expression in endothelial cells at both the protein and mRNA levels (20). Taken collectively, these studies suggest that NO regulates the expression of inflammatory mediators (i.e. adhesion molecules and chemokines) and that the mechanism by which NO suppresses inflammatory mediator expression may involve an inhibition of the cytosolic-to-nuclear translocation of the redox-sensitive transcription factor NF-κB.

Several recent studies aimed at unraveling the mechanism(s) by which exogenous NO may inhibit NF-κB translocation, however, are in conflict. Katsuyama *et al*. (21) observed that in rat vascular smooth muscle cells, the inhibitory effect of NO on NF-κB activation occurs through inhibition of IκB-α phosphorylation (21). On the other hand, Peng *et al*. (22) reported that NO donors inhibit TNF-α-induced NF-κB activation in human saphenous

vein endothelial cells by induction and stabilization of IκB-α (22). In addition, NO also increased the mRNA expression of IκB-α. This group of investigators subsequently reported that the inhibition of NF-κB activation by NO occurs via increases in cytoplasmic and nuclear concentrations of IκB-α. The discrepancies in these observations may be accounted for by the different cell types, species, cytokines, and NO donors used (21, 23). Recent *in vitro* studies published by Umansky *et al*. (24) indicate that the effect of NO on NF-κB activation may be concentration dependent. Low concentrations of the NO-generating compound glycerol trinitrate (GTN) enhanced TNF-α or PMA-induced NF-κB binding activity and transactivation in endothelial TC 10 cells which was associated with increased activation of IκB-α kinase (IKK-α) (24). NO at high concentrations (>250 μM) on the other hand, repressed NF-κB-dependent transactivation almost to the basal level. Since iNOS expression itself is activated by NF-κB these data illustrate that low concentrations of NO provide a self-amplifying signal in the inflammatory response (23, 25).

Our observation that NO blunts TNF-α and IL-1β-induced fluctuations in intracellular-reduced glutathione pools provides additional evidence in favor of the hypothesis that NO-mediated modulation of endothelial MCP-1 expression occurs through a redox-sensitive mechanism. In an *in vitro* analysis of the role of NO in modulation of neutrophil-endothelial interactions, Niu *et al*. (26) reported that exposure of HUVECs to NO results in a decrease in ICAM-1 expression (26). Conversely, incubation of HUVECs with the NOS inhibitor L-NAME resulted in an increase in both platelet-activating factor and ICAM-1-mediated neutrophil adhesion (26). Through the use of 2′, 7′-dichlorodihydro-fluorescein diacetate-loaded HUVECs and a series of intracellular and extracellular oxygen radical scavengers these researchers concluded that NO-mediated effects on endothelial cell adhesion molecule expression occurred via a redox-sensitive mechanism (26). Our previous studies in a rat model of glucan-induced granulomatous vasculitis suggest that neutrophil-derived reactive oxygen intermediates modulate the expression of MCP-1 in pulmonary blood vessel wall cells (7). The *in vivo* data presented here complement the larger body of *in vitro* data suggesting a pathway by which NO may delimit angiocentric granuloma formation. Exposure of unactivated endothelial cells at the margins of a developing granuloma to the relatively high local concentrations of NO produced by recruited monocytes and macrophages may render these endothelial cells less responsive to proinflammatory stimuli such as TNF-α and IL-1β, This hypothesis is supported by the *in vitro* data that indicate that preexposure, but not simultaneous or later exposure, of HUVECs to NO renders them less responsive to TNF-α and IL-1β with respect to MCP-1 expression. This action of NO may be mediated by resistance to changes in EC redox status.

In the context of an evolving granuloma these observations and the report by Umansky *et al*. (24) suggest that low concentrations of NO released by recruited, activated monocytes may amplify the expression of inflammatory mediators (24). As monocyte numbers increase to a critical mass, high local concentrations of NO may down-regulate the inflammatory response. This study supports the idea that by down-regulating the expression of critical inflammatory mediators (i.e. adhesion molecules, chemokines, and cytokines) NO may play an important role in processes that lead to the delimitation and resolution of granulomatous lesions in the lung.

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