L-Arginine attenuates lipopolysaccharide-induced lung chemokine production

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Chemokines stimulate the influx of leukocytes into tissues. Their production is regulated by nuclear factor-κB (NF-κB), an inducible transcription factor under the control of inhibitory factor κB-α (IκB-α). We have previously demonstrated that L-arginine (L-Arg) attenuates neutrophil accumulation and pulmonary vascular injury after administration of lipopolysaccharide (LPS). We hypothesized that L-Arg would attenuate the production of lung chemokines by stabilizing IκB-α and preventing NF-κB DNA binding. We examined the effect of L-Arg on chemokine production, IκB-α degradation, and NF-κB DNA binding in the lung after systemic LPS. To block nitric oxide (NO) production, a NO synthase inhibitor was given before L-Arg. LPS induced the production of chemokine protein and mRNA. L-Arg attenuated the production of chemokine protein and mRNA, prevented the decrease in IκB-α levels, and inhibited NF-κB DNA binding. NO synthase inhibition abolished the effects of L-Arg on all measured parameters. Our results suggest that L-Arg abrogates chemokine protein and mRNA production in rat lung after LPS. This effect is dependent on NO and is mediated by stabilization of IκB-α levels and inhibition of NF-κB DNA binding.

nitric oxide; nitric oxide synthase; cytokine-induced neutrophil chemoattractant-1; macrophage inflammatory protein-2; acute lung injury; nuclear factor-κB; inhibitory factor κB

may inhibit CINC-1 and MIP-2 production and attenuate LPS-induced ALI. In the resting state, NF-κB is bound to inhibitory factor IκB-α (IκB-α), which confines the complex to the cytoplasm. Inflammatory stimuli such as LPS or ischemia lead to dissociation of IκB-α from NF-κB; the latter element is able to translocate to the nucleus and induce transcription. We hypothesized that in vivo administration of l-Arg would prevent the LPS-induced decrease in IκB-α levels and prevent NF-κB-mediated expression of CINC-1 and MIP-2. The purpose of this study was to determine the effect of l-Arg on LPS-induced CINC-1 and MIP-2 protein and mRNA expression, NF-κB DNA binding, and IκB-α protein in the lung. We found that treatment with l-Arg before LPS attenuated both CINC-1 and MIP-2 protein and mRNA production and NF-κB DNA binding. This mechanism was mediated through preservation of IκB-α protein levels after LPS.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (body weight 300–350 g) were quarantined and maintained on a standard pellet diet for 2 wk before the initiation of experimental protocols. All animal experiments were approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee. Animals received humane care in accordance with the revised Guide for the Care and Use of Laboratory Animals (7th ed.; Washington, DC: Natl. Acad. Press, 1996).

Experimental protocol. Awake rats were administered saline or LPS (0.5 mg/kg LPS; Salmonella typhimurium, lot no. 47H4093, Sigma, St. Louis, MO) freshly prepared in 5% saline via intraperitoneal injection. The optical density at 540 nm was determined spectrophotometrically with the use of a microplate reader (Bio-Rad, Hercules, CA) and plotted against a standard curve. Results are expressed in micromoles.

Nitrite/nitrate assay. Nitrite plus nitrate (NOx) production was measured in rat plasma as previously described (39) with a kit provided by R&D Systems (Minneapolis, MN). Briefly, the plasma was first diluted (1:2) in HEPES buffer and ultrafiltered to exclude proteins >10 kDa. The nitrate component of the filtrate was then reduced to nitrite by incubation with nitrate reductase and NADH at 37°C for 30 min. The total nitrite concentration in the samples was then measured by the addition of 100 μl of the Griess reagent (0.1% naphthylenediamine dihydrochloride in H2O and 1% sulfanilamide in 5% concentrated HCl) to 50 μl of sample. The optical density at 540 nm was determined spectrophotometrically with the use of a microplate reader (Bio-Rad, Hercules, CA) and plotted against a standard curve. Results are expressed in micromoles.

Electrophoretic mobility shift assay. NF-κB (5'-AAGTCGGGCACCTTCCAGGC-3') binding site underlined) consensus oligonucleotide was 5'-end labeled with [γ-32P]dATP with T4 polynucleotide kinase. Unincorporated nucleotide was removed with a NucTrap probe purification column (Stratagene, La Jolla, CA). Five micrograms of nuclear protein were incubated with labeled oligonucleotide [100,000–200,000 counts/min (cpm)] in binding buffer [10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl2, 0.5 μg of poly(dI-dC)-poly(dI-dC), 1% Nonidet P-40, and 4% glycerol] for 25 min at room temperature in a final volume of 25 μl. Free oligonucleotide and oligonucleotide-bound proteins were then separated by electrophoresis on a native 4% polyacrylamide gel. The gel was subsequently dried and exposed to X-ray film between two intensifying screens overnight at −70°C. For supershift studies, antibody (5 μg) to the p65 subunit of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) was added before the addition of the labeled oligonucleotide. Binding of the antibody to the DNA-protein complex was indicated by a supershift in the electrophoretic mobility shift assay (EMSA). To further demonstrate specificity, excess unlabeled (cold probe) oligonucleotide was used as a specific competitor.

Immunoblotting. Lung tissue was homogenized with a tissue homogenizer (Virtishear homogenizer; Virtis, Gardiner, NY) in 5 volumes of homogenization buffer (25 mM Tris-HCl, 2 mM EGTA, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). After centrifugation (3,000 g at 4°C for 20 min), the supernatant was collected. Total protein concentration was determined with the Coomassie Plus protein assay kit (Pierce, Rockford, IL). Lungs were then homogenized in 5 volumes of homogenate buffer (10 mM HEPES, 10 mM KCl, 0.5 M sucrose, 0.1 mM EGTA, and 1 mM dithiothreitol). Homogenates were then centrifuged at 750 g for 10 min at 4°C to isolate crude nuclei. The supernatants, which contained the cytosolic fraction, were stored at −70°C and used for CINC-1 and MIP-2 protein determination. The crude nuclear pellet was then resuspended in 100 μl of ice-cold nuclear extraction buffer (20 mM HEPES, 0.4 M NaCl, 1 mM EGTA, and 1 mM dithiothreitol). Samples were incubated on ice for 30 min with brief, gentle vortexing every 5–10 min. The nuclear extract was then centrifuged at 12,000 g for 5 min at 4°C, and the supernatant was collected and stored at −70°C. Protein was quantified in both cytosolic and nuclear extracts with the Coomassie Plus protein assay kit (Pierce, Rockford, IL).
which 50% of the DNA probe-RNA hybrids melt, \[\text{Tm}_{50}\]. All prehybridization, hybridization, and high-stringency wash were terminated in a Mini-6 hybridization oven (Hybaid, Franklin, MA). Prehybridization, hybridization, and high-stringency wash temperatures were calculated based on a previously published method (22) that employed the equation \[\text{Tm}_{50} = 16.6 (\log [\text{Na}^+] + 81.5 + 0.41 \times (\% \text{GC} \text{ content}) - 675 \times \text{(no. of bases in probe)}, where \text{Tm}_{50} \text{ is the temperature at which 50\% of the DNA probe-RNA hybrids melt, [Na}^+] \text{ is the molar concentration of Na}^+ \text{ in the hybridization solution and %.GC is the percentage of guanidine and cytosine residues in the probe. The hybridization solution consisted of 1× SSPE, 2× Denhardt’s solution, 1% nonfat dry milk, 10% dextran sulfate, 2% SDS, 200 \mu g/ml of salmon sperm DNA, 200 \mu g/ml of yeast tRNA, and 200 \mu g/ml of polyadenylic acid. Prehybridization was carried out at the probe-approximate temperature for 8–12 h. The prehybridization solution was then decanted, and 10 ml of hybridization solution were added and incubated for 18 h. The hybridization solution was identical to the prehybridization solution except for the addition of labeled probe at a final concentration of 5.0 × 10^6 cpm/ml. Both CINC-1 and MIP-2 probes were single-strand 30-mer DNA oligonucleotides synthesized by Gibco BRL. The CINC-1 probe sequence (5′-GGGGTACCTTTGCCAAACTCT-GGATGTTCT-3′) was complementary to nucleotides 170–199 of the CINC-1 cDNA (14). The MIP-2 probe had the sequence 5′-GGGGTACCTTTGCCAAACTCT-GGATGTTCT-3′ and was complementary to rat MIP-2 cDNA nucleotides 304–333. Oligonucleotide probes were labeled by terminal deoxynucleotidyltransferase (TdT). The labeling reaction was set up by adding 2 \mu l of a 10 ng/\mu g solution of oligonucleotide, 2 \mu l of 1 mg/ml of bovine serum albumin, 4 \mu l of 5× TdT buffer (Gibco BRL), 10 \mu l (33 pmol) of [\alpha-32P]dATP (DuPont NEN, Boston, MA), and 2 \mu l of 15 U/\mu l of recombinant TdT (Gibco BRL). The reaction was incubated for 1 h at 37°C. Unincorporated nucleotide was removed by a NucTrap probe purification column (Stratagene). The activity of the probe was determined by scintillation.

After hybridization, blots were washed in 1× SSPE buffer containing 0.5% SDS and 0.1% nonfat dry milk at room temperature. A second set of washes was carried out in 0.2× SSPE with 1% SDS at room temperature. A final high-stringency wash was done in 0.1× SSPE with 0.5% SDS at the calculated temperature. The membrane was then dried, covered in plastic wrap, and exposed to X-ray film (BioMax-1 MR-1, Kodak) in the presence of two intensifying screens at −70°C. Quantification of specific mRNA species was accomplished by densitometry (NIH application 1.599b4) and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA. Membranes were stripped with 2 mM EDTA (pH 8.0), 2 mM Tris (pH 8.0), and 10× Denhardt’s solution for between 2 and 3 h at 65°C and then reprobed for the alternate chemokine or GAPDH.

Rat chemokine protein ELISA. CINC-1 and MIP-2 were measured in lung homogenates (cytoplasmic fraction as described in Nuclear and cytoplasmic protein isolation) with a rat-specific ELISA system. Kits were obtained from Immuno-Biological Laboratories (CINC-1; Fujioka City, Japan) and BioSource International (MIP-2; Camarillo, CA). The absorbancy of standards and samples was determined spectrophotometrically with a microplate reader (Bio-Rad). Results were recorded as optical densities and plotted against the linear portion of the standard curve.

Results. All antibodies for immunoblotting and EMSA were obtained from Santa Cruz Biotechnology. The IκB-α antibody was a rabbit polyclonal IgG against the carboxy terminus of human IκB-α that cross-reacts with rat IκB-α. The NF-κB antibodies were both goat polyclonal IgGs raised against the carboxy terminus of the human p65 subunit of NF-κB, and both cross-reacted with the rat NF-κB subunits. All other chemicals, unless stated, were obtained from Sigma.

Statistical analysis. Statistical analyses were performed with StatView software (Brain Power, Calabasas, CA). Data are presented as means ± SE. Statistical evaluation used standard one-way ANOVA with post hoc Bonferroni-Dunn test. Significance was accepted at a confidence limit of 95%.

Results. I-Arg plasma determinations. To determine the effect of I-Arg supplementation and LPS on plasma I-Arg concentrations, plasma was harvested from rats 1 h after saline, LPS, or I-Arg treatment and analyzed for I-Arg (n = 3 rats/group). Intrapertoneal I-Arg supplementation resulted in an increase in plasma I-Arg compared with saline control (I-Arg, 660.23 ± 129.29 nmol/ml vs. saline, 166.97 ± 9.05 nmol/ml; P < 0.05). LPS led to a decrease in circulating plasma I-Arg levels compared with saline control (saline, 166.97 ± 9.05 nmol/ml vs. LPS: 83.80 ± 14.43 nmol/ml; P < 0.05).

Nitrate/nitrite production. To determine the effect of LPS, supplemental I-Arg administration, and NOS inhibition on plasma nitrate and nitrite production, rat plasma was assayed for total NOx (saline, 22.14 ± 0.21 mol vs. LPS, 16.67 ± 0.23 mol; P < 0.05). LPS abolished the LPS-induced increase in NOx (AMT-LPS, 35.80 ± 1.34 mol; P < 0.01). A NOS inhibitor (AMT) given before LPS abolished the LPS-induced increase in NOx (AMT-LPS, 7.47 ± 0.23 mol; P < 0.05 compared with LPS) and had an inhibitory effect on baseline NOx levels (AMT alone, 9.11 ± 0.25 mol; P < 0.05 compared with saline).

Effect of I-Arg on lung CINC-1 and MIP-2 protein. To determine the effect of I-Arg on chemokine protein production after LPS, rat lung tissue was examined 2 h after LPS for the presence of CINC-1 (Fig. 2A) and MIP-2 (Fig.
**Fig. 1.** Plasma nitrate/nitrite (NO\textsubscript{x}) levels in rats (n = 4 rats/group) treated with saline, *Salmonella typhimurium* lipopolysaccharide (LPS; 0.5 mg/kg ip 2 h before harvest), L-arginine (L-Arg)-LPS (300 mg/kg of L-Arg ip 30 min before LPS), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT)-LPS (0.2 mg/kg of AMT 30 min before LPS), and AMT alone (0.2 mg/kg). Compared with saline, LPS caused an increase in NO\textsubscript{x} 2 h after LPS administration. However, L-Arg administration before LPS induced an even greater increase in NO\textsubscript{x} compared with both saline and LPS. The nitric oxide synthase (NOS) inhibitor AMT given before LPS abolished the LPS-induced increase in NO\textsubscript{x} and had an inhibitory effect on baseline NO\textsubscript{x} levels. *P < 0.05 vs. saline. †P < 0.05 vs. LPS.

**Effect of L-Arg on lung CINC-1 and MIP-2 mRNA.** To examine the effect of L-Arg on chemokine mRNA transcript expression after LPS, rat lung tissue was examined 1 h after LPS. L-Arg before LPS attenuated the effect of L-Arg. Administration of AMT alone had a similar effect to that in saline-treated animals.

**Effect of L-Arg on lung IκB-α.** Compared with saline, LPS caused a significant decrease in IκB-α within 1 h, which returned to saline control levels by 4 h (Fig. 4, A and B). Densitometry of Western blots from three separate experiments revealed no significant difference between the 0.5- and 4-h time points in the LPS-treated animals and no difference between saline control and the LPS-treated 4-h time point (data not shown). Therefore, to determine the effect of L-Arg on IκB-α after LPS, rat lung tissue was examined for IκB-α 1 h after LPS. L-Arg before LPS attenuated the LPS-induced decrease in IκB-α at 1 h (Fig. 4, C and D). D-Arg had no effect on the level of IκB-α protein after LPS. NOS inhibition prevented the effects of L-Arg on IκB-α levels after LPS. AMT alone had a similar effect to saline-treated animals (data not shown).
DISCUSSION

Sheridan et al. (51) previously found that L-Arg decreases lung PMN accumulation and pulmonary vascular injury after LPS (51). Our present studies tested the effect of L-Arg treatment before LPS on chemokine production in the lung. Northern blot and ELISA analysis of lung homogenates confirmed that a low dose of LPS caused an increase in lung CINC-1 and MIP-2 proteins and mRNAs as early as 2 h after LPS. L-Arg attenuated both chemokine mRNA expression and protein production in the lung after LPS. Furthermore, we attempted to determine the mechanism whereby L-Arg decreases chemokine production in this model. The attenuation of chemokine production by L-Arg was associated with maintenance of IκB-α levels and a decrease in NF-κB DNA binding. Finally, inhibition of NOS abolished the effects of L-Arg on LPS-induced chemokine mRNA and protein production, IκB-α levels, and NF-κB DNA binding.

L-Arg is the sole substrate for NO synthesis in mammalian systems. The protective anti-inflammatory role of L-Arg or NO in the lung has been evaluated in a number of studies. In animals, previous investigations have demonstrated that L-Arg or NO alleviates the pulmonary hypertension seen after mesenteric ischemia-reperfusion (16). Furthermore, L-Arg or NO decreases alveolar macrophage proinflammatory cytokine (tumor necrosis factor-α, IL-1β) production (34), prevents alveolar edema (25), and attenuates PMN

Fig. 3. A: Northern blot analysis for CINC-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to reflect equal RNA loading. B: respective densitometry. C: Northern blot analysis for MIP-2 and GAPDH to reflect equal RNA loading. D: respective densitometry. Rats were treated with saline, LPS (0.5 mg/kg ip 2 h before harvest), D-Arg-LPS (300 mg/kg of D-Arg 30 min before LPS), L-Arg-LPS (300 mg/kg of L-Arg ip 30 min before LPS), and AMT-L-Arg-LPS (0.2 mg/kg of AMT 30 min before L-Arg and 1 h before LPS). Representative gels of 3 separate experiments are shown. Densitometry is means ± SE for 3 separate experiments and was calculated as relative density compared with GAPDH. LPS caused a significant increase in both CINC-1 and MIP-2 mRNAs in lung homogenates within 2 h. L-Arg but not D-Arg attenuated the LPS-induced increase in both CINC-1 and MIP-2 messages. NOS inhibition before L-Arg and LPS abolished the L-Arg-associated attenuation of CINC-1 and MIP-2 mRNA production. *P < 0.05 vs. saline. †P < 0.05 vs. LPS.

Fig. 4. Western blot analysis for inhibitory factor κB-α (IκB-α) protein in LPS- (A) and saline-treated (B) rats at indicated times. C: experimental groups 1 h after injection. Lane 1, saline; lane 2, LPS (0.5 mg/kg ip); lane 3, D-Arg-LPS (300 mg/kg of D-Arg ip 30 min before LPS); lane 4, L-Arg-LPS (300 mg/kg of L-Arg ip 30 min before LPS); lane 5, AMT-L-Arg-LPS (0.2 mg/kg of AMT 30 min before L-Arg and 1 h before LPS). D: band densitometry. Values are means ± SE of 3 separate experiments. Representative gels of 3 separate experiments are shown. LPS caused IκB-α degradation within 1 h after LPS. IκB-α levels returned to saline control levels 4 h after LPS. L-Arg before LPS attenuated the LPS-induced degradation of IκB-α. D-Arg had no effect on the LPS-induced decrease in IκB-α. NOS inhibition prevented the effects of L-Arg on IκB-α stabilization after LPS. \*P < 0.05 vs. saline. †P < 0.05 vs. LPS.
The effect of L-Arg. AMT alone had no effect on NF-κB DNA binding, and AMT-L-Arg-LPS (0.2 mg/kg of AMT 30 min before LPS) abolished the effect of L-Arg and LPS. We chose AMT given its reported 20-fold selectivity for iNOS inhibition over endothelial NOS (eNOS) (55). However, the constitutive NOS enzymes may act by a negative feedback mechanism to inhibit the production of chemokines and thereby attenuate lung PMN accumulation. Indeed, one group of investigators (1) has evaluated the role of iNOS in PMN accumulation in a murine model of peritonitis induced by zymosan. In iNOS(−/−) mice, increased PMN accumulation after zymosan injection was associated with increased peritoneal levels of MIP-2 and KC (a murine C-X-C chemokine) (1). Furthermore, in a murine model of endotoxin-induced ALI, NOS inhibition increased proinflammatory cytokine protein and mRNA expression and NF-κB DNA binding (57).

The data in this study suggest that the anti-inflammatory effects of L-Arg are mediated by NOS because NOS inhibition in the presence of supplemental L-Arg prevented the attenuation of chemokine production after LPS. We chose AMT given its reported 20-fold selectivity for iNOS inhibition over endothelial NOS (eNOS) (55). However, the constitutive NOS enzymes may regulate PMN accumulation after a proinflammatory stimulus. Indeed, Lefer et al. (31) found that acute respiratory distress syndrome (ARDS), inhaled NO decreases PMN β2-integrin (CD11b/CD18) expression and bronchoalveolar lavage fluid IL-8 levels (10). The in vivo physiological implications of these data are not completely understood. Thus data from the current study, previous work by this laboratory (16, 51), and that of other investigators (20, 45) support the hypothesis that the l-Arg/NO pathway regulates PMN tissue sequestration by downregulation of both chemokine production and adhesion molecule expression.

Despite the beneficial role of NO in ALI, NO may contribute to lung injury by generation of oxidant by-products. Generation of peroxynitrite from the reaction of NO and superoxide can contribute to tissue damage after sepsis (18, 44, 58). Specifically, there is evidence to suggest that the liberation of NO by inducible NOS (iNOS) leads to tissue damage and contributes to lung injury (24). In fact, iNOS inhibition attenuates LPS-induced ALI in rabbits (36) and dogs (41) and hemorrhagic shock-induced ALI in mice and rats (24). However, experiments utilizing iNOS knockout (iNOS−/−) mice to substantiate the pathological role of iNOS in systemic inflammation give conflicting results. In support of iNOS as an effecter of sustained inflammation and tissue damage, iNOS(−/−) mice demonstrate reduced levels of NF-κB and signal transducer and activation of transcription-3 (STAT3) activation in the lung after hemorrhagic shock (24). Furthermore, Kristof et al. (28) found that LPS injection in iNOS(−/−) mice elicited no significant change in lactate dehydrogenase activity, lung wet-to-dry weight ratio, or pulmonary nitrotyrosine staining compared with wild-type mice (28).

On the other hand, iNOS inhibition or gene deletion may increase tissue PMN accumulation. Hickey et al. (23) found that leukocyte accumulation in the lung was elevated in iNOS(−/−) mice given systemic LPS, suggesting that induction of iNOS is a homeostatic regulator for leukocyte recruitment. Thus, early in the course of LPS-induced pulmonary inflammation, NO may act by a negative feedback mechanism to inhibit the production of chemokines and thereby attenuate lung PMN accumulation. Indeed, one group of investigators (1) has evaluated the role of iNOS in PMN accumulation in a murine model of peritonitis induced by zymosan. In iNOS(−/−) mice, increased PMN accumulation after zymosan injection was associated with increased peritoneal levels of MIP-2 and KC (a murine C-X-C chemokine) (1). Furthermore, in a murine model of endotoxin-induced ALI, NOS inhibition increased proinflammatory cytokine protein and mRNA expression and NF-κB DNA binding (57).

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transmigration of PMNs into the peritoneum of neuronal NOS(−/−) and eNOS(−/−) mice was increased after thioglycolate injection. Additionally, NO derived from eNOS may play a protective role in LPS-induced ALI (19). Despite the reported high degree of selectivity of AMT for iNOS, it is likely that AMT also inhibited the constitutive NOS isoform. This is further substantiated by the fact that AMT inhibited the production of NO in the saline control animals.

Transcription factor activation is a pivotal step in the pathophysiology of ARDS. Indeed, consensus sequences for NF-κB have been identified in the promoter regions of several genes (TNF-α, IL-1, and ICAM-1) that have been implicated in the pathogenesis of ARDS (37). NF-κB is activated and bound to DNA in the nucleus and affecting gene transcription. Loss of NF-κB by preventing the latter from translocating into the nucleus may play a protective role in LPS-induced ALI (19). Despite the reported high degree of selectivity of AMT for iNOS, it is likely that AMT also inhibited the constitutive NOS isoform. This is further substantiated by the fact that AMT inhibited the production of NO in the saline control animals.

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