Nitric Oxide as a Noninvasive Biomarker of Lipopolysaccharide-Induced Airway Inflammation: Possible Role in Lung Neutrophilia

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ABSTRACT

Lipopolysaccharide (LPS) is known to generate nitric oxide (NO) in the airway through the activation of nitric-oxide synthase (NOS). The functional consequences of this on the inflammatory response are not clear, with conflicting data published. In the clinic, exhaled NO (ex-NO) is used as a noninvasive biomarker to assess the extent of airway inflammation. It is proposed that monitoring levels of ex-NO could be a useful guide to determining the effectiveness of disease modifying therapies. The aim was, using pharmacological tools, to determine the role of NO in an aerosolized LPS-driven animal model of airway inflammation by assessment of ex-NO, neutrophilia, and inflammatory biomarkers, using a nonselective NOS inhibitor, \( N\)-G-nitro-L-arginine methyl ester (L-NAME), and a selective inducible NOS (iNOS) inhibitor, \( N\)-3 (aminomethyl)benzylacetamidine (1400W). Real-time mRNA analysis of the lung tissue indicated an increased gene expression of iNOS following LPS challenge with minimal impact on constitutive NOS isoforms. LPS induced an increase in ex-NO, which appeared to correlate with the increase in iNOS gene expression and airway neutrophilia. Treatment with L-NAME and 1400W resulted in comparable reductions in ex-NO, a reduction in airway neutrophilia, but had little impact on a range of inflammatory biomarkers. This study indicates that the LPS-induced rise in ex-NO is due to enhanced iNOS activity and that NO has a role in airway neutrophilia. Additionally, it appears using ex-NO as a guide to monitoring airway inflammation may have some use, but data should be interpreted with caution when assessing therapies that may directly impact on NO formation.
tion after airway instillation of LPS compared with wild-type mice. The reason for this discrepancy is not clear but may be related to varying developmental issues inherent with using genetically modified animals.

The aim of this study was to initially demonstrate that exposure to aerosolized LPS increases levels of NOS gene expression in the airway tissue and levels of ex-NO and then, using pharmacological tools, to determine which NOS isoform is involved in the LPS-induced increase in ex-NO. The compounds utilized were N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, N\textsuperscript{G}-nitro-D-arginine methyl ester (D-NAME), the inactive isomers of L-NAME, and N-(3-(aminomethyl)benzyl)acetamidine (1400W), an iNOS selective inhibitor (5000-fold more selective for iNOS than eNOS and 200-fold more selective for iNOS than nNOS; Garvey et al., 1997) and then, by having demonstrated the impact of this dosing regimen on ex-NO determine, with greater confidence, the role of NO in the LPS-induced airway inflammation seen in this model. Additionally, animals were included that were dosed with dexamethasone, a compound known to inhibit LPS-induced airway inflammation (Haddad et al., 2001), to explore the possibility of using ex-NO as a noninvasive biomarker of airway inflammation in this model.

We believe that, using other disease-relevant models, approaches similar to this will be very useful in determining the role of NO in respiratory diseases such as asthma and chronic obstructive pulmonary disease. Additionally, this profiling technique may be valuable in deciding the relevance of using ex-NO as a noninvasive biomarker for monitoring the effectiveness of possible disease-modifying therapies preclinically and, if these observations can be extrapolated to the situation in man, clinically.

Materials and Methods

Animals

Male Wistar rats (150–180 g) were purchased from Harlan UK Limited (Bicester, Oxon, UK) and kept for at least 5 days before initiating experiments. Food and water were supplied ad libitum. UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed. Experiments were performed on male rats.

LPS-Induced Airway Inflammation

Effect of LPS Concentration on Exhaled NO Release in the Wistar Rat. Male Wistar rats were challenged with inhaled endotoxin-free saline or LPS (0.003–3 mg/ml for 30 min). Four hours after challenge, exhaled NO was measured. Six hours after challenge, lung samples were taken for measurement of NOS isoform gene expression and cellular inflammation.

Effect of LPS on Exhaled NO Production over Time in the Wistar Rat. Wistar rats were challenged with inhaled endotoxin-free saline or LPS (1 mg/ml for 30 min). At 2, 4, 6, 8, and 24 h after challenge, exhaled NO was measured.

Effect of L-NAME, 1400W, and Dexamethasone on LPS-Induced Exhaled NO Production in the Wistar Rat. Wistar rats were challenged with an aerosol of endotoxin-free saline or LPS (1 mg/ml for 30 min). Vehicle (saline, 1 ml/kg), L-NAME, or 1400W (30 or 100 mg/kg) was administered i.p. 2 h prior to and 1 h after challenge. The negative control for L-NAME, D-NAME (100 mg/kg i.p.), was administered at the same time points. The positive standard, dexamethasone (1 mg/kg), was dosed orally 24 and 1 h prior to challenge. Six hours after challenge, cellular inflammation, MPO activity, MMP-9 activity/release, iNOS gene expression, and protein levels were determined.

Assessment of Gene Expression in the Lung Tissue following LPS Challenge

RNA Extraction and Reverse Transcription. Total cellular RNA was isolated from rat lungs using TRI Reagent (Sigma Chemical, Poole, Dorset, UK). Briefly, 1 ml of TRI Reagent was added to 100 mg of ground-up tissue (pizzle and mortar). Samples were centrifuged at 15,000 rpm in a bench-top microcentrifuge (Sigma 2K15; Sigma Chemical) at 4°C for 15 min. The clear supernatant was collected, and 200 μl of chloroform was added. Samples were centrifuged again, the aqueous fraction was collected, and 500 μl of isopropanol was added to the sample to cause precipitation of RNA. Samples were centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant discarded, leaving the RNA pellet. This was washed with 70% (v/v) ethanol and centrifuged at 12,000 rpm for 5 min at 4°C. The ethanol was removed, taking care not to disturb the pellet, and the samples were left to dry. RNA was resuspended in 50 μl of nuclease-free water. The purity and integrity of the RNA samples was assessed by A\textsubscript{260}/A\textsubscript{280} spectrophotometric measurements on the BioTec Universal Microplate Spectrophotometer (Fisher Scientific UK Ltd., Leicestershire, UK).

RNA samples (1 μg) were reverse transcribed using a master mix (Taqman reverse transcription reagents; Applied Biosystems, Warrington, UK) containing 1× Taqman reverse transcription buffer, 5.5 mM MgCl\textsubscript{2}, deoxyNTPs mixture (500 μM per NTP), 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl Multiscribe reverse transcriptase in a final reaction volume of 50 μl. The RNA samples were incubated in a PerkinElmer 480 thermal cycler (PerkinElmer Life and Analytical Sciences, Boston, MA) at 25°C for 10 min, then reverse transcribed at 48°C for 30 min, and finally the enzyme was denatured at 95°C for 5 min. Samples were then stored at −80°C until required for analysis.

Lung Tissue mRNA Expression. Transcriptional expression of target mRNA transcripts in RNA samples were detected by polymerase chain reaction (PCR) amplification and quantified by 5′-nuclease assay using fluorescent labeled Taqman probes and analyzed using real-time quantitative PCR with ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Oligonucleotide primers and TaqMan probes for target genes (iNOS, eNOS, and nNOS) were designed from published GenBank databases of mRNA sequences, using the Primer Express version 1.6 software (Applied Biosystems). The primers for the target gene used for real-time PCR were located in two different exons of each gene to avoid amplification of any contaminating genomic DNA. The TaqMan probe had a fluorescent reporter dye (FAM) covalently linked to its 5′-end and a downstream quencher dye (TAMRA) linked to its 3′-end. Fluorescence quenching depends on the spatial proximity of the reporter and quencher dyes. Reactions were internally controlled with the 18s rRNA internal control (Applied Biosystems). Validations were performed to ensure the reactions were efficient.

PCR reactions were performed in a 25-μl reaction volume containing 3 μl of sample cDNA (2 ng/μl), with forward and reverse primers, TaqMan probe, 2× TaqMan universal master mix, and 18-s internal
control. Amplification and detection of specific products were carried out using an amplification protocol consisting of 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Results were analyzed using Sequence Detection Software (Applied Biosystems), and the relative amount of target gene transcript was normalized to the amount of 18S internal control transcript in the same cDNA sample. The data were then compared with levels in the saline/vehicle control group and are presented as fold increase over this group.

**Quantification of Airway Inflammation.** Six hours after saline or LPS challenge, animals were euthanized with sodium pentobarbital (200 mg/kg i.p.) and the trachea cannulated. Bronchoalveolar lavage (BAL) cells were recovered from the airway lumen by flushing the airways with 10 ml/kg RPMI 1640 (Invitrogen, Paisley, UK) delivered through the tracheal cannula and removed after a 30-s interval. This procedure was repeated, and samples were then pooled for each animal.

The inflammatory cells were extracted from the lung tissue by collagenase digest as described by Underwood et al. (1997). Total white cell numbers in the BAL and lung tissue samples were determined on the Sysmex F820 hematogy analyzer (Linford Wood, Milton Keynes, UK). Cytospins of these samples were prepared by centrifugation of 100–1000 aliquots in a cytosin (Shandon Scientific, Cheshire, UK) at 700 rpm for 5 min, low acceleration at room temperature. Slides were fixed and stained on a Hematek 2000 (Spectron Corp., Kirkland, WA) with modified Wright’s-Giemsa stain. Four-part differential counts on 200 cells per slide were performed following standard morphological criteria, and the percentage of eosinophils, lymphomononuclear cells, and neutrophils was determined.

**Measurement of Exhaled NO Production from Wistar Rats following LPS Challenge.** Exhaled NO was measured using the Sievers NO analyzer (Analytix Ltd., Durham, UK). Animals were placed in sealed 3-l Perspex boxes, which have an NO scrubber attached to the bottom port of the chamber. The top valve of the box was connected by a tube to a pump that when turned on pulled NO-free air through the scrubber into the box. NO was purged from the box for a period of 10 min, then the pump was turned off and the valve closed, allowing the ex-NO to build up for a period of 20 min. At the end of this time, an NO measurement was taken using a sampling tube that was connected to the analyzer and could be attached to a port on the front of the box. Exhaled NO production was expressed as parts per billion.

**Measurement of Neutrophil MPO Activity following LPS Challenge.** Myeloperoxidase activity was measured in BAL supernatants using o-dianisidine hydrochloride as the substrate/hydrogen donor. A mixture of 240 μl of substrate solution (0.167 mg/ml o-dianisidine hydrochloride added to 50 mM phosphate buffer 0.2M monobasic sodium phosphate and 0.2M dibasic sodium phosphate, pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide) and 10 μl of 0.0005% hydrogen peroxide was added to a 96-well plate. Ten microliters of samples or standards (human leukocyte MPO, 0–5 U/ml in saline) was then added to the wells and incubated at room temperature in the dark for 10 min. The plate was read after 30 min on the Multiskan MCC/340 plate reader at 450 nm, and the standard curve was used to determine the MPO content of each sample.

**Cytokine Determination in the BAL and Lung Tissue following LPS Challenge.** For the determination of cytokine protein levels in the lung tissue, approximately 200 mg of lung tissue was homogenized in phosphate-buffered saline using an Ultraturrax T25 homogenizer. The samples were then spun in a bench-top microcentrifuge at 13,000g for 20 min at 4°C [MSE Micro Centaur, Jencons (Scientific) Ltd., Leighton Buzzard, Bedfordshire, UK]. The resulting supernatants were used for cytokine protein quantification.

Protein levels for IL-1α, CINC-2, and CINC-3 were determined by enzyme-linked immunosorbent assay (ELISA) using rat Duosets according to the manufacturer’s instructions. For lung tissue, cytokine levels were further corrected for total protein content, which was measured using the Bradford assay.

**Effect of LPS Challenge on MMP-9 Activation/Release in the BAL.** The presence of MMP-9 in the lavage samples was determined by zymography in accordance with the manufacturer’s instructions (Invitrogen). Briefly, the standard (0.04 ng/μl MMP 9 active enzyme) and samples were denatured by diluting 1:1 with Tris-glycine SDS sample buffer and left to stand for 10 min at room temperature. Samples, standards, and a rainbow molecular weight marker were then loaded onto the gels in a volume of 10 μl. The gels were run for 90 min, at constant voltage of 125 V, an approximated current of 30 to 40 mA/gel at the start, and ending with 8 to 12 mA/gel. After 90 min, the gelatin gels were removed from their casing and incubated in the renaturing buffer for 30 min at room temperature, with gentle rocking. The samples and standards on the gels were then activated in a developing buffer, and the gels were incubated overnight at 37°C. Eighteen hours later, the gels were stained for protein using a 0.1% Serva Blue R stain powder in 40% ethanol, 10% glacial acetic acid, and 75% H2O solution. Gelatinase activity in the samples and standards corresponded to areas on the gels without protein and were exposed with de-stain (19% ethanol, 6% acetic acid, and 50% H2O) and captured on the Grab-IT, Annotating grabber 2.55 package (Ultra Violet Products Ltd., Cambridge, UK). Optical densities of bands were determined by Gel Works 1D Intermediate package (Ultra Violet Products Ltd.).

**Materials.** Sodium pentobarbitone (200 mg/kg) was obtained from Rhône Merieux (Harlow, UK). RPMI 1640, buffers and gels for zymography and fetal bovine serum were from Invitrogen. Roche Diagnostics (Lewes, UK) supplied the DNAse and collagenase. LPS, dexamethasone, t-NAME, d-NAME, Wright’s-Giemsa stain, and reagents for RNA extraction were purchased from Sigma Chemical. 1400W was a gift from GlaxoSmithKline (Stevenage, UK). All ELISA Duoset kits were from R&D Systems Europe (Oxford, UK). All reverse transcription-PCR reagents and primers and probes were obtained from Applied Biosystems. MMP-9 standard was purchased from Merck Biosciences (Nottingham, UK). Endotoxin-free saline was bought from Fresenius Kabi (Warrington, UK).

**Analysis.** Values are expressed as mean ± S.E.M. of n independent observations. Statistical analysis was made using analysis of variance with a correction for multiple comparisons. P < 0.05 was considered to be statistically significant.

**Results**

**Effect of Increasing Doses of Aerosolized LPS on NOS Gene Expression, ex-NO, and Airway Neutrophilia.** The saline-exposed control animals had low levels of expression of all three NOS isoforms in the lung tissue. Exposure to LPS caused a dose-related increase in iNOS gene expression that became significant from 0.3 mg/ml (Fig. 1A). Conversely, exposure to LPS had relatively little impact on eNOS and nNOS gene expression (Fig. 1, B and C).

Aerosolized LPS caused a dose-related increase in ex-NO and airway neutrophilia that reached significance from 0.3 to 1.0 mg/ml (Fig. 2, A–C). The submaximal dose of 1 mg/ml LPS was chosen to complete the remainder of the study.

**Effect of Aerosolized LPS (1 mg/ml) on ex-NO: Time Course.** Exposure to the submaximal dose of LPS (1 mg/ml) caused a time-dependent increase in ex-NO that reached significance at 4 h, peaked at 6 h, and returned to near-normal levels 24 h after challenge (Fig. 3). From these data, it was
decided to determine the impact of the pharmacological tools on ex-NO and airway inflammation at 6 h after challenge.

**Effect of L-NAME, 1400W, and Dexamethasone on LPS-Induced ex-NO.** Negligible amounts of NO were measured from the chambers containing the non-LPS-exposed, vehicle-treated animals compared with the levels measured in the same chambers without the rats present (8.6 versus 6.7 ppm, respectively). Both of the NOS inhibitors reduced basal levels of NO back to empty chamber levels of NO (Fig. 4). In the chambers containing the LPS-exposed, vehicle-treated rats, there was a significant increase in NO. Treatment with L-NAME or 1400W caused a significant dose-related reduction in the LPS-induced increase in NO levels. Dexamethasone significantly inhibited the LPS-induced increase in NO, although not to the same extent as the NOS inhibitors (Fig. 4).

**Effect of L-NAME, 1400W, and Dexamethasone on LPS-Induced Airway Inflammation.** LPS challenge caused a significant increase in airway neutrophilia, BAL MPO levels, iNOS gene expression, and amounts of inflammatory cytokines in the airways (Figs. 5–7). 1400W and L-NAME caused similar small dose-related inhibition of BAL and tissue neutrophilia and BAL MPO levels (Fig. 5). D-NAME did not have a significant impact on these indices (2509 ± 271 10^3 cells/ml; 4933 ± 271 10^3 cells/mg of tissue; 1.7 ± 0.2 U/ml, respectively). Treatment with dexamethasone significantly inhibited airway neutrophilia. The magnitude of the inhibition seen with dexamethasone was in excess of that achieved with the NOS inhibitors. None of
the treatments had any impact on the total volume of BAL recovered.

Of the NOS inhibitors, only the nonselective inhibitor had any impact on the LPS-induced increase in IL-1β, MMP-9, and CINC-3 levels in the airways. Although the inhibition by L-NAME did not reach significance, these data would imply constitutive NOS was involved in the increase in these inflammatory mediators after exposure to LPS. Both 1400W and L-NAME inhibited levels of CINC-2. D-NAME did not have a significant impact on IL-1β, MMP-9, CINC-2, and CINC-3 levels (4082 ± 11006 pg/mg; 5332 ± 1169 optical density of bands; 1141 ± 131 pg/mg; and 123 ± 9 pg/mg, respectively). All inflammatory biomarkers measured were significantly inhibited by treatment with dexamethasone.

LPS exposure significantly increased iNOS gene expression in the lung tissue; both NOS inhibitors caused a small, dose-related reduction in gene expression (Fig. 7). D-NAME did not have a significant impact on iNOS expression (17- ± 2-fold difference from control group). Treatment with dexamethasone significantly inhibited the LPS-induced increase in iNOS gene expression (Fig. 7).

Discussion

It is known that exposing rodents to aerosolized LPS causes marked lung inflammation involving an up-regulation of iNOS expression, an increase in inflammatory biomarkers, and neutrophilia (Kobzik et al., 1993; Haddad et al., 2001). Here, for the first time, we have shown that there appears to be a correlation between the LPS-induced increase in iNOS gene expression and increased levels of NO produced. This would suggest that the increased level of iNOS expression is responsible for the raised NO production. Further evidence implicating iNOS as the primary enzyme involved in the production of NO in this model comes from the fact that both the nonselective NOS inhibitor and the selective iNOS inhibitor blocked the LPS-induced increase in NO production. One possible caveat to this statement is whether 1400W, at these doses, is still selective; however, from data in a previous study, we have shown that using this dosing regimen in rats, 1400W is still selective (Birrell et al., 2003).

Although it is not possible to definitely state that the increase in NO levels in the chambers containing LPS challenged rats is exhaled NO, it seems likely. Other non-airway sources of NO, using this noninvasive method, could be from intestinal mucosa and flora (Hoffman et al., 1997) and bacterial skin flora (Dippel et al., 1994) but are unlikely given that aerosolized LPS produces a lung-specific inflammatory response with no increase in systemic biomarkers, e.g., plasma tumor necrosis factor α (unpublished data). NOS-independent mechanisms (Zweier et al., 1995) could be responsible but would seem doubtful given that the levels of ex-NO are modulated by NOS inhibitors. Another limitation to this technique of measuring ex-NO is the inability to separate the contributions of the upper and...
lower airway; however, a recent study by Weicker et al. (2001) showed that the lower airways, and not the upper airways, were the source of ex-NO using a similar technique in mice.

The small increase in ex-NO levels in the non-LPS-exposed rats, compared with the levels of NO in the chambers without the rats, was completely abolished by L-NAME and 1400W. This would suggest that iNOS activity is the principal source of ex-NO in rats under normal conditions, although an impact of the exposure to aerosol-
ized, endotoxin-free saline cannot be ruled out. These data concur with similar findings published by Steudel et al. (2000) using knockout mice.

Aerosolized LPS caused a significant rise in airway neutrophilia that was inhibited by both L-NAME and 1400W, which would suggest that the iNOS-derived NO plays an inflammatory role in this model. This finding agrees with Shanley et al. (2002) and Okamoto et al. (2004) but not Speyer et al. (2003), all of whom compared the effect of topical airway exposure of LPS in wild-type and iNOS knockout mice. As mentioned in the introduction, it is not clear why there are these discrepancies. Unlike treatment with dexamethasone, which caused almost complete inhibition of the LPS-induced airway neutrophilia; the NOS inhibitors only had a marginal impact (30–40%) on neutrophil numbers in the airways. This limited effect of the NOS inhibitors on lung neutrophilia was accompanied by a nearly complete abolition of ex-NO; this would imply that although NO is involved in LPS-induced airway inflammation in this model, its role accounts for only 30 to 40% of the influx of neutrophils.

The mechanism involved in NO-mediated neutrophil recruitment into the airways after exposure to aerosolized LPS is not known. One possibility is that NO is thought to act as a direct or indirect chemoattractant for a variety of cell types including neutrophils (Belenky et al., 1993; Corriente et al., 1998). Another explanation is that NO has been reported to modulate inflammatory cytokine production (Okamoto et al., 2004); indeed, in this study, L-NAME appeared to cause a small dose-related inhibition of the
inflammatory mediators. 1400W, however, did not impact on the same mediators, suggesting that the impact of l-NAME on these cytokines is due to inhibition of constitutive NOS or is a compound-specific effect. Both NOS inhibitors caused approximately the same percent inhibition of airway neutrophilia; this would imply that, if both NOS inhibitors share a common inhibitory mechanism, the impact of l-NAME on these mediators has little biological relevance in this model. Interestingly, both the selective and nonselective NOS inhibitors appeared to reduce levels of CINC-2. On a search of the literature, the authors were unable to find any reasons why 1400W would selectively inhibit CINC-2 production. One possible explanation is that the reduction in CINC-2 protein levels is a consequence of the inhibition of neutrophilia rather than a cause; indeed, the profiles of neutrophil number and CINC-2 levels are strikingly similar. In addition, because NOS inhibitors are not reported to impact on iNOS gene expression directly, the inhibition of neutrophilia could also be the reason for the nonsignificant reduction in iNOS gene expression observed in the NOS inhibitor treatment groups.

Other possible NO-dependent mechanisms that could be involved in airway neutrophilia are the possible role of NO in microvascular leakage (Kuo et al., 1992), regulation of airway tone (Nijkamp et al., 1993), and the effect of NO on blood pressure and heart rate (Rees et al., 1990).

This is the first study, using pharmacological tools, to determine the role of NO in topically administered LPS-induced airway inflammation. This study shows that the principal source of ex-NO is as a consequence of inducible NOS enzyme activation, and blocking NO production is anti-inflammatory. Additionally, treatment with dexamethasone inhibited iNOS formation, ex-NO, and airway inflammation to a similar extent, suggesting that ex-NO could be a useful noninvasive tool when monitoring the effectiveness of potential disease modifying therapies on airway inflammation. Unfortunately, because only one high dose of steroid was used in this study, it is not possible to determine whether the impact of different doses of dexamethasone on ex-NO correlates with the inhibition of inflammation. Determining the usefulness of ex-NO as a noninvasive monitor of the effectiveness of a therapy will have to be done in this, or any preclinical model, for each class of compound because it will depend on the impact of the therapy directly on NO formation and whether that correlates with the anti-inflammatory effects. Additionally, in the clinic, patients usually receive therapies once or twice a day, so there may be no single dose that will allow monitoring of inflammation to a similar extent, suggesting that ex-NO could be a useful noninvasive tool when monitoring the effectiveness of potential disease modifying therapies on airway inflammation. Unfortunately, because only one high dose of steroid was used in this study, it is not possible to determine whether the impact of different doses of dexamethasone on ex-NO correlates with the inhibition of inflammation.

We believe that, using other disease-relevant models, approaches similar to this will be very useful in determining the role of NO in respiratory diseases such as asthma and chronic obstructive pulmonary disease. Additionally, this profiling technique may be valuable in deciding the relevance of using ex-NO as a noninvasive biomarker for monitoring the effectiveness of possible disease-modifying therapies preclinically and, if these observations can be extrapolated to the situation in man, clinically.

References

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