Expression of Inducible Nitric Oxide Synthase in Lungs of Broiler Chickens Following Intravenous Cellulose Microparticle Injection

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ABSTRACT

Intravenous microparticle (MP) injection is a patented method used to select broilers with a robust pulmonary capacity and improved resistance to pulmonary hypertension syndrome (PHS, ascites). Injected MP become entrapped in the terminal pulmonary arterioles where they elicit an increase in pulmonary arterial pressure attributable to vascular occlusion and focal thrombocyte aggregation. Within 2 to 48 h postinjection perivascular mononuclear cell aggregates begin to form around MP-occluded vessels. Nitric oxide (NO) has been shown to modulate the pulmonary arterial pressure response to MP entrapment, but a role of NO during the more chronic (2 to 48 h) focal inflammatory response has not been evaluated. In this study we determined the time-course of inducible nitric oxide synthase (iNOS) expression in the lungs of MP-injected broilers from PHS-resistant (RES) and PHS-susceptible (SUS) lines. Four-week-old broilers (10 broilers/line per time point) were injected i.v. with a minimally lethal dose of MP, and the right lung was collected at 0 h (no MP) and 2, 24, and 48 h postinjection. Immunohistochemistry revealed that macrophage infiltration increased over time in both lines and was higher in the RES line than the SUS line (P < 0.0001) at all time points. Nicotinamide adenine dinucleotide phosphate diaphorase staining showed nitric oxide synthase activity around MP-occluded vessels and in the perivascular mononuclear cell aggregates. Relative iNOS expression in lung tissue was examined by 2-step reverse transcription PCR. Lines differed in relative iNOS mRNA expression only at 24 h (P < 0.001; RES > SUS line). For the RES line iNOS mRNA expression increased consistently from 0 to 48 h, but for the SUS line iNOS mRNA expression increased at 2 h, decreased to baseline at 24 h, and increased again by 48 h. The decline in iNOS expression in the SUS line between 2 and 24 h coincides with the interval when most of the MP-induced mortality occurs, which suggests that NO synthesized by iNOS may contribute to lower MP-induced mortality in the RES line when compared with the SUS line.

Key words: ascites, chicken, microparticle, macrophage, reverse transcription-polymerase chain reaction

INTRODUCTION

Intravenous injection of cellulose microparticles (MP) having a size suitable for occluding pulmonary arterioles is a patented method (US patent no. 6,720,473) used by commercial broiler geneticists to screen for the susceptibility of broilers to pulmonary hypertension syndrome (PHS, ascites; Wideman and Erf, 2002; Wideman et al., 2002). The pathophysiology of PHS is initiated by the onset of pulmonary arterial hypertension (PAH) and progresses to the development of right-sided congestive heart failure and terminal ascites (fluid accumulation in the abdominal cavity). After MP are injected i.v. they are carried to the lungs by the venous blood where they occlude the precapillary arterioles resulting in PAH due to increased pulmonary vascular resistance (Wideman and Erf, 2002). A suitable MP injection dose causes broilers with the lowest pulmonary vascular capacity to succumb within 48 h to PAH and respiratory insufficiency; those with marginal pulmonary vascular capacity develop terminal ascites within 2 wk; and those with the most robust pulmonary vascular capacity thrive as clinically healthy PHS-resistant survivors (Wideman and Erf, 2002; Wideman et al., 2002).

The PAH response to MP entrapment has been attributed, in part, to the synthesis and release of vasoactive compounds produced and released by vascular endothelial cells, thrombocytes, and leukocytes (Wideman, 2001; Wideman et al., 2007). Nitric oxide (NO) is one of the principal vasodilators known to modulate the onset of PAH (Grabarevic et al., 1997). Nitric oxide is synthesized by the enzyme nitric oxide synthase (NOS) from the oxidation of L-arginine in the presence of O2, nicotinamide adenine dinucleotide phosphate (NADPH), and other cofactors (Zapol et al., 1994). Three isoforms of NOS have been identified so far: neuronal NOS (nNOS), endothelial...
NOS (eNOS), and inducible NOS (iNOS; Moncada et al., 1997). The inducible isoform, iNOS, is expressed by avian macrophages following activation by lipopolysaccharides (LPS), cytokines, and other factors (Hauschildt et al., 1991; Nathan, 1992) resulting in the production of large quantities of NO (Chang et al., 1996; Hussain and Qureshi, 1997). The competitive inhibitor N-nitro-l-arginine methyl ester (l-NAME) inhibits all NOS isoforms, and injecting l-NAME into broilers results in an acute increase in pulmonary arterial pressure (PAP) that can be counteracted by injecting a NOS-independent NO donor (Weidong et al., 2002; Chapman and Wideman, 2006). When broilers were preinjected with l-NAME the increase in PAP elicited by a subsequent MP injection was amplified 2-fold when compared with the responses of control broilers. Similarly, the mortality triggered within 48 h after injecting MP was more than doubled when l-NAME was combined with microparticle injection doses that otherwise caused relatively low mortality in the absence of l-NAME (Wideman et al., 2005). The modulatory efficacy of NO likely reflects its ability to dilate the vasculature directly, as well as its ability to inhibit the synthesis or release of key vasoconstrictors such as serotonin and endothelin-1 (Wideman et al., 2007).

The MP entrapped in the lungs initiate a vigorous, focal inflammatory response. Within minutes thrombocytes aggregate around MP lodged in pulmonary arterioles. This is followed by infiltration and aggregation of mononuclear cells (monocytes/macrophages and lymphocytes) in the perivascular region surrounding MP-occluded arterioles (Wideman et al., 2002, 2007; Wang et al., 2003). It is our hypothesis that activation of monocytes/macrophages leads to increased iNOS expression, resulting in increased NO synthesis, and that this focally produced NO assists in dilating (relaxing) the adjacent vascular smooth muscle as well as in modulating the release of vasoconstrictors. Subsequent to the acute PAH response induced by MP entrapment, the expression and activation of iNOS in recruited macrophages should serve as the major source of NO production contributing to the broilers’ ability to survive MP-induced PAH (Wideman et al., 2007). Accordingly, macrophages recruited to MP-occluded vessels in the lung would be expected to be activated and express iNOS, and the macrophages of broilers that are resistant to PHS may be infiltrating sooner, in greater numbers, or express more iNOS, or both, than the macrophages of broilers that are susceptible to PHS. Hence, the objectives of this study were to demonstrate the time course of pulmonary macrophage infiltration, iNOS mRNA expression and NO production following i.v. injection of MP in PHS-susceptible and PHS-resistant broiler lines.

MATERIALS AND METHODS

Broiler Management and Lines

Newly hatched male broiler chicks were transported from the hatchery to the Poultry Environmental Research Lab at the University of Arkansas Poultry Research Farm, Fayetteville. The broilers were progeny from the PHS-resistant (RES) and PHS-susceptible (SUS) lines that were developed by divergent selection in a hypobaric chamber (Pavlidis et al., 2007). At the time of this study the lines were at their ninth generation of selection, and when reared under conditions of hypobaric hypoxia they exhibited ascites mortalities of 7.5 and 75% for RES and SUS lines, respectively. One hundred male chicks per line were wing-banded and reared together on fresh wood shavings in environmental chambers (8 m² of floor space). Chicks were brooded at 33°C on d 1 to 5, at 29°C on d 6 to 10, and at 23.8°C, d 11 onwards. Chicks were fed corn-soybean meal broiler ration formulated to meet National Research Council (1994) recommendations for all the ingredients. Feed and water was provided ad libitum. Lights were on 24 h/d through d 5, 23 h/d through d 19, and 16 h/d from d 20 onwards.

Experimental Design and Tissue Collection

Fifty male broilers per line were injected with MP as described previously (Wideman and Erf, 2002; Wideman et al., 2002; Wang et al., 2003). Heparinized saline was prepared by dissolving 150 units of ammonium heparin (Sigma-Aldrich Inc., St. Louis, MO) per milliliter of 0.9% NaCl. Microgranular CM-32 ion exchange cellulose (Fisher Scientific, St. Louis, MO) having 30-μm average particle diameter was suspended at the rate of 0.02 g/mL in the heparinized saline solution. This suspension was vortexed continuously on a magnetic stirring plate to keep the MP evenly distributed and was injected to the broilers via the left wing vein at the dose of 0.30 mL/broiler using a 1-mL tuberculin syringe (Becton Dickinson). Lungs collected from 10 broilers per line that had not been injected with MP were designated as 0 h (uninjected control) samples. Lungs also were collected from 10 broilers per line at 2, 24, and 48 h post-MP injection.

Lung tissues were used for histology, immunohistochemistry, NADPH diaphorase histochemical staining, and iNOS mRNA gene expression analysis. At each time point 10 broilers per line were exsanguinated by decapitation. The portion of the right lung between the first and second anterior rib indentation was collected aseptically and was embedded in Tissue-Tek OCT freezing medium (Sakura Finetek Inc., Torrance, CA), snap frozen in liquid nitrogen, and stored at −80°C until analysis. An adjacent segment from each lung was immersed in 10% buffered formalin for histological evaluation. This tissue was embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and assessed microscopically to confirm that each of the lungs collected at 2, 24, and 48 h postinjection had entrapped substantial numbers of MP.

Immunohistochemistry for Macrophages

Immunohistochemical staining of the lung tissue for macrophages was done as described previously (Wang
et al., 2003). Briefly, a 5-μm-thick transverse section of frozen lung tissue was cut using a cryostat (Microm Laborkerätte GmbH, Waldorf, Germany) at −22°C, mounted on poly-L-lysine-coated slides, fixed in acetone (Burdick and Jackson, Muskegon, MI) for 5 min, and air-dried. The lung sections were incubated overnight at room temperature (RT) with blocking buffer containing 10% horse serum, and 90% PBS (0.01 M, pH 7.2) in a humid chamber to inhibit nonspecific binding of immunoreagents. After overnight incubation, the lung sections were washed 5 times with PBS (this wash step was repeated after each incubation step) and the sections were incubated with 150 μL of primary antibody (1:5 dilution in blocking buffer) for 30 min at RT. The primary antibody was a mouse monoclonal antibody specific for chicken monocytes/macrophages (KUL01, Southern Biotechnology Associates, Birmingham, AL). After the 30 min incubation, the sections were washed with PBS and incubated with 150 μL of secondary antibody (1:100 dilution in blocking buffer) for 30 min at RT. The secondary antibody was biotinylated polyclonal horse anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA). To detect binding of the primary and secondary antibody, the sections were incubated in 150 μL of ABC reagent for 30 min at RT. The ABC (Avidin and biotinylated-horseradish-peroxidase complex) reagent was prepared by adding 10 μL of reagent A and 10 μL of reagent B (Vector Laboratories Inc.) in 1 mL of PBS according to the manufacturer’s instructions. Finally, the sections were incubated with 150 μL of substrate (3,3′-diaminobenzidine (Sigma-Aldrich Inc.) for 5 to 10 min depending on the color development. The substrate was charged by adding 3.3 μL of 0.3% H2O2 per milliliter of substrate prior to adding to the sections. After sufficient color development, the tissue sections were washed with PBS, counterstained with MethylGreen stain (EMD Chemicals Inc. Gibbstown, NJ), dehydrated, and covered with aqueous mounting medium (Aquamount, Lerner Laboratories, Pittsburgh, PA) and a cover slip (VWR International, West Chester, PA) for microscopic observations. Immunostained lung sections were examined microscopically for macrophages using a computerized image analysis system comprised of a Cool SNAP cf digital camera (Image Processing Solutions Inc., North Reading, MA) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD). A minimum of 6 microscope fields per section were evaluated at 40× magnification using an Olympus BX50 microscope (Olympus America Inc., Center Valley, PA). Macrophage counts were expressed as the percentage of the total tissue area examined that was occupied by immunostained (brown) cells (macrophages, % area).

**NADPH Diaphorase Histochemistry**

The NADPH diaphorase activity has been used as a histochemical marker for NOS enzyme localization (Mitchell et al., 1992; Beesley, 1995). The NADPH diaphorase histochemistry was performed as described by Moreno de Sandino and Hernandez (2003). Briefly, three 5-μm-thick sections per tissue were cut from frozen lung tissue using a cryostat, mounted on poly-L-lysine-coated slides, and were fixed in freshly prepared 2% buffered paraformaldehyde (TCI, Portland, OR) in PBS with 0.5% glucose (EMD Chemicals Inc.) for 10 min. Sections were then washed with wash buffer containing 0.1 M Tris HCl (Shelton Scientific Inc., Shelton, CT), and 0.05% Triton X-100 (Sigma-Aldrich Inc.). Three tissue sections (1 each for stain proper, negative control, and positive control) per lung tissue were incubated with 150 μL of negative control, positive control, and stain proper solution, for 60 min at 37°C in a humid chamber. Stain proper solution had 0.25 mg/mL of nitroblue tetrazolium, and 1 mg/mL of NADPH (Sigma-Aldrich Inc.) in 0.05% Triton X-100 in 0.1 M Tris buffer. The negative control solution was stain proper solution without NADPH, and the positive control solution contained stain proper solution and 15 mM p-nitrophenylphosphate (Southern Biotechnology Associates). After 60 min, the sections were washed with wash buffer, air-dried, and covered with cover slips after addition of aqueous mounting medium. The tissue sections were then evaluated for NOS localization as evidenced by blue staining.

**Real-Time Reverse Transcription-PCR**

**RNA Isolation and Quantification.** Ten to fifteen 100-μm-thick frozen lung sections/lung (24 to 26 mg of tissue) were used for RNA isolation. The RNA was isolated using RNeasy mini kit including an additional DNA digestion step for 15 min with RNase-free DNase (Qiagen Inc., Valencia, CA). The concentration of isolated RNA was determined using the Ribogreen RNA quantitation kit (Molecular Probes, Eugene, OR) following the high range assay protocol (20 ng/mL to 1 μg/mL of RNA). Briefly, the standards for high range assay were prepared as shown in Table 1. The RNA samples were diluted 1:200 in 1× Tris EDTA buffer. Blanks, standards, and samples (100 μL each) in duplicate were pipetted into their designated wells in a 96-well microtiter plate. Ribogreen reagent (100 μL; 1:200 dilutions) was then added to each well, and the plate was incubated at RT for 5 min. The fluorescence was recorded in a fluorescence microplate reader (BioTek Instruments Inc., Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The concentration of RNA was calculated based on the standard curve (fluorescence vs. RNA concentration) generated from the set of RNA standards included in each plate.

**cDNA Synthesis.** The RNA (200 ng/sample) was reverse transcribed to cDNA using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s instruction. The reaction was carried out in a 20-μL reaction volume having the following reagents at final concentration: 1× Taqman RT buffer, 5.5 mM MgCl2, 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 unit/μL of RNase inhibitor, and 1.25 unit/μL of Multiscribe reverse transcriptase. The re-
verse transcription was performed in a Biometra personal cycler (Biometra GmbH I. L., Göttingen, Germany). The incubation steps used were one cycle of 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

**Real Time PCR and Quantification of Relative mRNA Expression.** Real-time PCR was performed using the Taqman PCR Core Reagent Kit (Applied Biosystems) and an ABI PRISM 7700 sequence detection system (Applied Biosystems). The PCR was performed in a reaction volume of 25 µL containing the following reagents at final concentration: 1× Taqman buffer A, 5.5 mM MgCl₂, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, forward primer 200 nM, reverse primer 200 nM, Probe 100 nM, 0.01 unit/µL of AmpErase UNG, 0.025 unit/µL of AmpliTaq Gold DNA polymerase, and 2 µL of cDNA sample. The cycling profiles used were one cycle at 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s, and 60°C for 60 s.

Previously published primers and probes for 28S and chicken iNOS (Smith et al., 2005) were used for PCR. The primer and probe sequences are given in Table 2. In each plate, a no template control (no cDNA, master mix only), a calibrator sample, cDNA samples, a set of 5 standards for iNOS (dilutions: 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵), and 28S (dilutions: 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, and 10⁻⁶) were used. The 28S was used as an endogenous control. The cDNA used for standards was synthesized from RNA isolated from an LPS-stimulated chicken macrophage cell line (MQ-NCSU) 6 h post-LPS stimulation. The relative quantification of iNOS mRNA expression was carried out by using the cycle threshold value by relative standard curve method as described in ABI PRISM 7700 sequence detection system, user bulletin no. 2 (Applied Biosystems).

**Statistical Analysis**

For the macrophage count (% area) and the relative expression level of iNOS mRNA, 1-way ANOVA was carried out to determine line difference within a time point and also the time point difference within a line using SYSTAT statistical software (version 10.2, Systat Software Inc., San Jose, CA). Differences among the group means were determined by Fisher’s LSD multiple mean comparison test. Data were expressed as mean ± SEM, and the differences were considered to be significant at P ≤ 0.05.

### Table 2. Primers and probes used for real time PCR

<table>
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<th>Target</th>
<th>Primer¹ or probe¹,²</th>
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<td>Reverse</td>
<td>5’-GACGACCGATTTGACGTC-3’</td>
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<td></td>
<td>Probe</td>
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<td>U46504</td>
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<td></td>
<td>Reverse</td>
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<td></td>
<td>Probe</td>
<td>5’-TCACAGACGATAGGCCCTTCTTT-3’</td>
<td></td>
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</table>

¹Primers and probes used were obtained from MWG Biotech, High Point, NC.
²Probes were labeled with TAMRA (6-carboxyfluorescein) at the 5’ end, and with SYTO18 (6-carboxytetramethylrhodamine) at the 3’ end.
³Previously published sequences (Smith et al., 2005) for primers and probes were used.
⁴Subtype of ribosomal RNA.
⁵Inducible nitric oxide synthase (iNOS).
RESULTS AND DISCUSSION

Macrophage Infiltration

In the lungs of un.injected control (0 h) broilers, immunohistochemical staining with the KUL01 antibody revealed only few evenly distributed monocytes/macrophages (Figure 1A). Following MP injection, immunohistochemistry revealed macrophage infiltration in the vicinity of entrapped MP in all of the lung tissues (Figure 1A). By 2 h the MP-occluded pulmonary arterioles were outlined by very strong KUL01 staining, which may be due to monocytes surrounding the particle inside the vessels as well as macrophages infiltrating the perivascular region of the occluded arterioles. Moreover, small macrophage foci were clearly evident in the perivascular region of the occluded arterioles. By 24 h the infiltration of macrophages was more intense, and many more macrophages were seen in the perivascular area of MP-occluded arterioles. By 48 h, the macrophage infiltration became more generalized and incorporated almost the whole area of the lung sections (Figure 1A).

The mean macrophage count (% of tissue area examined) was higher (P = 0.0001) in the RES line than in the SUS line at all time points examined (Figure 2). For the RES line, the macrophage count increased steadily from a control level of 4.61 ± 0.16% at 0 h to 8.60 ± 0.14% at 48 h, whereas for the SUS line the macrophage count increased from 3.58 ± 0.14% at 0 h to 6.13 ± 0.15% at 24 h but did not increase further by 48 h (5.78 ± 0.16%; Figure 2). The present study demonstrates the time-course of macrophage infiltration following MP injection and confirms the results of a previous study (Wang et al., 2003) demonstrating that cellulose MP entrapped in the pulmonary vasculature acutely initiate marked, focal inflammatory responses within the lung parenchyma of broilers. The higher macrophage count and the sustained increase in macrophage infiltration in the RES line, compared with the SUS line over the 48-h time period examined, suggests that the RES line’s ability to better cope with and control the MP-induced PAH may be due, in part, to an inherent superiority in mounting a fast and appropriate inflammatory response able to modulate the vascular challenge initiated by the i.v. MP injection.

NOS Activity

In the context of MP-induced PAH, NO produced by NOS may be critical for counteracting the MP-induced vasoconstriction. The NOS activity in the lung tissue of MP-injected broilers from the RES and SUS line was examined by NADPH diaphorase histochemistry as described by Moreno de Sandoino and Hernandez (2003). In this assay, blue color development (seen as dark staining in the black and white pictures) indicates the location of NOS activity, which was the primary objective using this staining method (Figure 1B). The NOS activity was not observed at 0 h in uninjected controls, but within 2 h after MP injection dark staining could be observed around the vessels occluded with the MP. The staining was also observed in the area of the perivascular macrophage infiltrate, but the intensity of the stain was highest in the immediate vicinity of the occluded vessels. Furthermore, the intensity as well as the area involved in NOS activity increased consistently from 2 to 48 h (Figure 1B). The NADPH diaphorase histochemistry is a marker for the localization of NOS enzyme, rather than a quantitative estimate of NOS activity (Mitchell et al., 1992; Beesley, 1995), and therefore could not be used for quantitative comparison between RES and SUS birds in this study. Additionally, this staining procedure clearly showed that the location, intensity, and extent of NOS activity coincided with the location and amount of macrophage infiltration (% area) from 0 to 48 h and hence would be primarily indicative of iNOS activity.

Relative iNOS mRNA Expression

To specifically address the involvement of iNOS in the response to MP lodged in the pulmonary vasculature, the relative iNOS mRNA expression in the broiler lung tissue was examined by real time RT-PCR methodology, and the results were expressed as fold change. Line differences were observed in the relative iNOS mRNA expression at 24 h post-MP injection (P = 0.001) but not at the 0, 2, and 48 h sampling intervals (Figure 3). For the RES line the iNOS mRNA expression increased consistently from 0 h (2.70 ± 0.08) to 48 h (24.90 ± 6.47), but for the SUS line iNOS mRNA expression was biphasic and increased at 2 h (10.80 ± 2.58), decreased to base line value at 24 h (2.38 ± 0.62), and increased again at 48 h (26.20 ± 4.40) (Figure 3). This study demonstrates for the first time in situ iNOS mRNA expression in lungs within 2 h post i.v. MP injection. Maximal levels in iNOS expression were achieved at 48 h post-MP injection for both the RES and SUS lines. In an in vitro study, Hussain and Qureshi (1997) examined the expression of iNOS by cells from the MQ-NCSU macrophage cell line and cephadex-elicited abdominal macrophages from 3 different chicken strains [Cornell K strain, GB1 (B15B15), and GB2 (B6B6)] following in vitro stimulation with LPS. They reported that iNOS expression in the macrophages peaked at 6 h post-LPS stimulation and that the amount of NO produced by the macrophages was directly proportional to iNOS mRNA expression (i.e., high in high NO-producing Cornell K-strain and MQ-NCSU macrophages; low in low NO-producing GB1 and GB2 macrophages). In another study, Bowen et al. (2007) reported that plasma NO concentrations in response to i.v. LPS injection in male broiler chickens peaked between 5 to 6 h post-LPS injection; however, following i.v. MP injection plasma NO concentrations did not increase throughout the 12-h study period. The difference in the plasma NO concentration following the i.v. injection of LPS compared with MP may be due to the fact that LPS is able to systemically activate monocytes/macrophages, whereas the MP act locally within the vicinity of the obstructed pulmonary microvasculature. Hence,
**Figure 1.** Monocyte/macrophage infiltration (A) and nitric oxide synthase (NOS) activity (B) in the lungs of broilers from a pulmonary hypertension syndrome (PHS)-resistant (RES) and PHS-susceptible (SUS) line at 0 h (not injected) and 2, 24, and 48 h post i.v. injection of microparticles (MP).

A. To detect monocytes/macrophages, frozen lung tissues sections (5 μm) were immunohistochemically stained using mouse anti-chicken monocyte/macrophage monoclonal antibody (KUL01) as the primary antibody, biotinylated polyclonal horse anti-mouse IgG as the secondary antibody, and avidin-biotin-peroxidase detection reagents. The immunostained sections were counterstained with methyl-green stain. Dark stained cells are KUL01-positive.

B. NOS activity (dark stained areas) in frozen lung sections was identified by NADPH diaphorase histochemical staining. Five-micrometer-thick transverse sections of frozen lung tissue were fixed in 2% buffered paraformaldehyde in PBS with 0.5% glucose for 10 min and then incubated with staining solution consisting of 0.25 mg/mL of nitroblue tetrazolium and 1 mg/mL NADPH in 0.1 M Tris buffer containing 0.05% Triton X-100 for 60 min. The NOS activity resulted in a blue (dark) staining. Images (A and B) were captured at 100× magnification.
Macrophage counts (% area) in the lungs of broilers from a pulmonary hypertension syndrome (PHS)-resistant (RES) and PHS-susceptible (SUS) line at 0 (not-injected) and 2, 24, and 48 h post i.v. microparticle injection. Monocytes/macrophages in frozen lung tissues sections were identified by the indirect immunoperoxidase staining using the KUL01 mouse anti-chicken monocyte/macrophage monoclonal antibody. Stained sections were examined microscopically for monocytes/macrophages (KUL01, brown stained cells) by a computerized image analysis system composed of a Cool SNAP cf digital camera (Image Processing Solutions Inc., North Reading, MA) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD). A minimum of 6 fields per section were evaluated at magnification of 40× and data were expressed as the percentage of brown cells in the total area of the tissue sections examined (% area). Data shown are the mean ± SEM based on the macrophages count (% area) from 6 lungs per line/time point. Letters a and b indicate line differences between each time point and letters w, x, y, and z indicate difference among the time points within each line. For each analysis, means without common letters are considered different (P ≤ 0.05).

Figure 2. Macrophage counts (% area) in the lung may be sufficient to elicit a biological response but not sufficient to cause an increase in plasma NO concentrations. Previous studies suggest that NO produced in the immediate vicinity of pulmonary vascular smooth muscle can vary significantly biological impact by dilating the local vasculature without necessarily elevating total plasma NO concentrations (Chapman and Wideman, 2006; Bowen et al., 2007). Moreover, as the MP are entrapped in the pulmonary vasculature the stimulus of entrapped MP persists for a long time and macrophage recruitment and activation continue for >48 h, whereas, for LPS, the single i.v. dose likely dissipates and thus the stimulus for iNOS activation subsides rapidly. For the 12 h sampling window used by Bowen et al. (2007), the local pulmonary iNOS expression and macrophage recruitment in response to the MP injection was in the initial stages, and hence NO production was likely not sufficiently high to be noted in the circulation. Whether local pulmonary NO production in MP injected broilers may be reflected in the plasma at later time points when peak macrophage infiltration is in progress remains to be determined.

In summary, this experiment was conducted to study the time course and extent of iNOS mRNA expression in the lung of broilers injected with MP and to compare iNOS expression profiles of PHS-resistant and PHS-susceptible broilers. Immunohistochemistry showed that macrophages infiltrated the perivascular area of MP entrapped in the pulmonary vasculature as early as 2 h postinjection, and thereafter macrophage infiltration continued to increase over time in both lines. Based on the overlap of location, timing, and extent of NOS activity with that of the observed macrophage infiltration, we
Figure 3. Fold change in inducible nitric oxide synthase (iNOS) mRNA expression in the lung tissue of the broilers from pulmonary hypertension syndrome (PHS)-resistant (RES) and PHS-susceptible (SUS) line at 0 (not-injected) and 2, 24, and 48 h post i.v. microparticle injection. Relative iNOS mRNA level was quantified by real time reverse transcription PCR that was performed in Applied Biosystems 7700 sequence detection system (Applied Biosystems, Foster City, CA). Data are expressed as means ± SEM, and means are based on relative iNOS mRNA levels from 8 lung samples per line per time point (0, 2, 24, and 48 h). Letters a and b indicate line differences between each time point, and letters w, x, y, and z indicate differences among the time points within each line. For each analysis, means without a common letters are considered different ($P \leq 0.05$).

concluded that NOS activity, as determined by NADPH diaphorase histochemistry, at 2, 24 and 48 h post-MP injection was due to activation of iNOS in both lines. Subsequent quantitative estimation of iNOS mRNA expression revealed a consistent increase from 0 to 48 h in the RES line, but a biphasic expression for the SUS line, with iNOS mRNA expression increasing at 2 h, returning to baseline levels at 24 h before increasing again at 48 h. It is likely that the steady increase in pulmonary iNOS mRNA expression and resulting NO production over the first 48 h post-MP injection is critical for the lower morbidity and mortality observed in broilers from the RES line compared with those of the SUS line. The modulatory role of NO in counteracting pulmonary vasoconstriction in broilers is well documented (Grabarevic et al., 1997; Weidong et al., 2002) and the sustained vasoconstriction initiated by MP injection would require a sustained vasodilatory effort to control the MP-induced elevation of PAP. The biphasic nature of iNOS expression in lungs from MP-injected SUS line broilers would indicate a disruption in NO production at a time when vasodilation and the modulation of vasoconstrictors by NO is critical for survival.

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REFERENCES


