Differential expression of vasoactive mediators in microparticle-challenged lungs of chickens that differ in susceptibility to pulmonary arterial hypertension

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Am J Physiol Regul Integr Comp Physiol 298: R235–R242, 2010. First published November 11, 2009; doi:10.1152/ajpregu.00451.2009.—Pulmonary hypertension syndrome (PHS; ascites) in fast-growing meat-type chickens (broilers) is characterized by the onset of idiopathic pulmonary arterial hypertension (IPAH) leading to right-sided congestive heart failure and terminal ascites. Intravenous microparticle (MP) injection is a tool used by poultry geneticists to screen for the broilers that are resistant (RES) or susceptible (SUS) to IPAH in a breeding population. MPs occlude pulmonary arterioles and initiate focal inflammation, causing local tissues and responding leukocytes to release vasoactive mediators such as serotonin (5-HT), endothelin-1 (ET-1), and nitric oxide (NO). RT-PCR was used to examine the differences between RES and SUS broilers in terms of gene expression of ET-1, ET receptor types A and B (ETA and ETB), the serotonin transporter (SERT), serotonin receptors (5-HT1A, 5-HT2A, 5-HT2B), endothelial NO synthase (eNOS), and inducible NOS (iNOS) in the lungs of these broilers before (0 h) and after (2, 6, 12, 24, and 48 h) MP injection. In SUS broilers MP injection elicited higher (P < 0.05) pulmonary expression of 5-HT1A, 5-HT2B, and ET-1, which promote vasoconstriction and proliferation of pulmonary arterial smooth muscle cells (PASMC). In RES broilers the MP injection elicited higher expression of eNOS, iNOS, and ETB, which promote vasodilation and inhibit PASMC proliferation. These observations support the hypothesis that the resistance of broiler chickens to IPAH may be due to the higher expression of vasoactive mediators that favor enhanced vasodilation and attenuated vasoconstriction during MP injection challenges to the pulmonary vasculature.

vasoactive mediators, such as endothelin-1, serotonin (5-HT), and nitric oxide that can alter the PVR either by constricting (serotonin, endothelin-1) or dilating (nitric oxide) nearby blood vessels (6, 7, 18, 54, 57, 62).

Endothelin-1 has an important role in the pathogenesis of IPAH (15, 32). It is produced by endothelial cells (23, 24) and acts by binding to two endothelin receptors, ETA and ETB (19, 40). The ETA receptors are expressed exclusively on pulmonary arterial smooth muscle cells (PASMC) (21), whereas ETB receptors are expressed predominantly on endothelial cells and at lower levels on PASMC (36). Binding of endothelin-1 to ETA and ETB receptors on the PASMC leads to vasoconstriction (38) and PASMC proliferation (9, 43). Binding of endothelin-1 to ETB receptors leads to vasodilation through the release of nitric oxide and prostacyclin (20). In chickens, endothelin-1 produced dose-dependent contractile responses in pulmonary artery rings, and these responses were modulated by nitric oxide (29, 48). The ETA receptor antagonist BQ123 attenuated the development of pulmonary hypertension and right ventricular hypertrophy in broilers chronically exposed to low ambient temperatures (63). The lungs of broilers with advanced IPAH had higher expression of endothelin-1 but lower expression of ETAA when compared with the healthy flock mates (17).

Serotonin and the serotonin transporter contribute to the pathogenesis of human IPAH (14, 25) and PAH induced by hypoxia (12) and by serotonergic appetite suppressant drugs (1, 13). Serotonin causes vasoconstriction by interacting with its receptors 5-HT1A, 5-HT2A, and 5-HT2B, which are expressed on PASMC (8, 28, 47, 51). A role for the 5-HT1A receptor subtype in IPAH pathogenesis had not been demonstrated. Serotonin transporter is abundantly expressed in the lung and is primarily located on PASMC (35). Serotonin transported into the intracellular compartment of PASMC causes smooth muscle cell proliferation and vascular remodeling (11, 26). In broilers, MP entrapment within the pulmonary vasculature stimulates thrombocytes to release serotonin, resulting in potent pulmonary vasoconstriction and pulmonary hypertension, which can be prevented by pretreatment with methiothepin, a nonselective 5-HT1 antagonist (4, 5, 6, 7). Methiothepin pretreatment also reduced by 60% the postinjection mortality caused by injecting MP into SUS broilers, demonstrating a central role for serotonin during the MP challenge (5, 6, 7).

Nitric oxide is synthesized in broiler lungs by endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) expressed by activated macrophages (18, 22, 33, 61). The competitive inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) blocks NOS, acutely increases the PVR and PAP, and doubles...
the mortality caused by MP injections in broilers (50, 52, 57, 58, 60, 61, 62). Repeated intraperitoneal injections of L-NAME caused PAH and ascites in broilers (16), whereas dietary supplementation with L-arginine elevated plasma nitric oxide levels, attenuated pulmonary arteriole remodeling, induced vasodilation, and reduced IPAH-related mortality in broilers (44, 52, 53). Nitric oxide plays a key role in broiler lungs as the primary flow-dependent vasodilator and modulator of vasoconstriction (18, 57, 61, 62). When compared with healthy flock mates, broilers with advanced IPAH had either reduced eNOS expression in pulmonary arterioles (30, 31) or unaltered pulmonary eNOS and iNOS expression (46). Previously, we demonstrated that intravenous MP injections elicited increased pulmonary iNOS expression in broilers from SUS and RES genetic lines and that at 24 h postinjection RES broilers had higher pulmonary iNOS expression than SUS broilers (18). Expression of eNOS in response to MP injection was not evaluated in that study.

In this study, MP were injected intravenously into clinically healthy broilers from the RES and SUS lines. Clinically healthy SUS broilers typically have higher PVR and PAP and are more susceptible to MP-induced mortality than the RES broilers (2, 6, 56). Our objective was to examine the expression of genes for key vasoactive mediators, such as endothelin-1 and its receptors, serotonin receptors and transporter, and NOS enzymes, that favor enhanced vasodilation in combination with attenuated vasoconstriction during hypertensive challenges to the pulmonary vasculature.

MATERIALS AND METHODS

Broiler Management

All animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee. The RES and SUS broiler lines used for this study were developed by divergent selection based on rearing them in a hypobaric chamber. At the time of this study the lines were at the ninth generation of their selection and exhibited ascites mortalities of 7.5% (RES line) and 75% (SUS line) when reared under hypobaric conditions (37).

Fifty male chicks per line were reared on fresh wood shavings in environmental chambers (8- m² floor space). Chicks were brooded at 33.2°C on days 1 to 3, at 31.1°C on days 4 to 6, at 29.4°C on days 7 to 10, at 25.5°C on days 11 to 14, and at 23.9°C, day 15 onwards. Feed and water were provided ad libitum, and light schedules were 24 h/day for days 1 to 4 and 16:8-h light-dark from day 5 onward.

Microparticle Injection, Lung Collection, and Quantification of Microparticles

Broilers (4 wk old) were injected with MP as described previously (49, 55). Briefly, CM-32 ion exchange cellulose (Fisher Scientific, St. Louis, MO) was suspended at the rate of 0.02 g/ml in heparinized saline. This suspension (0.35 ml/broiler) was injected via the wing vein using a 22-gauge needle attached to 1-ml tuberculin syringe (Becton Dickinson, Franklin Lake, NJ).

The portion of the right lung between the first and second anterior rib indentation (costal sulcus) was collected from six broilers per line at 0 (uninjected control lung), 2, 6, 12, 24, and 48 h post-MP injection. The lungs were sliced into 5–6 pieces, immersed in 3 ml of RNAlater RNA preservation buffer (Qiagen, Valencia, CA), stored at 4°C overnight and at −20°C until RNA extraction. An adjacent segment from each lung was immersed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. To ensure that each lung had entrapped a similar number of MP, the number of MP in each lung section was quantified. For this, hematoxylin and eosin-stained lung sections (6/line/time point) were examined microscopically for MP by using a computerized image analysis system comprising a Cool SNAP cf digital camera (Image Processing Solutions, North Reading, MA) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD). For each section, four randomly selected microscopic fields were evaluated at ×10 magnification by using an Olympus BX50 microscope (Olympus America, Center Valley, PA). The number of MP in the each lung section was calculated by counting the MP in four microscopic fields per section and averaging the number of MP per microscopic field. At each time point, the average number of MP was similar (P > 0.05) in both lines. The average number of MP per microscopic field per lung section was 5.5.

Real-Time RT-PCR

RNA isolation. RNA was isolated from the RNAlater preserved lung tissues (91 to 99 μg per sample) using the Aurum total RNA fatty and fibrous tissue kit (Bio-Rad, Hercules, CA) and following the spin format protocol with slight modification. To remove contaminating DNA, if any, an additional DNA digestion step was performed. The RNA samples were aliquoted into four subsamples and stored at −80°C until analysis.

Assessment of quality and quantity of RNA. The quality and quantity of RNA were examined using an Experion automated electrophoresis system and the Experion RNA StdSens analysis kit (Bio-Rad) following the manufacturer’s protocol with some modifications. Automated electrophoresis provided the RNA concentration for each sample, a virtual gel, and an electropherogram showing the 18S and 28S band peak. Intact and crisp 18S and 28S bands indicate good quality of RNA (virtual gel not shown).

Reverse transcription. cDNA (2.5 μg/sample) was reverse transcribed to cDNA by using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol in a Biometra personal cycler (Biometra, Gottingen, Germany). Briefly, 40 μl of each reaction mixture contained 4 μl of 10X RT buffer, 1.6 μl of 25X dNTP mix (100 mM), 4 μl of 10X RT random primers, 2 μl of MultiScribe reverse transcriptase (50 U/ml), and 2.5 μg total RNA with nuclelease-free H2O added to bring the volume to 40 μl. The incubation steps used were: one cycle of 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s. After cDNA synthesis, the cDNA samples were aliquoted into four subsamples of 10 μl each and stored at −80°C until analysis.

Quantification of relative gene expression of vasoactive mediators. Real-time PCR was performed using TaqMan Universal PCR master mix in an ABI PRISM 7300 sequence detection system (Applied Biosystems). The PCR was performed in a reaction volume of 25 μl containing the reagents at the following final concentrations: 1X TaqMan Universal PCR Master Mix (2×), forward primer 200 nM, reverse primer 200 nM, probe 100 nM, and 1 μl of cDNA sample. The cycling profiles used were 1 cycle at 50°C for 2 min, 95°C for 10 min, and 40 cycles (95°C for 15 s, and 60°C for 60 s). Previously published primers and probes for 28S and chicken iNOS were used for PCR (41). The primers and probes for the remaining target genes were designed by the author using primers express software version 2.0 (Applied Biosystems). The sequences for the primers and probes used are listed in Table 1. In each plate, a no-template control (no cDNA, master mix only), a calibrator sample, cDNA samples, and endogenous control (28S) were included. Endogenous controls (28S) were listed in Table 1. In each plate, a no-template control (no cDNA, master mix only), a calibrator sample, cDNA samples, and endogenous control (28S) were included. Endogenous controls (28S) were analyzed in duplicate, and the target genes were analyzed in triplicate. The calibrator sample was cDNA from the 0-h lung samples that had not been injected with MP. The relative gene expression was quantified by the ΔΔCt method. The fold change in gene expression was calculated by comparing the gene expression of the sample with the
expression of the calibrator sample from a broiler that was not MP injected.

Statistical Analysis

Using JMP Statistical Software (version 7.0.1; SAS Institute, Cary, NC), one-way ANOVA was carried out to determine differences in the fold change of target genes in the lungs of broilers from RES and SUS groups at any of the time points and between samples collected at the various time points within each line. Differences among the group means were determined by Fisher’s least significant difference multiple mean comparisons test. Data were expressed as means ± SE, and the differences were considered significant at *P* ≤ 0.05.

RESULTS

Endothelin-1 and Its Receptors, ET\textsubscript{A} and ET\textsubscript{B}

Endothelin-1 expression increased in the lungs of both RES and SUS broilers by 2 h postinjection (3.3-fold increase) and remained elevated thereafter. Compared with the lungs from RES broilers, the lungs from SUS broilers had higher expression of endothelin-1 at 2 h (P = 0.0016), 6 h (P = 0.0024), and 12 h (P = 0.0119) postinjection (Fig. 1A).

In both lines, ET\textsubscript{A} receptor expression more than doubled by 6 h postinjection and remained elevated throughout the 48-h period (Fig. 1B). The SUS and RES lungs did not differ in expression of ET\textsubscript{A} at any of the time points examined (Fig. 1B). In both lines, ET\textsubscript{B} receptor expression almost tripled by 10.220.32.246 on April 9, 2017 http://ajpregu.physiology.org/ Downloaded from
time points in both lines. Lungs from both lines did not differ in 5-HT1B expression at any of the time points (Fig. 2C). Expression of 5-HT2B increased within 6 h postinjection in RES lungs (2.4-fold increase) and within 2 h postinjection in SUS lungs (3.4-fold increase) (Fig. 2D). Lungs from SUS broilers had higher expression of 5-HT2B receptors at 2 h ($P = 0.016$), 6 h ($P = 0.0088$), and 12 h ($P = 0.0056$) postinjection compared with the lungs from RES broilers (Fig. 2D). Serotonin transporter expression increased within 6 h postinjection in RES lungs (2.3-fold increase) or within 2 h postinjection in SUS lungs (2.2-fold increase) and remained elevated at all subsequent time points in both lines. The lines did not differ in serotonin transporter expression at any time points (Fig. 2E).

eNOS and iNOS

Expression of eNOS in the lungs of RES broilers increased within 6 h after MP injection (5.4-fold increase), continued to increase up to 12 h postinjection, and remained elevated thereafter. In the lungs of SUS broilers, eNOS expression increased by 2 h postinjection (4.4-fold increase), continued to increase up to 6 h, and remained elevated throughout subsequent time points (Fig. 3A). The lungs of RES broilers had higher eNOS expression at 12 h ($P = 0.008$), 24 h ($P = 0.0001$), and 48 h ($P < 0.0001$) postinjection when compared with the lungs from SUS broilers (Fig. 3A). Expression of iNOS in RES lungs increased by 6 h postinjection (4.7-fold increase), continued to increase up to 24 h, and remained elevated at 48 h, whereas in SUS lungs iNOS expression increased by 6 h, remained at that level at 12 h, and increased again at 24 and 48 h (Fig. 3B). Lungs from RES broilers exhibited higher iNOS expression at 12 h ($P < 0.0001$), 24 h ($P < 0.0001$), and 48 h ($P < 0.0001$) postinjection when compared with the lungs from SUS broilers (Fig. 3B).

DISCUSSION

Endothelin-1 elicits vasoconstriction and proliferation of PASMC by binding to ETA and ETB receptors (19, 40). Plasma levels of endothelin-1 are elevated 3 to 4 times above normal levels in human patients with various forms of PAH including IPAH (39). Lungs of SUS broilers had higher expression of endothelin-1, but the lines did not differ in expression of ETA, which indicates that susceptibility to IPAH in broilers is related to higher endothelin-1 expression rather than to ETA availability, in agreement with previous studies in which the lungs of broilers with advanced IPAH exhibited higher endothelin-1 expression but lower ETA expression than the healthy flock mates (17). The potential impact of ETB expression depends on its location. Stimulation of ETB receptors on smooth muscle cells results in vasocostriction (38), and on endothelial cells results in vasodilation (20). In this study, RNA was isolated from whole lung tissue so the ETB expression might be from either smooth muscle cells or endothelial cells or both. ETB receptors are exclusively involved in the clearance of the circulating endothelin-1 from the blood (10), which reduces the bioavailability of endothelin-1, thereby minimizing its pulmonary vasoconstrictor and mitogenic effects.

Expression of all serotonin receptor types evaluated in this study increased in the lungs of broilers following MP injection. Broilers from the SUS line had a higher expression of 5-HT1A.
and 5-HT$_{2B}$ receptors over the first 12 h postinjection, but the lines did not differ in expression of other receptors. The role of serotonin receptors 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{2B}$ in vasoconstriction and in proliferation of PASMC has been well established in mammalian IPAH pathogenesis (8, 28). To the best of our knowledge, this is the first evaluation of 5-HT$_{1A}$ receptor expression in broiler lungs, and the first indication that 5-HT$_{1A}$ receptors may play a role in pathogenesis of IPAH. The higher expression of 5-HT$_{2B}$ receptors in the lungs of SUS broilers suggests that this receptor contributes to pulmonary vasoconstriction and PASMC proliferation that accompany susceptibility to IPAH in broilers (5, 61, 62), a phenomenon defined in mammals (34).

The expression of serotonin transporter increased similarly in the lungs of both SUS and RES broilers after the MP injection. Increased serotonin transporter expression enables PASMC to internalize additional serotonin and thereby increases the clearance of serotonin from the circulation and hence reduces serotonin mediated vasoconstriction; but once internalized, serotonin has a mitogenic effect on PASMC leading to vascular remodeling, arterial muscularization, and increase in PVR (11, 26).

Higher expression of eNOS and iNOS should elevate production of nitric oxide, a potent pulmonary vasodilator in mammals and birds (3, 27, see above). Nitric oxide also has an antimitogenic effect on PASMC (44, 45). Blockade of the
Activities of eNOS and iNOS by L-NAME decreased the biosynthesis of nitric oxide leading to vasoconstriction and pulmonary hypertension (50, 52, 57, 58). Higher expression of iNOS in the lungs of MP-injected RES broilers compared with SUS broilers supports our previous finding (18). Reduced eNOS expression in the pulmonary arterioles of broilers developing IPAH during chronic exposure to hypobaric hypoxia (30, 31) further underscores the role of eNOS as a modulator of IPAH in broilers. Higher expression of eNOS and iNOS by the lungs of RES broilers provides the distinctive advantage of counteracting multiple pathways through which MP entrapment induces IPAH in SUS broilers. Prostacyclin and prostaglandin E2 are important pulmonary vasodilators in mammals but are ineffective as vasodilators in broiler chickens (42, 59), leaving nitric oxide as the only known vasodilator, which has a modulatory role in the pathogenesis of IPAH in broilers. The eNOS and iNOS expression patterns following MP injection support the capacity of nitric oxide to play a key role in the lungs of RES broilers by counteracting vasoconstriction and exerting antimitogenic effect to inhibit PASMC proliferation.

In conclusion, in RES broilers the MP elicited higher pulmonary expression of eNOS, iNOS, and ETB, which promote vasodilation and inhibit PASMC proliferation through the action of nitric oxide. In SUS broilers, the MP elicited higher pulmonary expression of endothelin-1 and the 5-HT2B receptor, which enhance vasoconstriction and PASMC proliferation. These results support our hypothesis that the resistance of broiler chickens to IPAH may be due to the higher expression of those vasoactive mediators (ETB, eNOS, iNOS) that favor enhanced vasodilation, attenuated vasoconstriction, and reduced PASMC proliferation during MP challenges to the pulmonary vasculature.

**Perspectives and Significance**

The pathogenesis of PHS in broiler chicken is very similar to human IPAH. Availability of RES and SUS genetic lines of broilers presents an excellent opportunity to study PHS pathogenesis in broiler chickens to further substantiate their use as an animal model for human IPAH. The role of endothelin-1, serotonin, and nitric oxide in PHS in broiler chickens is well known. However, gene expression of these vasoactive mediators in the lungs of broilers regarding the pathogenesis of PHS had not been studied. This study demonstrated differential gene expression of key vasoactive mediators in the lungs of clinically healthy broilers from RES and SUS lines following intravenous MP injection. In this study there was no direct evidence that the observed expression of vasoactive mediators translated into protein and functional consequences; nevertheless, these patterns of gene expression are consistent with numerous observations from previous studies. For example, the ETa receptor antagonist BQ123 attenuated the development of PH and right ventricular hypertrophy in broilers chronically exposed to low ambient temperature (63). Furthermore, blockade of 5-HT1/2 receptors with methiothepin substantially reduced the mortality triggered by injection of MP into SUS broilers (5, 6, 7). Finally, inhibition of eNOS and iNOS with L-NAME increased the mortality triggered by MP injection in broilers (50, 61, 62). This study lays a solid foundation for evaluating the gene and protein expression patterns underlying the development and pathogenesis of PHS in broilers and for validating them as an animal model for IPAH in humans.

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**DISCLOSURE**

No conflicts of interest are declared by the author(s).

**REFERENCES**

EXPRESSION OF VASOACTIVE MEDIATORS IN THE CHICKEN LUNG


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