Evaluation of the Serotonin Receptor Blockers Ketanserin and Methiothepin on the Pulmonary Hypertensive Responses of Broilers to Intravenously Infused Serotonin

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ABSTRACT The pathogenesis of pulmonary hypertension remains incompletely understood. Many factors have been implicated; however, there has been great interest in the potent pulmonary vasoconstrictor serotonin (5-HT) due to episodes of primary pulmonary hypertension in humans triggered by serotoninergic appetite-suppressant drugs. Pulmonary hypertensive patients have elevated blood 5-HT levels and pulmonary vasoconstriction induced by 5-HT is believed to be mediated through 5-HT1B/1D and 5-HT2A receptors that are expressed by pulmonary smooth muscle cells. The vascular remodeling associated with pulmonary hypertension also appears to require the serotonin transporter. We investigated the roles of 5-HT receptor blockers on the development of pulmonary hypertension induced by infusing 5-HT i.v. in broilers. For this purpose, we treated broilers with the selective 5-HT2A receptor antagonist ketanserin (5 mg/kg of BW) or with the nonselective 5-HT1/2 receptor antagonist methiothepin (3 mg/kg of BW). Receptor blockade was followed by infusion of 5-HT while recording pulmonary arterial pressure and pulmonary arterial blood flow. The results demonstrate that methiothepin, but not ketanserin, eliminated the 5-HT-induced pulmonary hypertensive responses in broilers. The 5-HT2A receptor does not, therefore, appear to play a role in the 5-HT-induced pulmonary hypertensive responses in broilers. Methiothepin did not inhibit pulmonary vascular contractility per se, because the pulmonary hypertensive response to the thromboxane A2 mimetic U44069 remained intact in methiothepin-treated broilers. Methiothepin will be a useful tool for evaluating the role of 5-HT in the pathogenesis of pulmonary hypertension syndrome (ascites) as well as the onset of pulmonary hypertension triggered by inflammatory stimuli such as bacterial lipopolysaccharide.

Key words: pulmonary hypertension, broiler, serotonin, ketanserin, methiothepin

INTRODUCTION

Fast-growing broilers are susceptible to pulmonary hypertension leading to pulmonary hypertension syndrome (PHS, ascites) when the right ventricle must progressively elevate the pulmonary arterial pressure (PAP) to propel the requisite cardiac output through lungs having a marginally inadequate pulmonary vascular capacity (Peacock et al., 1989; Julian, 1993; Wideman and Bottje, 1993; Wideman, 2000; Wideman et al., 2001). Any factor that increases the cardiac output, reduces the pulmonary vascular capacity, or triggers pulmonary vasoconstriction can contribute to the pathogenesis of PHS in broilers (Wideman, 2000). The pathogenesis of pulmonary hypertension remains incompletely understood. Factors synthesized by or acting upon the pulmonary arterial vasculature such as transforming growth factor-β, serotonin (5-hydroxytryptamine, 5-HT), thromboxane A2 (TXA2), and endothelin have been implicated (Eddahibi et al., 2002). There has been considerable interest in the role of 5-HT due to episodes of primary pulmonary hypertension (PPH) in humans linked to serotoninergic appetite-suppressant drugs (Abenhaim et al., 1996). Increased plasma 5-HT levels also have been recorded in pulmonary hypertensive patients (Hervé et al., 1995).

Serotonin is a potent pulmonary vasoconstrictor that is synthesized from the essential amino acid tryptophan, actively accumulated by mammalian platelets and avian thrombocytes, and released into the plasma during platelet or thrombocyte aggregation (Meyer and Sturkie, 1974; Cox, 1985; Lacoste-Eleaume et al., 1994). Serotonin has been implicated in the mechanisms responsible for pulmonary hypertension in several human, animal, and broiler studies (Seiler et al., 1974; Douglas et al., 1981; Brenot et al., 1993; Abenhaim et al., 1996; Chapman and Wideman, 2002). The majority of circulating 5-HT is produced by endocrine cells in the gastro-intestinal tract, and is released into the blood passing through the gut. Platelets and thrombocytes accumulate 5-HT via membrane transporter proteins and store it intracellularly in the form of dense granules. When platelets and thrombo-
cytes aggregate, they release several physiologically active substances including 5-HT, which causes proliferation of pulmonary vascular smooth muscle and stimulates vasoconstriction, thereby reducing the blood flow at the site of injury (McGoon and Vanhoutte, 1984; Lee et al., 1994; Pitt et al., 1994; Fanburg and Lee, 1997). The pulmonary vasoconstriction triggered by 5-HT is believed to be mediated through the 5-HT_{1B/1D} and 5-HT_{2A} receptors expressed by pulmonary smooth muscle cells (Choi and Maroteaux, 1996; MacLean et al., 1996). Vascular remodeling and smooth muscle proliferation appear to require the serotonin transporter (5-HTT; MacLean et al., 2000; Eddahibi et al., 2002).

In this study, we investigated the impact of 5-HT receptor blockers on the development of pulmonary hypertension induced by infusing 5-HT i.v. in broilers. For this purpose, we treated broilers with the selective 5-HT_{2A} receptor antagonist ketanserin (Barnes and Sharp, 1999) or with the nonselective 5-HT_{1/2} receptor antagonist methiothepin. Ketanserin has high affinity for the serotonin 5-HT_{2A} receptor, but also binds less potently to the 5-HT_{2C}, 5-HT_{2B}, 5-HT_{1D} receptors, adrenergic receptor, and dopamine receptors. In humans, ketanserin has been used as a vasodilator to lower blood pressure in hypertensive patients (Vanhoutte et al., 1988). To evaluate the role of the 5-HT_{2A} receptor in PHF, broilers in Experiment 1 were treated with ketanserin or saline followed by infusion of 5-HT while recording PAP. Methiothepin is a nonselective 5-HT_{1} and 5-HT_{2}, as well as a 5-HT_{5-7} receptor antagonist with varying degrees of selectivity; however, it displays high affinities for 5-HT_{1A} and 5-HT_{1B} receptor subtypes in rats (Engel et al., 1986). In Experiment 2, broilers were treated with methiothepin or saline followed by infusion of 5-HT while recording PAP. To further evaluate the hemodynamic responses to 5-HT after treatment with methiothepin, blood flow and PAP were recorded in broilers in Experiment 3. Hemodynamic responses to repeated 5-HT infusion and TXA2 mimetic U44069 injection after treatment with methiothepin were also evaluated in Experiment 3.

**MATERIALS AND METHODS**

Male chicks hatched at a commercial hatchery were wing-banded and shipped on the day of hatch (d 1) to the Poultry Environmental Research Laboratory at the University of Arkansas. They were placed on fresh wood shavings in environmental chambers (8 m² of floor space)
and were brooded at 33°C from d 1 to 5, 29°C from d 6 to 10, and 27°C from d 11 to 17. Thereafter, the broilers were maintained at 21°C until the experiment was terminated. The photoperiod was 24L:0D from d 1 to 5, and 23L:1D thereafter. Water was provided ad libitum via nipple-type waterers. A corn-soybean meal starter ration (22.7% CP, 3,059 kcal of ME/kg, 1.5% arginine, and 1.43% lysine) was provided ad libitum and was formulated to meet minimum NRC (1994) standards for all ingredients. The diet was provided as crumbles during wk 1 and 2, and as pellets thereafter.

**Experiment 1**

Male broilers (n = 24; 2,623 ± 54 g of BW, mean ± SEM) were anesthetized to a light surgical plane with intramuscular injections of allobarbital (3.0 mL, 25 mg/mL 5,5-diallybarbituric acid (Sigma Chemical Co., St. Louis, MO) and ketamine HCl (1.0 mL, 100 mg/mL, Henry Schein Inc., Melville, NY). The birds were placed on a heated surgical board (30°C) and restrained in dorsal recumbency. A wing was extended and feathers removed from the ventral surface as needed to uncover the skin over the basilica vein. After intracutaneous injections of 2% lidocaine HCl (Abbott Laboratories, Chicago, IL) were administered as a local anesthetic, an incision was made to expose the vein, which was then cannulated with a Silastic catheter (0.03 cm i.d., 0.094 cm o.d., Konigsberg Instruments Inc., Pasadena, CA) filled with 0.9% sodium chloride solution containing 200 IU of heparin/mL. The catheter was attached to a blood pressure transducer interfaced through a Transbridge preamplifier (World Precision Instruments) to a Biopac MP 100 data acquisition system using AcqKnowledge software (Biopac Systems, Inc., Goleta, CA). The catheter was advanced through the right atrium and ventricle into a pulmonary artery while monitoring the characteristic pulse pressures to identify the location (Wideman et al., 1996; Chapman and Wideman, 2001). All PAP readings were made with the transducer at the level of the thoracic inlet. Before recording, the system was calibrated in millimeters of mercury (mmHg) using a mercury manometer. The left basilica vein was also cannulated with PE-50 polyethylene tubing filled with heparinized saline for i.v. infusions.

After surgical preparations were complete and a stabilization period of 10 min had elapsed, control data were recorded for 10 min. Broilers were then injected with ketanserin tartrate salt (Sigma Chemical Co., St. Louis,
Figure 3. Pulmonary arterial pressure (PAP, upper panel) and blood flow through the left pulmonary artery (Flow, lower panel) for male broilers (n = 8; 2,147 ± 112 g BW, mean ±SEM) in experiment 3, at the start of data collection and at 5-min intervals thereafter (St, S5, S10), within 30 s after the beginning of serotonin (5-HT) infusion and at a 5-min interval thereafter (Se, Se5), within 30 s after ending 5-HT infusion (E), within 30 s after methiothepin injection and at 5-min intervals thereafter (M, M5, M10), within 30 s after second beginning the second 5-HT infusion and at a 5-min interval thereafter (2Se, 2Se5), within 30 s after ending the second 5-HT infusion (2E), within 30 s after injecting the thromboxane A2 mimetic U44069 (Tx) and within 30 s thereafter (3E). Letters (a-g) represent differences across sample intervals (P \leq 0.05).

MO) at 5 mg/kg of BW in 0.9% saline (ketanserin group, n = 12) or 0.9% saline (saline group, n = 12), and data were recorded for a further 10 min. The 5 mg/kg of BW dose used in this study is similar to the levels of ketanserin used to effectively block 5-HT2A receptors in mammals (Miao et al., 2003; Ni et al., 2004; Deuchar et al., 2005).

Both treatments contained 20% acetic acid to dissolve the ketanserin. Treatment was followed by infusing 5-HT (0.1 mg of 5-hydroxytryptamine maleate salt in 20 mL of 2.5% mannitol, Biopac Systems, Inc.) at 0.1 mL/min per kg of BW through the polyethylene tubing in the left basilica vein for 10 min, after which data were recorded for a
Figure 4. Cardiac output (upper panel) and heart rate (lower panel) for male broilers (n = 8; 2,147 ± 112 g of BW, mean ± SEM) in experiment 3, at the start of data collection and at 5-min intervals thereafter (St, S5, S10), within 30 s after the beginning of serotonin (5-HT) infusion and at a 5-min interval thereafter (Se, Se5), within 30 s after ending 5-HT infusion (E), within 30 s after methiothepin injection and at 5-min intervals thereafter (M, M5, M10), within 30 s after beginning the second 5-HT infusion and at a 5-min interval thereafter (2Se, 2Se5), within 30 s after ending the second 5-HT infusion (2E), within 30 s after injecting the thromboxane A2 mimetic U44069 (Tx) and within 30 s thereafter (3E). Letters (a-c) represent differences across sample intervals (P ≤ 0.05).

Further 10 min. Pilot studies were conducted previously to survey effective i.v. infusion dosages for 5-HT; infusion of from 1.0 to 0.5 mg of 5-HT in 20 mL of 2.5% mannitol triggered massive pulmonary vasoconstriction leading to an immediate (within 30 s) >90% reduction in cardiac output and death (Chapman and Wideman, 2002). Thereafter, 0.1 mg of 5-HT in 20 mL of 2.5% (wt/vol) mannitol was established as an efficacious intermediate dosage that permitted 100% survival and postinfusion recovery toward control values by the experimental animals. After a satisfactory recording was obtained, the birds were euthanized with 10 mL (i.v.) of 0.1 M KCl.
Figure 5. Stroke volume (upper panel) and pulmonary vascular resistance (lower panel) for male broilers (n = 8; 2,147 ± 112 g of BW, mean ± SEM) in experiment 3, at the start of data collection and at 5-min intervals thereafter (St, S5, S10), within 30 s after the beginning of serotonin (5-HT) infusion and at a 5-min interval thereafter (Se, Se5), within 30 s after ending 5-HT infusion (E), within 30 s after methiothepin injection and at 5-min intervals thereafter (M, M5, M10), within 30 s after beginning the second 5-HT infusion and at a 5-min interval thereafter (2Se, 2Se5), within 30 s after ending the second 5-HT infusion (2E), within 30 s after injecting the thromboxane A2 mimetic U44069 (Tx) and within 30 s thereafter (3E). Letters (a-f) represent differences across sample intervals (P ≤ 0.05).

**Experiment 2**

Male broilers (n = 20; 2,704 ± 68 g of BW, mean ± SEM) were anesthetized, and cannulated for measurement of PAP and i.v. infusions as described above. After surgical preparations were complete and a stabilization period of 10 min had elapsed, control data were recorded for 10 min. Broilers were then injected with 0.9% saline (saline group, n = 10) or methiothepin mesylate salt (Biopac Systems, Inc.) at 3 mg/kg of BW (methiothepin
Figure 6. Pulmonary arterial pressure (PAP, upper panel) and blood flow through the left pulmonary artery (Flow, lower panel) for 2 individual male broilers in experiment 3, at the start of data collection and at 5-min intervals thereafter (St, S5, S10), within 30 s after beginning the first serotonin (5-HT) infusion and at a 5-min interval thereafter (Se, Se5), within 30 s after ending the first 5-HT infusion (E), within 30 s after beginning the second 5-HT infusion and at a 5-min interval thereafter (2Se, 2Se5), within 30 s after ending the second 5-HT infusion (2E), within 30 s after methiothepin injection and at 5-min intervals thereafter (M, M5, M10), within 30 s after the third 5-HT infusion and at a 5-min interval thereafter (3Se, 3Se5), within 30 s after ending the third 5-HT infusion (3E), within 30 s after injecting the thromboxane A2 mimetic U44069 (Tx) and within 30 s thereafter (4E).

Experiment 3

Male broilers (n = 8; 2,147 ± 112 g of BW, mean ± SEM) were anesthetized, and cannulated for measurement of PAP and i.v. infusions as described above. After 2% (wt/vol) lidocaine HCl had been administered intracutaneously as a local anesthetic, an incision was made to
open the thoracic inlet, a transonic ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY) was positioned on the left pulmonary artery, and the probe was connected to a Transonic T206 blood flow meter (Transonic Systems Inc.) to confirm signal acquisition. The thoracic inlet was then sealed with stainless steel wound clips.

After surgical preparations were complete and a stabilization period of 10 min had elapsed, control data were recorded for 10 min. First, 5-HT (0.1 mg in 20 mL of 2.5% mannitol) was infused at 0.1 mL/min per kg of BW through the polyethylene tubing in the left basilica vein for 10 min, after which methiothepin (3 mg/kg of BW) was injected, and data recorded for a further 10 min. The 5-HT was then infused for a second 10-min period followed by a 5-min stabilization period. Finally, 0.3 mL of the thromboxane mimetic U44069 (1 mM in 2.5% mannitol) was injected and data recorded for 5 min. Two of the broilers in Experiment 3 were given a second infusion of 5-HT before methiothepin was injected to confirm the responsiveness of the pulmonary vasculature to repeated 5-HT infusions. After a satisfactory recording was obtained, the birds were euthanized with 10 mL (i.v.) of 0.1 M KCl.

Data Analysis

The primary channels recorded by the Biopac MP 100 data acquisition system included PAP (mmHg), blood flow (Experiment 3) through the left pulmonary artery (mL/min), and heart rate (beats/min) obtained by counting systolic peaks over time in the PAP recording (Experiment 3).

These primary data were averaged electronically during representative sample intervals, while accommodating for the influences of pulse pressure and respiratory cycles on PAP (Wideman et al., 1996). In Experiment 3, the primary data were used to calculate cardiac output, stroke volume, and pulmonary vascular resistance. Based on the assumption that cardiac output (mL/min) is normally divided equally between the lungs, cardiac output was calculated as $2 \times$ blood flow. The cardiac output is the product of heart rate $\times$ stroke volume (mL/beat); consequently, stroke volume was calculated as cardiac output divided by heart rate. Assuming the pressure gradient across the pulmonary circulation is essentially equal to PAP (Wideman et al., 1996, 1998), then the relationships between pressure gradients, flow rates, and resistances are summarized by the equation: PAP = cardiac output $\times$ pulmonary vascular resistance. Thus, pulmonary vascular resistance was calculated in relative resistance units as PAP (mmHg) divided by cardiac output (mL/min) as described by Besch and Kadono (1978), Sturkie (1986), and Wideman et al. (1996, 1998).

Data were analyzed by t-test (comparison between 2 groups) or within a group over time (across sample intervals) using the SigmaStat repeated measures ANOVA procedure (1994 version, Jandel Scientific, San Rafael, CA), by using the Student-Newman-Keuls method for the separation of treatment means. The threshold for significance was $P \leq 0.05$.

RESULTS

Experiment 1

Pulmonary arterial pressure increased nonsignificantly by approximately 2 mmHg after the injection of saline containing acetic acid, with or without ketanserin (Figure 1). Infusion of 5-HT triggered rapid increases in PAP that averaged approximately 7 mmHg above preinfusion baseline values in both the ketanserin and saline groups. Pulmonary arterial pressure remained elevated above baseline values throughout the interval of 5-HT infusion and returned to preinfusion levels upon cessation of 5-HT infusion.

Experiment 2

In the saline group, 5-HT infusion triggered rapid increases in PAP of approximately 15 mmHg above preinfusion baseline values (Figure 2). The pulmonary hypertensive response declined as the 10-min infusion of 5-HT progressed, but PAP remained elevated above baseline values throughout the interval of 5-HT infusion. Pulmonary arterial pressure returned to preinfusion baseline values upon cessation of 5-HT infusion. In the methiothepin group, PAP decreased by approximately 4 mmHg after injection of methiothepin and returned to preinjection baseline values 5 min postinjection. Infusing 5-HT did not trigger an increase in PAP, which remained below preinjection baseline values for the duration of the experiment.

Experiment 3

Infusing 5-HT triggered rapid increases in PAP accompanied by decreases in pulmonary arterial blood flow (Figure 3). The 5-HT infusions increased PAP by approximately 10 mmHg above preinfusion baseline values. These pulmonary hypertensive responses were associated with reductions in pulmonary arterial blood flow (Figure 3, lower panel) and cardiac output (Figure 4, upper panel) that were not associated with reductions in heart rate (Figure 4, lower panel) but instead were associated with reductions in stroke volume (Figure 5, upper panel) and increases in pulmonary vascular resistance (Figure 5, lower panel). Pulmonary arterial pressure, cardiac output, stroke volume, and pulmonary vascular resistance returned to preinfusion baseline values upon cessation of 5-HT infusion.

Methiothepin injection triggered rapid decreases in PAP accompanied by a slight decrease in pulmonary arterial blood flow. Methiothepin decreased PAP to approximately 5 mmHg below preinfusion baseline values (Figure 3). This hypotensive response was associated with reductions in pulmonary vascular resistance (Figure 5). Pulmonary arterial pressure and pulmonary vas-
cular resistance remained below baseline values subsequent to methiothepin injection and throughout a second 5-min infusion of 5-HT. Blood flow, cardiac output, stroke volume, and heart rate remained at baseline values throughout the postmethiothepin 5-HT infusion (Figures 3 to 5).

Injecting the TXA₂ mimetic U44069 triggered rapid increases in PAP accompanied by slight decreases in pulmonary arterial blood flow. The U44069 infusions increased PAP by approximately 6 mmHg above preinfusion baseline values (Figure 3). This pulmonary hypertensive response was associated with increased pulmonary vascular resistance (Figure 5). A slight contemporaneous reduction in cardiac output (Figure 4) was associated with reductions in stroke volume (Figure 5), but not with a reduction of heart rate (Figure 4).

Two individual broilers in experiment 3 showed that a second infusion of 5-HT before methiothepin injection was efficacious in eliciting a hypertensive response by the pulmonary vasculature (Figure 6).

DISCUSSION

Infusing 5-HT triggered rapid increases in PAP in broilers treated with ketanserin and saline. The inclusion of acetic acid to dissolve the ketanserin presumably accounts for the slight postinjection increase in PAP observed in both the ketanserin and saline groups. Previous studies suggested that bolus acid injections cause pulmonary hypertension by stimulating increased synthesis of TXA₂ (Wideman et al., 1999). Pulmonary hypertensive responses induced by 5-HT were accompanied by decreases in cardiac output associated with increased pulmonary vascular resistance (pulmonary vasoconstriction). The peak PAP attainable was inadequate to propel the normal cardiac output through the elevated pulmonary vascular resistance. Consequently, the impeded venous return to the left ventricle caused dependent reductions in stroke volume and cardiac output without corresponding changes in heart rate. Treatment with methiothepin triggered decreases in PAP that returned toward preinjection baseline values 5 min postinjection; however, subsequent 5-HT infusion did not trigger an increase in PAP or pulmonary vascular resistance, which remained below preinjection baseline values for the duration of the experiment. It is evident that methiothepin did not inhibit pulmonary vascular contractility per se, because the pulmonary vasculature remained responsive to a second 5-HT infusion before treatment with methiothepin (Figure 6), and to a second vasoconstrictor, the TXA₂ mimetic U44069, following treatment with methiothepin.

Ketanserin, a selective 5-HT₂A antagonist (Barnes and Sharp, 1999), has been used successfully in humans as a vasodilator to lower blood pressure in hypertensive patients (Vanhoutte et al., 1988). Nevertheless, it seems that the 5-HT₂A receptor is not important in modulating the hypertensive response to 5-HT in broilers. The nonselective 5-HT₁/₂ receptor antagonist methiothepin eliminated the pulmonary hypertensive response to 5-HT in broilers and, in fact, reduced PAP below baseline values. Several recent studies point to the 5-HT₁B receptor, rather than the 5-HT₂A receptor as the mediator of 5-HT-induced pulmonary vasoconstriction in mammals (MacLean et al., 2000). It has been shown that circulating 5-HT measured in humans with PPH contracts isolated human arterioles, and that this effect is mediated by the 5-HT₁B receptor (Morecroft et al., 1999). Knockout mice unable to express 5-HT₁B receptors developed less severe pulmonary hypertension than did wild-type control subjects (Keegan et al., 2001). In contrast, Launay et al. (2002) recently postulated that 5-HT₂B might mediate 5-HT-induced vasoconstriction. Indeed, the active metabolite of dexfenfluramine, which is a serotoninergic appetite-suppressant drug related to an increased risk of developing PPH in humans (Abenhaim et al., 1996), is a selective 5-HT₂B receptor agonist. In addition, mice with inactive 5-HT₂B receptors did not develop pulmonary hypertension when exposed to hypoxia (Launay et al., 2002). This observation would explain why dexfenfluramine is associated with PPH when inhibiting effects on the 5-HT should protect against pulmonary arterial remodeling.

The present study provides direct evidence that methiothepin, but not ketanserin, can modulate the hypertensive response to 5-HT in broilers. The 5-HT₂A receptor apparently does not, therefore, play a role in the 5-HT-induced pulmonary response in broilers. Furthermore, methiothepin will be a useful tool for evaluating the role of 5-HT in the pathogenesis of PHS (ascites) as well as the onset of pulmonary hypertension triggered by inflammatory stimuli such as bacterial lipopolysaccharide.

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