Immune Modulation of the Pulmonary Hypertensive Response to Bacterial Lipopolysaccharide (Endotoxin) in Broilers

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ABSTRACT The lungs of broilers are constantly challenged with lipopolysaccharide (LPS, endotoxin) that can activate leukocytes and trigger thromboxane A_2 (TxA_2)- and serotonin (5HT)-mediated pulmonary vasoconstriction leading to pulmonary hypertension. Among broilers from a single genetic line, some individuals respond to LPS with large increases in pulmonary arterial pressure, whereas others fail to exhibit any response to the same supramaximal dose of LPS. This extreme variability in the pulmonary hypertensive response to LPS appears to reflect variability in the types or proportions of chemical mediators released by leukocytes. Our research has confirmed that TxA_2 and 5HT are potent pulmonary vasoconstrictors in broilers and that broilers hatched and reared together consistently exhibit pulmonary hypertension after i.v. injections of TxA_2 or 5HT. Previous in vitro studies conducted using macrophages from different lines of chickens demonstrated innate variability in the LPS-stimulated induction of nitric oxide synthase (iNOS) followed by the onset of an LPS-refractory state. The NOS enzyme converts arginine to citrulline and nitric oxide (NO). It is known that NO produced by endothelial NOS serves as a key modulator of flow-dependent pulmonary vasodilation, and it is likely that NO generated by iNOS also contributes to the pulmonary vasodilator response. Accordingly, it is our hypothesis that the pulmonary hypertensive response to LPS in broilers is minimal when more vasodilators (NO, prostacyclin) than vasoconstrictors (TxA_2, 5HT) are generated during an LPS challenge. Indeed, inhibiting NO production through pharmacological blockade of NOS with the inhibitor N^{ω}-nitro-L-arginine methyl ester modestly increased the baseline pulmonary arterial pressure and dramatically increased the pulmonary hypertensive response to LPS in all broilers evaluated. Innate differences in the effect of LPS on the pulmonary vasculature may contribute to differences in susceptibility of broilers to pulmonary hypertension syndrome (ascites).

(*Key words*: ascites, prostacyclin, macrophage, nitric oxide, thromboxane)

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PULMONARY VASCULAR CAPACITY AND PULMONARY HYPERTENSION

The pulmonary vascular capacity of modern broiler chickens is only marginally adequate to accommodate the cardiac output (CO) required to support the metabolic demands incurred by fast growth and the extremes of environmental temperatures (Julian, 1989; Peacock et al., 1989; Wideman and Bottje, 1993; Owen et al., 1995; Wideman et al., 1998c; 2003a,b; Wideman, 2000, 2001; Wideman and Tackett, 2000). In this context, the "pulmonary vascular capacity" can be broadly defined to encompass metabolic limitations related to the tone (degree of contraction) maintained by the primary resistance vessels (precapillary arterioles) within a broiler's lungs, as well as anatomical constraints related to the compliance and effective volume of the blood vessels. Hemodynamic evaluations of broilers have confirmed that their pulmonary blood vessels have a low compliance (are poorly distensible), the vascular channels function as if they are fully engorged with blood (additional vascular volume cannot be recruited), and the precapillary arterioles are the primary sites of excessive resistance to pulmonary blood flow (Wideman and Kirby, 1995a,b; Wideman et al., 1996a,b, 1999b; Forman and Wideman, 1999, 2001; Chapman and Wideman, 2001). Accordingly, broilers are susceptible to the onset of pulmonary hypertension leading to pulmonary hypertension syndrome (PHS, ascites) whenever

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Abbreviation Key: CO = cardiac output; ET-1 = endothelin 1; eNOS = endothelial nitric oxide synthase; IL = interleukin; iNOS = inducible nitric oxide synthase; L-NAME = N^{ω}-nitro-L-arginine methyl ester; LPS = lipopolysaccharide; mCD14 = membrane bound CD14 receptor; NO = nitric oxide; NOS = nitric oxide synthase; PAF = platelet activating factor; PAP = pulmonary arterial pressure; PGI_2 = prostacyclin; PHS = pulmonary hypertension syndrome; PIM = pulmonary intravascular macrophages; PVR = pulmonary vascular resistance; TLR4 = toll-like receptor 4; TxA_2 = thromboxane A_2; 5HT = 5-hydroxytryptamine (serotonin).

their right ventricle must develop an elevated pulmonary arterial pressure (PAP) to propel the CO through lungs having a inadequate pulmonary vascular capacity and thus an elevated pulmonary vascular resistance (PVR) (Wideman and Bottje, 1993; Wideman, 2000, 2001). Indeed, experimental procedures designed specifically to reduce the pulmonary vascular capacity and elevate the PVR have been shown to reliably initiate the characteristic pathogenesis leading to PHS in susceptible broilers (Wideman and Kirby, 1995a, 1996; Wideman et al., 1997, 2002; Ruiz-Feria et al., 1999; Wideman and Erf, 2002). In contrast, some broilers are genetically resistant to PHS and are capable of thriving in spite of substantial reductions in their pulmonary vascular capacity (Wideman and French, 1999; Wideman et al., 2002). Resistant male and female breeder parents selected for their robust pulmonary vascular capacity produced progeny exhibiting a cumulative 90% reduction in susceptibility to PHS when grown as rapidly as possible during exposure to cool temperatures (Wideman and French, 2000). The rapid progress achieved in selecting PHS-resistant broiler lines demonstrates that selection pressure rigorously focused to chronically challenge the pulmonary vasculature succeeded in virtually eliminating a gene coding for a highly significant proportion of the PHS susceptibility in commercial broilers (Wideman, 2001).

LIPOPOLYSACCHARIDE (ENDOTOXIN) AND PULMONARY HYPERTENSION

All factors contributing to increases in the PVR theoretically can initiate or accelerate the pathogenesis of PHS if the right ventricle of the heart is forced to increase the PAP to propel the requisite CO through the lungs (Wideman and Bottje, 1993; Wideman, 2000). Bacterial lipopolysaccharide (LPS, endotoxin) is an integral component of the cell wall of gram-negative bacteria, including *Escherichia coli* and a number of other pathogens (e.g., *Salmonella*, *Bordetella*, *Campylobacter*). In mammals, LPS stimulates pulmonary vasoconstriction and pulmonary hypertension (accompanied by increases in PVR and PAP, respectively) mediated by endothelin-1 (ET-1) and thromboxane A_2 (TxA_2) (Morel et al., 1989; Longworth et al., 1994; Staub, 1994; Faltin et al., 1996; Snapper et al., 1998; Wanecek et al., 2000). Our research has confirmed that LPS can cause pulmonary vasoconstriction and pulmonary hypertension in broilers. As shown in Figure 1, clinically healthy broilers injected i.v. with a supramaximal (1 mg) dose of LPS exhibited a delayed-onset (≥15 min) pulmonary vasoconstriction that increased the pulmonary arterial pressure within 20 to 25 min followed by a gradual decline by 60 min postinjection. Subsequent to the onset of pulmonary hypertension, the same birds became refractory or tolerant (unresponsive) to injections of ≥ 4 mg LPS (Wideman et al., 2001). The time-course and magnitude of the pulmonary hypertensive responses of individual broilers to LPS varied widely for reasons that were not apparent based on attempts to maintain uniformity across all aspects of the experiment. Among broilers

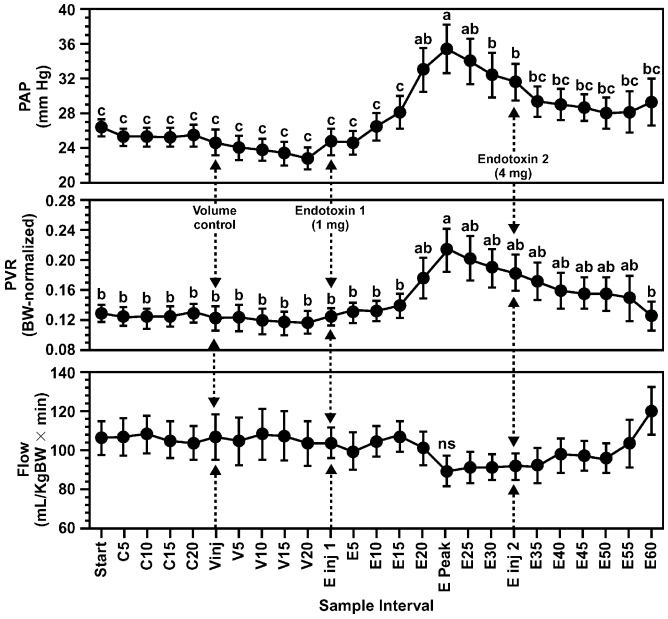

FIGURE 1. Pulmonary arterial pressure (PAP, upper panel), pulmonary vascular resistance (PVR, middle panel), and blood flow through the right pulmonary artery (Flow, lower panel) for male broilers (mean ± SEM, n = 7) at the start of data collection (Start), at 5-min intervals during the control period (C5 to C20), within 30 s after injecting the volume control (Vinj), at 5-min intervals during the volume control period (VC5 to VC20), within 30 s after the injection with 1 mg of endotoxin (Einj 1), at 5-min intervals after the injection with 1 mg of endotoxin (E5 to E30), during the maximum PAP response to 1 mg of endotoxin (E Peak), within 30 s after the injection with 4 mg of endotoxin (Einj 2), and at 5-min intervals thereafter (E35 to E60). [a–c]Different letters designate differences between means over time ($P \leq 0.05$); ns = not significant ($P > 0.05$) (adapted from Wideman et al., 2001).

from a single genetic line that had been hatched and reared together, some hyperresponsive individuals reacted to LPS with large increases in PAP, whereas the same supramaximal dose of LPS failed to elicit pulmonary hypertension in nonresponsive individuals (Figure 2) (Wideman et al., 2001). Subsequent research confirmed that broilers are equally variable in their responses to similar doses of LPS purified from *E. coli* and *S. typhimurium*, and doses of LPS ranging from 20 μg/kg BW to 10 mg/kg BW were capable of triggering maximal pulmonary hypertension in hyper- but not hyporesponsive individuals (R. F. Wideman, M. E. Chapman, W. Wang, and G. F. Erf, unpublished observations). Attempts to reduce the individual variability in the pulmonary hypertensive response to LPS were not effective. Broilers reared in clean stainless steel cages from which the fecal and dander material were removed daily nevertheless exhibited pulmonary hypertensive responses to LPS that were as variable as those observed in broilers whose pulmonary gas exchange capacity had been compromised by their being reared at a higher density on floor litter (Wang et al., 2002b). Rearing broilers on litter increases the oxidative stress, structural damage to the lungs, and the incidence of birds found to have viable intrapulmonary microorganisms when compared with broilers reared in cages or on raised netting floors (Madelin and Wathes, 1989; Bottje et al., 1998). Broilers whose lungs were primed 48 h pre-

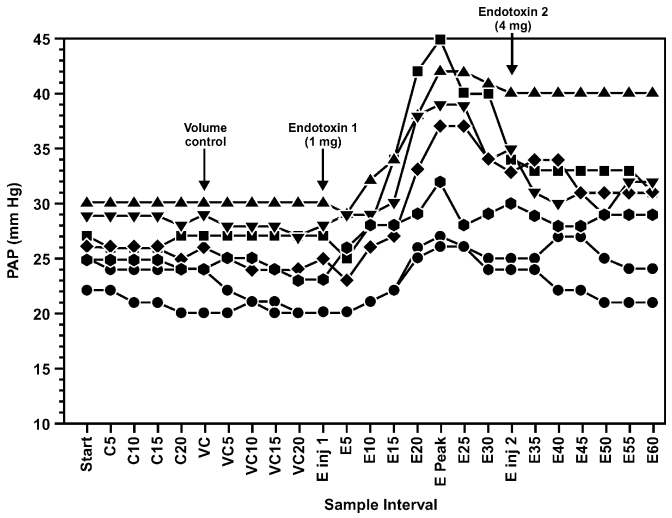

FIGURE 2. Individual pulmonary arterial pressure (PAP) values for 7 male broilers at the start of data collection (Start), at 5-min intervals during the control period (C5 to C20), within 30 s after injecting the volume control (VC), at 5-min intervals during the volume control period (VC5 to VC20), within 30 s after the injection with 1 mg of endotoxin (E inj 1), at 5-min intervals after the injection with 1 mg of endotoxin (E5 to E30), during the maximum PAP response to 1 mg of endotoxin (E Pk), within 30 s after the injection with 4 mg of endotoxin (E inj 2), and at 5-min intervals thereafter (E35 to E60) (adapted from Wideman et al., 2001; also see Wang et al., 2002a,b).

viously by intravenously injecting a low dose (minimally occlusive) of cellulose microparticles did not differ in the time of onset, amplitude, or variability in their pulmonary hypertensive response to LPS when compared with unprimed controls (Wang et al., 2002a). Intravenously injected cellulose microparticles become lodged in the pulmonary precapillary arterioles and initiate acute focal inflammatory responses within the surrounding lung parenchyma (Wideman et al., 2002; Wang et al., 2003). These observations suggest the variation in pulmonary vascular responsiveness to LPS among individuals within a broiler population may reflect the innate rather than acquired characteristics of those individuals.

IMMUNE MODULATION OF LPS-INDUCED PULMONARY HYPERTENSION

The lungs of chickens are constantly challenged with airborne gram-negative bacteria and LPS, as well as LPS translocated from pathogens resident in the intestine (Whyte, 1993, 2002; Sander, 1994; Martensson, 1995; Brown et al., 1997; Alexander and Rietschel, 2001). Approximately 40% of the gram-negative bacteria in poultry house dust reside on particles of a respirable size (<5 μm in diameter) capable of reaching the air sacs and gas exchange regions (parabronchi) of avian lungs. Substantial quantities of aerosolized LPS (e.g., 0.31 $\mu g/m^3$ of air) are consistently detected in the air of poultry houses (Hayter and Besch, 1974; Sander, 1994). Our current understanding of the avian intrapulmonary immune responses to aerosolized gram-negative bacteria and LPS is limited. Most aerosolized particulates are trapped by mucus in the conducting airway system which includes the trachea and primary and secondary bronchi. These particulates are prevented from entering the lung parenchyma by the mucociliary "escalator" present in the conducting airways and at the lung-air sac junction of chickens. The mucus and entrapped particulates are propelled by cilia to the pharynx for elimination or ingestion (McLelland, 1989a,b; Jeurissen et al., 1994; Fedde, 1998). The lymphoid tissues of bronchi primarily consist of demarcated oval lymphoid nodules present directly underneath the bronchiolar epithelial layer (Sminia et al., 1989; Jeurissen et al., 1994). This bronchus-associated lymphoid tissue can be most frequently observed at the junction of primary and secondary bronchi. The tissue has defined T and B cell areas, germinal centers, and accessory cells required for antigen presentation, suggesting a role of this tissue in the initiation of mucosal immune responses. Exposure to ammonia inhibits mucociliary transport, contributes to deciliation of the conducting airways, promotes leukocyte infiltration into the lung parenchyma, and increases the susceptibility of chickens to airborne pathogens (Anderson et al., 1964, 1966; Al-Mashhadani and Beck, 1985). In broilers treated with infectious bronchitis virus to inhibit mucociliary transport and permit the bacteria to penetrate through the conducting airways to the lung parenchyma, intratracheal inoculation with *E. coli* increased the incidence of PHS 5-fold (Tottori et al., 1997; Yamaguchi et al., 2000).

Macrophages and neutrophils play a central role in the mammalian response to LPS, and mammals have alveolar macrophages as a first line of defense at their gas exchange surfaces. Birds do not appear to have resident macrophages or other resident leukocytes at their gas exchange surfaces or within the air sacs. However, during respiratory infection or aspiration of particulates, leukocytes (primarily phagocytic macrophages and heterophils) are present in lavage fluid from the avian respiratory tract, indicating mechanisms do exist that allow these cells to enter the gas-filled spaces when necessary (e.g., inflammation) (Ficken et al., 1986; Toth and Siegel, 1986; Toth et al., 1987, 1988; Qureshi et al., 1993; Klika et al., 1996; Kunkle and Rimler, 1996; Pruimboom et al., 1996; Brown et al., 1997). Lung tissues surrounding the noncartilagenous, nonmucociliary tertiary bronchi (parabronchi) contain dentritic cells that may be important in the uptake and presentation of airborne antigens penetrating the parabronchus (Jeurissen et al., 1994). Immune cells are also present in the parenchyma of the lung. These include T and B cells as well as dentritic cells, macrophages, and mast cells (Jeurissen et al., 1994; Wang et al., 2003). In view of the normal dearth of macrophages on the gas surfaces of the air sac and parabronchial epithelium, it has been proposed that in birds the initial phagocytic function may reside in the pulmonary epithelial cells themselves followed by exocytosis to the underlying interstitium (Brown et al., 1997). For example, respirable microparticulates reaching the lower respiratory tract of birds were trapped in the surfactant (trilamellar) layer, engulfed by the gas-exchange epithelial cells, and translo-

cated to the interstitium where they were phagocytosed by macrophages (Bretz and Schmidt-Nielsen, 1971; Bland et al., 1985; Stearns et al., 1987; Brown et al., 1997). A role of the surfactant and epithelium of mammalian lungs in the local defense against LPS is also supported by recent evidence (Sano et al., 1999; Dentener et al., 2000; Song and Phelps, 2000).

The lungs also respond immunologically to bloodborne particulates and antigens. An important yet under-appreciated function of the pulmonary vasculature is to filter and clear the returning venous blood of micro- and macroparticulate matter including bacteria, immune complexes, cellular debris, aged red blood cells, and emboli. In addition to particulates entering the blood stream directly, materials engulfed by lymphatic capillaries throughout the body subsequently flow through major lymph trunks to empty into the vena cava immediately proximal to the right atrium (Berens and Rautenfeld, 1993). The pulmonary vasculature of broilers therefore can be challenged by a wide variety of substances, and the ability of the pulmonary vasculature to clear these substances from the blood serves to defend sensitive tissues such as the brain and heart. In several mammalian species, bloodborne antigens and intravenously injected microparticulates circulating through the pulmonary capillaries are primarily removed from the blood stream by pulmonary intravascular macrophages (PIM) which are large mature macrophages bound to the pulmonary capillary endothelium. The PIM of sheep are responsible for 40 to 100% of uptake of intravenously injected particulates and have been shown to occupy 15% of the intravascular volume of pulmonary capillaries. Collectively PIM provide an extensive surface area for contact with bloodborne antigens and constitute an important part of the mononuclear phagocytic system (Warner et al., 1986). However, resident PIM capable of removing tracer particulates and bacteria evidently are not present in rats and chickens (Lund et al., 1921; Malik, 1983; Warner et al., 1986; Winkler, 1988; Staub, 1994; Dantzker, 1997; Heffner and Repine, 1997; Brain et al., 1999; Weidner and Lancaster, 1999). The absence of PIM does not leave the chicken's lungs immunologically unresponsive to bloodborne antigens because CO values for broilers demonstrate that the entire blood volume, and thus all of the circulating leukocytes, flow through the lungs every 30 s (Sturkie, 1986; Wideman, 1999). Indeed, intravenously injected microparticulates and LPS induce dynamic intrapulmonary inflammatory responses in broilers. Preliminary histopathology of lung tissues obtained after LPS injections revealed vascular congestion, endothelial cell swelling, and notable increases in both large and small mononuclear cells within in the pulmonary microvasculature. Intravenous injections of microparticulates and LPS cause acute reductions in circulating monocyte concentrations that coincide with the appearance of monocytes/macrophages in the lung parenchyma of broilers (Wideman et al., 2002; Wang, 2003; Wang et al., 2003). Intravenous LPS administration greatly enhances the pulmonary mononuclear cell uptake of circulating particles and pathogens in the rat (Warner et al., 1994), and pretreatment of chickens with LPS apparently activated circulating leukocytes and enabled the microparticulate tracer Monastral blue to trigger a profound pulmonary hypertension that could not be elicited with injections of the tracer alone (Weidner and Lancaster, 1999). Similarly, LPS injected into chicken skin resulted in uptake of carbon particles by mononuclear cells in venules near the injection site, a process not observed in skin injected with saline alone. Other effects of LPS injury in chicken skin included increased vascular permeability for up to 30 min postinjection and leukocyte emigration (Katiyar et al., 1992). Avian thrombocytes are phagocytic toward bacteria and microparticulates (Glick et al., 1964; Carlson et al., 1968; Sterz and Weiss, 1973; Chang and Hamilton, 1979a,b; Awadhiya et al., 1980; Ohata and Ito, 1986; DaMatta et al., 1998; Roland and Birrenkott, 1998), and in mammals LPS causes an acute intrapulmonary platelet aggregation accompanied by the release of 5-hydroxytryptamine (5HT, serotonin), a potent pulmonary vasoconstrictor (Shibazaki et al., 1996, 1999). The pulmonary hypertensive response to LPS in broilers may implicate intrapulmonary thrombocyte accumulation and 5HT release (Wang, 2003). Hence it is likely that in chickens, as in the rat, LPS causes the release of mediators by pulmonary endothelial cells and circulating leukocytes leading to an influx of other inflammatory cells into the lung parenchyma.

The LPS-initiated intrapulmonary inflammatory responses having a major impact on the pulmonary vasculature are summarized in Figures 3 to 5. The biological activity characteristic of endotoxin resides in the Lipid A component of the outer membrane of gram-negative bacteria. LPS entering the blood first binds to LPS binding protein in the plasma which in turn transfers the LPS to evolutionarily ancient CD14 "pattern recognition receptors" that are part of the innate (genetically predetermined, non-clonal) immune system. The binding protein transfers LPS to membrane-bound CD14 receptors (mCD14) on the surface of monocytes/macrophages and neutrophils/heterophils. Binding of LPS to mCD14 receptors facilitates the recognition of LPS by membrane-associated toll-like receptors (TLR4), and mCD14 receptors may transfer LPS to TLR4 receptors. LPS also can bind to soluble CD14 receptors that are synthesized by monocytes/macrophages and then released into the plasma (Figure 3). The LPS-soluble CD14 receptor complex appears to be capable of directly activating local endothelial cells and platelets/thrombocytes (Figures 3 and 4). The ensuing cascade of intracellular signaling events culminates in transcription and translation of genes associated with the innate immune responses; production and local release of inflammatory cytokines (e.g., interleukin(IL)-1, IL-6, and tumor necrosis factor-α, ET-1, and platelet activating factor (PAF); release of 5HT from platelets; and synthesis of a wide range of eicosanoid metabolites consisting of leukotrienes, prostaglandins (prostacyclin, PGI_2), and TxA_2 (Figures 3 and 4) (Shibazaki et al., 1996, 1999; Chilton et al., 1997; Arditi, 1999; Kuijpers and Van

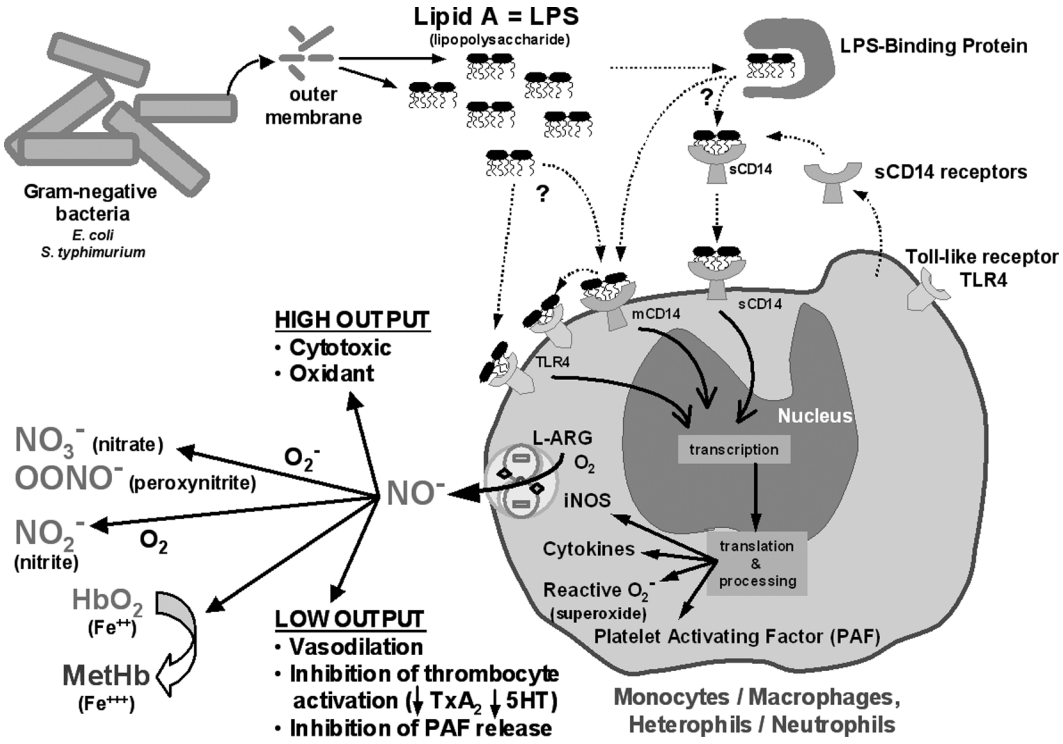

FIGURE 3. Characteristic endotoxin activity resides in the lipid A component (lipopolysaccharide, LPS) of the outer membrane of gram-negative bacteria. LPS binds to LPS binding protein which in turn transfers the LPS to CD14 "pattern recognition receptors" that are membrane-bound (mCD14) on the surface of monocytes/macrophages and neutrophils/heterophils or that are synthesized and released by monocytes/macrophages into the plasma as soluble CD14 receptors (sCD14). Binding of LPS to CD14 receptors facilitates the recognition of LPS by membrane-associated toll-like receptors (TLR4). The ensuing cascade of intracellular signaling events culminates in transcription and translation of genes associated with the innate immune responses, production and local release of inflammatory cytokines and platelet activating factor (PAF), and induction of the gene for nitric oxide synthase (iNOS). Once activated, iNOS produces copious quantities of nitric oxide (NO) from L-arginine. NO and its derivative reactive oxygen/nitrogen species (e.g., nitrate, peroxynitrite, nitrite) are nonspecifically cytotoxic to pathogens but also can damage tissues in the immediate vicinity of the inflammatory response. NO and its metabolites also combine with hemoglobin to form nitrosyl-hemoglobin complexes that are oxidized to methemoglobin and therefore reduce the oxygen carrying capacity of blood. NO also promotes flow-dependent pulmonary vasodilation, inhibits activation of thrombocytes by platelet activating factor (PAF), and inhibits thrombocyte release of thromboxane (TXA_2) and serotonin (5-hydroxytryptamine, 5HT) (figure adapted from Raetz and Whitfield, 2002).

der Poll, 1999; Tobias, 1999; Alexander and Rietschel, 2001; Gryglewski et al., 2001).

Lipopolysaccharide also induces leukocytes to express the gene for nitric oxide synthase (NOS) (Figure 3) (Chang et al., 1996; Hussain and Qureshi, 1997, 1998; Dil and Qureshi, 2002a,b; Janeway and Medzhitov, 2002; Qureshi, 2003). Previously NOS had been considered a "constitutive" (constantly expressed) enzyme in endothelial cells (NOS-3, eNOS) and an "inducible" (acutely upregulated) enzyme in monocytes/macrophages and neutrophils/heterophils (NOS-2, iNOS). Current evidence indicates that NOS can be induced in a variety of cells including endothelial cells. The eNOS enzyme tends to be activated transiently due to its readily reversible binding of the Ca^{2+}/calmodulin complex, whereas the iNOS enzyme binds its Ca^{2+}/calmodulin complex so tightly that, once activated, iNOS produces copious quantities of nitric oxide (NO) (Davis and Matalon, 2001). The NOS enzyme forms the free radical NO from the guanidine nitrogen of L-arginine, an essential amino acid for birds and a limiting substrate for avian NOS and macrophage function (Taylor et al., 1992; Dietert et al., 1994; Wideman et al., 1995; Martinez-Lemus et al., 1999; Kidd et al., 2001; Ruiz-Feria et al., 2001; Villamor et al., 2002). Evidence that supplemental L-arginine can reduce PAP and PVR during endotoxemia in mammals may indicate that LPS can stimulate sustained rates of NO production that are high enough to deplete extracellular reserves of the L-arginine substrate (Weitzberg, 1993). NO and its derivative reactive oxygen/nitrogen species (e.g., nitrate, peroxynitrite, nitrite) are nonspecifically cytotoxic to pathogens, but also can damage tissues in the immediate vicinity of the inflammatory response, thereby contributing to the pathogenesis of PHS in broilers (Bottje and Wideman, 1995; Gaston and Stamler, 1997). NO and its metabolites also combine with hemoglobin to form nitrosyl-hemoglobin complexes that are oxidized to methemoglobin and therefore reduce the oxygen carrying capacity of blood (Gaston and Stamler, 1997). Hemoglobin is capable of binding NO in quantities sufficient to inhibit NO-mediated influences on the systemic arterial vasculature of chickens (Hasegawa et al., 1993) (Figure 3). As shown in Figure 5, endothelial-derived NO diffuses into the vascular smooth muscle where it activates soluble guanylate cyclase to increase intracellular concentrations of guanosine 3′,5′-cyclic monophosphate and promote flow-dependent pulmonary vasodilation (relaxation of vascular smooth muscle). NO relaxes pre-constricted pul-

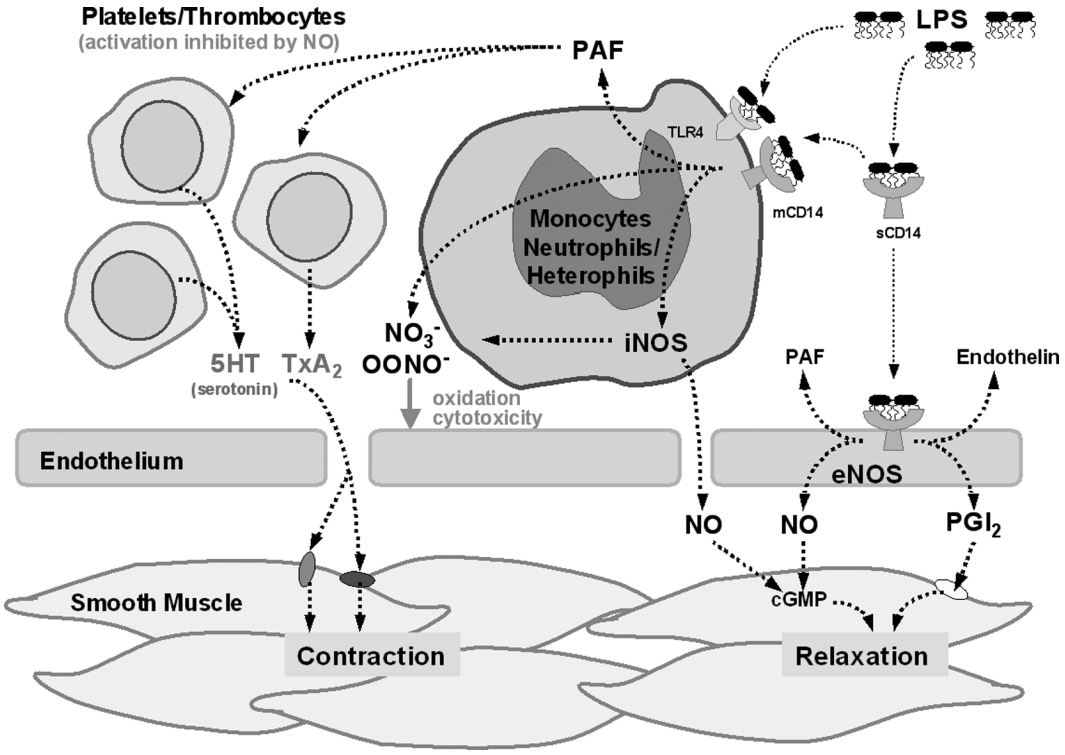

FIGURE 4. Lipopolysaccharide (LPS) binds to membrane-bound receptors (mCD14) on the surface of monocytes/macrophages and neutrophils/heterophils or to soluble receptors (sCD14) that can activate the endothelium. The CD14 receptors and toll-like receptors (TLR4) trigger a cascade of intracellular signaling events including the release of platelet activating factor (PAF), endothelin, and induction of nitric oxide synthase (iNOS). PAF causes thrombocytes to release substances that constrict pulmonary vascular smooth muscle (e.g., thromboxane: TXA_2, and serotonin: 5-hydroxytryptamine, 5HT). The iNOS enzyme produces copious quantities of nitric oxide (NO) and derivative reactive oxygen/nitrogen species (e.g., NO_3-, OONO-) that are nonspecifically cytotoxic. NO and prostacyclin (PGI_2) relax pulmonary vascular smooth muscle, NO inhibits PAF activation of thrombocytes, and NO and PGI_2 inhibit platelet aggregation and the formation of obstructive microthrombi.

monary artery rings isolated from broiler and Leghorn chickens, and NO minimizes the onset of pulmonary hypertension when broiler lungs are challenged in vivo with disproportionate increases in CO (Wideman et al., 1995, 1996a, 1998b; Martinez-Lemus et al., 1999; Villamor et al., 2002). Therefore it is reasonable to assume that NO generated by leukocytes in response to LPS can dilate the smooth muscle of the pulmonary vasculature and airways, thereby helping to attenuate the pulmonary hypertensive response to LPS (Figure 5).

Processes that initiate intrapulmonary inflammatory responses are recognized as potentially being profoundly deleterious to pulmonary hemodynamics and gas exchange in mammals (Malik, 1983; Dantzker, 1997; Heffner and Repine, 1997). Once an intrapulmonary inflammatory response has been initiated, the extent of the subsequent lung injury is determined by the proportions of "damaging" vs. "protective" factors released by leukocytes and local tissues. Factors that promote lung damage and cause excessive constriction of pulmonary blood vessels and airways include PAF, pro-inflammatory cytokines (e.g., IL-6), reactive oxygen species, ET-1, TxA_2, and 5HT. The leukocytes and endothelial cells also produce vasodilators, PGI_2 and NO, that are considered "protective" of pulmonary function to the extent that they promote smooth muscle relaxation and attenuate pulmonary hypertension. For example, when mammals are infused i.v. with LPS, both the vasoconstrictor TxA_2 and the vasodilator PGI_2 normally are produced and can exert dynamically antagonistic influences on pulmonary vascular resistance. PGI_2 and NO inhibit both platelet aggregation and the formation of vascular microthrombi that otherwise can physically occlude the vascular bed and progress to the disseminated intravascular coagulation, ischemia, and tissue destruction observed in sepsis (Radomski et al., 1987; Wanecek et al., 2000). NO also reduces or inhibits the concurrent local LPS-stimulated release of PAF, ET-1, TxA_2, and 5HT, thereby minimizing the tissue damage and vasoconstriction attributable to leukocyte activation (Figures 3 and 4) (Longworth et al., 1994; Frank et al., 1996; Gaston and Stamler, 1997; Teder and Nobel, 2000; Davis and Matalon, 2001; Gryglewski et al., 2001; Miyata et al., 2001; Lauer et al., 2002). For example, pharmacological blockade of NOS with the inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) permitted LPS to stimulate unrestricted platelet and neutrophil activation leading to vascular and airway constriction, acute microvascular lung injury, and hemorrhagic lung edema (Gryglewski et al., 2001). Naturally occurring NO donors, such as S-nitrosoglutathione, reduce the pulmonary vasoconstrictive response to 5HT through possible chemical modification by NO of the $5HT_2$ G protein-coupled receptor system (Nozik-Grayck et al., 2002). NO currently is recognized as a key protective modulator of leukocyte-mediated

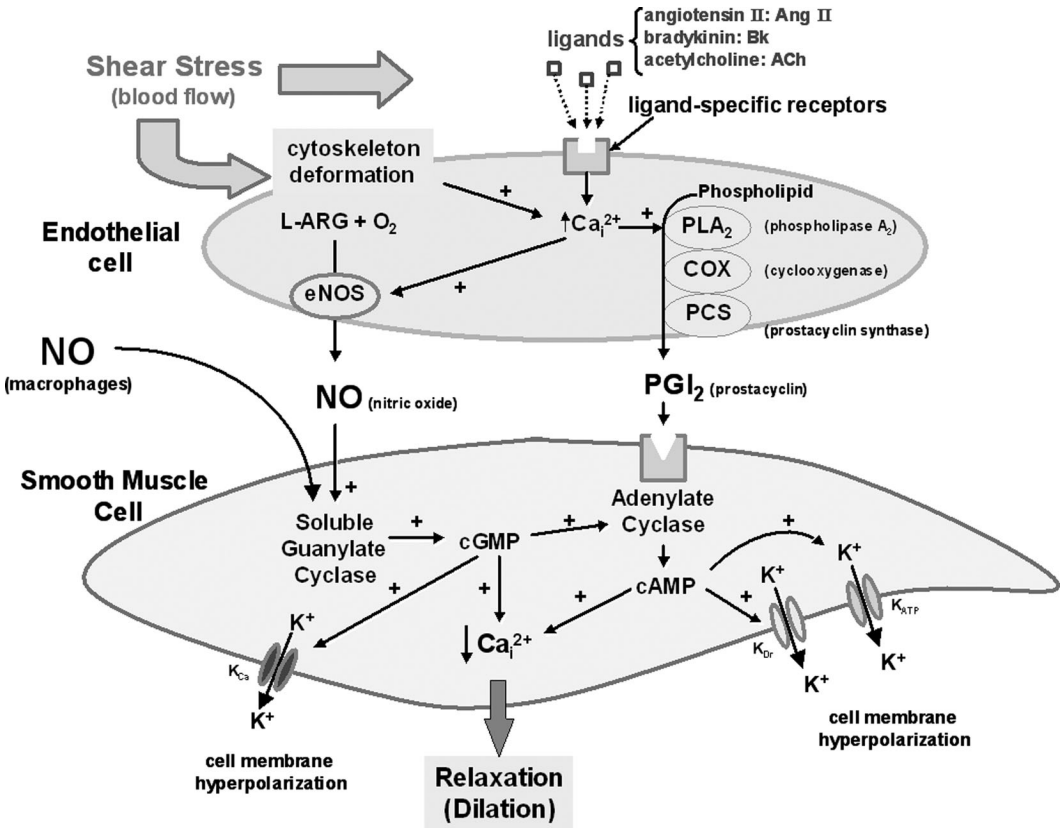

FIGURE 5. Elevated blood flow rates apply shear stress that deforms the cytoskeleton of endothelial cells causing intracellular free calcium ion (Ca_i^{2+}) concentrations to increase. The Ca_i^{2+} concentrations also increase in response to binding of endothelium-dependent pulmonary vasodilators (e.g., angiotensin II, Ang II; bradykinin, Bk; and acetylcholine, ACh) to their specific membrane receptors. The Ca_i^{2+} activates endothelial nitric oxide synthase (eNOS) and phospholipase A_2 (PLA_2) leading to increased synthesis of nitric oxide (NO) and prostacyclin (PGI_2), respectively. NO derived from the endothelium or from leukocytes (e.g., macrophages) readily diffuses into vascular smooth muscle cells where it activates soluble guanylate cyclase to increase intracellular concentrations of guanosine 3′,5′-cyclic monophosphate (cGMP). PGI_2 binds to its receptor on the smooth muscle cell membrane and activates adenylate cyclase leading to the production of adenosine 3′,5′-cyclic monophosphate (cAMP). Both cGMP and cAMP promote Ca^{2+} and K^+ efflux from smooth muscle cells, thereby preventing calmodulin-mediated actin-myosin interactions (reduced Ca_i^{2+}) and hyperpolarizing the cell (K^+ efflux). The result is pulmonary vasodilation (relaxation of vascular smooth muscle throughout the lungs). K_{Ca} = Ca^{2+}-dependent K^+ channel; K_{ATP} = ATP-dependent K^+ channel; K_{Dr} = delayed rectifier K^+ channel (figure adapted from Hecker, 2000; also see Villamor et al., 2002).

damage and thrombosis within the pulmonary circulation of mammals, and it appears likely that NO rather than PGI_2 serves as the primary intrapulmonary modulator of the detrimental effects of PAF and TxA_2 (Grabarevic et al., 1997). NO also may play a protective role in chickens. The addition of L-NAME causes chicken pulmonary artery rings to contract, thereby revealing a sustained role of NO in reducing the basal tone of the vascular smooth muscle. Endothelium-dependent relaxation of chick pulmonary artery rings primarily depends on NO rather than PGI_2 production (Villamor et al., 2002). Supplemental L-arginine and NO attenuated the response of chicken pulmonary artery rings to receptor-mediated endothelium-dependent vasoconstrictors such as ET-1 and the TxA_2 mimetic U-46619 (Martinez-Lemus et al., 1999; Villamor et al., 2002). Injecting L-NAME i.v. into intact broilers caused a modest pulmonary hypertension that was counteracted by administering the NOS-independent NO donor sodium nitroprusside (Weidong et al., 2002), and repeated i.p. injections of L-NAME caused PHS in broilers (Grabarevic et al., 1997).

CURRENT KNOWLEDGE, HYPOTHESES, AND ONGOING RESEARCH

The evidence currently available supports the hypothesis that variability in the pulmonary hypertensive responses of broilers to LPS may reflect variability in the proportions or profiles of chemical mediators released during the ensuing inflammatory response. Broilers are more likely to exhibit profound pulmonary hypertension when LPS elicits the production of substantially more vasoconstrictors (TxA_2, 5HT, PAF, ET-1, IL-6) than vasodilators (PGI_2, NO). The pulmonary vasculature may appear hyporesponsive to LPS if substantially more vasodilators than vasoconstrictors are produced or if hyper-expression of iNOS causes NO to be generated in sufficient quantities to inhibit vasoconstrictor production or release. Additional research is needed to reveal key indices in the LPS-initiated inflammatory cascade that are correlated with the magnitude of the evoked pulmonary hypertensive response. These indices may prove useful in selecting broilers capable of thriving in spite of ongoing

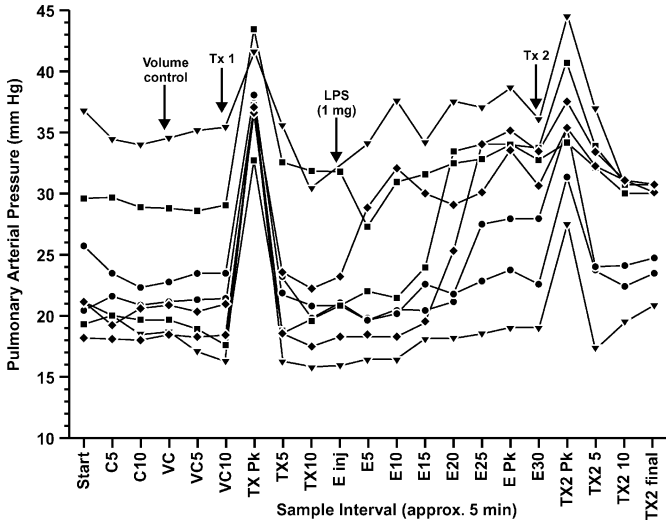

FIGURE 6. Individual pulmonary arterial pressure (PAP) values for male broilers (mean ± SEM, n = 8) at the start of data collection (Start), at 5-min intervals during the control period (C5 and C10), within 30 s after injecting the volume control (VC), at 5-min intervals during the volume control period (VC5 and VC10), during the maximum PAP response within 90 s after the first injection (Tx1) of the thromboxane A_2 mimetic U44069 (TX Pk), at 5-min intervals after the first thromboxane mimetic injection (TX5 and TX10), within 30 s after endotoxin injection (E inj), at 5-min intervals after endotoxin injection (E5 to E30), during the maximum PAP response to endotoxin (E Pk), during the maximum PAP response within 90 s after the second injection (Tx2) of thromboxane mimetic (TX2 Pk), and at 5-min intervals after the second thromboxane mimetic injection (TX2 5 to TX2 final). Different letters (a,b,c) designate differences between means over time ($P \leq 0.05$) (adapted from Wideman et al., 2001).

exposure to aerosolized LPS, and the potential exists that similar criteria may be useful in selecting broilers for resistance to PHS. Uncovering the mechanisms responsible for the individually variable pulmonary hypertensive responsiveness to LPS will likely contribute to our understanding of the multi-factorial pathogenesis of PHS (Wideman, 2001). Our current knowledge of the roles of several relevant vasodilators and vasoconstrictors in broilers can be summarized as follows.

Thromboxane

TxA_2, whether administered i.v. as the potent TxA_2 mimetic U44069 or produced by circulating thrombocytes in response to bolus acid injections, increases the PVR and the PAP in broilers (Wideman et al., 1998a, 1999a, 2001). The TxA_2 mimetic U44069 triggered uniformly high pulmonary hypertension in broilers that subsequently exhibited a typically variable range of pulmonary vasoconstriction in response to LPS (Figure 6). The pulmonary hypertensive response to U44069 could also be elicited during the refractory or tolerant period when broilers become unresponsive to subsequent LPS injections (Figure 6). In contrast, the magnitude of the pulmonary vasoconstriction induced by bolus acid injections varied widely among individual broilers (Wideman et al., 1998a, 1999a, 2001). These observations suggest broilers vary relatively little in their pulmonary vascular responsiveness to TxA_2; nevertheless, innate factors responsible for initiating (e.g., ET-1), modulating (e.g., NO), or synthesizing (e.g., phospholipase A_2, cyclooxygenase 2) TxA_2 may contribute to variability in the individual responsiveness to LPS (Wideman et al., 1998a, 2001; Martinez-Lemus et al., 1999; Villamor et al., 2002). For example, LPS-induced hyperexpression of the prostaglandin G/H 2 gene has been correlated with amplified TxA_2 production and pulmonary hypertension in rabbits (Conary et al., 1994; Delong et al., 1999).

Serotonin

In domesticated avian species, biogenic amines (e.g., epinephrine, norepinephrine, phenylephrine, 5HT) can cause pulmonary vasoconstriction and thrombocyte aggregation and degranulation (Belamarich et al., 1968; Wideman, 1999; Villamor et al., 2002). 5HT is actively accumulated by mammalian platelets and avian thrombocytes and is released into the plasma during platelet/thrombocyte activation in response to PAF (Meyer and Sturkie, 1974; Cox, 1985; Lacoste-Eleaume et al., 1994). 5HT is an extremely potent pulmonary vasoconstrictor capable of eliciting a sustained pulmonary hypertension when infused i.v. into broilers. In fact, 5HT is singularly the most potent pulmonary vasoconstrictor we have evaluated in broilers, capable of causing extensive pulmonary vasoconstriction leading to rapid, terminal suffocation unless injection dosages are carefully titrated to 100-fold lower than equivalently hypertensive doses in mammals (Chapman and Wideman, 2002). However, the accumulation of 5HT by thrombocytes, the in vivo release of 5HT, and plasma levels of 5HT have not been evaluated in broilers during the inflammatory response initiated by LPS. In humans, plasma 5HT levels increase markedly during gram-negative sepsis, and elevated plasma concentrations of 5HT have been implicated in the pulmonary hypertension associated with acute respiratory distress syndrome (Heffner and Repine, 1997). LPS triggers rapid intrapulmonary platelet aggregation and 5HT release in mice (Shibazaki et al., 1999). Elevated circulating levels of 5HT have been implicated in the initiation of acute and chronic pulmonary hypertension in several human and animal studies, including the pulmonary hypertensive response to appetite suppressant drugs (Seiler et al., 1974; Douglas et al., 1981; Herve et al., 1990; Brenot et al., 1993; Abenhaim et al., 1996; Weir et al., 1996; Egermayer et al., 1999; Kereveur et al., 2000). Polymorphisms of the 5HT membrane transporter or the transporter gene promoter may contribute to the susceptibility of mammals to pulmonary artery smooth muscle cell proliferation during the pathogenesis of pulmonary hypertension (Eddahibi et al., 2000; Simonneau et al., 2001).

Nitric Oxide

As summarized above, NO functions as a cytotoxic free radical (Gaston and Stamler, 1997; Bottje and Wideman, 1995), but it also plays an essential protective role that

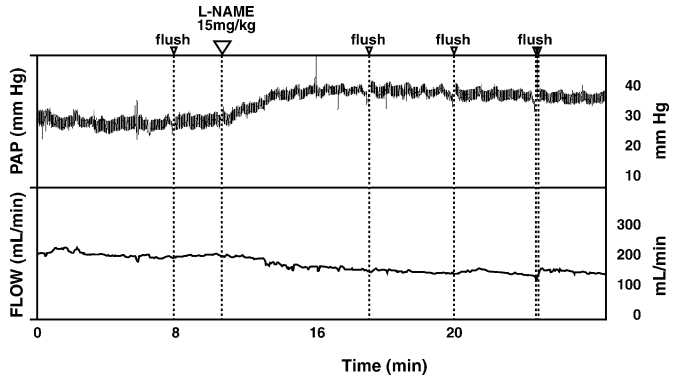

FIGURE 7. Physiograph recording from an individual male broiler showing continuous values for pulmonary arterial pressure (PAP) and blood flow through the right pulmonary artery (FLOW) during an initial 10-min control period and after an i.v. injection of the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) at 15 mg per kg BW to inhibit nitric oxide (NO) synthesis. Flush markers identify points where heparinized saline was injected to clear blood from the pulmonary arterial cannula. The moderate pulmonary hypertensive response to L-NAME developed rapidly and, as indicated by the trend toward a reduced FLOW, can be attributed to constriction of the pulmonary vasculature after inhibition of NO synthesis (R. F. Wideman and M. E. Chapman, unpublished).

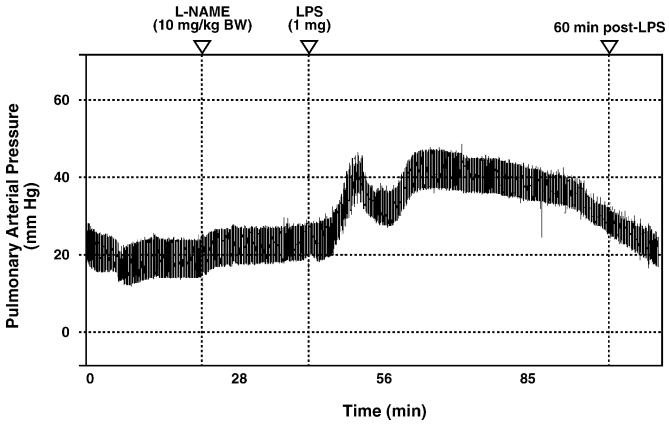

FIGURE 8. Physiograph recording from an individual male broiler showing continuous values for pulmonary arterial pressure (PAP) during an initial 15-min control period, for 10 min following an i.v. injection of the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) at 10 mg/kg BW, and for > 60 min after an i.v. injection of 1 mg lipopolysaccharide (LPS). The L-NAME alone modestly increased the PAP; thereafter the response to LPS was biphasic, high in amplitude (peak > 40 mm Hg) and prolonged in duration (R. F. Wideman and M. E. Chapman, unpublished).

is directly relevant to hemodynamic challenges (flow-dependent vasodilation) and pulmonary inflammatory responses (modulation of TxA_2, 5HT, and PAF release) (vide supra). LPS induces expression of the NOS gene in chickens (Chang et al., 1996), and different genetic lines of chickens can exhibit substantial innate variability in their LPS-mediated iNOS responsiveness coupled with corresponding variability in the levels of NO produced by their macrophages (Hussain and Qureshi, 1997). The characteristic level of LPS-induced iNOS expression in hyper- and hyporesponsive lines of chickens does not depend on the bacterial source of LPS, rather the magnitude of iNOS expression may be due to proportional macrophage expression of mCD14 and TLR4 receptors (Figures 3 and 4) (Hussain and Qureshi, 1997, 1998; Dil and Qureshi, 2002a,b; Qureshi, 2003). Pilot experiments were recently conducted to evaluate the modulatory role of NO using our standard protocol for assessing the pulmonary hypertensive response to LPS (Figures 7 and 8) (Wideman et al., 2001; Wang et al., 2002a,b). Broilers in the control group were injected with saline (volume/carrier vehicle control), and broilers in the L-NAME group were injected with 10 to 50 mg L-NAME/kg BW to block NO production. This dosage range for L-NAME has been demonstrated to be effective without causing acute toxicity in broilers (Grabarevic et al., 1997; Weidong et al., 2002). L-NAME inhibition of NO synthesis caused a rapid modest increase in PAP (Figure 7), confirming previous reports that tonic/basal NO synthesis promotes pulmonary vasodilation in chickens (Wideman et al., 1995; Wideman et al., 1996a; Villamor et al., 2002; Weidong et al., 2002). When both groups were injected with LPS, the L-NAME group exhibited an early pulmonary hypertensive peak that rarely develops in broilers in the absence of L-NAME (Wideman et al., 2001; Wang et al., 2002a), and that has been correlated with PAF-induced TxA_2 synthesis in mammals (Snapper et al., 1998; Chlopicki et al., 1999; Wanecek et al., 2000; Gryglewski et al., 2001). The subsequent, more prolonged pulmonary hypertensive response to LPS was greater in duration in the L-NAME group when compared with the control group, and was higher in amplitude when the high dose of 50 mg of L-NAME/kg BW was used (Figure 8). In mammals, the delayed, more sustained pulmonary hypertensive response to LPS has been correlated with induction and transcription of the gene for pre-pro-endothelin, after which ET-1 constricts the pulmonary vasculature either directly via specific receptors on the smooth muscle cells or indirectly by stimulating leukocytes to produce TxA_2 (Weitzberg, 1993; Faltin et al., 1996; Snapper et al., 1998; Wanecek et al., 2000). Chicken pulmonary artery rings exhibit dose-dependent contractions in response to ET-1, and NO attenuates this contractile response (Martinez-Lemus et al., 1999; Villamor et al., 2002). In this context, it is relevant that the capacity of i.v. injected LPS to elicit the delayed (sustained) phase of the pulmonary hypertensive response varies among mammalian species, and this variability appears to be positively correlated with species differences in the abundance of PIM available to promote TxA_2 synthesis (Faltin et al., 1996). PIM are absent from avian lungs, and the pulmonary hypertensive response to LPS in broilers was not altered in magnitude or duration when an intrapulmonary inflammatory response (e.g., induced intrapulmonary monocyte/macrophage accumulation) was initiated 48 h prior to injecting LPS (Wang et al., 2002a, 2003).

Prostacyclin

As shown in Figure 9, infusing PGI_2 i.v. into clinically healthy broilers caused concurrent reductions in mean

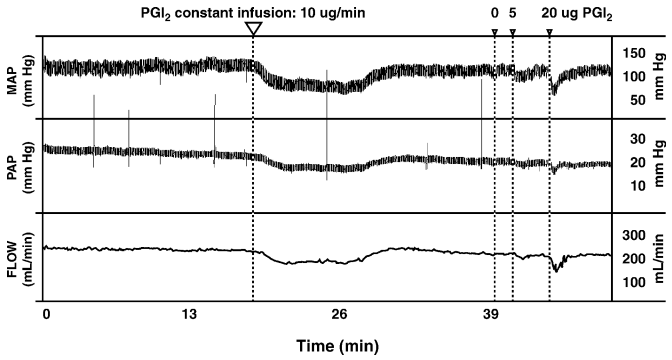

FIGURE 9. Physiograph recording from an individual male broiler showing continuous values for mean systemic arterial pressure (MAP), pulmonary arterial pressure (PAP), and blood flow through the right pulmonary artery (FLOW). After an initial control period, prostacyclin (PGI_2) was infused i.v. at 10 μg/min for 10 min and then the PGI_2 infusion ceased. Beginning approximately 40 min into the recording, the broiler was injected with 0 (volume control), 5, and 20 μg PGI_2 i.v. to evaluate responses to different doses. In all cases, PGI_2-induced reductions in PAP coincided with reductions in MAP and FLOW (R. F. Wideman and M. E. Chapman, unpublished).

systemic arterial pressure, PAP, and CO. Preliminary analyses of these studies indicate that the reduction in CO represents the primary response to PGI_2, with the systemic and pulmonary hypertension primarily reflecting reduced flow rather than PGI_2-induced vasodilation (R. F. Wideman and M. E. Chapman, unpublished). These preliminary experiments were conducted on clinically healthy broilers having PAP values within the normal range. More dramatic vasodilation might be anticipated following PGI_2 infusion into the pulmonary vasculature of broilers exhibiting pre-ascitic pulmonary hypertension or clinical PHS (Wideman and Tackett, 2000; Wideman et al., 2000).

CONCLUSIONS

Broilers have a marginal pulmonary vascular capacity that renders them susceptible to the onset of pulmonary hypertension whenever any factor increases the PVR and forces the right ventricle to elevate the PAP to propel the CO through the lungs. The focal intrapulmonary inflammatory response to inhaled or bloodborne microparticulates and LPS triggers the release of a cascade of substances known to constrict the pulmonary vasculature. At the local tissue level, pulmonary vasoconstriction may serve to isolate and restrict the distribution of the offending antigen, permit inflammatory mediators and cytotoxic substances to accumulate in regionally high concentrations to neutralize the antigen, and shunt blood flow away from regions of the lungs where gas diffusion has been compromised by focal inflammatory damage to the endothelium and epithelium. These events apparently help preserve essential organ function (e.g., optimized blood-gas exchange) at the expense of subjecting focal tissues at the sites of inflammation to enhanced damage mediated by activated leukocytes. Problems arise from the scenario outlined above when the offending antigen is widely distributed throughout the lungs, causing constriction of a significant proportion of the resistance vessels. The resulting increase in PVR constitutes the greatest threat when overlaid upon a preexisting incipient pulmonary vascular inadequacy, as in modern broilers. Innate mechanisms do exist to modulate life-threatening bouts of immune-mediated pulmonary hypertension. For example, following acute pulmonary vasoconstriction in response to LPS, a refractory or tolerant phase ensues, during which pulmonary hypertension cannot again be elicited even with very large doses of LPS. During this period of LPS tolerance, autocrine inflammatory stimuli (e.g., 5HT and TxA_2 stimulation of additional 5HT and TxA_2 release from platelets) presumably reach a crescendo and then subside. It remains to be determined whether broilers are more susceptible to invasion by gram-negative bacteria during the period of LPS tolerance. In addition, although NO itself clearly can have cytotoxic efficacy, NO generally is considered to modulate the tissue damage caused by activated leukocytes, modulate pro-inflammatory autocrine responses, and attenuate the pulmonary hypertension triggered by a disseminated pulmonary inflammatory response in mammals.

The results of pilot experiments provide evidence that in some broilers sufficient NO can be produced during an LPS challenge to modulate the production and biological impact of concurrently induced vasoconstrictors. In these individuals, pulmonary vasoconstriction in response to LPS is revealed only following pharmacological blockade of NOS with L-NAME. Blocking LPS-induced synthesis of NO caused higher-amplitude and longer-duration pulmonary hypertensive responses to LPS overall. These observations are consistent with the hypothesis that a pulmonary hypertensive response to LPS is not observed in some broilers because they innately generate more vasodilators (NO, PGI_2) than vasoconstrictors (TxA_2, 5HT, PAF, IL-6, ET-1) during the LPS challenge. Variability among individuals in their pulmonary hypertensive responses to LPS clearly may reflect different innate characteristics of monocytes/macrophages in broilers (Chang et al., 1996; Hussain and Qureshi, 1997, 1998; Dil and Qureshi, 2002a,b). We now are poised to explore the possibility that LPS can serve as a relevant "tool" for evaluating the basis for pulmonary hypertension in broilers.

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