In Vitro Hypoxia Differentially Affects Constriction and Relaxation Responses of Isolated Pulmonary Arteries from Broiler and Leghorn Chickens


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ABSTRACT Under normoxic conditions in vitro, isolated pulmonary arteries from broilers exhibit reduced endothelium-dependent relaxation responses when compared with Leghorns. In vivo, hypoxia increases the susceptibility of broiler chickens to pulmonary hypertension syndrome (PHS), whereas Leghorns are considered resistant to PHS. Because L-arginine supplementation decreases the incidence of PHS in vivo and improves the relaxation responses of broiler isolated pulmonary arteries in vitro, we hypothesized that in vitro hypoxia would further reduce the relaxation responses of broilers to endothelium-derived nitric oxide (EDNO)-dependent vaso-dilators and that L-arginine supplementation would alleviate this impairment. As a test of this hypothesis, pulmonary arteries from broiler and Leghorn chickens were isolated and exposed to normoxia or hypoxia in the presence or absence of L-arginine while their constriction and relaxation responses to vasoactive compounds were recorded. In broilers, hypoxia did not affect the constriction responses of isolated pulmonary arteries but decreased EDNO-dependent acetylcholine-induced relaxation responses. In contrast, in Leghorns hypoxia increased endothelin-1-induced vasoconstriction responses and reduced the EDNO-dependent relaxation responses only to the lowest concentration of acetylcholine used. L-Arginine supplementation augmented the relaxation responses to acetylcholine in broilers and Leghorns under normoxia but failed to augment them under hypoxia. Relaxation responses to the NO donor, sodium nitroprusside, were not affected by hypoxia in Leghorns but were increased by hypoxia in broilers. These results suggest that the increased incidence of PHS in broiler chickens reared under hypoxia may be associated with a hypoxia-induced reduction in the synthesis or activity of EDNO in the pulmonary circulation.

(Key words: acetylcholine, ascites, endothelin, nitric oxide, pulmonary hypertension)

INTRODUCTION

Broiler chickens are highly susceptible to pulmonary hypertension, right-sided congestive heart failure, and ascites (Wideman, 2000). This pathological aggregate of signs known as pulmonary hypertension syndrome (PHS) was originally described in broiler chickens reared at high altitudes where available levels of oxygen for respiration are reduced (Cueva et al., 1974). Later, as genetic selection improved growth rate and meat yield, PHS became prevalent in broilers reared at any altitude (Peacock et al., 1989; Wideman et al., 1997). PHS, however, continues to be associated with subnormal levels of arterial blood oxygenation or hypoxemia (Peacock et al., 1989, 1990; Reeves et al., 1991; Julian and Mirmalidi, 1992; Wideman and Kochera-Kirby, 1995; Wideman et al., 1997, 1998), and its incidence is inversely related to the level of available oxygen for respiration (Cueva et al., 1974; Owen et al., 1990; Peacock et al., 1990; Beker et al., 1995; Odom et al., 1995; Owen et al., 1995).

Reduced levels of oxygen for respiration (hypoxia) elevate pulmonary arterial pressure by increasing pulmonary vascular resistance (Peacock et al., 1989; Wideman et al., 1997). In most species studied so far, hypoxia induces pulmonary arterial vasoconstriction (Peake et al., 1981), and although this phenomenon has not been directly evidenced in chickens, the facts that unilateral pulmonary bronchus occlusion (Wideman et al., 1997) and reduced levels of respiratory oxygen increase pulmonary arterial

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Abbreviation Key: Ach = acetylcholine; ENDO = endothelium-derived nitric oxide; ET-1 = endothelin-1; PHS = pulmonary hypertension syndrome; SNP = sodium nitroprusside.
pressure (Peacock et al., 1989; Owen et al., 1995) suggest that hypoxia-induced vasoconstriction may also be the mechanism through which pulmonary vascular resistance is increased in hypoxic chickens. Failure to reduce pulmonary arterial pressure through vasodilation is believed to be the mechanism leading to chronic pulmonary hypertension, right ventricular hypertrophy, right-sided congestive heart failure, and death in hypoxia-induced PHS. A comparison between laying hens and broiler chickens indicated that broilers exhibit a greater increase in pulmonary vascular resistance in response to hypoxia than laying hens despite both lines of chickens exhibiting a similar level of hypoxemia. (Peacock et al., 1989). In mammals, a concomitant release of endothelium-derived nitric oxide (EDNO) counterregulates acute hypoxia-induced pulmonary arterial vasoconstriction (Shaul et al., 1993; Dumas et al., 1999; Weissmann et al., 2001; Emery et al., 2003). Evidence thus indicates that EDNO synthesis is an important mechanism for reducing the elevated pulmonary vascular resistance associated with hypoxia-induced pulmonary hypertension and that alterations in EDNO activity and that L-arginine supplementation would ameliorate it. Consequently, the present study was designed to determine the effects of acute in vitro hypoxia on the constriction and relaxation capacity of the pulmonary circulation of broilers and Leghorn chickens. We hypothesized that hypoxia by increasing the constriction ability and reducing the relaxation capacity of the pulmonary vasculature.

Endothelial cells synthesize nitric oxide from L-arginine in a reaction catalyzed by the enzyme nitric oxide synthase (Palmer et al., 1987, 1988). In vivo, L-arginine supplementation reduces the incidence of PHS (Wideman et al., 1995), suggesting that availability of endogenous L-arginine may be an important limiting factor for EDNO synthesis in the broiler chicken (Martinez-Lemus et al., 1999). Furthermore, relaxation responses to EDNO-dependent vasodilators are smaller in pulmonary arteries isolated from broilers than from Leghorns (Martinez-Lemus et al., 1999, 2003), and although in vitro L-arginine supplementation improves relaxations in both chicken lines, the improvement is greater in broilers than in Leghorns. All these findings suggest that reduced availability of L-arginine and reduced EDNO activity in the pulmonary circulation of broilers may be associated with the development of PHS. We hypothesized that hypoxia would exacerbate this reduced pulmonary artery EDNO activity and that L-arginine supplementation would ameliorate it. Consequently, the present study was designed to determine the effects of acute in vitro hypoxia on the constriction and relaxation capacity of broiler and Leghorn isolated pulmonary arteries with and without L-arginine supplementation.

**MATERIALS AND METHODS**

Male 1-d-old slow-growing Single Comb White Leghorn and fast-growing broiler chickens were acquired from Hy-Line 2 and Ideal 3 respectively. From 1 d of age, all chickens were kept under a 24-h light regimen in a temperature-controlled brooder unit and provided ad libitum access to water and a standard corn-soybean starter ration (3,190 kcal of ME/kg of diet, 22% CP, and 1.62% arginine) formulated to meet or exceed the NRC (1994) requirements for broilers. The Texas A&M University’s Animal Care Committee approved the use of chickens in all experimental protocols.

Totals of 28, 31, and 6 broilers and 26, 24, and 4 Leghorn chickens were weighed at 1, 2, and 3 wk of age, respectively, after euthanasia by cervical dislocation. The heart and lungs were exposed via thoracotomy, and the pericardial sac was removed in situ. A 0.5- to 1.0-cm section of the left pulmonary artery immediately distal to the bifurcation of the main pulmonary artery trunk was removed and placed in cold, aerated buffer solution. Excessive connective tissue was carefully removed from the pulmonary artery section, and a ring approximately 3 mm in length was cut with dissecting scissors. The heart was also removed, and the ventricles were dissected from the atria along with the atrioventricular fat. The right ventricular free wall was then dissected from the left ventricle and interventricular septum. Immediately after dissection, wet weights of both ventricles were obtained, and the ratio of right ventricular free wall weight to total ventricular weight was calculated as an index of right ventricular hypertrophy and pulmonary hypertension (Burton et al., 1968).

Each pulmonary artery ring was suspended between 2 stainless steel hooks, vertically mounted in a 20-mL water-jacketed organ bath containing a bicarbonate buffer solution (pH 7.40) at 41°C, and aerated with 95% oxygen and 5% carbon dioxide for normoxia or 95% nitrogen and 5% carbon dioxide for hypoxia. The bicarbonate buffer consisted of 142 mM NaCl, 5.4 mM KCl, 11.0 mM dextrose, 2.0 mM CaCl2, 1.2 mM MgCl2, and 18.0 mM NaHCO3. plus 10−5.5 M phenolamine to eliminate any potential effects of released catecholamines (Ashbrook et al., 1980; Mostaghim et al., 1986). The lower hook was attached to a stainless steel rod mounted within the organ chamber, and the upper hook was connected to the lever of a force-displacement transducer to record isometric contractions and relaxations as previously described (Martinez-Lemus et al., 1999, 2003). Increments in force were quantified as millimeter deflections recorded on a polygraph. 3

Once mounted, the pulmonary arteries were allowed to equilibrate for 2 h while maintaining a preload tension of 1 g. Vessels were constricted twice during this equilibration period with a KCl-substituted buffer. This buffer contained an additional 40 mM KCl (45.4 mM total) substituted equimolarly for NaCl (102 mM total).

Individual concentration response relationships for all vasodilators agents were completed in a cumulative manner without any intervening washout of bath chambers. After the equilibration period, the pulmonary arteries were submaximally constricted with an approximately 75%-effective concentration of endothelin-1 (ET-1, 10−7.5).
M). Once constriction to ET-1 had reached a plateau, relaxation responses to the receptor-mediated endothelium-dependent vasodilator acetylcholine (ACh, $10^{-5}$, $10^{-6}$, and $10^{-5}$ M) or the endothelium-independent vasodilator sodium nitroprusside (SNP; $10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5}$ M) were recorded. After the maximal relaxation response to the greatest concentration of each of the above vasodilators was reached, papaverine ($10^{-4}$ M) was added to define the maximal relaxation attainable in each pulmonary artery ring. To determine any potential involvement of prostaglandins in these experiments, prostaglandin formation was prevented in a series of experiments using pulmonary arteries from both chicken lines by adding indomethacin ($10^{-5}$ M) 30 min before the addition of ET-1. In another series of experiments, the pulmonary arteries were pretreated with L-arginine ($10^{-3}$ M, 15 min) after equilibration and before the addition of ET-1. Subsequently, relaxation responses to increasing concentrations of ACh were obtained as described above.

The ACh, ET-1, SNP, and indomethacin were obtained from Sigma Chemical. L-Arginine was purchased from Research Biochemicals, and phenolamine was obtained from Ciba-Geigy. All drugs were dissolved in double-distilled water, except for indomethacin, which was initially dissolved in ethanol. The final concentration of ethanol in the organ bath buffer solution never exceeded 0.15%.

Vasoconstriction responses were normalized to the weight of the pulmonary artery tissue and presented as means ± standard errors in milligrams of tension per milligram of pulmonary artery tissue. Relaxation responses induced by ACh and SNP are expressed as mean percentages ± standard errors of maximal ET-1 vasoconstriction. As previously reported, these normalizations are performed in order to make comparisons across individuals and across vessels of different sizes (Martinez-Lemus et al., 1999, 2003). The data were analyzed by the general linear model procedure of the Statistical Analysis System (SAS Institute, 1988). Partitioning of significantly different means was accomplished using Duncan’s multiple range test (Steel and Torrie, 1980).

**RESULTS**

The mean BW of all broiler chickens over the 3 ages sampled was significantly greater than that of Leghorn chickens ($279 ± 15.6$ vs. $120 ± 18.7$ g, $P < 0.05$) reflecting the rapid somatic growth of the broiler chicken line. The ratio of right ventricular free wall weight to total ventricular weight was also significantly greater in broiler than in Leghorn chickens ($0.28 ± 0.006$ vs. $0.24 ± 0.007$). The constriction responses of the pulmonary arteries to KCl (45.4 mM) and ET-1 ($10^{-7.5}$ M) are reported in Table 1. Constriction responses to KCl were not affected by hypoxia in either of the chicken lines, but overall Leghorn pulmonary arteries exhibited greater KCl-induced constriction responses than broilers ($124.5 ± 6.6$ vs. $93.4 ± 5.3$ mg/mg, respectively, $P < 0.05$). In contrast, ET-1-induced constriction responses were not different between the chicken lines but were significantly increased by hypoxia in Leghorns.

Blockade of prostaglandin synthesis by addition of 10 μM indomethacin 30 min before ET-1 vasoconstriction had no effect on any of the responses to ET-1, ACh, or SNP (data not shown). Vasodilator responses to ACh in broiler and Leghorn pulmonary arteries are shown in Figures 1 and 2. Pulmonary arteries from broiler and Leghorn chickens in response to KCI and endothelin-1 (ET-1).

**TABLE 1. Vasoconstriction force (mg of tension/mg of tissue) of isolated pulmonary arteries from broiler and Leghorn chickens in response to KCl and endothelin-1 (ET-1)**

<table>
<thead>
<tr>
<th>Item</th>
<th>Broiler</th>
<th>Leghorn</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normoxia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 20)</td>
<td>(n = 14)</td>
<td></td>
</tr>
<tr>
<td>KCl (mg/mg)</td>
<td>$98 ± 8$</td>
<td>$126 ± 9$</td>
</tr>
<tr>
<td>Hypoxia (n = 20)</td>
<td>(n = 12)</td>
<td></td>
</tr>
<tr>
<td>ET-1 (mg/mg)</td>
<td>$39 ± 6$</td>
<td>$45 ± 8$</td>
</tr>
<tr>
<td><strong>Hypoxia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 14)</td>
<td>(n = 12)</td>
<td></td>
</tr>
<tr>
<td>KCl (mg/mg)</td>
<td>$88 ± 8$</td>
<td>$72 ± 10$</td>
</tr>
<tr>
<td>ET-1 (mg/mg)</td>
<td>$39 ± 6$</td>
<td>$45 ± 8$</td>
</tr>
</tbody>
</table>

$a,b$cMeans ± SEM within a row lacking a common superscript differ ($P < 0.05$).
Leghorn chickens demonstrated a concentration-dependent relaxation to ACh after ET-1 constriction. Hypoxia significantly reduced the endothelium-dependent relaxation response of Leghorn pulmonary arteries only at the lowest concentration of ACh used (10^{-7} M). At higher concentrations of ACh (10^{-6} and 10^{-5} M), hypoxia had no significant effects on the endothelium-dependent relaxation responses of Leghorn pulmonary arteries. L-Arginine supplementation significantly increased the relaxation responses of Leghorn pulmonary arteries to ACh (10^{-4} and 10^{-5} M) in normoxia, whereas in hypoxia, L-arginine supplementation reduced the relaxation responses to all concentrations of ACh. In contrast to the responses of Leghorns, hypoxia significantly reduced the endothelium-dependent relaxation responses of broiler pulmonary arteries at all concentrations of ACh. L-Arginine supplementation in broilers as in Leghorns increased the relaxation responses in normoxia, but decreased them in hypoxia.

The concentration-response curves to the nitric oxide donor SNP in Leghorn and broiler pulmonary arteries are shown in Figure 3. SNP caused concentration-dependent relaxation responses in all pulmonary arteries. Under normoxia, relaxation responses to SNP were not different in broiler as compared with Leghorn pulmonary arteries up to a SNP concentration of 10^{-5} M. At the highest concentration of SNP (10^{-4} M), however, relaxation responses in broilers were greater than those in Leghorns. Hypoxia significantly increased the relaxation responses to 10^{-5} and 10^{-4} M SNP in broilers but had no effect on the SNP-induced relaxation responses of Leghorn pulmonary arteries.

**DISCUSSION**

This study provides evidence that in vitro hypoxia differentially affects the constriction and relaxation responses of broiler and Leghorn isolated pulmonary arteries. Hypoxia significantly increased the constriction responses of Leghorn pulmonary arteries to ET-1 but had no effect on those of broilers. The increased vasoconstriction response in Leghorns was specific for ET-1 and not related to an increased intrinsic ability of the pulmonary arteries to constrict during hypoxia, which was indicated by the fact that KCl-induced constrictions were not affected by hypoxia. However, as previously reported, KCl-induced constrictions were greater in Leghorns than in broilers (Martinez-Lemus et al., 2003). As in the present study, we previously showed that under normoxic conditions, pulmonary artery vasoconstriction responses to ET-1 are not different between broiler and Leghorn chickens (Mar-
Further, nitric oxide synthesis inhibition with N\textsuperscript{G}-nitro-\textit{l}-arginine methyl ester similarly increases the constriction response of broiler and Leghorn pulmonary arteries to ET-1, indicating that a concomitant release of EDNO occurs during ET-1-induced vasoconstriction in both chicken lines (Martinez-Lemus et al., 1999). Because hypoxia has been associated with a reduction in EDNO activity of the pulmonary vasculature (Adnot et al., 1991; Liu et al., 1991; Eddahibi et al., 1992; Emery et al., 2003; Shaul et al., 1993), we expected hypoxia would magnify the constriction response to ET-1 in both chicken lines, but to a greater degree in broilers than in Leghorns by reducing the production of EDNO via stimulation of ET\textsubscript{B} receptors. In contrast, we observed that hypoxia increased ET-1 vasoconstriction only in Leghorns but not in broilers. A reduction in EDNO activity may not be the cause of this increased vasoconstriction response to ET-1 in Leghorns, because Leghorn relaxations in response to the EDNO-dependent vasodilator ACh were not affected by hypoxia. Thus, the mechanism for this increased responsiveness to ET-1 in the Leghorn pulmonary artery warrants further investigation. ET-1 has been previously implicated in chronic and acute hypoxia-induced pulmonary hypertension (Elton, 1992; Stelznner et al., 1992; Giaid et al., 1993; Li et al., 1994). In acute hypoxia, the mechanism by which ET-1 participates in increasing pulmonary arterial pressure appears to be an increase in blood circulating levels of the peptide (Allen et al., 1993). Our results, however, indicated that other mechanisms by which ET-1 may be implicated in acute hypoxia-induced pulmonary hypertension exist because isolated pulmonary artery preparations were excluded from any influence from blood circulating vasoactive compounds.

In contrast to the effects of hypoxia on ET-1-induced vasoconstriction, hypoxia reduced endothelium-dependent relaxation responses of isolated pulmonary arteries from broilers but had no significant effect on those from Leghorns. That the relaxations in response to the nitric oxide donor, SNP, were not reduced in the hypoxic broiler pulmonary arteries suggests that the hypoxia-induced reduction on the endothelium-dependent relaxation responses of pulmonary arteries from broilers was not caused by a reduced responsiveness of the vascular smooth muscle to nitric oxide, but was related to an impairment of the ACh-induced EDNO synthesis pathway. Thus, the cause of the increased susceptibility of broilers to PHS may be associated with reduced EDNO activity that is exacerbated by hypoxia, which could explain why under the same levels of hypoxemia broilers exhibit greater pulmonary vascular resistance than layers (Peacock et al., 1989). Impairment of the pulmonary EDNO synthesis pathway in mammals by acute hypoxia has been extensively documented (Archer et al., 1989; Johns et al., 1989; Rodman et al., 1990; Shaul et al., 1993). The mechanisms leading to the impairment, however, have remained elusive, but possible mechanisms include reduced availability of oxygen, \textit{l}-arginine, nitric oxide synthase enzyme, and enzyme cofactors. As we have previously shown, under normoxia, in vitro supplementation of \textit{l}-arginine improves the relaxation responses of pulmonary arteries from broilers to a greater extent than those from Leghorns (Martinez-Lemus et al., 1999). Thus, we expected \textit{l}-arginine supplementation would reverse the endothelium-dependent impairment caused by hypoxia in broiler pulmonary arteries. In vitro \textit{l}-arginine supplementation, however, did not improve the relaxation responses of pulmonary arteries from either chicken line under hypoxia. Reduced \textit{l}-arginine uptake by endothelial cells could be the cause of this, as in vitro hypoxia has been shown to inhibit endothelial \textit{l}-arginine uptake (Block et al., 1995; Zharikov et al., 1997; Zharikov and Block, 2000). The apparent discrepancy between these results and the fact that \textit{l}-arginine supplementation ameliorates PHS in vivo could be explained by the degree of hypoxia to which endothelial cells are actually exposed under in vivo and in vitro situations, by the differential in vivo and in vitro metabolism of \textit{l}-arginine, or both (Carville et al., 1993).

Differential responses of the pulmonary circulation to hypoxia between strains or lines of the same animal species have been previously documented (Salameh et al., 1999). The Madison strain of Sprague-Dawley rats exhibits increased pulmonary vasoconstriction and increased relaxation responses to ACh under hypoxia in comparison with the Hilltop strain (Salameh et al., 1999). The increased hypoxia-induced vasoconstriction of isolated pulmonary arteries from the Madison strain is associated with a reduction in EDNO activity, which appears paradoxical when one considers that the same strain exhibits greater relaxations in response to ACh. In the present study, the increased ET-1-induced constriction observed in Leghorn chickens under hypoxia may also appear paradoxical when one considers that their relaxation responses to ACh were not affected by hypoxia. As mentioned above, one explanation of these paradoxical results may be that the increased ET-1-induced vasoconstriction may not be associated with a reduction in EDNO activity in Leghorns. Another possibility is, however, that the mechanisms leading to EDNO synthesis via activation of ET\textsubscript{B} receptors by ET-1 or via activation of muscarinic receptors by ACh may be differentially affected by hypoxia.

No significant hypoxia-induced vasoconstriction was observed in pulmonary arteries from either chicken line in the present study. This finding, however, does not necessarily mean that chickens do not exhibit hypoxia-induced pulmonary vasoconstriction. Although in most species studied hypoxia-induced pulmonary vasoconstriction occurs both in conduit arteries as well as in small arteries and arterioles, the most dramatic hypoxia-induced vasoconstriction occurs in the resistance vasculature (Dumas et al., 1999). Therefore it is possible that in the large conduit pulmonary artery of the chicken, hypoxia-induced vasoconstriction may not be significant but that at the resistance arteriolar level it may take place to increase the pulmonary vascular resistance as observed in whole animals when exposed to reduced levels of avail-
Hypoxia-induced pulmonary vasoconstriction is observed in pulmonary arteries that have basal EDNO activity. In the isolated pulmonary artery preparation from the chicken, we have previously shown that no basal EDNO activity can be detected (Martinez-Lemus et al., 1999). However, in vivo inhibition of EDNO synthesis causes increased pulmonary arterial pressure (Wang et al., 2002; Weidong et al., 2002), indicating that basal EDNO activity is present in the pulmonary circulation of the chicken.

Although hypoxia reduced the relaxation responses to ACh in broiler pulmonary arteries, it also appeared to increase the relaxation responsiveness of vascular smooth muscle to exogenous nitric oxide as the relaxation responses to SNP were increased in the broiler pulmonary arteries under hypoxia. This increased relaxation response to SNP was not caused by a greater intrinsic tone of the broiler hypoxic pulmonary arteries as hypoxia did not induce pulmonary artery vasoconstriction, and maximal relaxation responses to the endothelium-independent non specific vasodilator, papaverine, were not different between hypoxic and normoxic pulmonary arteries.

In conclusion, hypoxia affected the vasoactive performance of isolated pulmonary arteries from broiler and Leghorn chickens. In Leghorns, hypoxia increased the vasoconstriction induced by ET-1 without affecting ACh-induced EDNO-dependent relaxation responses, whereas in broilers hypoxia did not affect ET-1-induced vasoconstriction but decreased the ACh-induced EDNO-dependent relaxation responses. Thus, the increased susceptibility to PHS observed with broilers exposed to hypoxia may be related to a hypoxia-induced reduction in the capacity of the pulmonary circulation to relax and reduced vascular resistance due to diminished EDNO activity of the pulmonary circulation.

REFERENCES


