Adventitial growth and lung connective tissue growth factor expression in pulmonary arterioles due to hypobaric hypoxia in broilers

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ABSTRACT Forty broilers maintained under natural hypobaric hypoxia (2,638 m above sea level) and 20 maintained under relative normoxia (460 m above sea level) were selected as pulmonary hypertensive (**PHB**) and nonpulmonary hypertensive (**NPHB**), to estimate the degree of the adventitial vascular thickness in lung arterioles and connective tissue growth factor (**CTGF**) expression in lung. In each group, the adventitial thickness (**%AT**) of 20 arterioles with 100 to 250 μm of external diameter was measured in lung samples of 24 and 42-day-old broilers. Also, mRNA extraction and real-time reverse transcription-PCR analysis were used to measure lung CTGF expression. The %AT was higher in PHB at 42 D as compared to NPHB at both ages and PHB at 24 D; however, the same differences were not evidenced at 24 D. In the 2 ages evaluated, differences were observed in the %AT between broilers under hypobaric hypoxia (PHB and NPHB) and under relative normoxia ($P < 0.01$). In broilers subjected to relative normoxia, no significant differences were found at any of the 2 ages. The expression levels of CTGF mRNA were higher in PHB compared to NPHB at the 2 ages. The %AT was higher in PHB with high levels of expression of CTGF mRNA than those NPHB with low expression of CTGF mRNA. This study showed that adventitial thickening is part of the pulmonary hypertension (**PH**) physiopathology in broilers exposed to hypobaric hypoxia, in which CTGF appears to be a fibrosis enhancer. Although present data suggest that adventitial engrossment could be a time-dependent process, individual susceptibility and the variable time-course of PH pathophysiology have to be considered.

Key words: hypobaric hypoxia, pulmonary hypertension, vascular remodeling, broiler

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INTRODUCTION

Avian species and mammals can develop pulmonary hypertension (**PH**) when they reside in high altitudes (Meyrick and Reid, 1978; Cogo et al., 2004; Reeves and Grover, 2005). Several pathological processes contribute to the development and progression of PH. These begin with pulmonary vasoconstriction, followed by remodeling of the pulmonary vessels, notably through muscularization of smaller arteries and arterioles and adventitial thickening (Rabinovitch et al., 1986; Thompson and Lawrie, 2017). Vascular remodeling in severe chronic PH is driven to a large degree by autocrine and paracrine factors released by endothelial and smooth muscle cells (Coll-Bonfill et al., 2015); however, the role of the adventitia is not sufficiently known (Stenmark et al., 2013).

The cells of the 3 layers of the vascular walls are responsible for the maintenance of vascular homeostasis and the correspondent response to stress or injury in processes such as overdistension or hypoxia. In recent years the adventitial compartment of blood vessels has attracted attention on these functions, because it has the capacity to store and release important factors in different physiological and pathological states (Stenmark et al., 2011, 2013). Some authors postulate the adventitia as "the main injury-sensing tissue of the vessel wall" (Stenmark et al., 2011), which entails fibroblast induced expression of contractile and extracellular matrix proteins (Lv et al., 2013), the release

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Abbreviations: AT, adventitial thickness; CI, cardiac index; CTGF, connective tissue growth factor; ET-1, endothelin-1; HPRT, hypoxanthine guanine phosphoribosyltransferase; masl, meters above sea level; NO, nitric oxide; NPHB, nonpulmonary hypertensive broilers; PH, pulmonary hypertension; PHB, pulmonary hypertensive broilers; TGF-β, transforming growth factor-beta

of factors that affect the medial smooth muscle cells (Wang et al., 2001) and the signal processing of vascular inflammation (Li et al., 2016).

In hypoxic vascular remodeling, the fibroblasts, smooth muscle and endothelial cells proliferation, and the overproduction of matrix proteins occur mediated by vascular fibroblasts in the adventitia. As a result, the vessels become nondistensible by dilators, thus leading to a fixed state of PH (Welsh et al., 1998). In this context, it should be noted that both, myoblasts and fibroblasts originate from mesenchymal cells.

Endothelial cells are involved in the pulmonary vascular response to hypoxia through the release of vasoactive factors, such as nitric oxide (**NO**) (Shaul et al., 1995; Xu et al., 1995) and endothelin-1 (**ET-1**) (Kourembanas et al., 1991)**.** Nitric oxide is a potent vasodilator and inhibits the proliferation of vascular smooth muscle cells (Inagami et al., 1995), whereas ET-1 is a powerful vasoconstrictor and vascular smooth muscle cell mitogen (Giaid et al., 1993). Previous observations suggest that a decrease of NO synthase gene and increased of ET-1 gene expression secondary to physical factors such a hypoxia or sustained shear stress in pulmonary vascular arterioles is related to vascular remodeling (Giaid and Saleh, 1995; Moreno de Sandino and Hernandez, 2006; Gomez et al., 2008). The connective tissue growth factor (**CTGF**) is a fibrotic mediator over expressed in experimental models of hypertension. It is a member of the CCN family, including the early response genes ctgf/cyr61/nov genes, similar to elm1, cop1, and WISP-3 (Bork, 1993). Endothelin-1 activates transforming growth factor-beta (**TGF-β**) and increases the deposition of fibronectin and collagen (Ammarguellat et al., 2002). Both, CTGF and TGF-β promote chronic fibrosis. The former ensues apoptosis and fiber formation, induced by TGF-β (Perbal, 2004). The latter also mediates the fibrotic effects of angiotensin II in smooth muscle cells (Ruperez et al., 2003).

Susceptible chickens when subjected to chronic hypoxia develop PH and arterioles muscularization (Moreno de Sandino and Hernandez, 2006). The mechanisms underlying hypoxia-induced pulmonary vasoconstriction and remodeling are not completely understood in broilers. All published works on vascular remodeling in chickens, as approached by morphometric analysis, mainly relate to the muscle layer of arterioles (Sillau and Montalvo, 1982; Enkvetchakul et al., 1995). In current literature, information is lacking on the participation of the pulmonary vascular adventitial tissue in the remodeling process in the broiler chicken. The pertinent knowledge in this context might serve to look for therapeutic agents, as proposed in humans (Thompson and Lawrie, 2017) and also, to identify possible genes which can be employed to identify resistant and nonresistant chickens to develop hypoxic PH.

This study was aimed to establish possible morphometric differences in the adventitial remodeling process in lung arterioles between pulmonary hypertensive broilers (**PHB**) and nonpulmonary hypertensive broilers (**NPHB**) and the expression of lung CTGF expression.

MATERIALS AND METHODS

Animals and Tissue Samples

A total of 400 Cobb male chicks used in a previous investigation were kept as follows:

1) Two hundred chicks under natural hypobaric hypoxia at 2,638 m above sea level (**masl**) in Bogotá, Colombia (barometric pressure, P_B: 560 mmHg; oxygen partial pressure, PO_2: 117 mmHg; inspired PO_2, 105 mmHg). Temperature was automatically controlled in Bogotá, to assure a minimal temperature of 20°C after the third week of the growing period.
2) Two hundred chicks under relative normoxia at 460 masl in Villavicencio, Colombia (P_B: 739 mmHg; PO_2: 155 mmHg; inspired PO_2: 143 mmHg). Temperature records were daily assessed to guarantee that lower readings never went below 20°C.

The mass cardiac index (**CI** = right ventricular weight/total ventricular weight × 100) was used to identify PHB and NPHB. Broilers with CI values above 25 are taken as PHB and those with CI values below 25 as NPHB. Based on observations previously carried out in Bogota, using the same strain of chicken, temperature and management conditions to present ones, it is clear that higher 25 values for CI is a reliable parameter to identify NPHB broilers resident in Bogota (Areiza et al., 2012; Vásquez and Hernández, 2012; Monroy and Hernández, 2013).

Lung samples were obtained from all animals in which ET-1 mRNA expression was measured by real-time reverse transcription-PCR (Gomez et al., 2008). A total of 10 broilers chosen from the abovementioned 400 chickens were employed to compare the degree of adventitial thickness (**%AT**) in pulmonary arterioles and relative expression of CTGF mRNA. Each one of these broilers were assigned to the following groups:

Group 1: PHB with high relative expression level of ET-1 mRNA.
Group 2: NPHB kept under hypoxic conditions as described for group 1, with low ET-1 mRNA expression.
Group 3: NPHB maintained under relative normoxia with low relative expression of ET-1 mRNA.

The measurements were taken at 24 and 42-day-old. These ages were selected in order to ensure that a reasonable number of PHB would be available for analysis, because PH occurrence goes up with age advancement.

Table 1. Mean (±SD) values of cardiac index (**CI**) and adventitial thickness (**%AT**; adventitial width ÷ external vessel diameter × 100) of pulmonary arterioles in nonhypertensive [NH; expressing high mRNA levels of endothelin-1 (**ET-1**)] and hypertensive (H; expressing low mRNA levels of ET-1) broilers at 24 and 42-day-old, maintained under relative normoxia at 460 m above sea level (**masl**) and natural hypobaric hypoxia at 2638 masl.

	Age					
	24 D			42 D		
	H under hypobaric hypoxia (group 1)	NH under hypobaric hypoxia (group 2)	NH under relative normoxia (group 3)	H under hypobaric hypoxia (group 1)	NH under hypobaric hypoxia (group 2)	NH under relative normoxia (group 3)
Cardiac index (%)	39.72 ± 5.69[a]	16.57 ± 2.26[b]	14.02 ± 2.15[b]	41.27 ± 6.75[a]	16.76 ± 3.38[b]	15.49 ± 1.72[b]
Adventitial thickness (%)	8.58 ± 0.76[a]	9.08 ± 1.14[a]	7.17 ± 0.72[b]	10.34 ± 1.11[c]	6.32 ± 0.51[d]	8.03 ± 0.62[b]

[a–d]Means lacking a common superscript are significantly different ($P < 0.01$).

H: pulmonary hypertensive broilers. NH: nonhypertensive broilers. Hypobaric hypoxia: 2638 masl. Relative normoxia: 460 masl. Cardiac index values are from a previous report (Gomez et al., 2008).

Adventitial Thickness Analysis

Lung samples from each bird were taken for morphometric studies with a computerized program (Leco Instruments Ltd., St. Joseph, MI), using 5 μm slices stained with a standard Masson´s trichrome staining for light microscopy. The vascular %AT for each animal was calculated in 20 interparabronchial arterioles with external diameter between 100 and 250 μm. This diameter was taken as the mean of 2 measurements made at right angles to each other, and %AT was estimated as the mean of 4 measurements of the %AT around the circumference of each vessel, as previously employed in morphometric studies in the muscle layer of arterioles (Moreno de Sandino and Hernandez, 2006). The %AT for each arteriole was expressed as adventitial width: external vessel diameter ratio ×100. A total mean value of %AT for each broiler was obtained by summing up all %AT results calculated for individual arterioles and then dividing this value by the number of arterioles (Sillau and Montalvo, 1982). Adventitial thickness is shown as the mean ± SD. Comparisons among experimental groups were made by ANOVA (GraphPad Instat, GraphPad Software Inc., San Diego, CA). The assumption that the data were sampled from populations that follow Gaussian distributions was tested using the Kolmogorov and Smirnov test. Statistical differences were considered to be significant at $P < 0.05$.

Molecular Methods

Total RNA was extracted from 150 mg of lung tissue with the Trizol Reagent according to the manufacter´s instructions (Invitrogen Corp., Carlsbad, CA). Then, mRNA was isolated using Dynabeads mRNA Direct kit (Invitrogen Corp.). The mRNA concentration of each of the samples was quantified using light absorption at 260 and 280 nm (A260/280) with an UV-visible spectrophotometer (Spectronic BioMate 3 UV-Vis Spectrophotometer, Thermo Electron Corp., Waltham, MA). cDNA synthesis was performed from 1 μg of mRNA using 200 U MoML-reverse transcriptase, 20 U ribonuclease inhibitor RNase-Out, and 1 nm random hexamer primers (Invitrogen Corp.) in a total volume of 30 μL. Reverse-transcription reactions were incubated at 37°C for 45 min and 42°C for 15 min and were terminated by heating at 92°C for 2 min. Connective tissue growth factor and hypoxanthine guanine phosphoribosyltransferase (**HPRT**) mRNA expression was determined in the lung from the cDNA obtained by reverse transcriptase, with the real-time PCR using the Light Cycler thermocycler (Roche, Mannheim, Germany) and the detection methodology of SYBR green PCR core reagents (Roche). PCR was carried out using specific primers for CTGF (5′-GAA GAC ACT TAC GGC CCA GA-3′and 5′-AAA CTT GAT GGG CTT GGA GA-3′) and HPRT (5′-TCC AAA GAT GGT GAA AGT GG-3′and 5′-GCT TCC CCG TCT CAC TGA T-3′), designed with the Primer3 program from the sequences of the *Gallus gallus* genes reported in GenBank (45,383,589 y 45,382,332) and with product sizes of 252 and 179 bp, respectively. The final reaction volume was 20 μL. Reactions were carried out under the following conditions: 95°C for 3 min, 40 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 15 s. The mRNA levels were normalized with the expression levels of HPRT. The specificity of the products was confirmed with the dissociation curves. The size of the products obtained was verified in the electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

RESULTS

Adventitial Thickness

All results are presented in Table 1 and Figure 1. In PHB, %AT was lower at 24 than at 42 D of age ($P < 0.01$). No differences were encountered when results from PHB and NPHB at 24 D were compared ($P > 0.05$). However, differences were found between these 2 groups at 42 D ($P < 0.01$). The %AT was higher in PHB than the correspondent one in group 2, at 42 D ($P < 0.01$). In addition, %AT was higher in PHB than in NPHB maintained under relative normoxia, at 24 and 42 D of age ($P < 0.01$). In NPHB, the %AT was

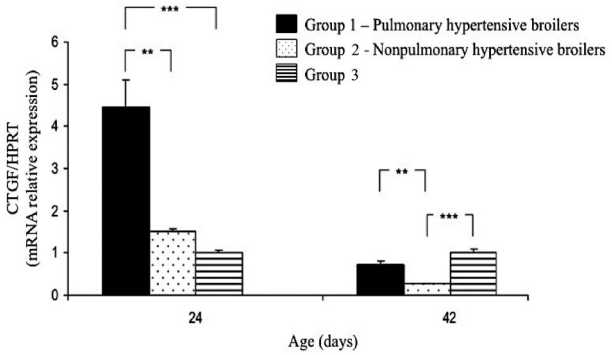

Figure 1. Comparative mean values and ± standard deviations of CTGF mRNA expression in the lung of nonpulmonary hypertensive and pulmonary hypertensive broilers raised under hypoxic and relative normoxia. $^{***}P < 0.001$, $^{*}P < 0.05$. CTGF: Connective Tissue Growth Factor. HPRT: Hypoxanthine Guanine Phosphoribosyltransferase.

higher at 24 than at 42 D ($P < 0.01$). This value was also higher in NPHB than in NPHB maintained under relative normoxia at 24 D, and lower in NPHB than in group NPHB maintained under relative normoxia at 42 D ($P < 0.01$). In the latter group, no differences were encountered in %AT values, when the 2 age-groups were compared ($P > 0.05$).

Relative Expression of CTGF mRNA

The expression of CTGF mRNA in the group exposed to hypobaric hypoxia was higher in PHB than in NPHB at both ages studied ($P < 0.01$). It was higher at 24 than at 42 D of age in the group exposed to hypobaric hypoxia ($P < 0.001$). In the group under relative normoxia, differences were not detected ($P > 0.05$) when comparing the expression levels of CTGF mRNA in the 2 ages studied. The expression levels in the lungs of PHB exposed to hypobaric hypoxia at 24 D were higher than those detected in chickens maintained under relative normoxia at the same age ($P < 0.001$). No differences were found in the expression of this gene when comparing PHB exposed to hypobaric hypoxia at 42 D and broilers under relative normoxia at the same age ($P > 0.05$). The expression levels of CTGF mRNA were significantly lower in NPHB exposed to hypobaric hypoxia at 42 D than those detected in the lungs of broilers kept under relative normoxia ($P < 0.001$) at the same age.

DISCUSSION

In previous studies a link between remodeling pulmonary arterioles, decreased endothelial NO production (Moreno de Sandino and Hernandez, 2006), and increased ET-1 mRNA expression levels was found (Gomez et al., 2008). The results of our current study show that there were no significant differences in the %AT in broilers kept under relative normoxia (NPHB) in both ages studied, whereas, chickens maintained under hypoxic conditions (both PHB and NPHB) had the highest values of %AT at 24 D. Hypoxia has a direct effect on the secretion of autocrine or paracrine factors or both that regulate adventitial fibroblast proliferation (Stenmark et al., 2002; Reyes et al., 2018). Overexpression of factors such as hypoxia-inducible factor-1α and other genes up-regulated by the hypoxia-responsive element can stimulate adventitial fibroblast proliferation by itself or by synergia with other growth factors (Krick et al., 2005; Kakudo et al., 2015).

In the present study, the highest %AT was detected in PHB under hypoxic conditions at 42 D of age. In hypoxia-induced PH in nonavian species, there is evidence of adventitial remodeling and the activation and proliferation of adventitial fibroblasts (Meyrick and Reid, 1978**;** Stenmark et al., 2002), even earlier than the other types of vascular wall cells (Belknap et al., 1997). Adventitial fibrosis was previously encountered in some pulmonary arteries of PHB (Hernández and de Sandino, 2011), and present results give a systematic approach to the abovementioned process. Hypoxia stimulates proliferation of pulmonary artery fibroblasts, but not systemic circulation fibroblasts; however, the molecular mechanisms that mediate these processes are not completely known (Welsh et al., 1998). Some molecules as the mitogen-activated protein kinases, Erk1, and Erk2, and the related stress-activated kinases, Jnk and p38 mitogen-activated protein kinase can be implicated in response to hypoxic stress (Kusuhara et al., 1998; Hood et al., 2017).

Overexpression of CTGF in PHB exposed to hypobaric hypoxia suggests this factor as a mediator of fibroblast proliferation and fibrotic effects of genes such as ET-1 in PH (Shi-Wen et al., 2004; Biasin et al., 2014), possibly due to the stimulus exerted by both ET-1 and hypobaric hypoxia on the promoter of this factor. This fact coincides with a previous finding of immunohistochemical ET-1 expression in the adventitia of PHB arterioles (Monroy and Hernández, 2017). However, it is important to consider that the cells of the vascular wall can release several factors which are responsible for the structural alterations that occur in the hypoxic PH.

Hypoxia-induced PH is also associated with early and dramatic increases in the differentiation of adventitial fibroblasts into a myofibroblast phenotype (Short et al., 2004; Yi et al., 2015). Activated fibroblasts and myofibroblasts contribute to the structural remodeling through increased production of collagen and another extracellular matrix proteins including fibronectin, tenascin, and elastin (Stenmark et al., 2006). Myofibroblast accumulation can also contribute to the abnormalities of vascular tone observed in chronic hypoxic PH. The contractile properties of myofibroblasts appear to differ from those of smooth muscle cells with a slower onset of contraction to vasoactive stimuli (Desmouliere et al., 2005).

Current findings indicate that the time of exposure influences the development of fibrosis, given the encountered differences between PHB 24 and the

42-day-old ones. Moreover, it is clear that individual time-responses vary in susceptible chickens. In fact, cardiac malfunction could ensue at any time from 1 to 10 D of age, but some individuals die in 2 or 3 D, whereas, in some others, death occurs 2 or 3 wk later (Pulido, 1996). In this context, it is feasible that after muscularization of arterioles, fibrosis ensues, although both processes might occur simultaneously. Also, the degree of fibrosis appears to vary on individual basis. From present findings it is tempting to suggest that broilers dying earlier develop arterioles fibrosis as a final effort to enhance the degree of PH.

In conclusion, we demonstrated that over expression of lung's CTGF appears to be associated with pulmonary adventitial vascular remodeling induced by chronic hypoxia in broilers. Also, that adventitial growth progresses in a time-dependent manner, which might resemble a similar process in affected mammals, in connective tissue pathologies such as pulmonary fibrosis (Farkas et al., 2011) and scleroderma (Morse et al., 2002). In the chicken, we have found an apparent increment of collagen, associated with PH in several cases (Hernández and de Sandino, 2011) which might have a link with the well-known presence of cartilage and bone neo-formations in the lung of some PHB (Alvarado et al., 1989), given the common progenitor cell in connective tissues. Connective tissue growth factor might be acting in harmony with ET-1 and diverse molecules such as growth factors, cytokines, receptors, and matrix proteins to provoke media muscle layer and adventitial connective tissue engrossment (Lipson et al., 2012). PH involves complex and heterogeneous processes, among which are the genetic and molecular abnormalities that interact and generate functional and structural changes in the pulmonary circulation. Pulmonary vasoconstriction and vascular remodeling are 2 essential mechanisms in the pathophysiology of the disease in broilers, involving endothelial cells, smooth muscle cells, and fibroblasts.

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