Endothelin 1, its Endothelin Type A Receptor, Connective Tissue Growth Factor, Platelet-Derived Growth Factor, and Adrenomedullin Expression in Lungs of Pulmonary Hypertensive and Nonhypertensive Chickens

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ABSTRACT Twenty-four 1-d-old broilers were distributed in 2 groups, pulmonary hypertensive broilers (PHB) and pulmonary nonhypertensive broilers (NPHB), to estimate possible differences between them in the expression of endothelin 1 (ET-1) and its type A receptor, connective tissue growth factor, platelet-derived growth factor, and adrenomedullin expression in the lungs. For this purpose, total RNA extraction and real-time PCR analysis were used. Endothelin 1 mRNA levels in the lungs of PHB were significantly higher than the corresponding level in NPHB ($P < 0.001$). In contrast, the opposite was true for ET-1 type A receptor mRNA levels ($P < 0.001$). Connective tissue growth factor mRNA levels in the lungs of PHB were significantly higher than in the lungs of NPHB ($P < 0.01$). However, no differences were encountered between the 2 groups of broilers in platelet-derived growth factor mRNA expression ($P > 0.05$). Adrenomedullin mRNA levels in the lungs of PHB were significantly higher than in NPHB ($P < 0.01$). It has been demonstrated for the first time that ET-1, connective tissue growth factor, and adrenomedullin are upregulated in the lungs of PHB. Furthermore, it is suggested that these peptides may play a major role in pulmonary hypertension pathophysiology. Present data might provide clues for future research directions such as genetic selection and therapeutic intervention to revert the process of pulmonary vasoconstriction and vascular remodeling. Major research goals could be to find endothelium-derived factors that probably trigger endothelial dysfunction, as well as possible interactions with already identified molecules which also intervene in the pulmonary response to hypoxia.

Key words: pulmonary hypertension, endothelin 1, connective tissue growth factor, platelet-derived growth factor, adrenomedullin

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INTRODUCTION

High-altitude hypoxia is a known cause of pulmonary hypertension (**PH**) in humans and broilers residing at high altitudes (Meyrick and Reid, 1978; Currie, 1999; Cogo et al., 2004; Bartsch et al., 2005; Kanazawa et al., 2005; Reeves and Grover, 2005; Remillard and Yuan, 2005; Rhodes, 2005). Pulmonary vascular resistance is enhanced by constriction of pulmonary vascular smooth muscle and structural remodeling of the vascular bed (Reid, 1979; Stenmark and Mecham, 1997). Several studies have revealed changes in serum levels and expression profile of molecules associated with pulmonary vasoconstriction and vascular remodeling in both humans and animals exposed to acute or chronic hypoxia (Yoshibayashi et al., 1991; Elton et al., 1992; Goerre et al., 1995). Endothelins (**ET**) are vasoconstrictor peptides that have been shown to contribute to various physiological functions in different tissues (Inagami et al., 1995). Endothelin 1 (**ET-1**) is the most abundant ET produced by endothelial cells (Yanagisawa et al., 1988). It acts through ET type A (**ETA**) and B (**ETB**) receptors to induce contraction of blood vessels and growth (Yanagisawa et al., 1988). Both ETA and ETB receptors are expressed in vascular smooth muscle cells (**SMC**), where ET-1 plays its vasoconstrictor, proliferative, and hypertrophic actions. Adrenomedullin (**AM**) is a factor to induce changes in the vascular tone; it is a long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma (Kitamura et al., 1993). Adrenomedullin has several effects on the vasculature such as vasodilation (Ishimitsu et al., 1994) and inhibition of endothelial apoptosis (Kato et al., 1997). In addition, AM has a protective effect against vascular injury, including oxidative stress (Kawai et al., 2004). Intravenous administration of AM decreases systemic and pulmonary arterial pressure and induces diuresis and natriuresis (Rademaker et al., 1997; Nagaya et al., 1999, 2000). Platelet-derived growth factor (**PDGF**) is a potent mitogen that has also been implicated in pulmonary vascular remodeling. The PDGF receptor antagonist STI571 (ima-

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tinib mesilate) reverses pulmonary vascular remodeling in 2 different animal models of PH (Shermuly et al., 2005). Connective tissue growth factor (**CTGF**) promotes fibroblast proliferation, migration, adhesion, and extracellular matrix formation. Its overproduction is proposed to play a major role in fibrosis (Moussad and Brigstock, 2000). There are no previous reports of expression profiles for PDGF, CTGF, AM, and ET-1 in pulmonary hypertensive broilers (**PHB**) and pulmonary nonhypertensive broilers (**NPHB**) subjected to hypobaric hypoxia under natural environmental conditions.

The aim of this study was to detect possible differences in ET-1 and its ETA receptor, AM, PDGF-C, and CTGF mRNA expression in the lung of PHB and NPHB subjected to chronic hypobaric hypoxia.

MATERIALS AND METHODS

Birds and Tissue Samples

Two hundred 1-d-old Cobb 308 male broilers were obtained from a commercial hatchery and reared on floor. The broilers were maintained under natural hypoxic conditions, at 2,638 m above sea level in Bogotá, Colombia, using standard nutritional and management procedures for commercial operations. Feed and water were provided for ad libitum consumption, and lighting was continuous. Temperature was initially 32°C and gradually was allowed to drop to a range from 18 to 21°C after the third week of the growing period.

At 24 d, 30 broilers were chosen and equally distributed in 2 groups according to their cardiac index (**CI**) values and clinical signs as follows: PHB and NPHB. To evaluate PH, the CI was calculated according to the procedure used by Alexander and Jensen (1959; CI = right ventricular weight/total ventricular mass weight × 100). Chickens with CI above 30 were allocated in PHB group and those with CI below 26 in the NPHB one. The mentioned values were obtained from previous studies carried out in Bogotá (Hernández, 1987; de Sandino and Hernández, 2006). It should be noted that CI is widely recognized as a valid parameter of PH (Burton and Smith, 1967; Cueva et al., 1974; Hernández, 1979). The apical regions of left lungs were obtained in all samples, frozen in liquid N, and stored at –80°C before the RNA extraction period. Blood samples were obtained from the jugular vein. Protein concentration in serum was determined by Lowry's method (Bio-Rad, Hercules, CA). All experimental procedures used in this study were reviewed and approved by the Ethic's Committee of the National University of Colombia, in accordance to international normative for the care and use of experimental animals.

Total RNA Extraction and Reverse-Transcription PCR Analysis

Gene mRNA expression of ET-1, ETA, AM, PDGF-C, and CTGF in the lungs was determined by reverse-transcription PCR (**RT-PCR**), as previously described (Steuerwald et al., 1999; Nogueiras et al., 2003; Hazari et al., 2004). Total RNA was extracted from 200 mg of the lung using Trizol reagent according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA) and then mRNA was isolated by using a Dynabeads mRNA Direct kit (Invitrogen Corp.). Messenger RNA was quantified using absorption of light at 260 and 280 nm (A260/280) with an ultraviolet-visible spectrophotometer (Spectronic BioMate 3 UV-Vis Spectrophotometer, Thermo Electron Corp., Waltham, MA). First-strand cDNA was synthesized from 1 μg of mRNA using 200 U of MoML-reverse transcription (Invitrogen Corp.), 20 U of ribonuclease inhibitor RNase-Out (Invitrogen Corp.), and 1 n*M* of random hexamer primers (Invitrogen Corp.), in a total volume of 30 μL. Reverse-transcription reactions were carried out at 37°C for 45 min and at 42°C for 15 min, followed by heating at 92°C for 2 min. Reverse-transcription reactions without addition of reverse transcriptase served as negative controls to ensure PCR amplification specificity. The cDNA was used for RT-PCR amplification using specific sense and antisense primers for chicken ET-1, ETA, AM, PDGF-C, and CTGF cDNA sequences (Table 1). For each target gene, primers were designed spanning intron-exon boundaries using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). Polymerase chain reaction amplification of the generated cDNA was carried out in 50 μL of 1× PCR buffer in the presence of 1.25 U of *Taq*-DNA polymerase (Invitrogen Corp.) and 1 nM forward and reverse primers (Invitrogen Corp.). The amplification profile for chicken genes was as follows: denaturation at 96°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The final step was extension at 72°C for 10 min. Thirty-eight PCR cycles were chosen for analysis of these genes in the experimental groups. The amplified products were resolved in 1.5% agarose gels and visualized with ethidium bromide. Hypoxanthine phosphoribosyltransferase (**HPRT**) was used as a control housekeeping gene (Table 1).

Real-Time RT-PCR Analysis

Endothelin 1, ETA, AM, PDGF-C, CTGF, and HPRT expression levels were quantified by real-time RT-PCR using a Light Cycler thermocycler (Roche, Mannheim, Germany). The PCR was performed using the primers shown in Table 1 and SYBR green PCR core reagents (Roche) according to the manufacturer's instructions and as previously described (Nogueiras et al., 2003). The final volume was 20 μL, which contained 2 μL of RT reaction products, 0.5 μ*M* of each primer, and 1× LightCycler DNA Master SYBR Green I mix (Roche). All reactions were brought about using the following cycling parameters: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 15 s, and extension at 72°C for 15 s. At the end of each run, melting curve profiles were produced (cooling the sample to 68°C and heating slowly to 95°C, with continuous measurement of fluorescence) to confirm am-

Table 1. Primer pairs sequence used for reverse-transcription PCR (RT-PCR) and real-time PCR analysis of endothelin 1 (ET-1), endothelin type A (ETA), adrenomedullin (AM), platelet-derived growth factor C (PDGF-C), and connective tissue growth factor (CTGF) transcripts in lung samples

Target[1]	Forward and reverse primers (5′-3′)	Length (bp)	GenInfo Identifier (gi)	Sequence
ET-1	GGA CGA GGA GTG CGT GTA TT GCT CCA GCA AGC ATC TCT G	141	50733669	ggacgaggag tgcgtgtatt tctgccacct ggatatcatc tggatcaaca cccccgagaa gactgttccc tatggtcttg gaggcccttc tcggtccaga agatcactga aggacataat gccagagatg cttgctggag c
ETA	GTG GCC TTT TGG AGA TTC TG GAT TCC GAT TCC CTG AAC AC	160	45383867	gtggcctttt ggagatttcg aatttgggca gtttctttgc aaattcttc cctttataca gaaggcatca gtgggaatca cagtccttaa tctctgtgca cttagtgtgg acaggtatag agcagttgcc tcctggagcc gtgttcaggg aatcggaatc
AM	GCC ACT TCG GAT CGC TAC TA CTC AGT ACC CAG CTC GTC CA	190	50747749	gccacttcgg atcgctacta cgagagcatt tggctcagtg gattatcctc actgccagga gttcagccag aatgaaactg gttcacgtag cgctactctg tctcggctcc gcgaccttct tcgggtggga tgctgcgagg gtggacgtag ccacagagtt caaacgaaaa tggacgagct gggtactgag
PDGF	TGA AAG ATT TGG GCT TGA GG TGC AGA ATC CAG GTT GAG AA	200	45382628	tgaaagattt gggcttgagg acccagaaga tgatatttgc aagtatgact ttgtagaagt tgaagaacct agtgatggca ctgttttagg gcgctggtgt ggttccagta gtgtgccaag tagacaaatc tccaaaggaa accagatcag aataagattt gtgtctgatg aatatttcc ttctcaacct ggattctgca
CTGF	GAA GAC ACT TAC GGC CCA GA AAA CTT GAT GGG CTT GGA GA	252	45383589	gaagacactt acggcccaga cccacccatg atgcgtgcca attgcctggt gcagactact gagtggagtg cttgctccaa gacctgtggc atgggcatct cgaccagggt caccaacgat aatgctttct gcagactgga gaagcagagc agactgtgca tggtcagacc ttgcgaagct gacctggagg agaacatcaa gaaaggcaaa aagtgcattc gcacccaaa aatctccaag cccatcaagt tt
HPRT	TCC AAA GAT GGT GAA AGT GG GCT TCC CCG TCT CAC TGA T	179	45382332	tccaaagatg gtgaaagtgg ccagtttgtt ggtcaaaaga actcctcgaa gtgtgggata tcggccagac tttgttggat ttgaagtgcc agacaaattt gttgttggat acgccctcga ctacaatgaa tacttcagag atttgaatca tatctgtgtg atcagtgaga cggggaagc

[1]HPRT = hypoxanthine phosphoribosyltransferase. For each target, the primer pair used for amplification is included. In addition, the expected size of the generated cDNA products and the sequence identification number is indicated for each signal [GenInfo identifier (gi) of chick cDNA, available online at http://www.ncbi.nlm.nih.gov/].

Table 2. Cardiac index and serum proteins levels in hypertensive and nonhypertensive broilers at 24 d old[1]

Item	Nonhypertensive	Hypertensive
Cardiac index	16.57 ± 2.26	39.72 ± 5.69***
Serum proteins (mg/mL)	0.91 ± 0.15	0.61 ± 0.11***

[1]Values are mean SD; n = 15 chickens/group of hypertensive and nonhypertensive broilers at 24 d old.

***$P < 0.001$.

plification of specific transcripts. Fluorescence emission readings were quantified using the second derivative maximum method of the LightCycler software package (Roche). This method determines the crossing points of individual samples by an algorithm that identifies the first turning point of the fluorescence curve. This turning point corresponds to the first maximum of the second derivative curve and correlates inversely with the log of the initial template concentration (Nogueiras et al., 2003). Messenger RNA levels were normalized with respect to chick HPRT level in each sample. Product purity was confirmed by dissociation curves.

Statistical Analysis

Cardiac index, protein concentration in serum, and mRNA levels are shown as the means ± SE. Significant differences ($P < 0.05$) between groups were determined using Student's 2-tailed unpaired *t*-test (GraphPad Instat, GraphPad Software Inc., San Diego, CA).

RESULTS

Clinical Signs

The PHB had depression, cyanosis, ascites, and generalized congestion. These broilers had enlarged hearts and dilation of the atria and right ventricles. Lungs and livers were congested. The NPHB did not have any gross lesions.

Cardiac index values in NPHB were from 12 to 20%. In PHB, CI values ranged from 34 to 51%. Differences in CI values between the 2 groups were highly significant ($P < 0.001$, Table 2). Total serum protein values were lower in PHB as compared with those obtained for NPHB ($P < 0.001$, Table 2).

Expression of ET-1 and ETA mRNA in PHB Lungs

Representative images of ethidium bromide-stained gel electrophoresis of the specific amplicons of ET-1 and ETA are presented in Figure 1. Endothelin 1 mRNA levels in the lung of PHB were significantly higher than the corresponding ones in NPHB ($P < 0.001$, Figure 2). In contrast, ETA mRNA levels in the lung of PHB were lower than those detected in NPHB ($P < 0.001$, Figure 3).

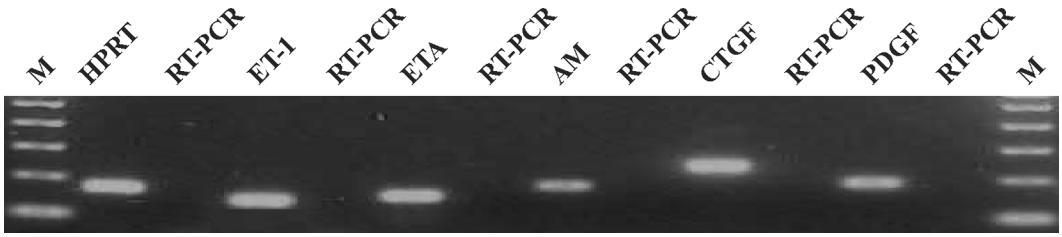

Figure 1. Representative reverse-transcription PCR (RT-PCR) for hypoxanthine phosphoribosyltransferase (HPRT; 179 bp), endothelin 1 (ET-1; 141 bp), ET receptor type A (ETA; 160 bp), adrenomedullin (AM; 190 bp), connective tissue growth factor (CTGF; 252 bp), and platelet-derived growth factor (PDGF; 200 bp) mRNA in lung samples from nonhypertensive broilers at 24 d old. A 100-bp molecular weight marker (M) was used. The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide and examined with ultraviolet light and visualized with a Gel Doc system (Bio-Rad, Hercules, CA). In addition, negative controls are shown and resulted in no bands after amplification.

Growth Factors mRNA Expression in PHB Lungs

Expression of the genes encoding CTGF and PDGF was evaluated in the lung (Figure 1). In addition, mRNA extracted from the lungs was investigated using a real-time RT-PCR. Connective tissue mRNA lung levels in PHB were significantly higher than in the lungs of NPHB ($P < 0.01$, Figure 4). However, no differences were encountered between the 2 groups of broilers in PDGF mRNA expression ($P > 0.05$, Figure 5).

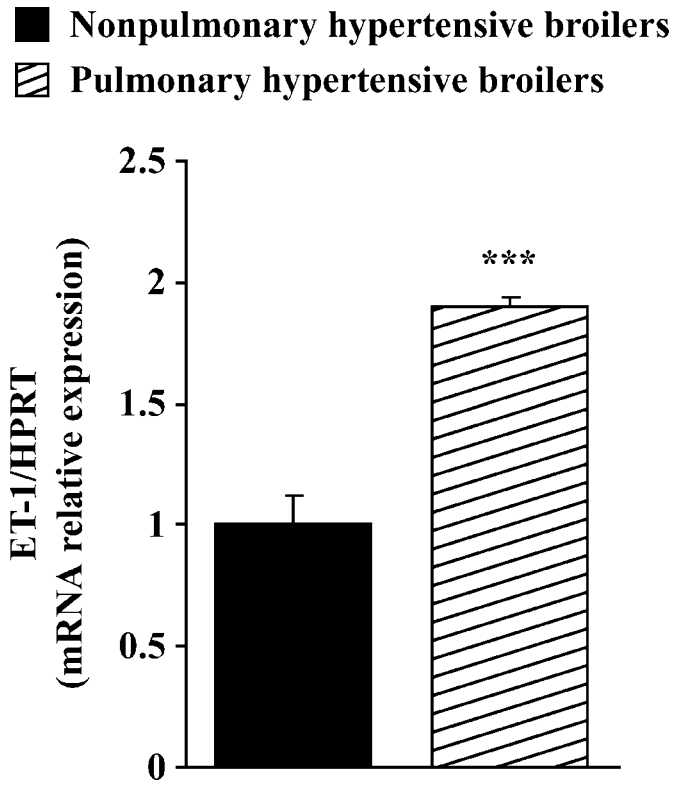

Figure 2. Comparison by using real-time reverse-transcription PCR analysis of lung endothelin 1 (ET-1) mRNA levels in nonpulmonary hypertensive and pulmonary hypertensive chickens subjected to chronic hypobaric hypoxia. Semiquantitative data of ET-1 mRNA expression levels were normalized to those of the internal control hypoxanthine phosphoribosyltransferase (HPRT). Data are represented as mean ± SEM (n = 15/group). ***$P < 0.001$.

AM mRNA Expression in the Lungs of PHB

Adrenomedullin mRNA was detected in the lungs (Figure 1). Adrenomedullin mRNA was analyzed with real-time RT-PCR and related to the endogenous control HPRT, and it was found that AM mRNA levels in the lung of PHB were significantly higher than in NPHB ($P < 0.01$, Figure 6).

DISCUSSION

To contribute to a better understanding of the molecular pathophysiology of PH, comprehensive gene expression analysis was performed in the lung of broilers, under chronic hypobaric hypoxic conditions by using real-time PCR analysis. In the present study, it was shown for the first time that ET-1 mRNA expression is higher in the lung of PHB. This result is in agreement with previous observations made in humans and in induced PH in various animal models (Mortensen and Fink, 1992; Rabelink et al., 1994; Potter et al., 1997; Cardillo et al., 1999), in which it has been shown that ET-1 could be involved in the pathogenesis of PH. It was demonstrated that ET-1 induces vasoconstriction, promotes fibrosis, has mitogenic potential, and is important in the regulation of vascular tone, arterial remodeling, and vascular injury. However, its role in normal cardiovascular homeostasis and PH is unclear (Touyz and Schiffrin, 2003). Endothelin 1 acts through binding to ETA and ETB receptors; ETA receptors on SMC are responsible for the vasoconstrictor effect of ET-1, whereas ETB receptors on endothelial cells mediate vasodilatation (Sakurai et al., 1990). Contrary to previous reports in the lungs of hypoxic rats (Li et al., 1994) and in cells from the pulmonary artery of PH sheep (Balyakina et al., 2002), it is presently reported that there is a decrease in ETA mRNA levels in the lungs of PHB.

In studies with ET-1 receptors, antagonist differences were found in expression levels, according to the experimental models used (McCulloch and MacLean, 1995; Maxwell et al., 1998; Luscher and Barton, 2000). The distribution and density of ET-1 receptors on vascular SMC varies between species and their location in the corresponding blood vessel (Nishimura et al., 1995; Chen and Oparil, 2000; Balyakina et al., 2002). Balyakina et al. (2002) showed that both ETA and ETB receptors in inner medial

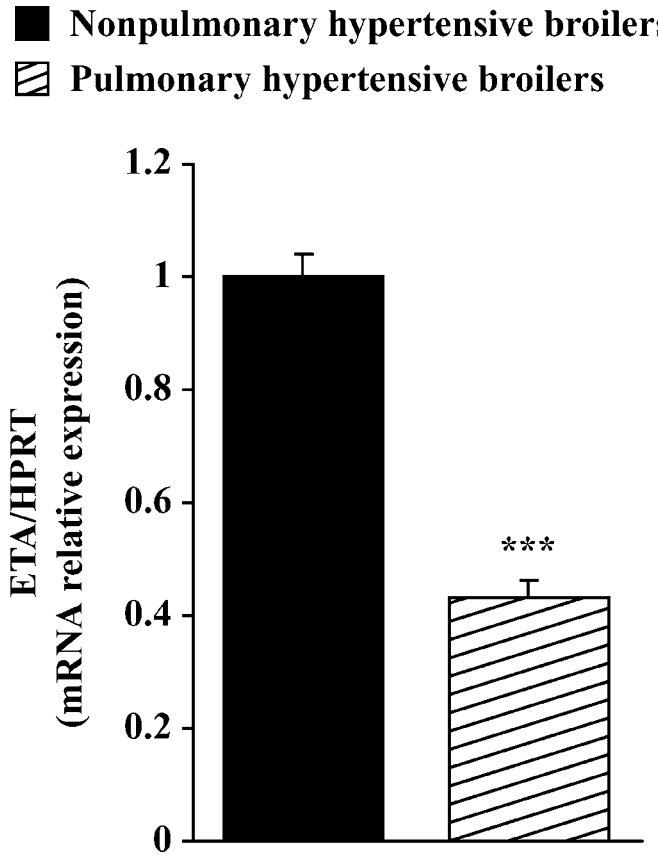

Figure 3. Relative quantification of the regulation of lung endothelin type A (ETA) mRNA expression in pulmonary hypertensive and nonpulmonary hypertensive chickens subjected to chronic hypobaric hypoxia by using real-time reverse-transcription PCR analysis. Data of ETA mRNA expression levels were normalized to those of the internal control hypoxanthine phosphoribosyltransferase (HPRT). Data are represented as mean ± SEM (n = 15/group). ***$P < 0.001$.

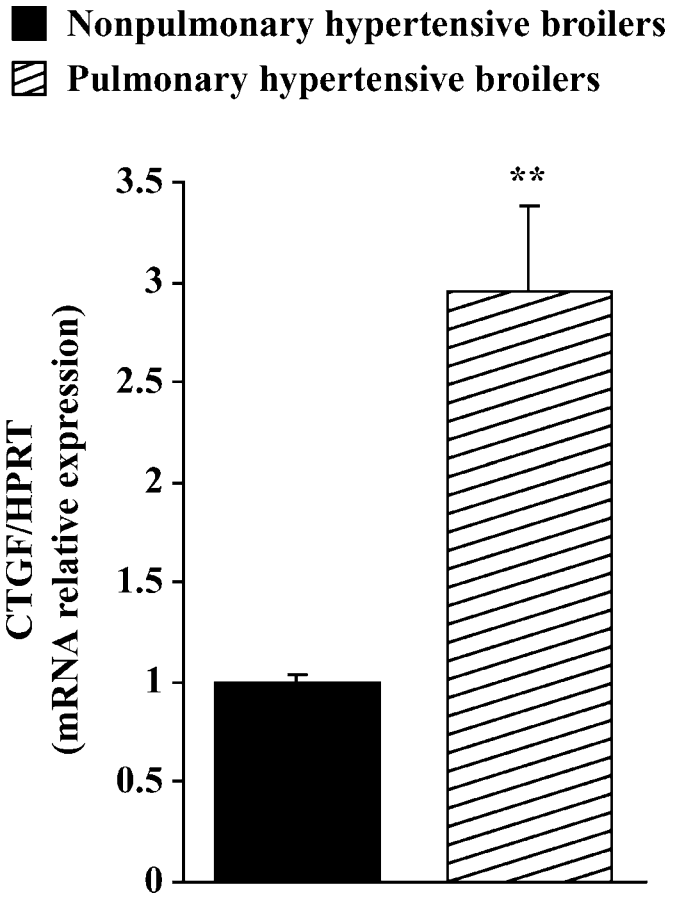

Figure 4. Comparison of the connective tissue growth factor (CTGF) mRNA expression in lung samples from pulmonary hypertensive and nonpulmonary hypertensive chickens subjected to chronic hypobaric hypoxia. Lung CGTF mRNA expression was evaluated using semiquantitative real-time reverse-transcription PCR. The CTGF mRNA levels have been standardized by hypoxanthine phosphoribosyltransferase (HPRT) mRNA levels, and the results are expressed as arbitrary units. Data are represented as mean ± SEM (n = 15 /group). **$P < 0.01$.

cells from the main pulmonary artery of sheep bind and are responsible for internalization of exogenous ET-1 after 18 h of exposure. Nevertheless, the role of other ET-1 receptors that mediate the vasoconstrictor response in this animal model requires further study.

Endothelin 1 is regulated by angiotensin II, catecholamines, cytokines, growth factors, hypoxia, and mechanical stress (Rubanyi and Polokoff, 1994). On the other hand, ET-1 stimulates the production of tumor necrosis factor-α, vascular endothelial growth factor, and basic fibroblast growth factor-2 (Matsuura et al., 1998) and strengthens the effects of transforming growth factor-β and PDGF (Rodriguez-Vita et al., 2005). The pathophysiology of PH includes endothelial cell dysfunction and proliferation and migration of SMC. As PDGF has been implicated in these processes, Schermuly et al. (2005) hypothesized that altered PDGF signaling may be involved in vascular remodeling; therefore, they found that administration of STI571, a PDGF receptor inhibitor, reversed pulmonary vascular changes. However, we did not find differences in PDGF mRNA expression levels between PHB and NPHB. Endothelin 1 also increases CTGF mRNA expression, promoter activity, and protein production (Rodriguez-Vita et al., 2005). Connective tissue growth factor regulates cell proliferation and apoptosis, angiogenesis, migration, adhesion, and fibrosis (Brigstock, 1999; Perbal, 2004). It was presently found that there is an increase in the CTGF mRNA expression levels in hypertensive chickens, which suggests that CTGF could be a mediator of fibrotic effects of ET-1 in hypoxic PH. Connective tissue growth factor might be considered as a new target for therapeutic interventions in PH (Rodriguez-Vita et al., 2005). This is supported by the present results.

It was also shown, in the present work, that AM mRNA expression augments in PHB, which is in agreement with other findings (Nakayama et al., 1998; Wang et al., 2001; Xu et al., 2002). Intravenous AM administration decreases systemic and pulmonary arterial pressure (Nagaya et al., 1999, 2000, 2005), suggesting its involvement in the regulation of vascular tone (Nishikimi et al., 2003; Okumura et al., 2004). Adrenomedullin actives the PI3K/Akt-dependent pathway in vascular endothelial cells (Nishimatsu et al., 2001), which is considered to regulate angiogenesis (Jiang et al., 2000). In an in vitro study, it was demonstrated that AM is upregulated by the hypoxia inducible factor-1 under hypoxic conditions (Garayoa et

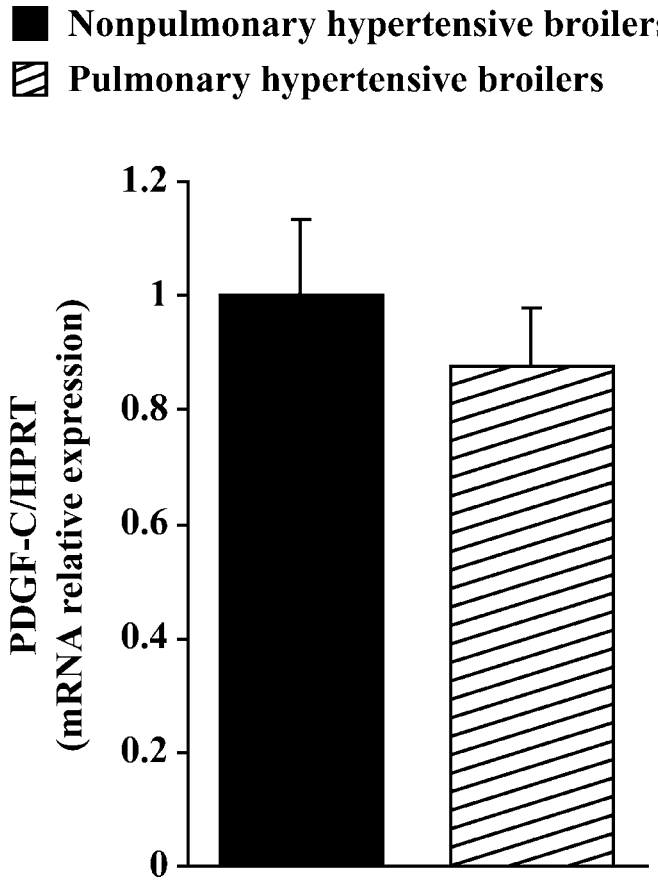

Figure 5. Platelet-derived growth factor C (PDGF-C) mRNA levels in lung tissues of pulmonary hypertensive and nonpulmonary hypertensive chickens subjected to chronic hypobaric hypoxia, as measured by real-time reverse-transcription PCR. The PDGF-C mRNA levels have been standardized by hypoxanthine phosphoribosyltransferase (HPRT) mRNA levels, and the results are expressed as arbitrary units. Data are presenteded as mean ± SEM (n = 15/group). There were no significant differences between both groups ($P > 0.05$).

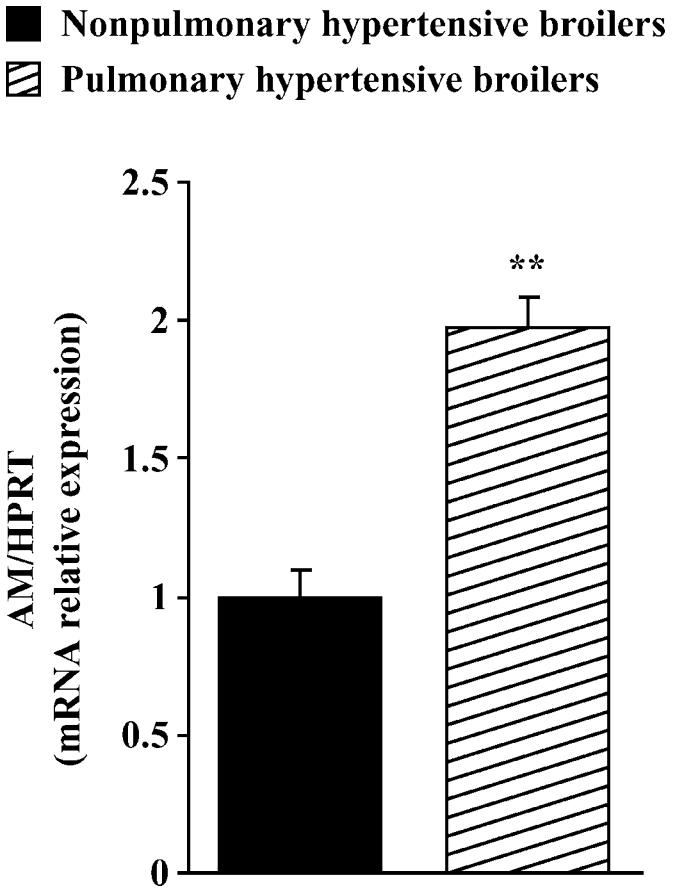

Figure 6. Comparison of adrenomedullin (AM) mRNA levels in lung samples from pulmonary hypertensive and nonpulmonary hypertensive chickens subjected to chronic hypobaric hypoxia, as measured by real-time reverse-transcription PCR. Adrenomedullin mRNA levels have been standardized by hypoxanthine phosphoribosyltransferase (HPRT) mRNA levels, and the results are expressed as arbitrary units. Data are presented as mean ± SEM (n = 15/group). $^{**}P < 0.01$.

al., 2000). This results suggest that hypoxia is effective in promoting AM synthesis and that this peptide plays an important regulatory role in pulmonary circulation and vascular remodeling (Wang et al., 2001) and represents a compensatory mechanism as an angiogenic factor promoting neovascularization under hypoxic conditions (Nagaya et al., 2005). It should be noted that several other molecules appear to participate in the vascular response to hypoxia, such as nuclear factor interleukin-6 and early growth response-1 (Semenza, 2000), although a possible interaction with presently studied molecules is not currently evident. It is not to be overlooked that various molecules have been implicated in the regulatory mechanism of pulmonary vascular tone, such as serotonin (Chapman and Wideman, 2002), nitric oxide (de Sandino and Hernández, 2003), thromboxane (Wideman et al., 1999), among others.

In summary, it has been shown for the first time that there are differences in the expression of ET-1, CTGF, and AM in the lungs of PHB and NPHB subjected to chronic hypobaric hypoxia for 24 d. The abovementioned molecules are probably upregulated in PH. Furthermore, it is postulated that these peptides may play a major role in the PH pathophysiology. Present data might provide clues for future research directions such as therapeutic intervention to revert the process of pulmonary vasoconstriction and vascular remodeling. Major research goals could be to find endothelium-derived factors, which probably trigger endothelial dysfunction, as well as possible interactions with already identified molecules, which also intervene in the pulmonary response to hypoxia.

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