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Hypoxic Pulmonary Arterial Hypertension in the Chicken Model

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1. Introduction

Pulmonary hypertension (PH) in modern genetic strains of chicken broilers is a world wide distributed entity, which has a recognized economical impact (Maxwell et al., 1997; Pavlidis et al., 2007). Also, the commercial chicken has been a model to study PH in man, because encountered pathological changes in that animal closely resemble the human condition.

Under natural atmospheric conditions, two determining causes for PH are known: hypobaric hypoxia and low temperatures exposure (i.e. birds exposure to temperatures below 16° C (Pakdel et al., 2005; Pan et al., 2005).

The incidence and clinical evolution of PH in various strains of commercial chickens maintained under a natural hypoxic tropical environment in the Bogotá plane at 2638 m above sea level (masl) have been evaluated over several years, using direct observation of animals, electrocardiography, morphometric studies, hematocrit and hemoglobin changes, histochemical, immunohistochemical and molecular procedures.

Morphometric cardiac and pulmonary changes allowed in post-mortem studies to define mass cardiac index values (CI) to differentiate non-pulmonary hypertensive chickens (NPHC) from the pulmonary hypertensive ones (PHC) (Hernández, 1982, 1987; Cárdenas et al., 1985; Areiza, 2010, Vásquez, 2010). CI is defined as the right ventricular muscle mass weight expressed as a percentage of the total ventricular muscle weight (Alexander and Jensen, 1959). It is now clear that PHC have a CI value of 25 and higher and, below 22, birds can be safely allocated in the NPHC group (Gómez et al., 2007; Areiza, 2010; Vásquez, 2010). The remodeling process of pulmonary arterioles has been studied by morphometric analysis of smooth muscle and adventitia layers in PHC (Useche et al., 1981 Sandino and Hernández, 2005).

Electrocardiography was found to have a predictive value to define which animals would develop PH. Also, it determined that there is not a defined time course in the evolution of PH in chickens. The latter has been corroborated with daily field observations (Pulido, 1996).

Several studies showed that low temperatures and pulmonary diseases have an enhancing effect in the occurrence of PH (Mejía, 1982; Hernández, 1984).

Hypobaric hypoxia and low temperature are determinants of PH occurrence in the pulmonary vasculature of broilers. Sensitivity is species dependent and the domestic chicken appears to be

most prone to develop that condition, whereas South American camelids and the bovine yak are highly resistant, among studied species. Man is believed to occupy a low place in this context (Grover et al., 1983; Monge and León-Velarde, 1991). However, there is a genetic component within species, which is responsible for susceptibility or resistance, as seen in human native Tibetans and the bovine yak (Durmowicz et al., 1993; Moore et al., 1998). The bar-headed goose and the Himalayan domestic chicken are well adapted avian species to altitudes above 3000 masl. The bar-headed goose can reach altitudes of 9000 masl (Graham and Milsom, 2007). The modern broiler chicken´s strains also differ in their susceptibility (Hernández, 1982; Huchzermeyer et al, 1988).

There is a large amount of scientific data which give capital importance to the energy density of feed as causative of PH, but, at least under low altitude and respiratory healthy conditions, commercial chickens do not develop PH (Useche et al., 1981, Cárdenas et al, 1985; Gómez et al, 2007; Vásquez, 2010). Nevertheless, the energy content is in fact an undeniable adjuvant factor to increase incidence of PH when low temperatures and/or hypobaric hypoxia are present, although its mechanism has not been clearly elucidated (Tarquino et al., 1990). Interesting theories and well carried studies have been advanced, which include thyroid hormones metabolism (hypothyroidism) and hypoxemia (Camacho-Fernández et al, 2002; Hassanzadeh et al., 2005), but they do not focus on the principal issue which is pulmonary vasoconstriction due to low temperature and/or hypobaric hypoxia. In fact, they attribute PH to a metabolic disarrangement which results in hypoxemia. Another element to enhance or probably cause PH, most likely through hypoxia, is the presence of pulmonary obstructive diseases (Huchzermeyer et al., 1987; Guzmán et al., 2001), which are common entities in the broiler´s industry nowadays.

It is important to note that when both temperature and energy density of feed are controlled, hypobaric hypoxia *per se* is sufficient to cause PH (Tarquino et al, 1990; Vásquez, 2010).

2. Compensatory mechanisms and development of PH

Elevation of pulmonary artery pressure is an immediate response to hypobaric hypoxia. If the level of this increment is maintained above physiological limits, that is, in susceptible (non- adapted) individuals, PH ensues (Cueva et al., 1974). From there on, cardiac output is maintained due to ventricular adaptation to high pulmonary vascular resistance. There is right ventricular mass increment through hypertrophy and hyperplasia (Bernal et al., 1984) and the II Q wave in electrocardiographic readings becomes progressively more negative, until ventricular dilatation determines cardiac failure (table 2). This process does not have a defined time pattern and may begin as early as 10 days post-hatching, ranging from 4 to 20 days (Pulido, 1996).

Another compensatory mechanism is augmentation of hematocrit (Ht) and hemoglobin (Hb) content (Table 2; Cárdenas et al, 1985; Colmenares et al, 1990). Hypoxia induces the expression of the hypoxia inducible factor 2α (HIF-2α) which stimulates renal secretion of erythropoietin by cortical fibroblasts in the kidney, a molecule responsible for hematocrit increment, by diminishing apoptosis in erythrocyte progenitor cells (Paliege et al., 2010). As a consequence, blood viscosity is augmented, a change believed to increase resistance in the pulmonary vasculature, which can be considered as an aggravating factor in the development of PH. One compensatory mechanism is increased hemoglobin affinity for

oxygen, as exhibited by well adapted avian species such as the bar headed and Andean geese as well as the Tibetan chicken (Weber et al. 1993, Zhang et al, 2007).

Hypoxemia results from low pO2 in the air spaces in the lung (alveoli in mammals and respiratory capillaries in avian species). Oxygen and carbon dioxide low blood pressure sensing structures such as the carotid cell bodies (Lahiri et al., 2006), intrapulmonary and peripheral neural receptors elicit a rise in respiratory rate through vagal neural efferent inputs originated in neural respiratory centers (Glogowska et al., 1972).

3. Gross and light microscopic changes in pulmonary hypertension

The lungs appear congested, but no signs of edema are evident as known to occur in mammals. Microscopically, the most prominent finding relates to the engrossment of the medial muscle layer in arterioles (figures 9 and 10). This subjective appreciation has been thoroughly corroborated by morphometric methods in several studies (Sillau and Montalvo, 1982; Useche et al., 1981, Moreno de Sandino and Hernández, 2006). Cartilage and/or bone neo-formations can be seen in variable amounts within the pulmonary parenchyma (figure 3), together with fibrosis of the adventitial layer of arterioles. The degree of fibrosis varies (figure 2). Muscle hypertrophy is also found in peribronchial walls (Dalmau, 1997). Atelectasia can be encountered in many areas of the lung.

Fig. 1. Pulmonary hypertensive chicken. Ascites

In the heart, different degrees of right ventricular dilatation are present, which are presumably connected to the severity of PH. The increase in ventricular mass weight is due to both hyperplasia and hypertrophy (Bernal et al, 1984). No signs of vascular changes in the heart are detected, as seen with the light microscope.

As a result of right ventricular insufficiency secondary to increased pulmonary vascular resistance, detention of returning venous blood becomes increasingly difficult. Hence, generalized passive congestion is evident, which includes portal congestion. Ascites results from plasma leaking from the congested liver (Figure 1). Liver hypertrophy is a common finding in necropsies. Microscopically, some degree of fibrosis and sinusoidal dilatation can be seen.

In most cases of PH, hydropericardium is encountered. Some individuals may die with this lesion, and absence of ascites. Furthermore, a few animals die with none of the abovementioned lesions. The latter cases probably correspond to more susceptible individuals.
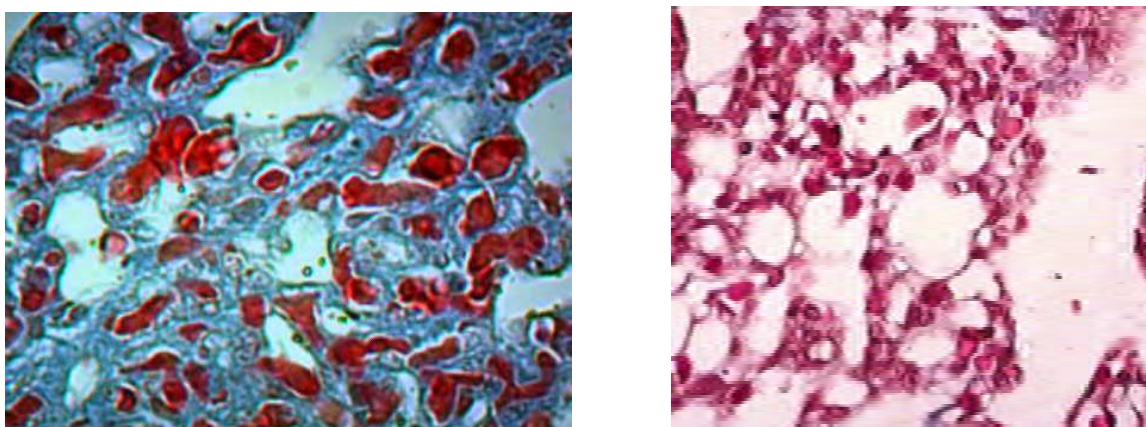

Fig. 2. Lung fibrosis (left). Pulmonary hypertensive chicken. Lung from a non-pulmonary hypertensive chicken (right). Masson´s trichromic stain
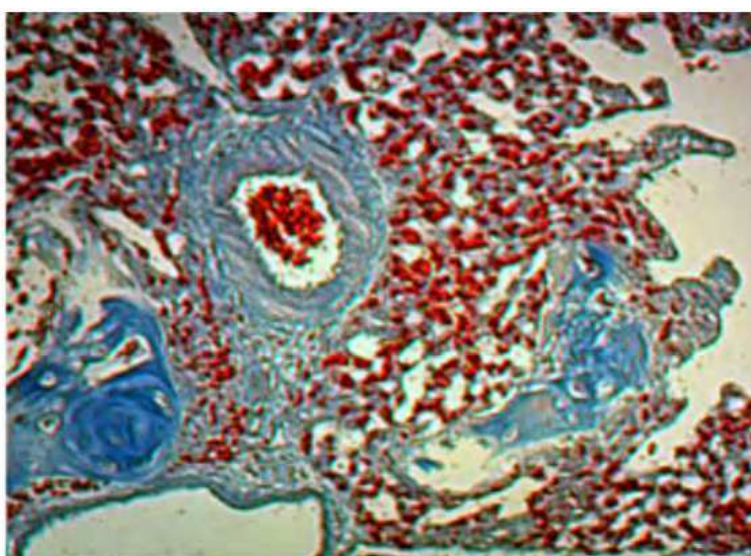

Fig. 3. Fibrosis and bone neo-formation. Pulmonary hipertensive chicken Masson's trichromic stain

Altitude m above sea level	Hemoglobine g/100 mm		Hematocrit % PVC		Red blood cells millions/cc *	
	Mean	SD	Mean	SD	Mean	SD
225	8.54	1.26	27.30	2.88	4.72	0.97
2638 NPHC	10.82	1.36	33.91	3.15	4.40	0.71
2638 PHC	13.15	2.30	40.10	8.30	2.43	1.31

SD: standard deviation. NPHC and PHC: non-pulmonary and pulmonary hypertensive chickens
* Red blood cells values should be multiplied by 10000
Cárdenas et al., 1985.

Table 1. Hemoglobine, hematocrit and red blood cells counts in healthy and pulmonary hypertensive broilers maintained under normoxia and hypobaric hypoxia

L(II) s (- mV) values at different age ranges (in days)

8-10	15-17	22-24	29-31	36-41 days	CI	D
0.20	0.40				35.2	15
0.35					42.9	17
0.40	0.40				38.5	22
0.10	0.30				37.5	22
0.30	0.20				33.3	22
0.05	0.15	0.30			38.7	22
0.35	0.60	0.65			41.7	23
0.30	0.60	0.65			36.4	23
0.15	0.40				37.5	24
0.25	0.25	0.50			34.8	29
0.15	0.25	0.60	0.80		37.0	30
0.35	0.60	0.75			40.0	30
0.10	0.10	0.20	0.50		36.2	30
0.25	0.15	0.45	0.65		37.5	31
0.30	0.10	0.50	1.00		48.9	31
0.20	0.20	0.30	0.50	0.90	40.5	37
0.30	0.40	0.30	0.35		35.4	37
0.20	0.10	0.20	0.25	0.30	41.3	41

CI= cardiac mass index. D= age-day of spontaneous animal´s death. Data taken and modified from Pulido, 1996

Table 2. Electrocardiographic wave 2. derivative II [L (II)s] (mV) and cardiac mass index in pulmonary hypertensive chickens

4. Pathogenesis

As already stated, chronic exposure to hypoxia leads to PH due to pulmonary vasoconstriction, structural remodeling of pulmonary vessels and increased blood viscosity in mammals and chickens (Bartsch et al., 2005; Burton and Smith, 1967; Burton et al., 1967; Cueva et al., 1974; Currie, 1999; Cogo et al., 2004; Huchzermeyer, 1988; Kanazawa et al., 2005; Meyrick and Reid, 1978; Remillard and Yuan, 2005; Reeves and Grover, 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).

The pathogenesis of PH involves a complex and multifactorial process. Vasoconstriction and remodeling of the pulmonary vessel wall contribute to increased pulmonary vascular resistance in PH (Voelkel et al., 1997). Hypoxic pulmonary vasoconstriction (HPV) is an important physiological property of the pulmonary circulation and can optimize the ventilation: perfusion ratio by diverting blood away from poorly ventilated areas of the lung. However, in global hypoxia, which is found in high altitude environments, HPV results in a rise in pulmonary vascular resistance (PVR) and concomitant PH. This severely increases the afterload on the right heart and, in combination with associated pulmonary vascular remodelling, leads to a right ventricular dilatation and heart failure. Sustained vasoconstriction in response to moderate hypoxia is unique to the pulmonary vasculature. In despite of the progress made from many studies, the precise mechanisms involved are elusive. Recent advances in the field have opened up new areas of investigation, particularly in regard to the role of the vascular endothelium.

The first modern observation of HPV was made in 1894 when Bradford and Dean (1894) described increases in pulmonary arterial pressure (PAP) in response to asphyxia. Half a century later, HPV was recognized as an adaptive phenomenon by von Euler and Liljestrand (1946). They ventilated anesthetized cats with either hypoxic (10% O_2) or hypercapnic gas mixtures and found that PAP increased, with minimal change in left atrial pressure, predominantly with the hypoxic challenge. They concluded that HPV might "increase the blood flow to better aerated lung areas, which leads to improved conditions for the utilization of alveolar air". HPV shunts blood from poorly oxygenated areas to better ventilated lung segments, thereby optimizing ventilation-perfusion matching, reducing shunt fraction and optimizing systemic O_2 delivery in conditions such as atelectasis and pneumonia (Brimioulle et al, 1996). HPV onsets within seconds of moderate hypoxia and reverses quickly on restoration of normoxic ventilation. Whereas, in pneumonia and atelectasis, HPV is a focal response limited to the diseased lung segment, with global hypoxia, as occurs at high altitude or with sleep apnea, HPV constricts PAs throughout the pulmonary circulation, increasing the PVR (Moudgi et al., 2005).

Vasoconstriction in response to hypoxia can occur in isolated pulmonary arteries, which leads to the conclusion that the oxygen sensor and subsequent constrictor mechanism(s) must be located in either the vascular smooth muscle or the endothelium. The discovery of K^+ channels that are depressed by hypoxia in pulmonary, but not systemic vascular smooth muscle cells, has provided a potential signal mechanism of transduction, which permits to propose a link between reduction in alveolar PO_2, depolarization of the cell membrane, and contraction via voltage-gated Ca^{2+} entry (Post et al., 1992). Several reports have also shown

that hypoxia can cause shortening in isolated pulmonary arterial smooth muscle cells (Madden et al., 1992) and this suggests that HPV is a function of the smooth muscle rather than the endothelium. However, other studies have shown that the endothelium is definitely required for sustained vasoconstriction (Demiryurek et al., 1993; Leach et al., 1994; Zhang and Morice, 1994). Although some differences in response might be ascribed to variations among species and variations in artery size (Leach et al.1994), a possibly more important aspect is the time course over which the experiment is conducted.

Most reports, in particular those on isolated cells, describe processes that occur during the first 10-15 min. However, isolated pulmonary arteries show a biphasic response to hypoxia when observed over longer periods (Bennie et al., 1991; Leach et al. 1994; Zhang and Morice, 1994; Robertson et al., 1995). This biphasic response consists of a rapid, transient increase in vascular tone over about 5-10 min (phase 1), which then falls towards the baseline. This is followed by a more slowly developing but sustained increase in tone (phase 2), which reaches a plateau about 40 min later on. The phase 1 of constriction is relatively unaffected by removal of the endothelium, while in most reports phase 2 is abolished (Hoshino et al., 1994; Leach et al. 1994; Zhang and Morice, 1994).

The relative physiological significance of the abovementioned phases is opened to question. Although HPV *in vivo* or in blood-perfused lungs is generally characterized by a rapid rise in pulmonary vascular resistance, this increase in resistance is sustained for long periods. It would seem most likely, therefore, that the second sustained phase of constriction in isolated arteries is more relevant to physiological HPV than the transient first phase. In the whole lung the effects may result in a rapid but sustained increase in resistance. The biphasic response to hypoxia suggests that HPV involves multifactorial mechanisms, which may or may not be interdependent.

HPV is intrinsic to the lung and, although modulated upstream by the endothelium and downstream by calcium sensitization of the contractile apparatus (rho kinase), the core mechanism involves a redox-based O_2 sensor (likely the mitochondria) that generates a diffusible redox mediator (likely H_2O_2) that is withdrawn during hypoxia, leading to hypoxic inhibition of certain voltage-gated K^+ channels (K_v) in pulmonary artery smooth muscle cells (PASMCs). Specific O_2-sensitive K_v channels, including $K_v1.5$ and $K_v2.1$, set membrane potential (E_M) and thus control Ca^{2+} influx, via voltage-gated Ca^{2+} channels, and therefore vascular tone (Moudgi et al., 2005).

Substantial work defining the properties of HPV has been performed by integrative physiologists studying normal and diseased humans. Ignorance of this integrative physiology and overreliance on reductionist models, using vascular cells and rings have created confusion in the quest for the molecular mechanism of HPV. In addition, because HPV is elicited by moderate airway hypoxia, rather than anoxia or low mixed- venous pO_2, it is worthwhile to define "hypoxia" as it pertains to HPV. Ascent to the summit of Mount Everest defines the limit of hypoxia tolerated by humans and thus serves as a practical guide to what constitutes "physiologically relevant" hypoxia. At the summit of Mount Everest, the inspired and arterial PO_2 values are approximately 43 Torr, whereas the arterial pCO_2 is 11 ± 2 Torr and arterial pH is 7.53 (Malconian et al., 1993).

HPV increases PVR by 50-300%. The response to hypoxia onsets in minutes, reaching a maximum within 15 min (Bindslev et al., 1985). In normal volunteers, inhalation of 12.5% O_2

decreases systemic pO_2 to below 50 Torr and increases PVR by 100–150% (Moore et al., 1998). HPV is not potentiated by repeated hypoxic challenges, nor does it decrease when sustained for hours (Dorrington et al., 1997). An interesting case report attests to the potential of segmental HPV to persist chronically. A patient with bronchial obstruction due to an adenoma had persistent, HPV-induced lung hypoperfusion that was normalized by resection of the tumor and restoration of distal airflow (Grant et al., 1980).

In an excellent demonstration that HPV depends on airway and not mixed-venous PO_2, unilateral graded hypoxia (12–5% inspired O_2) was delivered while the contralateral lung received 100% O_2. This reduced the perfusion of the hypoxic lung from a normoxic value of 52 ± 2 to 30 ± 8% of total lung flow, without a significant fall in mixed-venous PO_2 (Hambraeus-Jonzon et al., 1997). In healthy volunteers, 8 h of hypoxia increased PVR from 1.2 ± 0.3 to 2.9 ± 0.3 Wood Units at 2 h and thereafter PVR remained constant, reversing on return to normoxia (Dorrington et al., 1997). Systemic vascular resistance (SVR) decreased in parallel. The intrinsic nature of these opposing responses to hypoxia is demonstrated by the ability to demonstrate simultaneous HPV and renal vasodilatation in the isolated, serially perfused lung-kidney model and even in isolated arterial rings (Michelakis et al., 2002). HPV also occurs in children. In a study of children with congenital heart disease, mild hypoxic ventilation (15% inspired O_2) increased the PVR/SVR ratio from 0.33 to 0.40 (Waldman et al., 1983).

If alveolar pO_2 is maintained above 60 Torr, there is a little pulmonary vasoconstriction to hypoxemia, even when mixed- venous pO_2 is reduced to 10 Torr (Marshall and Marshall, 1983), confirming that alveolar O_2 tension, not blood PO_2, is the major determinant of HPV. Micropuncture studies confirm that the small resistance PAs (<200 μm) are directly exposed to the alveolar PO_2 and are the major site of HPV (Kato and Staub,1966). HPV can be demonstrated in salt-perfused isolated lungs (McMurtry et al., 1976) and resistance PA rings denuded of endothelium (Archer et al., 2004). Indeed, isolated pulmonary artery smooth muscle cell (PASMC) from resistance pulmonary arteries (PAs) constrict to hypoxia. In contrast, smooth muscle cells (SMCs) from carotid arteries or even conduit PAs do not constrict to hypoxia (Madden et al., 1992). This demonstrates that HPV is unique to the PA, particularly the resistance segment of the pulmonary circulation. As in humans, hypoxia dilates most systemic arteries in animals (Hampl et al., 1994).

The persistence of global hypoxia ultimately results in a selective downregulation of acute HPV, despite the occurrence of PH and the finding that constrictor responses to other stimuli are preserved or enhanced (Durmowicz et al., 1993; Reeve et al., 2001). Exposure to hypoxia for only 3 h can elicit this selective suppression of HPV (Greenlees and Tucker,1984). Animals and humans genetically adapted to life at high altitude, such as the yak (Durmowicz et al., 1993) or the native Tibetan (Moore et al., 1998) have weak or absent HPV.

The vascular endothelium is known to produce a wide range of active compounds, including various vasodilators and vasoconstrictors. Vasoconstriction could result from either a reduction in vasodilator activity or an increase in vasoconstrictor activity; several possible mediators have been examined, including nitric oxide (NO), cyclooxygenase and lipoxygenase products, endothelin-1 (ET-1) and serotonin. There is disagreement about the nature of HPV in isolated PA rings. In animals, HPV causes a rapid increase in PVR that gradually reaches a

plateau and is sustained, much as occurs in humans. However, in isolated PA rings, some research groups find that HPV is biphasic, consisting of an immediate, endothelium-independent constriction, which peaks in ~10 min (phase I) and a second, slowly developing endothelium-dependent sustained contraction that peaks at ~40 min (phase II) (Ward and Robertson, 1995). On the other hand, Archer et al., 2001 consistently found a monophasic PA constriction in resistance PA rings, even if they are denuded of endothelium. They believe that the role of the endothelium is to modulate HPV. Nitric oxide (NO), produced in response to pulmonary vasoconstriction, suppresses HPV. Endothelin-1 (ET-1), produced in response to hypoxia, enhances HPV (Platoshyn et al., 2000).

One of the problems associated with such examinations is that the release of many of these vasoactive agents is affected by the vascular tone, so that it may be difficult to determine whether changes in a particular agent are due to the enhanced vasoconstriction during hypoxia, or to hypoxia *per se* (Ignarro, 1990; Inagami et al, 1995).

4.1 Molecular mechanisms of pulmonary hypertension

It is now well known that, under physiological conditions, the vascular endothelium produces factors that maintain normal vascular tone and homeostasis. Two of the most important factors produced by the vascular endothelium are the endothelium-derived relaxing and antiproliferative factor NO, and the endothelium-derived vasoconstrictor and mitogenic factor endothelin-1 (ET-1) (Moncada et al., 1991; Yanagisawa, 1994). The interactions between NO and ET-1 in the control of vascular tone have been extensively studied (Lavallee et al., 2001).

Pulmonary vasoconstriction is believed to be an early component of the pulmonary hypertensive process. Excessive vasoconstriction has been related to abnormal function of expression of potassium channels, as well as to endothelial dysfunction. Endothelial dysfunction seems to play an integral role in alveolar hypoxic vasoconstriction. The endothelium mediates the structural changes in the pulmonary vasculature, which includes an altered production of various endothelial vasoactive mediators, such as NO, prostacyclin, endothelin-1 (ET-1), serotonin, and thromboxane. Endothelial dysfunction leads to chronically impaired production of vasodilators such as NO and prostacyclin along with prolonged over expression of vasoconstrictors such as ET-1. These changes affect vascular tone and promote vascular remodeling. Given that most of these mediators affect the growth of smooth muscle cells, an alteration in their production may facilitate the development of pulmonary vascular hypertrophy and the structural remodeling characteristic of pulmonary hypertension (Budhiraja et al., 2004; Humbert et al., 2004).

4.1.1 Nitric oxide

Originally identified as the reactive intermediate by which nitroprusside caused smooth muscle cell relaxation, it was not until nitric oxide (NO) was identified as the endothelium-derived relaxing factor that its role was explored in PH (Gruetter et al., 1979; Ignarro, 1989). NO was found to be a critical vasodilator that could also inhibit platelet aggregation and proliferation of vascular smooth muscle cells. Human studies reported variable production of NO in patients with idiopathic pulmonary arterial hypertension (IPAH). However,

important mechanistic insights into the role of NO in the control of pulmonary vascular tone and remodeling originated from animal models, which showed NO-mediated protection against HPV in lungs, inhibition of smooth muscle proliferation and platelet aggregation, and downregulation of ET-1 production (Perrella et al., 1992). NO has broader effects in that it contributes to angiogenesis, endothelial cell survival, and mobilization of bone marrow progenitor cells.

The hypothesized role of endothelial NO deficiency in contributing to PH was further strengthened by the modest but healthy effects of inhaled NO and NO donors such as L-arginine in patients with PH. NO stimulates the production of cGMP starting a regulatory cascade resulting in pulmonary vasodilatation. While inhaled NO is used as a clinical therapy only in patients with primary pulmonary hypertension of the newborn, sildenafil, an oral inhibitor of phosphodiestarase-5, has beneficial effects in both adult and childhood PAH, probably by means of increasing cyclic GMP through decreased breakdown (Sastry et al., 2004).

Synthesis of NO involves incorporation of molecular oxygen, and hypoxia might therefore be expected to reduce NO release. A reduction in NO-mediated relaxation has been proposed as the underlying mechanism for HPV (Rodman et al., 1990), and hypoxia has been shown to inhibit agonist-stimulated release of NO in pulmonary arteries (Demiryurek et al. 1993). Inhibitors of NO synthesis potentiate HPV both *in vivo* and in perfused lungs (Sprague et al., 1992). It has been suggested that endogenous NO opposes HPV and thus limits the reduction in blood flow to the hypoxic alveoli (Sprague et al. 1992). Thus, the NO is clearly a major regulator of pulmonary vascular tone and may be important modulator during hypoxia.

NO is synthesized endogenously by nitric oxide synthase (NOS) enzymes, which convert L-arginine to L-citrulline and NO in the presence of oxygen, NADPH, flavin adenine dinucleotide, flavin adenine mononucleotide, tetrahydrobiopterin, and calmodulin (Knowles and Moncada, 1994). Three NOS enzymes have been so far identified in the lung. Neuronal NOS or NOS1 and endothelial NOS (eNOS) or NOS3 are primarily expressed in neuronal and vascular endothelial cells, respectively, of the normal lung, whereas the inducible NOS (iNOS) or NOS2 is expressed in the airway epithelium (Barnes and Belvisi, 1993; Kobzik et al., 1993; Guo et al., 1995). Basal release of NO by isoform eNOS produces a consistent vasodilator and antimitogenic effect. Although eNOS is constitutively expressed, its expression can be modulated by a variety of chemical, physical, and developmental stimuli (Forstermann et al., 1998).

The effects of eNOS-derived NO are mediated directly and via secondary messengers. In the presence of NO, guanylate cyclase becomes activated and produces cyclic guanosine monophosphate (cGMP) from guanosine triphosphate in the vascular smooth muscle. These two NO dependent routes resulting in calcium sequestration and smooth muscle relaxation (Jia et al., 1996; Moncada, 1993; McDonald and Murad, 1995; Rafikova et al., 2002; Stamler et al., 2001). Endothelial NO-induced vascular smooth muscle relaxation has been termed endothelium dependent. Exogenous NO donors, such as nitroglycerin, can cause endothelium-independent vascular smooth muscle relaxation. In particular, NO seems to be important for maintaining normal blood pressure and matching ventilation-perfusion within

the lung in broiler chickens. This concept is supported by the finding of Wang et al. (2002), who found that intravenous infusion of Nv-L-arginine-methyl-ester (L-NAME), which inhibits NO synthesis by both the eNOS and iNOS forms, caused an immediately reduced plasma concentration of NO and continuously increased pulmonary arterial pressure in healthy broilers. However, the L-NAME-induced increase in pulmonary pressure can be reversed by sodium nitroprusside, a nitric oxide donor (Weidong et al., 2002).

Wideman et al (2006) suggested that pulmonary vasodilatation might be affected by the NO produced by iNOS in lungs exposed to an inflammatory stimulus. The endothelium dependent vasodilatation in chickens is partly mediated by NO, as reflected by the finding that L-NAME attenuates the concentration-dependent relaxation response of artery rings to acetylcholine (Martinez-Lemus et al., 1999). It has been considered that the endothelium-dependent relaxation in the pulmonary artery of broilers is impaired, which contributes to the susceptibility of broilers to PH (Martinez-Lemus et al., 1999).

In experimental studies the alteration of eNOS availability in PH was examined by exposing chickens to hypobaric hypoxia (Moreno de Sandino and Hernandez, 2003, 2006) and cold stress (Tan et al., 2007) to induce PH, right ventricular hypertrophy and pulmonary vascular remodeling, similar to that in natural PH cases. Those studies demonstrated that hypoxia-induced as well as cold-induced PH was associated with reduced eNOS expression in the pulmonary arterioles, and that eNOS expression was inversely related to pulmonary vascular remodeling, strengthening the hypothesis of Martinez-Lemus et al. (1999) that impaired NO synthesis contributes to the susceptibility of chickens to PH. Giaid and Saleh (1995) reported decreased eNOS expression in human pulmonary vascular preparations and concluded that the reduction of this vasodilator enzyme contributed to the development of PH. Using eNOS-deficient rats, Steudel et al. (1998) confirmed that impaired eNOS-derived NO enhanced hypoxia-induced PH. NO levels in exhaled breath are reduced in the humans with IPAH, and there is an inverse relationship between NO in exhaled breath and the degree of PH (Kaneko et al., 1998; Machado et al., 2004; Girgis et al., 2005).

The study by Richard et al. (1995) provided evidence that part of the hypertensive response in rats induced by inhibition of NO synthesis, which is generally considered to reflect removal of the tonic vasodilator influence of NO, is in fact due to the unmasking of an endothelin-induced vasoconstrictor response. With the progression of vascular disease and the gradual loss of the ability of vessels to dilate, vasoconstrictor processes can assume a greater importance, thereby causing further vascular dysfunction (Lavallee et al., 2001). In broilers, increased pulmonary pressure accompanied by elevated ET-1 concentration was observed following inhibition of NO synthesis by supplemental L-NAME to the diet (Wang et al., 2002). In this context, it is also probable that reduced production of the NO in hypertensive broilers may be only one manifestation of endothelial dysfunction, and that the vasoconstrictor role of the endothelium-derived vasoconstrictor ET-1 may become progressively important in the development of PH. Indeed, ETA receptor antagonist BQ123 has been shown to effectively prevent cold-induced PH in broilers (Yang et al., 2005).

Supplemental dietary L-arginine has been shown to reduce pulmonary arterial pressure as well as the incidence of PH in chickens exposed to cool environmental temperature without alteration of body weight (Wideman et al., 1995), and further studies revealed that dietary L-arginine supplementation permitted broilers to exhibit flow-dependent pulmonary

vasodilatation when a pulmonary artery snare was tightened to force the entire cardiac output through one lung. Those findings were supportive of a NO vasodilator effect. Tan et al (2005a) showed that supplemental L-arginine markedly increased NO production in cold-exposed broilers, coincident with reduced pulmonary blood pressure. This indicates that an increase in endogenous NO synthesis may relax the tone of the resistance vessels in the lung of broilers, thereby permitting the increased blood flow to pass through the lung at a lower pressure. Similarly, intravenous L-arginine was shown to reduce pulmonary arterial pressure in human patients with PH by increasing the endogenous production of NO (Mehta et al., 1995). In an *in vitro* study Eddahibi et al., (1992) provided evidence that L-arginine restores endothelium-dependent relaxation of pulmonary vessels from hypertensive rats.

Although the mechanisms for reduced expression of eNOS in hypertensive broilers remain to be identified, it has been considered reasonable to postulate that endothelium dysfunction significantly contributes to PH development through several mechanisms, including an imbalance between the release of vasoconstrictor agents like ET-1 and vasodilator substances such as prostacycline and NO (Adnot et al., 1991; Inagami et al., 1995: Stewart, 1994; Tozzi and Riley, 1990; Voelkel and Tuder, 1995). A decrease in the amount of endothelium-dependent relaxation has been observed in pulmonary vessels from hypertensive animals, although its precise mechanism in the development of PH has not been established (Dhin-Xuan, et al., 1989, 1992; Martinez-Lemus et al., 1999). Some forms of hypertension might be due to changes in NO synthesis or action in the vasculature (Rees et al., 1989). Differences in endothelial NO synthesis are likely to be the result of various levels of genetically determined susceptibility to chronic hypoxia. Understanding the pathogenesis of PH and its key cellular and molecular mechanisms may allow manipulations to diminish the incidence of PH.

Impairment of NO production in chronic hypoxia occurs in pulmonary hypertensive animals (Maruyama and Maruyama, 1994; Reddy et al., 1996;Tozzi and Riley, 1990) and humans (Cooper et al., 1996; Dinh-Xuan et al., 1992; Dinh- Xuan et al., 1989). However, the cause of this impairment remains unknown. A defect in NOS enzyme could cause reduction of NO production (Cooke et al., 1997; Huang et al., 1995). This hypothesis is supported by previous immunohistochemical analysis showing decreased expression of endothelial NOS (Giaid and Saleh, 1995). Alternatively, there may be inhibition of the enzymatic reaction of NO synthesis, due to deficiency of necessary cofactors or the L-arginine substrate (Ignarro, 1990; Mitzutani and Layon, 1996; Shaul and Wells, 1994). NOS blockade has been shown to worsen hypoxia-induced PH, and administration of NO (in the inhaled air) ameliorates hypoxia-induced PH (Kouyoumdjian et al., 1994). In addition, L-arginine supplementation reduced right ventricular hypertrophy and mortality due to PH in male broilers exposed to low environmental temperatures (Wideman et al., 1995) and intravenous injections of L-arginine reduced pulmonary arterial pressure in pulmonary hypertensive individuals by increasing endogenous production of NO (Mehta et al., 1995). L-arginine ameliorated changes associated with PH in rats, probably by enhancing endogenous NO production (Mitzutani and Layon, 1996).

To determine whether or not exposure to chronic hypoxia and subsequent development of PH induce alterations in endothelial NO production in broiler's pulmonary vascular bed, in our laboratory, we studied the expression of NOS in pulmonary endothelial cells in

healthy and hypertensive broilers raised at an altitude of 2638 m above sea level, using a nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemical staining reaction. In this study, we demonstrated that NOS is present in pulmonary arterial endothelial cells of healthy and pulmonary hypertensive broilers. NOS activity is reduced in pulmonary arterial endothelial cells of pulmonary hypertensive broilers. Pulmonary hypertensive animals had lower numbers of pulmonary arterioles with NADPH-diaphorase-positive-staining endothelial cells than non-hypertensive chickens (healthy chickens) (Figures 4 and 5). Furthermore, the number of arterioles expressing NOS in the endothelial cells was inversely correlated with the degree of PH. The degree of PH was also significantly and inversely associated with NADPH-diaphorase positive staining. In fact, all hypertensive broilers had fewer arterioles expressing NOS in endothelial cells compared with the non-hypertensive broilers. This finding implies that hypertensive chickens show lower NO production than the non-hypertensive ones. Therefore, it is possible that NO participates in the pathogenesis of PH in broilers (Moreno de Sandino and Hernandez, 2003). Low L-arginine in the diet could provoke PH on its own or concomitantly with decreased NOS expression in hypertensive broilers. Although L-arginine content in feed in our study was not quantified, the diet used was presumed to be adequate in this context.

The results of this work are in harmony with previous observations that the degree of PH was significantly and inversely associated with NOS inmunoreactivity (Giaid and Saleh, 1995). Others demonstrated that hypoxia-induced PH is associated with a loss of NO activity in pulmonary vessels (Adnot and Raffestin, 1991). Other mediators, such ET-1, are also likely to be involved in the regulation of pulmonary vascular resistance (Giaid, 1998; Giaid et al., 1993; Stewart, 1994; Stewart et al., 1991). Interactions among mediators and NO may be important.

Endothelial-derived NO production or activity or both are reduced in certain forms of chronic PH in several experimental animal models and in humans. Whether this impairment causes the disease, or merely reflects its severity remains to be elucidated. Hence, NO formation by pulmonary endothelial cells may represent a primary event in the pathogenesis of PH (Moreno de Sandino and Hernández, 2003).

Recent research (Tan et al., 2007) demonstrated that loss of eNOS expression could be prevented by supplemental L-arginine. This finding probably reflects the ability of L-arginine to ameliorate haemodynamic damage to the pulmonary endothelium in hypertensive broilers, and provides a proof that supplemental L-arginine increasing NO production is associated with elevated eNOS expression. Supplemental L-arginine reduced but did not eliminate mortality due to PH (Wideman et al., 1995; Tan et al., 2007), indicating that treatment with L-arginine reduced pulmonary pressure only to a certain extent. In one case, however, supplemental L-arginine did not affect PH mortality (Ruiz-Feria et al., 2001). It is well known that PH in fast-growing broilers is a complex, multi-factorial process. In addition to vascular dysfunction (e.g. remodeling and impaired endothelium-dependent relaxation), causes that may affect pulmonary pressure include an inherently low pulmonary vascular capacity, inadequate gas exchange area for blood cells, and a cardiac output that chronically increases in support of metabolic requirements (Wideman and Tackett, 2000; Wideman, 2001; Hassanzadeh et al., 2005). Therefore, it is difficult to eliminate PH in broilers solely by supplemental L-arginine.

Fig. 4. Endothelium NADPH-diaphorase positive reaction of pulmonary arteriole (Moreno de Sandino and Hernández, 2003).
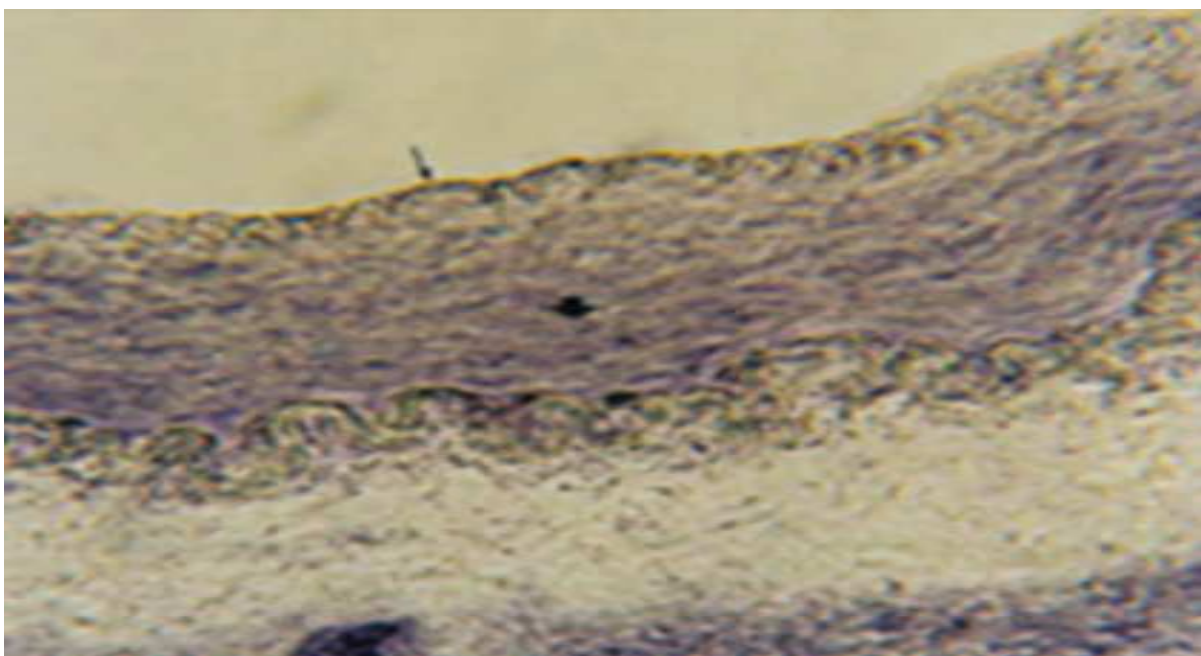

Fig. 5. Endothelium NADPH-diaphorase negative reaction of pulmonary arteriole (Moreno de Sandino and Hernández, 2003).

Several lines of evidence support a major role for NO and in particular for eNOS in the pulmonary circulation:

1. eNOS-deficient mice had mild PH (pulmonary artery pressure 19.0 ± 0.8 compared with 16.4 ± 0.6 mmHg in wild-type mice). In the same study, isolated pulmonary arteries of NOS3-deficient mice failed to show the normal vasodilator response to acetylcholine but were morphologically unaltered (Steudel et al., 1997).

2. In a complementary study by the same group of investigators, mice with congenital deficiency of eNOS (which exhibit major systemic and mild PH under normoxic conditions) developed more severe degrees of PH after a 3- to 6-wk period of hypoxia (11% oxygen). The NOS-deficient mice also showed greater increases in vascular remodeling and right ventricular hypertrophy than wild-type mice, and right ventricular hypertrophy was prevented by breathing at 20 parts per million NO (Steudel et al., 1997).

3. Deletion of the eNOS gene was associated with histological evidence of PH in both male and female mice during fetal life and at birth, but PH and right ventricular hypertrophy persisted only in the adult males. In neither sex did inducible or neuronal NOS compensate for the deletion of eNOS (Miller et al., 2005).

4. Lung tissue from patients with chronic PH showed decreased eNOS expression in vascular endothelium, especially in patients with severe histological abnormalities (i.e., with plexiform lesions); the intensity of the enzyme immunoreactivity correlated inversely with the severity of histological changes. The findings support the conclusion that the reduction of this vasodilator enzyme may contribute to the development of PH (Giaid A and Saleh, 1995).

5. Consistent with the above conclusion, mice deficient in the *de novo* production of tetrahydrobiopterin (BH4), a cofactor for NOS, selectively express a pulmonary hypertensive, but not systemic hypertensive phenotype (Nandi et al., 2005).

6. Inhaled NO attenuated PH and improved lung growth in infant rats after neonatal treatment with a VEGF receptor inhibitor (Tang et al., 2004).

7. In chickens reared at high altitude, NOS expression is lower in the endothelial cells of pulmonary arteries of hypertensive chickens compared with nonhypertensive chickens (healthy subjects) and lower NOS expression was associated with medial smooth muscle thickening (Moreno de Sandino and Hernández, 2003, 2006. See figures 4 and 5)

8. In chickens reared at high altitude, NOS mRNA expression is lower in the lungs of hypertensive chickens compared with nonhypertensive chickens (healthy subjects), and lower NOS mRNA expression was associated with medial smooth muscle thickening (non-published data).

4.1.2 Endothelin- 1

Endothelin (ET-1) is a family of four 21-amino-acid peptides (ET-1, ET-2, ET-3, ET-4). Of the four active endothelins, ET-1 is the predominant isoform in the cardiovascular system, which is generated through the cleavage of prepro-ET-1 to big ET-1 and then to ET-1 by the action of endothelin-converting enzymes (ECE). ET-1 is abundantly expressed in human lung´s vasculature and plays an important role in pulmonary vascular tone regulation (Giaid et al., 1993; Kourembanas et al., 1993). In mammals, both pulmonary vascular endothelial cells and vascular smooth muscle cells synthesize and release ET-1 (Wort et al., 2001). The vascular effects of ET-1 are mediated by two receptor types: ET receptor subtype A (ETA) and ET receptor subtype B (ETB) (Luscher and Barton, 2000). The ETA receptors are expressed on pulmonary vascular smooth muscle cells, while ETB receptors are located on both pulmonary vascular endothelial cells and smooth muscle cells. Stimulation of ETA receptors results in vasoconstriction and cell proliferation, whereas ETB receptors on the endothelial cells mediate clearance of ET-1, inhibition of endothelial cell apoptosis, release of NO and prostacyclin, and inhibit the expression of ECE-1. However, there is evidence that

the ETB receptors on smooth muscle cells may also play a major role in mediating ET-1-induced constriction of intrapulmonary conduit and resistance arteries in humans and rats (Wang et al., 2006; Sakurai et al., 1990).

Through its action on the endothelin receptor A (ET_A) on pulmonary artery smooth muscle cells, ET-1 leads to a rapid increase in intracellular calcium and sustained activation of protein kinase C. Early activation of the p42/p44 isoforms of mitogen-activated protein kinase and induction of the early growth response genes c-*fos* and c-*jun* are also observed (Jeffery and Morrell 2002). The mitogenic action of ET-1 on pulmonary artery smooth muscle cells occurs through the ET_A or endothelin receptor B (ET_B) subtype, depending on the anatomic location of cells. For instance, ET_A mediates mitogenesis in cells derived from the main pulmonary artery, whereas in cells from resistance arteries both receptor subtypes may contribute. There is strong evidence that endothelium-derived ET-1 is a major player in the vasodilator/vasoconstrictor imbalance characteristic of PH. Levels of lung and circulating ET-1 are increased in animals and patients with PH of various etiologies (Giaid et al., 1993). These observations indicate that ET-1 is likely to contribute to the vasoactive component of PH, as well as to the abnormal pulmonary vascular remodeling characteristic of the condition. Results of chronic ET receptor antagonist therapy support the relevance of this pathway in PH.

ET-1 is a potent vasoconstrictor peptide, and hypoxia has been demonstrated to increase ET-1 gene expression and secretion in cultured endothelial cells (Kourembanas et al., 1991). A study by Wang et al., (1995) has shown that the ET-1 antagonist BQ-123 can inhibit HPV in fetal lambs, but it is worth noting that the decline to baseline tension on return to normoxia was slow in this preparation. Although a role for ET-1 in HPV cannot be ruled out, it is more likely to be of importance in chronic hypoxia and it may well be involved in mediating some aspects of the pulmonary vascular remodelling seen under these conditions.

Gómez et al (2007, 2008), Davie et al (2002), Giaid et al (1993), Motte et al (2006) and Stewart et al (1991) showed convincing evidence which suggests that local ET-1 synthesis in vascular endothelial cells probably plays an important role in the pathophysiology of PH:

1. ET-1-like immunoreactivity and messenger RNA, rarely present in vascular endothelial cells from control subjects, were abundant in IPAH patients and were associated with medial thickening and intimal fibrosis.
2. There was a strong correlation between the intensity of ET-1-like immunoreactivity and pulmonary vascular resistance in patients with plexogenic pulmonary arteriopathy, but not in those with secondary PH.
3. ET-1 receptor density was considerably greater in smaller pulmonary arteries and lung parenchyma from pulmonary hypertensive patients than in control subjects.
4. ET-1 stimulated DNA synthesis in human PA smooth muscle cells.
5. Inhibition of endogenous ET-1 release or its action attenuated serum-stimulated proliferation of pulmonary vascular smooth muscle cells.
6. ET-1 mRNA expression is higher in the lung of pulmonary hypertensive chickens at high altitude compared with nonhypertensive chickens (healthy subjects), and high ET-1 mRNA expression was associated with medial smooth muscle thickness (Gómez et al., 2007, 2008).

7. In addition, studies have shown enhanced ET-1 and ECE-1 synthesis in humans and experimental animals with primary IPAH (Giaid et al., 1993; Giaid, 1998).

Similarly, increased ET-1 mRNA level in the lung of broilers with PH induced by chronic hypobaric hypoxia was observed in our laboratory; comprehensive gene expression analysis was performed in the lung of broilers under chronic hypobaric hypoxic conditions by using real-time PCR analysis. In this study, it was shown for the first time that ET-1 mRNA expression is higher in the lung of pulmonary hypertensive broilers (PHB) than nonhypertensive broilers (NPHB) (Figures 6,7 and 8; Gómez et al., 2007). 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. 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).

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), our work showed a decrease in ETA mRNA levels in the lungs of PHB (Figure 5). Thus, HPB showed high values of mRNA ET-1 and lowest values of mRNA of eNOS, and NHPB had opposite results (data non published). In studies with ET-1 receptors antagonists, 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 among species and their location in the corresponding blood vessel (Nishimura et al., 1995; Chen and Oparil, 2000; Balyakina et al., 2002). Nevertheless, the role of ET-1 receptors that mediate the vasoconstrictor response in this animal model requires further study.
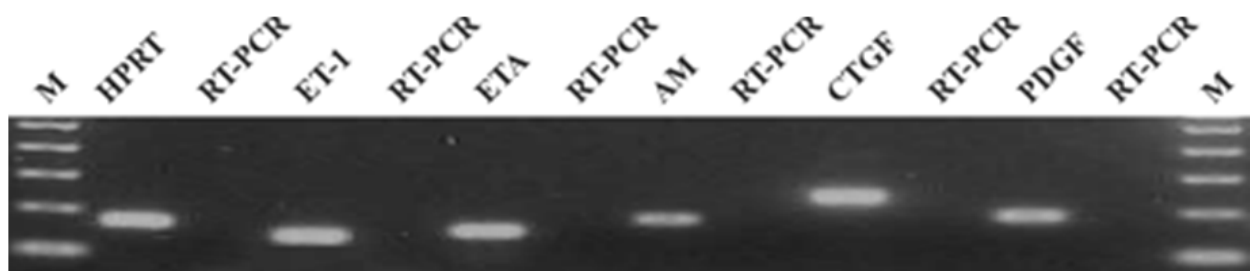

Fig. 6. Representative reverse-transcription PCR (RT-PCR) for hypoxanthine phosphoribosyltransferase (HPRT; 179 bp), endothelin 1 (ET-1; 141 bp), ET receptor type A (ETA; 160 bp), adrenomedullin (AM; 190 bp), connective tissue growth factor (CTGF; 252 bp), and platelet-derived growth factor (PDGF; 200 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 (Gómez et al., 2007).

Several studies have demonstrated the interaction between NO and ET-1 in the vascular endothelium in mammals. NO mitigates the vasopressor activity of ET-1 (Filep et al., 1993), inhibits translation of prepro-ET-1 mRNA (Bodi et al., 1995), augments the degradation of ET-1 protein, and reduces ET-1 formation (Kourembanas et al., 1993). A number of vasculopathies associated with impaired bioavailability of NO are linked to increased ET-1 synthesis (Alonso and Radomski, 2003).
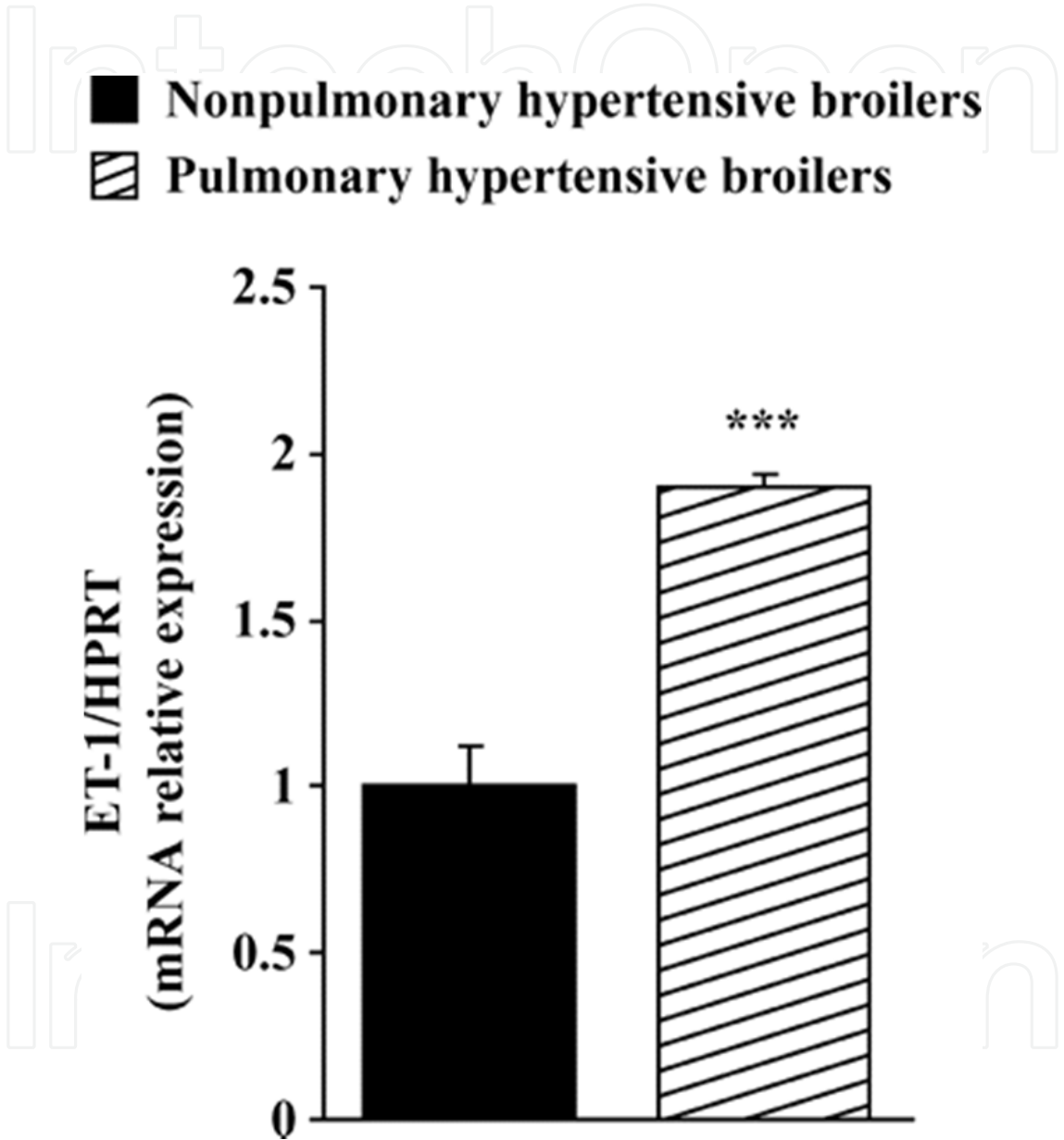

Fig. 7. Comparison by using real-time reverse-transcription PCR analysis of lung endothelin 1 (ET-1) mRNA levels in non-pulmonary hypertensive and pulmonary hypertensive chickens subjected to chronic hypobaric hypoxia. Semiquantitative data of ET-1 mRNA expression levels were normalized to those of the internal control hypoxanthine phosphoribosyltransferase (HPRT). Data are represented as mean ± SEM (n = 15/group). ***$P < 0.001$ (Gómez et al., 2007).
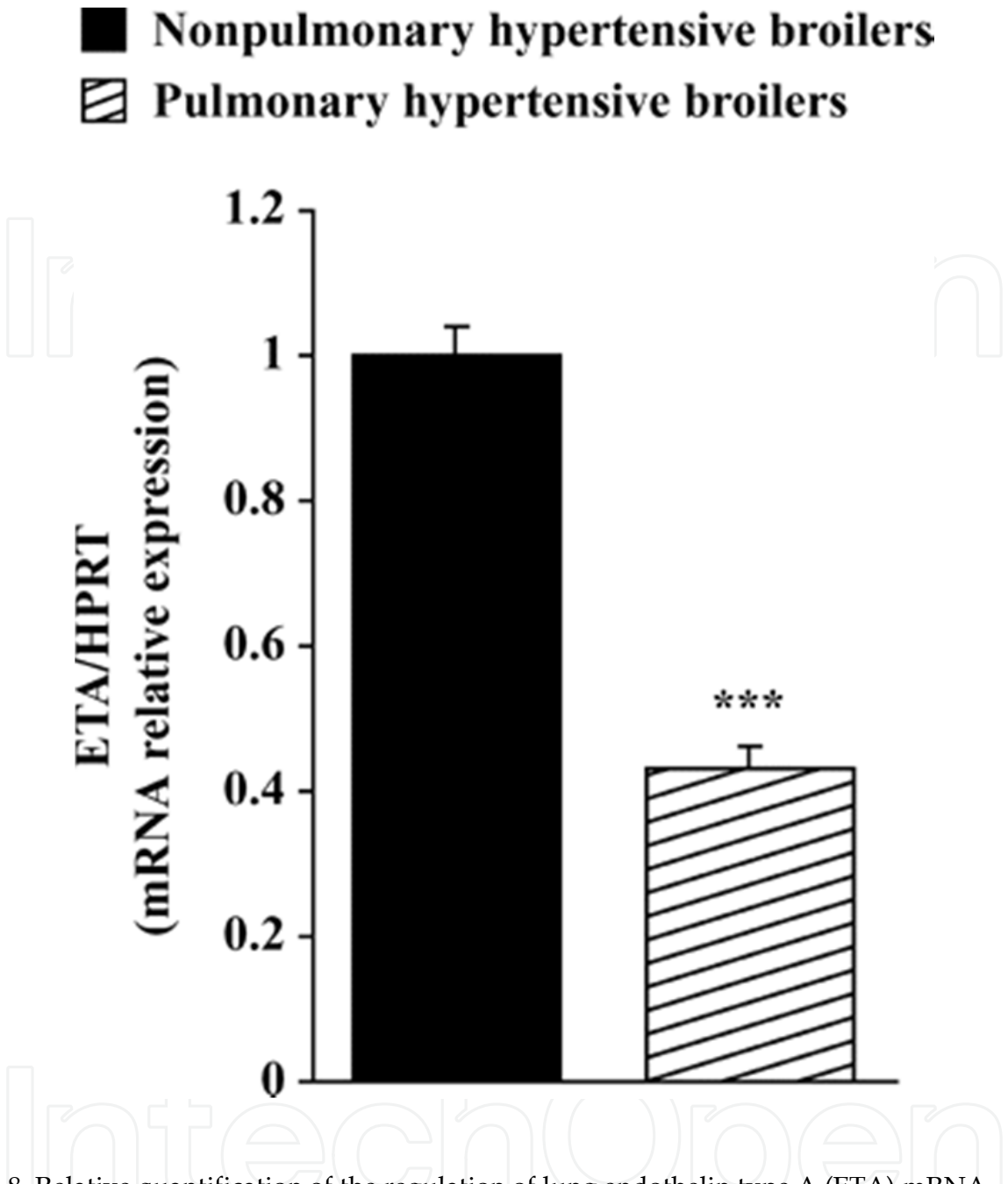
Fig. 8. Relative quantification of the regulation of lung endothelin type A (ETA) mRNA expression in pulmonary hypertensive and non-pulmonary hypertensive chickens subjected to chronic hypobaric hypoxia by using real-time reverse-transcription PCR analysis. Data of ETA mRNA expression levels were normalized to those of the internal control hypoxanthine phosphoribosyltransferase (HPRT). Data are represented as mean ± SEM (n = 15/group). ***P <0.001 (Gómez et al., 2007).

4.1.3 Potassium channels

Lessons relevant to PH can be learned from understanding the mechanism of HPV, although PH also involves cell proliferation and abnormalities of apoptosis (Archer and Rich, 2000). HPV is elicited when hypoxia inhibits one or more voltage-gated potassium channels (Kv) in

the pulmonary artery smooth muscle cells of resistance pulmonary arteries. The resulting membrane depolarization increases the opening of voltage-gated calcium channels, raising cytosolic calcium and initiating constriction. The Kv1.5 is downregulated in pulmonary artery smooth muscle cells in humans with PH (Yuan et al., 1998), and both Kv1.5 and Kv2.1 are downregulated in rats with chronic hypoxia-induced pulmonary hypertension (Michelakis et al., 2002).

Furthermore, deoxyribonucleic acid microarray studies have shown downregulation of Kv channel genes in PH lungs (Geraci et al., 2001; Weir and Olschewiski, 2006). The selective loss of these Kv channels leads to pulmonary artery smooth muscle cell depolarization, an increase in the intracellular calcium, and both vasoconstriction and cell proliferation. It is not clear whether these Kv channel abnormalities are genetically determined or acquired. However, it is clear that the appetite suppressants dexfenfluramine and aminorex directly inhibit Kv1.5 and Kv2.1 (Weir et al., 1996). Augmenting Kv pathways should cause pulmonary vasodilation and promote regression of pulmonary remodeling. Drugs including dichloroacetate and sildenafil may enhance the expression and function of these potassium channels.

4-Aminopyridine (4-AP), an inhibitor of K_v channels, but not ATP-sensitive K^+ channels, causes pulmonary vasoconstriction (Hasunuma et al., 1991). Post et al. (1992) showed that hypoxia inhibited K^+ current (I_K) and depolarized membrane (E_M) in canine PASMCs. This initiated extensive research to quantify the contribution of K^+ channels to HPV and determine the molecular identity of the O_2-sensitive K^+ channels. K^+ channels are proteins consisting of four transmembrane-bound α-subunits and four regulatory β-subunits. The ionic pore which determines the channel's intrinsic conductance and ionic specificity is created by the formation of tetramers of α-subunits. The K_v channels also have a voltage sensor in their S4 region. β-Subunits associate with many K^+ channels and alter their expression and kinetics. There are several types of K^+ channel α-subunits, including K_v, inward rectifier, and twin pore channels. The K_v channels have emerged as a possible effector in HPV (Reeve et al., 2001).

K_v channels are important determinants of equilibrium potential of vascular SMCs. Closure of K_v channels decreases the tonic efflux of K^+ that otherwise occurs because of the intra-/extracellular concentration gradient (145/5 mM). Channel closure renders the cell interior relatively more positive (depolarized). At these more positive potentials (positive to –30 mV), the probability of L-type voltage-gated Ca^{2+} channels to open increases. This augments intracellular Ca^{2+} influx (down a 20,000/1 concentration gradient). Although less important than in cardiomyocytes, Ca^{2+} influx also cause release of intracellular stores, so-called calcium-induced calcium release, effectively increasing total calcium levels inside the cell. Increased cytosolic Ca^{2+} activates contraction via the actin-myosin apparatus and also increases the activation of immediate early genes, inducing a proliferative response (Platoshyn et al., 2000). Thus, regulation of K^+ channel activity and the subsequent regulation of Ca^{2+} may be important to maintain the pulmonary circulation's low PVR and the thin-walled morphology of small pulmonary arteries.

Although all K^+ channels are somewhat sensitive to prolonged or severe O_2 deprivation (because most require some basal phosphorylation and thus ATP), certain K^+ channels are specially suited to O_2 sensing, by virtue of their content of key cysteine and methionine groups. Reduction or oxidation of these residues by a redox mediator such as ROS can cause conformational changes in the channel, thereby altering pore function (Archer et al.,1998). In

this regard, some K_v channels, including $K_v1.5$, respond to reduction and oxidation by changing their gating and open-state probability (Archer et al., 1998).

Hypoxia and redox agents may alter the function of K^+ channels, directly (Jiang and Haddad, 1994) or by modulating the levels of ROS, a diffusible redox mediator. It is unknown whether the electrophysiological effects of O_2 act directly or through a redox mediator. However, for a channel to respond to a diffusible redox mediator, it probably must be intrinsically redox sensitive.

Nine families of K_v channel have been identified, each with multiple isoforms. These channels activate in a nonlinear fashion with depolarization and many are inhibited by the pore-blocking drug 4-AP. A variety of putative O_2-sensitive channels exist in the PASMCs ($K_v1.2$, $K_v1.5$, $K_v2.1$, $K_v3.1b$, and $K_v9.3$) (Coppock et al., 2001). The importance of $K_v1.5$ and $K_v2.1$, 4-AP-, and redox-sensitive channels is emphasized by two models that manifest selective suppression of HPV: the chronic hypoxic hypertension model and the $K_v1.5$ knockout mouse.

In chronic hypoxia, impairment of HPV results from loss of $K_v1.5$, and to a lesser extent $K_v2.1$, expression with concordant suppression of O_2-sensitive I_K (Reeve et al., 2001). In chronic hypoxia, enhancing expression of $K_v1.5$ via $K_v1.5$ adenoviral gene transfer (Pozeg et al., 2003) restores K_v expression, O_2-sensitive I_K, and HPV. Mice with targeted $K_v1.5$ deletions also have impaired HPV and reduced PASMC O_2-sensitive I_K (Archer et al., 2001).

Archer et al. (2004) showed that preconstriction with the K_v blocker 4-AP eliminates subsequent HPV, although the pulmonary artery constricts vigorously to phenylephrine under the same conditions. The strong parallel between constriction to the selective $K_v1.x$ channel inhibitor correolide and hypoxia suggests that a $K_v1.x$ channel is central to the mechanism of HPV (Archer et al. 2004). Theoretically, correolide can inhibit $K_v1.2$; however, tityustoxin, an inhibitor of $K_v1.2/K_v1.3$, inhibits only a small portion of the hypoxia-sensitive I_K (Archer et al. 2004). Moreover, intracellular dialysis of PASMCs with antibodies against intracellular domains of $K_v1.5$ causes membrane depolarization and the combination of anti-$K_v1.5$ and anti-$K_v2.1$ blunts responsiveness to hypoxia (Archer et al., 1998, Archer et al. 2004). Finally, although mRNA of many K_v channels is more abundant in resistance vs. conduit pulmonary arteries, only $K_v1.5$ protein is expressed more abundantly in resistance pulmonary arteries, which are the main locus for HPV (Archer et al., 2004).

Human $K_v1.5$, cloned from normal pulmonary artery, generates an outward K_v current at –65 mV, which is inhibited by hypoxia (Archer et al., 2004). Thus the consequence of enriched $K_v1.5$ expression in resistance pulmonary arteries is a relative hyperpolarization of resistance vs. conduit pulmonary artery smooth muscle cells (⁓–60 vs. ⁓–35 mV) (Archer et al., 2004).

Intracellular Ca^{2+} plays an obligatory role in pulmonary vasoconstriction (Gelband and Gelband, 1997, Salvaterra and Goldman, 1993); the question is the extent to which influx of extracellular Ca^{2+} vs. release of intracellular Ca^{2+} initiates HPV. There is no doubt that, as in all types of smooth muscle cells, release of Ca^{2+} from intracellular pools, particularly the SR, is important to vasoconstriction. As with the differential function of O_2-sensitive K_v channels in pulmonary artery vs. renal arteries, there are differences in handling of intracellular Ca^{2+} between SMCs in these circulations. In PASMCs, inositol triphosphate (IP_3) and ryanodine-sensitive Ca^{2+} stores are organized into spatially distinct compartments.

Hypoxia causes intracellular Ca^{2+} increase, reaching maximum level in 1–2 min, and this is sustained during hypoxia, reversing on return to normoxia (Robertson et al., 2000). Urena et al. (1996) found that in conduit pulmonary artery smooth muscle cell, hypoxia reduced basal intracellular Ca^{2+} and decreased Ca^{2+} spikes (Urena et al., 1996), consistent with the lack of significant HPV in conduit pulmonary arteries (Archer et al., 1996). In resistance pulmonary arteries, two subsets of PASMCs were identified, one in which hypoxia increased cytosolic Ca^{2+}, a response mimicked by KCl and inhibited by nifedipine, or the removal of extracellular Ca^{2+}, and another in which hypoxia decreased Ca^{2+} (Urena et al., 1996). These findings of longitudinal heterogeneity in Ca^{2+} homeostasis are in keeping with the previously identified K^+ channel diversity in the pulmonary circulation (Albarwani et al., 1995; Archer et al., 1996). Nonetheless, the predominant source of Ca^{2+} for HPV appears to be extracellular and it enters the PASMCs via the L-type Ca^{2+} channels. For example, in 300-μm pulmonary arteries, hypoxia (PO_2 30–50 Torr) causes vasoconstriction and increases intracellular Ca^{2+} (Harder et al., 1985), both of which were blocked by verapamil, as occurs in humans (Burghuber, 1987) and rodents (McMurtry et al., 1976).

In conclusion, ionic channels play an important role in response to hypoxia in the pulmonary arteries. In addition to responding to membrane depolarization by hypoxia, there is an increase of calcium influx in the pulmonary artery via the L-type Ca channel which signal further release of calcium from the sarcoplasmic reticulum. Clearly, hypoxia increases calcium entry through stored-operated channels (SOCs), enhances calcium sensitivity, and increases vascular smooth muscle contraction and proliferation (Weir et al., 2005; Weir et al., 2006)

5. Pulmonary vascular remodeling

The pulmonary circulation is a low-pressure, high-flow system with a great capacity for recruitment of normally unperfused vessels. As a consequence, the walls of pulmonary arteries are thin, in keeping with their low transmural pressure. PH is a disease of the small pulmonary arteries, characterized by vascular narrowing and thickening, leading to a progressive increase in pulmonary vascular resistance. The consequence of this increased right ventricle afterload is the failure of the after load-intolerant right ventricle. The vascular remodeling is characterized by hypertrophy of the vascular media and the extension of smooth muscle to previously unmuscularized pulmonary arterioles (Hislop and Reid, 1976; Reid, 1979; Stenmark and Mecham, 1997). The process of pulmonary vascular remodeling involves all layers of the vessel wall and is complicated by the cellular heterogeneity which exists within the compartment of the pulmonary arterial wall (Jeffery et al., 2002). Indeed, each cell type (endothelial, smooth muscle, and fibroblast), as well as inflammatory cells and platelets, may play a significant role in PH. There is endothelial cell injury, migration of smooth muscle cells into the subintima, thickening of the medial layer, and increased production of collagen and elastin within the vessel wall (Gibbons and Dzau, 1994). Similar pathological changes are shown in most, if not all, broilers with chronic PH (Enkvetchakul et al., 1995; Xiang et al., 2002). Many factors are involved in the remodeling response in PH in humans and experimental animals (Jeffery and Wanstall, 2001).

In order to determine whether pulmonary arteriolar remodeling is related to eNOS expression in the vascular pulmonary bed of hypoxia-induced PH in broiler chickens, we investigated NOS expression and remodeling in lung vessels of hypertensive and nonhypertensive broilers

reared at high altitude (2638 masl). In this study, we demonstrated that normal broilers expressed greater amounts of NOS than PH broilers, and that NOS expression was inversely associated with vascular remodeling. PH broilers had thicker muscular and adventitial layers in pulmonary arterioles when compared with normotensive ones (Figures 9 and 10). We concluded that hypoxia-induced remodeling of pulmonary arterioles in broiler chickens appears to be associated with decreased endothelial NO production (Moreno de Sandino and Hernandez, 2006); the inhibition effect might be associated with decreased NO-induced apoptosis in PASMCs (Tan et al., 2005a). We concluded that the vascular remodeling could be mediated, at least in part, by decreased NO release by endothelial cells (Moreno de Sandino and Hernandez, 2006; Stenmark and Mecham, 1997).

Recent studies demonstrated that supplemental L-arginine inhibited remodeling in hypertensive broilers, an effect associated with NO-induced apoptosis of smooth muscle cells (Tan et al., 2005a) and NO-induced loss of expression of protein kinase C (Tan et al., 2006) in pulmonary arterioles. Other mechanisms involved in this process might include the direct anti-mitogenic effect of NO on smooth muscle cells (Thomae et al., 1995), NO-induced loss of production or expression in vasoconstrictors and mitogens such as ET-1 and platelet-derived growth factor (Kourembanas et al., 1993), and NO-induced loss of MMP activity in the lung (Souza-Costa et al., 2005). However, these mechanisms remain to be studied in broilers.
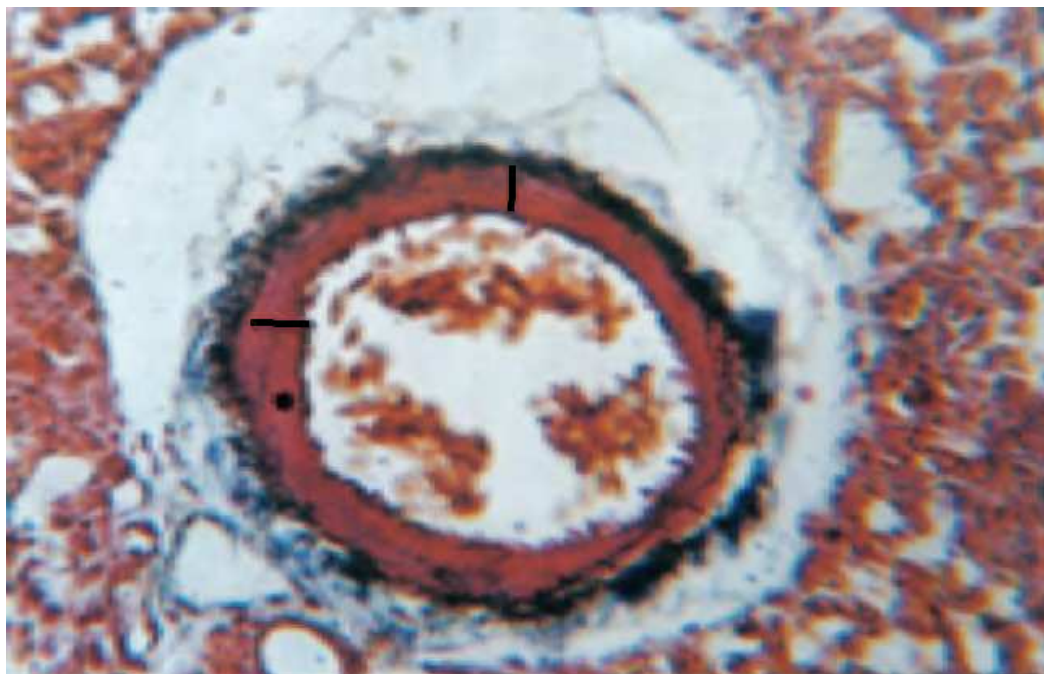

Fig. 9. Non-hypertensive chicken's arteriole. Thin smooth muscular (stained in red and indicated by black lines) and adventitial (dark blue) layers. Masson trichromic-stained.

Hypoxic arterial remodeling includes proliferation and migration of PASMCs. Platelet derived growth factor (PDGF) has also been implicated in these processes. Schermuly et al. (2005) found that administration of STI571, a PDGF receptor inhibitor, reversed pulmonary vascular changes in hypoxia-induced PH. Conversely, we did not find differences in PDGF mRNA expression levels between PH broilers and their controls (Gómez et al., 2007).
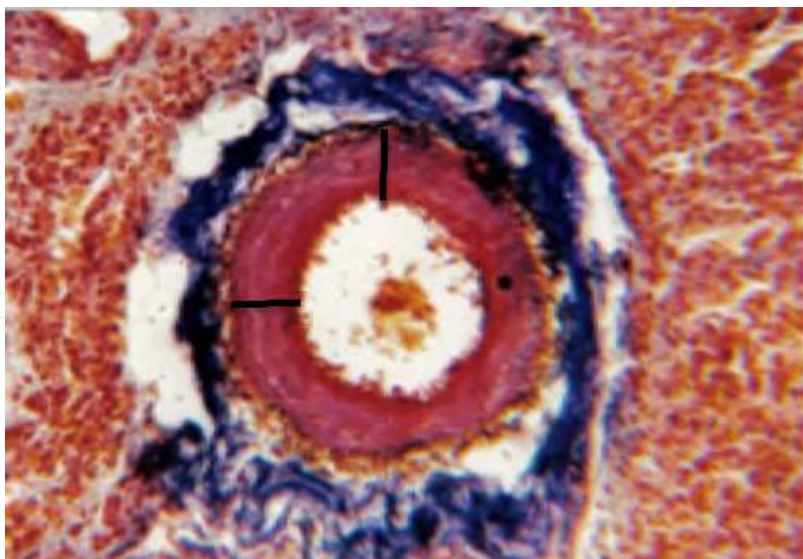
Fig. 10. Hypertensive chicken´s arteriole. Thick smooth muscular (stained in red and indicated by black lines) and surrounding adventitial (stained in dark blue) layers. Masson trichromic stain.

ET-1 participates in remodeling by increasing the connective tissue growth factor (CTGF) mRNA expression, promoter activity, and protein production (Rodriguez-Vita et al., 2005). CTGF regulates cell proliferation and apoptosis, angiogenesis, migration, adhesion, and fibrosis (Brigstock, 1999; Perbal, 2004). In order to determine if CTGF is involved in the PH in chickens, we investigated the CTGFmRNA in hypoxic hypertensive chickens and non hypertensive broilers reared in altitude (2638 masl). We demonstrated 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 (Gómez et al., 2007). Some investigators suggest that the CTGF might be considered as a new target for therapeutic interventions in PH (Rodriguez-Vita et al., 2005). This is supported by the results of our work (Gómez et al., 2007).

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (Kitamura et al., 1993). Immunoreactive AM has subsequently been detected in plasma and in a variety of tissues, including blood vessels and lungs (Ichiki et al., 1994; Sakata et al., 1994). It has been reported that there are abundant binding sites for AM in the lungs. Owji et al., 1995; Kakishita et al.,1999; Yoshibayashi et al., 1997 have shown that the plasma AM level increases in proportion to the severity of PH and that circulating AM is partially metabolized in the lungs. Interestingly, AM has been shown to inhibit the migration and proliferation of vascular smooth muscle cells (Horio et al., 1995; Kano et al., 1996). These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling. In fact, experimental studies (Heaton et al., 1995; Nossaman et al., 1996) have demonstrated that intralobar arterial infusion of AM induces pulmonary vasodilation in rats and cats. In humans, short-term intravenous infusion of AM significantly decreases pulmonary vascular resistance in patients with congestive heart failure (Nagaya et al., 2000a) or IPAH (Nagaya et al., 2000). Intravenously administered AM also decreases systemic arterial pressure in such patients, as well as in experimental models of PH (Nagaya et al., 1999, 2000, 2005; Nishikimi et al., 2003; Okumura et al., 2004), via a nonselective vasodilation of pulmonary and systemic vascular beds.

Nagaya et al (2003) showed that repeated inhalation of AM inhibited monocrotaline-induced pulmonary hypertension without systemic hypotension and improved survival in rats. Thus, they suggest that the long-term treatment with aerosolized AM may be a new therapeutic strategy for the treatment of PH.

We and others also showed that AM mRNA expression is decreased in pulmonary hypertensive broilers compared with non-hypertensive chickens (Gómez et al., 2007; Nakayama et al., 1998; Wang et al., 2001; Xu et al., 2002).

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, AM was upregulated by the hypoxia inducible factor-1 under hypoxic conditions (Garayoa et al., 2000). These results suggest that hypoxia simulates AM synthesis, which 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 serotonin (Chapman and Wideman, 2002) and thromboxane (Wideman et al., 1999), nuclear factor, interleukin-6, and early growth response-1 (Semenza, 2000).

Although little is known about the pathogenesis of remodeling in hypertensive broilers, evidence is emerging that there is reduced apoptosis in vascular smooth muscle cells (Tan et al., 2005a) and increased expression of protein kinase C (Tan et al., 2005b), a key enzyme in promoting the proliferation and differentiation of vascular smooth muscle cells and non-muscle cells (Assender et al., 1994; Haller et al., 1995; Wang et al., 1997; Fleming et al., 1998). Additionally, using an immunohistochemical technique, Ozyigit et al. (2005) found that the expression of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitors of metalloproteinase (TIMP)-1 were increased in the lung of broilers with salt-induced PHS, and that MMP-2 was localized mainly in pulmonary vascular endothelium whereas TIMP-1 was localized in the adventitia. MMPs are matrix-degrading enzymes involved in extracellular matrix turnover, smooth muscle cell and endothelial cell migration and proliferation. Increased MMP-2 activity in the pulmonary vessels of hypoxic rats was shown to be associated with the severity of PH (Frisdal et al., 2001). In addition, Herget et al. (2003) reported that inhibition of MMP activity attenuated pulmonary vascular remodeling as well as hypertension in chronically hypoxic rats, and suggested that the increased MMP activity might represent a substantial factor mediating the effect of hypoxia on the development of PH. Another study by Lepetit et al. (2005) demonstrated an imbalance between MMP-3 and TIMP-1 expression and an increase in MMP-2 expression in pulmonary arterial smooth muscle cells from patients with IPAH. These authors speculated that the imbalance between MMP-3 and TIMP-1 might lead to extracellular matrix accumulation and that the increased MMP-2 expression contributes to smooth muscle cell migration and proliferation (Lepetit et al., 2005). In this context, the results from mammals are indicative that the development of pulmonary vascular remodeling in hypertensive broilers may be related to alterations in the expression of MMPs and their TIMPs (e.g., MMP-2 and TIMP-1). However, definitive studies need to be designed to test this hypothesis.

Vascular remodeling will contribute to a sustained elevation in pulmonary arterial pressure and become progressively important as the disease advances because of an increased

pulmonary vascular resistance to blood flow and a decreased vascular compliance response. Therefore, inhibiting remodeling may be an important approach for prevention in PH (Jeffery et al., 2001). The development of a resistant genetic strain of commercial chickens to hypobaric hypoxia as a long-term goal should be a coherent strategy to diminish PH in chickens, with a concomitant economic impact. Furthermore, the avian model has been accepted as a model to study PH in mammals. Hence, future research is expected to support pharmacological approaches to be applied in another animal species, including man.

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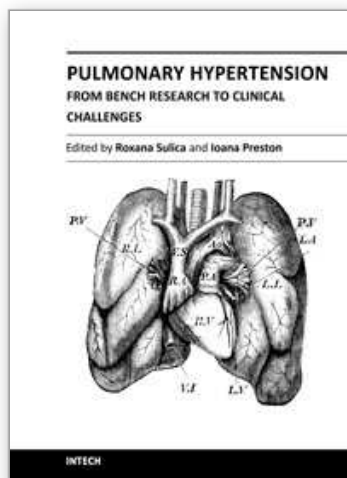
Pulmonary Hypertension - From Bench Research to Clinical Challenges

Edited by Dr. Roxana Sulica

The textbook "Pulmonary Hypertension - From Bench Research to Clinical Challenges" addresses the following topics: structure and function of the normal pulmonary vasculature; disregulated cellular pathways seen in experimental and human pulmonary hypertension; clinical aspects of pulmonary hypertension in general; presentation of several specific forms of pulmonary hypertension, and management of pulmonary hypertension in special circumstances. The textbook is unique in that it combines pulmonary and cardiac physiology and pathophysiology with clinical aspects of the disease. First two sections are reserved for the basic knowledge and the recent discoveries related to structure and cellular function of the pulmonary vasculature. The chapters also describe disregulated pathways known to be affected in pulmonary hypertension. A special section deals with the effects of hypoxia on the pulmonary vasculature and the myocardium. Other three sections introduce the methods of evaluating pulmonary hypertension to the reader. The chapters present several forms of pulmonary hypertension which are particularly challenging in clinical practice (such as pulmonary arterial hypertension associated with systemic sclerosis), and lastly, they address special considerations regarding management of pulmonary hypertension in certain clinical scenarios such as pulmonary hypertension in the critically ill.

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