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# The Role of ATPase in the Development of Idiopathic Pulmonary Arterial Hypertension in Broilers

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# The Role of ATPase in the Development of Idiopathic Pulmonary Arterial Hypertension in Broilers

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# Chapter 1:

## INTRODUCTION

Idiopathic pulmonary arterial hypertension, formerly known as primary pulmonary hypertension, is a rare, incurable, fatal disease of unknown cause (Naeiji, 2004; MacLean, et al., 2000; Wideman and Hamal, 2011; Xu, et al., 2007; Giad and Saleh, 1995). Each year, two new patients per million in the population are diagnosed with idiopathic pulmonary arterial hypertension. Patients rarely seek medical care in the early stages of the disease because they are asymptomatic (Wideman and Hamal, 2011). Once symptoms begin and patients are diagnosed, there are relatively few treatment options available. Roughly two to eight years after diagnosis, the disease progresses to right sided congestive heart failure and death (Runo and Lloyd, 2003; Cogolludo et al., 2006; Wideman and Hamal, 2011).

Idiopathic pulmonary arterial hypertension is characterized by increased pulmonary arterial pressure and pulmonary vascular resistance resulting from vascular remodeling and excess vasoconstriction, leading to the formation of plexiform lesions that occlude the pulmonary arterioles (Cogolludo et al., 2006; Eddahibi et al., 2006; Eddahibi et al., 2001; Marcos et al., 2004; Morrell et al., 2009; Xu et al., 2007; Wideman and Hamal, 2011; MacLean et al., 2000; Chapman and Wideman, 2000; Wideman et al., 2007; Burnstock, 1998; Lorenzi, Anthony, and Wideman, 2008; Humbert et al., 2004; Giad and Saleh, 1995; Zhang et al., 2004; Budhiraja, Tuder, and Hassoun, 2004; Voelkel and Tuder, 1997). These plexiform lesions are found in the lungs of approximately 80% of patients diagnosed with severe idiopathic pulmonary arterial hypertension (Wideman and Hamal, 2011).

Research on idiopathic pulmonary arterial hypertension has been hindered due to the previous lack of a model that spontaneously develops plexiform lesions (Wideman and Hamal, 2011; Humbert et al., 2004). However, broiler chickens bred for rapid growth and meat production have been found to spontaneously develop plexogenic arteriopathy, pulmonary vascular resistances, and pulmonary arterial pressures similar to those in humans with idiopathic pulmonary arterial hypertension (Wideman et al., 2007; Wideman and Hamal, 2011).

Research using chicken and mammalian models has found that increased levels of serotonin, a potent vasoconstrictor, act on pulmonary arterial smooth muscle cells and the endothelium to lead to cell proliferation and increased vasoconstriction, contributing to the elevated pulmonary vascular resistance characteristic of idiopathic pulmonary arterial hypertension (Wideman and Hamal, 2011; Herve et al., 1995; Cogolludo et al., 2006). Pilot data from Dr. Wideman's lab has demonstrated that the main sites of increased pulmonary vascular resistance in the vasculature of broiler lungs reside in the arterioles of the lung (Chapman and Wideman, 2001). More specifically, pilot data from Dr. Kluess' lab suggests that serotonin constricts these 1A and the 2A arterioles in the chicken lung, implying their role in increasing pulmonary vascular resistance. Furthermore, recent evidence implies that serotonin may interact with the purinergic P2 receptor for adenosine tri-phosphate (ATP) leading to excessive vasoconstriction (Dergachevea et al., 2008). These P2 receptors normally respond to ATP by causing vasoconstriction in the pulmonary arterial smooth muscle cells (Burnstock, 2002). However, the presence of such receptors in pulmonary vasculature of broilers has not yet been confirmed.

This study is part of a larger project with the overall purpose to elucidate mechanisms causing increased arteriole resistance in broilers susceptible to pulmonary arterial hypertension, so as better to understand the mechanisms leading to plexogenic arteriopathy in broilers and in humans. This will be accomplished through the manipulation of the amount of tryptophan, a rate limiting, essential amino acid precursor to the development of serotonin, in the diets of broilers susceptible to idiopathic pulmonary arterial hypertension (Corzo, et al., 2005; Morrel et al., 2009). It is expected that the broilers with the diet excess in tryptophan will develop more severe idiopathic pulmonary arterial hypertension than the control group due to increased pulmonary arterial pressure and pulmonary vascular resistance due to excess serotonin in circulation.

As a part of this larger project, the goal of this study is to assess the ATPase activity in the vascular walls of the pulmonary artery, 1A arteriole, and 2A arteriole of the broiler lung. Essentially, ATPase activity modulates the amount of vasoconstriction through the regulation of the amount of ATP acting on the outside wall of pulmonary vasculature. By breaking down ATP and preventing it from binding to the receptors on the vascular walls of pulmonary arterial smooth muscle cells, the vasoconstrictive effects of ATP are curbed. Further acting to curb the vasoconstriction, the breakdown products of ATP act to enhance vasodilatation. It is hypothesized that broilers on a diet of high tryptophan will have a worse case of pulmonary hypertension than those in the control group due to increased vasoconstriction leading to higher pulmonary arterial pressures and pulmonary vascular resistances. This increased vasoconstriction is possibly due to increased levels of serotonin in circulation, as well as lower ATPase activity. Therefore, it is hypothesized that the broilers fed the diet with high levels of tryptophan will have

lower ATPase activity associated with a worsening case of pulmonary hypertension. Whereas those on the control diet are hypothesized to have higher ATPase activity than those on the high tryptophan diet.

With respect to the vessels being examined, this study will also attempt to discern differences in ATPase activity between the 1A arteriole, 2A arteriole, and the pulmonary artery. Previous research has implicated vessel size as an important component of constriction; the smaller the vessel, the larger the purinergic component of constriction (Gitterman and Evans, 2009). Because ATP acts on purinergic receptors in the pulmonary vasculature, it is hypothesized that because they are smaller vessels, vasoconstriction will be higher in the arterioles, thus, leading to enhanced pulmonary arterial pressures and pulmonary vascular resistances. It is this increased stress that triggers the release of ATPase (Yegutkin et. al., 2009). Consequently, it is hypothesized that this higher amount of vasoconstriction in the smaller vessels correlates to increased ATPase activity in response to the stress. Therefore, the arterioles are hypothesized to have higher ATPase activity than the larger pulmonary arteries.

# Chapter 2:

# LITERATURE REVIEW

## Plexogenic Arteriopathy

The role of the endothelia in the vascular pathology of idiopathic pulmonary arterial hypertension is poorly understood. It has been identified that plexiform lesions are composed of aggregates of these endothelial cells (pulmonary arterial smooth muscle cells) and platelets (Tuder et al., 2009; Wideman and Hamal, 2011). In advanced stages of idiopathic pulmonary arterial hypertension, the plexiform lesions (plexogenic arteriopathy) develop in the endothelia of small arterioles downstream from branching points in lung vasculature (Wideman and Hamal, 2011; Tuder et al., 2009; Humbert, et al., 2004). It is postulated that as a consequence of increased pulmonary arterial pressure, localized turbulent blood flow causes shear stress, resulting in damage to the endothelium of small arterioles (Wideman and Hamal, 2011). This damage is believed to initiate disordered endothelial cell proliferation (Tuder et al., 2009; Humbert et al., 2004). This endothelial cell growth causes both the thickening and muscularization of the walls of pulmonary arterioles, as well as narrowing of the pulmonary artery lumen, effectively occluding the flow of blood through them (MacLean et al., 2000; Voelkel and Tuder, 1997; Giad and Saleh, 1995; Humbert et al., 2004). Overall, it is this vascular remodeling—the hyperplasia of pulmonary arterial smooth muscle cells and the medial hypertrophy of pulmonary arteries—that is the main underlying pathological change in idiopathic pulmonary arterial hypertension (Eddahibi et al., 2006; Eddahibi and Adnot, 2001; Marcos et al., 2004; Wideman and Hamal, 2011; Humbert et al., 2004).

The aggregates of endothelial cells and platelets build up and ultimately block the small pulmonary arterioles (Eddahibi et al., 2006; Marcos et al., 2004). The blood flow that would usually be directed through these blocked vessels must be pushed through the remaining vessels that are only partially occluded or unaffected by the proliferation of endothelia cells. Because the heart must pump blood through less pulmonary vasculature when the vessels are fully blocked, the cardiac output increases to make up for these anatomical inadequacies of pulmonary vascular capacity (Wideman et al., 2007). Also, because the resistance to flow through a blood vessel is primarily determined by the radius of that vessel, when blood is pumped through occluded vessels with decreased luminal radii, the cardiac output must compensate for the increased resistance (Wideman et al., 2007). Essentially, this causes an increase in pulmonary vascular resistance and pulmonary arterial pressure, where pulmonary arterial pressure is equal to cardiac output multiplied by pulmonary vascular resistance (Zhang et al., 2004; Wideman et al., 2007).

When both pulmonary vascular resistance and cardiac output increase, the pulmonary arterial pressure is increased by 10 to 15 mmHg up to a characteristic level for idiopathic pulmonary arterial hypertension greater than or equal to 25 mmHg at rest in the absence of other chronic lung or heart diseases (MacLean et al., 2000; Wideman and Hamal, 2011). Because the same amount of blood is being pumped through less vasculature at faster rates, a diffusion limitation occurs. When the erythrocytes (red blood cells) are forced to flow too rapidly past the pulmonary gas exchange surfaces in the lungs, full blood gas exchange of $O_2$ and $CO_2$ is not permitted (Wideman et al., 2007; Wideman and Tackett, 1999). This diffusion limitation creates the development of systemic arterial hypoxemia (Chapman and Wideman, 2000; MacLean et al., 2000). This

leads to hypoxic pulmonary vasoconstriction in the pulmonary arteries, which enhances the resistance in the vessels and causes increased shear stress on the vasculature (MacLean et al., 2000; Burnstock, 1998). Another contributor to idiopathic pulmonary arterial hypertension, is platelet dysfunction caused by the previously described vascular abnormalities such as hyperplasia of pulmonary arterial smooth muscle cells and medial hypertrophy of pulmonary arterioles. The shear stresses lead to the increased release of vasoactive and mitogenic mediators such as serotonin, a potent vasoconstrictor (Humbert et al., 2004). These vasoconstrictive actions of the endothelium and platelet dysfunctions lead to further increased pulmonary vascular resistance (Humbert et al., 2004). Overall, this increased pulmonary vascular resistance and vasoconstriction over time impedes the functioning of the right ventricle to pump blood, leading to right ventricle hypertrophy and ultimately to right ventricular failure (Marcos et al., 2004; Chapman and Wideman, 2000).

Vasodilator therapies have attempted to reverse this vasoconstriction, however, have been largely unsuccessful in reversing this cycle once the presence of plexiform lesions is identified (MacLean et al., 2000; Wideman and Hamal, 2011). Once patients have presented with plexogenic arteriopathy, they have a poor prognosis for survival (Wideman and Hamal, 2011). The normal pathophysiologial progression of idiopathic pulmonary arterial hypertension following the development of plexogenic arteriopathy includes the development of systemic arterial hypoxemia, systemic arterial hypotension due to reduced total peripheral resistance, regurgitation by the monocuspid right atrioventricular valve, cardiac decompensation, right-sided congestive heart failure, central venous hypertension, hepatic cirrhosis, accumulation of ascitic fluid in the

abdominal cavity, and eventually death (Wideman and Hamal, 2011; Chapman and Wideman, 2000). Despite having general information about the pathophysiological progression of idiopathic pulmonary arterial hypertension, further research is needed to understand the vascular mechanisms behind plexogenic arteriopathy. It is hoped that these research efforts will lead to better treatment solutions in the future. Several animal models such as rats, guinea pigs, and dogs, have been used in the research of idiopathic pulmonary arterial hypertension, however advances have been hindered by the previous absence of a model able to spontaneously recapitulate the histology of idiopathic pulmonary arterial hypertension especially the presence of plexiform lesions (Wideman and Hamal, 2011; Humbert et al., 2004).

**Broiler Chickens as Model of Idiopathic Pulmonary Arterial Hypertension**

Unlike other animal models used previously to research idiopathic pulmonary arterial hypertension, broiler chickens (domestic fowl bred for meat production) spontaneously develop semi-occlusive endothelial cell proliferation that progresses into the development of plexiform lesions (Wideman and Hamal, 2011). The two primary triggers of idiopathic pulmonary arterial hypertension in broilers are fast growth and cool temperatures (Wideman et al., 2002). In broilers, idiopathic pulmonary arterial hypertension is triggered by extremely rapid early growth and limitations due to their developmentally immature cardiovascular and pulmonary systems (Wideman and Hamal, 2011). Broiler chickens grow at a maximal rate in cold temperatures, however their lungs remain isovolumetric due to the anatomical constraints imposed by the unchanging size of the rib cage despite their increased metabolic requirement for oxygen (Wideman, Erf, and Chapman, 2001; Wideman et al., 2002; Wideman et al., 2007). The mismatch of

physical constraints with the high metabolic oxygen demands lead to increases in cardiac output blood levels proportional to the growth rate (Wideman, Erf, and Chapman, 2001; Chapman and Wideman, 2006). An oxygen/carbon dioxide diffusion limitation arises, just as in human idiopathic pulmonary arterial hypertension, when red blood cells flow too quickly past the gas exchange sites in the pulmonary vasculature to permit the hemoglobin to become fully saturated with oxygen (Wideman et al., 2002). The diffusion limitation leads to hypoxemia, and elevated blood pressure within the pulmonary circulation (Wideman et al., 2002; Wideman et al., 2007; Wideman, 2006; Bowen et al., 2006). The ensuing hypoxia initiates endothelial cell dysfunction, which leads to proliferation and formation of plexiform lesions. Endothelial cells are important regulators of vascular function, and as such, endothelial dysfunction denotes an imbalance in the production of vasoconstrictors versus vasodilators and activators (mitogens) versus inhibitors of pulmonary arterial smooth muscle cell growth and migration (Morrell et al. 2009). Specifically, a sustained vasoconstriction and medial hypertrophy of small pulmonary arterioles related to endothelial dysfunction lead to chronically enhanced production of or sensitivity to endothelium pro-mitogenic (stimulates proliferation) vasoconstrictors such as serotonin, and reduced production of or sensitivity to endothelium derived anti-mitogenic vasodilators such as nitric oxide (Humbert et al., 2004; Wideman and Hamal, 2011). This imbalance further causes the pulmonary arterial pressure and pulmonary vascular resistance to increase to levels characteristic of idiopathic pulmonary arterial hypertension in both humans and broilers (Chapman and Wideman, 2006).

There are several similarities between human idiopathic pulmonary arterial hypertension and broiler idiopathic pulmonary arterial hypertension in addition to the presentation of plexiform lesions and the mechanism leading to hypoxia. Broilers exhibit a medial hypertrophy at branch points within the pulmonary vasculature similar to those locations in humans (Wideman and Hamal, 2011). In addition, the development of pulmonary vascular lesions permanently obliterates small pulmonary arterioles, leading to reductions in the available pulmonary vasculature causing increases in pulmonary vascular resistance (Wideman and Hamal, 2011). This triggers a positive feedback cycle in which the arteriole occlusions are progressively increasing the pulmonary vascular resistance, thus, increasing the right ventricular after load, causing the hypertrophied right ventricle to increase the pulmonary arterial pressure to push the cardiac output through the decreasing numbers of unobstructed pulmonary vessels (Wideman and Hamal, 2011).

Despite the similarities between human and broiler idiopathic pulmonary arterial hypertension, there are also key differences. First, unlike mammals with compensatory mechanisms known to reduce the pulmonary vascular resistance (such as flow dependent pulmonary vasodilatation, arteriole distillation, capillary distention, or the recruitment of un- or under-perfused vascular channels), broilers do not have similar compensatory mechanisms to adapt when their pulmonary vasculature is incapable of accommodating the required cardiac output (Wideman and Chapman, 2000). Lacking pulmonary vasculature elasticity, broiler right ventricles and their pulmonary arterial pressures must respond to the increased cardiac output (Wideman and Chapman, 2000; Wideman and Hamal, 2011). Second, as broilers and humans with the disease age, the severity of plexogenic arteriopathy is significantly less in broilers than in humans (Wideman and

Hamal, 2011). This observation could be partially due to the early death of susceptible broilers with idiopathic pulmonary arterial hypertension due to terminal right-sided heart failure (Wideman and Hamal, 2011). These differences would be problematic if the goal were to discover therapeutic treatments for humans rather than studying the mechanisms behind idiopathic pulmonary arterial hypertension and plexogenic arteriopathy (Wideman and Hamal, 2011).

**Serotonin**

Research using this broiler model and mammal models has already been successful in uncovering some of the mechanisms of idiopathic pulmonary arterial hypertension, specifically with regard to advances in understanding of the roles of serotonin, nitric oxide, and ATP. There has been considerable interest in the role of serotonin in the pathogenesis of idiopathic pulmonary arterial hypertension due to the formation of plexiform lesions in the small pulmonary arteries and arterioles in humans using serotoninergic appetite suppressant drugs (anorectic drugs, anorexigens) (Chapman and Wideman, 2006; Wideman et al., 2007; Wideman and Hamal, 2011). Furthermore, there is an association between patients using anorexigens such as fenfluramine and aminorex, and the development of idiopathic pulmonary arterial hypertension (Naeiji, 2004; Eddahibi and Adnot, 2002). In fact, in patients who received these drugs for over a three-month period, there was a twenty three to thirty fold increase in the risk of developing idiopathic pulmonary arterial hypertension (Lawrie et al., 2005; Eddahibi and Adnot, 2002; Cogolludo et al., 2006). These anorexigens stimulate serotonin release in the blood stream and act through interactions with the serotonin transporter located on pulmonary arterial smooth muscle cells (Wideman and Hamal, 2011; Cogolludo et al.,

2006; Eddahibi and Adnot, 2002). Because of the research done to understand the correlation between anorexigens and idiopathic pulmonary arterial hypertension, the roles and mechanisms of serotonin in sustained vasoconstriction leading to increased pulmonary vascular resistance and in structural remodeling associated with pulmonary arterial smooth muscle cell proliferation are better understood.

In healthy broilers, serotonin is the most potent vasoconstrictor capable of triggering essentially instantaneous and full vasoconstriction within 30 seconds leading to an immediate reduction in cardiac output by 90% or more and terminal suffocation (Wieman and Hamal, 2011; Wideman et al., 2007). It is mainly produced in the enterochromaffin cells of the intestine (MacLean et al., 2000; Eddahibi et al., 2006; Herve et al., 1995). From there, serotonin is normally stored in large quantities within the platelets or thrombocytes (MacLean et al., 2000; Eddahibi et al., 2006). These nucleated thrombocytes are equivalent to mammalian platelets and are the most numerous leukocytes in avian blood (Wideman and Hamal, 2011; Wideman et al., 2007). In idiopathic pulmonary arterial hypertension, platelets dysfunction and aggregate within pulmonary arteries and arterioles and their ability to store serotonin becomes impaired (MacLean et al., 2000; Wideman et al., 2007; Pakala et al., 1994; Herve et al., 1995; Chapman and Wideman, 2006). This change in capacity for serotonin may be due in part to the lower numbers of platelets in patients with idiopathic pulmonary arterial hypertension, decreased pulmonary endothelial metabolism, or due to hypoxia in the airways of accumulating thrombocytes (MacLean et al., 2000; Herve et al., 1995; Bowen et al., 2006). This abnormal managing of serotonin causes an increased circulating level of serotonin (Herve et al., 1995; Humbert et al., 2004; Morrell et al., 2009; MacLean et

al., 2000; Naeiji, 2004; Cogolludo et al., 2006). Another cause for an increased level of serotonin is a mutation in the gene encoding the bone morphogenic protein receptor 2 found in cases of familial idiopathic pulmonary arterial hypertension (Naeiji, 2004). This mutation causes an up-regulation of angiopoetin-1, which further increases the amount of serotonin in circulation (Naeiji, 2004). Normally, free serotonin in the plasma is rapidly metabolized by the endothelial monoamine oxides enzymatic activity in the liver or the lungs, preventing excessive serotonin levels from entering the pulmonary bed (Herve et al., 1995). However, when the amount of serotonin in the plasma overwhelms the metabolic capabilities to break it down, excess serotonin enters the pulmonary vasculature. Once circulating in the pulmonary vasculature, serotonin targets receptors (specifically the serotonin$_{1b\text{-}1d}$, serotonin$_{2a}$, and serotonin$_{2b}$ receptors) on pulmonary arterial smooth muscle cells leading to membrane depolarization and vasoconstriction through the inhibition of $K^+$ channels (Humbert et al., 2004; Morrell et al., 2009; Wideman and Hamal, 2011; MacLean et al., 2000; Dergacheva et al., 2008; Naeiji, 2004; Cogolludo et al., 2006; Eddahibi and Adnot, 2002). These voltage gated $K^+$ channels play an essential role in regulating resting membrane potential, intracellular calcium concentration, and contraction of vascular smooth muscle (Cogolludo et al., 2006). Activation of $K^+$ channels leads to hyperpolarization of the pulmonary arterial smooth mucle cells, in contrast to conditions of hypoxia and serotonin binding to receptors that leads to the inhibition of the $K^+$ channel, and resulting in membrane depolarization of the pulmonary arterial smooth mucle cells (Cogolludo et al., 2006; Humbert et al., 2004). This leads to the opening of $Ca^{2+}$ channels, an influx of $Ca^{2+}$ into the pulmonary arterial smooth muscle cell, and vasoconstriction (Cogolludo et al., 2006; Humbert et al., 2004).

It is this direct vasoconstriction related to $K^+$ channel blockade or increased intracellular $Ca^{2+}$ levels in pulmonary arterial smooth muscle cells that contributes to sustained increases in pulmonary vascular resistance and pulmonary arterial pressure that are characteristic of idiopathic pulmonary arterial hypertension (Wideman and Hamal, 2011; Eddahibi and Adnot, 2002).

Not only is serotonin a potent vasoconstrictor, but it is also the most potent mitogen of all the endothelial and platelet derived growth factors, stimulating proliferation of vascular endothelial and pulmonary arterial smooth muscle cells (Wideman and Hamal, 2011). The mitogenic effects of serotonin require it to enter into pulmonary arterial smooth muscle cells through serotonin transporters (Morrell et al., 2009). When under hypoxic conditions characteristic of idiopathic pulmonary arterial hypertension, there is a 2.5 to 3 fold increase in the number and activity of these serotonin transporters (MacLean et al., 2000). After binding to a serotonin receptor, the serotonin transporter actively takes up serotonin into the endothelium (MacLean et al., 2000; Dergacheva et al., 2008; Naeiji, 2004; Marcos et al., 2004; Lawrie et al., 2005; Herve et al., 1995; Cogolludo et al., 2006). Once serotonin enters into the pulmonary arterial smooth muscle cells, it causes hypertrophy and proliferation of the medial muscle layer in pulmonary arterioles (Wideman and Hamal, 2011; Herve et al., 1995; Cogolludo et al., 2006). This medial hypertrophy of pulmonary arterioles is also enhanced by the decreased $K^+$ channel activity which inhibits apoptosis (Cogolludo et al., 2006). The resulting proliferation of pulmonary arterial smooth mucle cells and lack of apoptosis lead to the formation of plexiform lesions and result in a hypoxia that re-fuels the cycle (Eddahibi et al., 2006; Pakala et al., 1994; Marcos et al., 2004; Eddahibi et al., 2001).

**Nitric Oxide**

Normally in the maintenance of pulmonary vascular tone, vasoconstriction imposed by serotonin is balanced by vasodilatatory effects (MacLean et al., 2000). Vasodilators reduce the pulmonary arterial pressure required to push the necessary cardiac output through the pulmonary blood vessels, thus reducing pulmonary vascular resistance (Wideman et al., 1994). This reduction in pulmonary vascular resistance should delay the pathophysiological progression of pulmonary hypertension (Wideman et al., 1994). However, idiopathic pulmonary arterial hypertension results when the vasoconstrictors overwhelm the dilatory effects of vasodilators such as nitric oxide (Chapman and Wideman, 2006).

Nitric oxide is a potent, endothelium derived pulmonary vasodilator that is important in maintaining the low pressure in the normal pulmonary vasculature (Giad and Saleh, 1995). It is an important vasodilatory determinant of vascular resistance and vascular tone through the reduction of pulmonary arterial pressure (Sprague et al., 1996; Xu et al., 2007). It modulates pulmonary vasoconstriction and pulmonary hypertension through reducing the resistance to blood flow through lungs by relaxing vascular smooth muscle, modulating vasoconstrictor (serotonin) release, desensitizing endothelial responsiveness to vasoconstrictors, inhibiting pulmonary arterial smooth muscle cell proliferation and platelet aggregation in precapillary arterioles, and protecting against hypoxia-induced vasoconstriction (Bowen et al., 2006; Giad and Saleh, 1995; Sprague et al., 1996; Budhiraja, Tuder, and Hassoun, 2004; Wideman and Hamal, 2011; Wideman et al., 2007; Chapman and Wideman, 2006; Burnstock, 1998; Wideman et al., 1994). In broilers, nitric oxide also reduces the pulmonary vascular resistance by dilating the

vasculature and attenuating its responsiveness to endothelium dependent vasoconstrictors (Bowen et al., 2006). Nitric oxide is synthesized in the vascular endothelium by either constitutive (endothelial) or inductable (inflammatory) forms of nitric oxide synthase (Bowen et al., 2006; Sprague et al., 1996; Xu et al., 2007; Burnstock, 1998). Whereas the nitric oxide synthesized by endothelial nitric oxide synthase is produced in short bursts at low but effective concentrations, that synthesized by inflammatory nitric oxide synthase is produced in high quantities over a longer period of time before biologically effective concentrations can be produced (Bowen et al., 2006). Most of the nitric oxide generated *in vivo* is produced by endothelial nitric oxide synthase in response to shear stress on the endothelium, consequent to the increased viscosity and/or flow rate during the idiopathic pulmonary arterial hypertension response (Sprague et al., 1996). Specifically, it is the mechanical deformation of red blood cells leading to the release of ATP that is the primary stimulation of nitric oxide synthesis (Sprague et al., 1996). It is this relationship that results in the delivery of nitric oxide to peripheral pulmonary vasculature (Sprague et al., 1996). When the diffusion limitation occurs in which hemoglobin does not fully acquire oxygen due to increased flow rate of blood through the pulmonary vasculature, hemoglobin collects nitric oxide in the lungs and delivers it to the peripheral vasculature thus reducing vasoconstriction (Sprague et al., 1996). Overall, it is the release of ATP in response to deformation of red blood cells that is the basis for vasodilatory effects of nitric oxide that counteract the vasoconstrictive effects of serotonin (Sprague et al., 1996).

**ATP and ATPase**

ATP is a neurotransmitter as well as a nucleotide that can act both as an intracellular energy source and an extracellular signaling tool in blood vessels (Sprague et al., 2003; Zhang et al., 2004; Burnstock, 2006). It plays a significant role in transmitting signals from the neuron to the smooth muscle through a purinergic signaling system (Vizi and Burnstock, 1988; Burnstock, 2006). Essentially, a purinergic signaling system uses purine nucleotides as extracellular messengers released to mediate short term (acute) signaling functions in secretion, vasodilatation, and vascular tone, and long term (chronic) signaling functions in cell proliferation, differentiation, and death (Burnstock, 2006; Zhang et al., 2004). The role that ATP has on manipulating the vasculature depends largely on the site of release. This is due in part to different purinergic receptors present in different locations in the vasculature (in pulmonary arterial smooth muscle cells or the endothelium) (Sprague et al., 2003; Sprague et al., 1996). Depending on the site of ATP release (either luminally or abluminally), the interaction of ATP with receptors can result in either vasoconstriction or vasorelaxation in the pulmonary vasculature (Sprague et al., 2003). Specifically, ATP released abluminally from nerve terminals onto pulmonary arterial smooth muscle cells would interact with $P_{2x}$ purinergetic receptors producing vasoconstriction, whereas ATP released within the vascular lumen (intraluminally) would interact on the P2Y receptors on the endothelial cells, leading to vasodilatation (Sprague et al., 2003; Sprague et al., 1996).

ATP applied to the luminal side of a vessel is released from the red blood cells within the circulation (Sprague et al., 1996). These red blood cells normally contain

large amounts of ATP and release it in response to physiological stimuli such as deformation due to changes in flow rates, shear stress, or hypoxia (Sprague et al., 1996). When this ATP binds to the endothelial P2Y receptor, the synthesis of endothelium-derived relaxing factors—such as nitric oxide, a vasodilator—is initiated (Sprague et al., 1996; Sprague et al., 2003). Red blood cells can evoke nitric oxide synthesis in the vascular endothelium with only millimolar amounts of ATP, making ATP a crucial mechanism for decreasing vascular resistance and balancing vascular tone in normal pulmonary vasculature (Sprague et al., 2003). However, the red blood cells of humans with idiopathic pulmonary arterial hypertension fail to release ATP in response to mechanical deformation over successive increases in flow (Burnstock, 1998; Burnstock, 2006). This results in a decrease in the synthesis and production of nitric oxide, and thereby the excessive vasoconstriction remains unbalanced by vasorelaxation (vasodilatation) (Burnstock, 2006).

Whereas ATP applied luminally to P2Y receptors causes slow vasodilatation through the synthesis of nitric oxide, ATP applied abluminally (extracellularly) quickly enhances vasoconstriction (Sprague et al., 1996; Burnstock, 2006). Extracellular ATP is a potent vasoconstrictor and smooth muscle and endothelial cell mitogen (Zhang et al., 2004). It can be released from nerve terminals near vascular smooth muscle in vascular endothelial cells, damaged vessel walls, hypoxic myocardium, or aggregated platelets (Sprague et al., 2003). From here, ATP acts on P2X receptors, causing an influx of $Ca^{2+}$ which leads to a rapid vasoconstriction and increase in vascular resistance (Burnstock, 1999; Burnstock, 2008; Sprague et al., 2003). Like intracellular ATP responses, only small levels (millimolar amounts) of extracellular ATP are needed to play a significant

role in pulmonary arterial smooth muscle cell proliferation (Zhang et al., 2004). After a small level of extracellular ATP activates the $P_{2x}$ receptor on the plasma membrane of the pulmonary arterial smooth muscle cell, phosphorylation activates cyclic AMP response element binding protein, which increases the expression of canonical transient receptor potential, and this is what raises the $Ca^{2+}$ concentration leading to pulmonary arterial smooth muscle cell proliferation (Zhang et al., 2004; Burnstock, 2002).

Extracellular ATP not taken up by the purinergic receptors is rapidly metabolized by ecto-nucleotidases (ecto-ATPase; ATPase activity) (Vizi and Burnstock, 1988). The products of ATP breakdown include adenosine di-phosphate (ADP) and a free phosphate ion ($P_i$), or adenosine mono-phosphate (AMP) and two free phosphate ions (Sprague et al., 2003). Of these products, ADP is capable of activating endothelial purinergic receptors and causing vasodilatation, and adenosine inhibits smooth muscle cell proliferation (Sprague et al., 2003; Burnstock, 2002). Some research also suggests that the amount of ATPase increases extracelluarlly in response to shear stress (Yegutkin et. al, 2009). These effects of ATPase suggest its role as an important tool in normal vascular functioning to maintain the balance between vasoconstriction and vasodilatation.

Although there is minimal research on the role of ATPase in the pathology of idiopathic pulmonary arterial hypertension, its increase in response to shear stress would suggest that under conditions of decreased vessel diameter (due to plexiform lesions) and increased flow resistance—characteristic of idiopathic pulmonary arterial hypertension—extracellular ATPase would be increased. Thus, when deformed red blood cells are releasing ATP intracelluarly to trigger vasodilation, extracellular ATPase is also released to break down ATP that would act to increase vasoconstriction. Furthermore, the

products of the breakdown would act against the progression of the disease by combating the vasoconstrictive effects of extracellular ATP by triggering vasodilatory effects with ADP and act to prevent further proliferation of the endothelia.

**Wedge Pressures as a Determinant of Idiopathic Pulmonary Arterial Hypertension**

Wedge pressures estimate the downstream pressure in the pulmonary vasculature (Lorenzoni et al., 2008). These estimations are essential in differentiating between pulmonary venous hypertension and pulmonary arterial hypertension (Lorenzoni et al., 2008). Wedge pressures are found by inserting a catheter into a wing vein and advancing it into a pulmonary branch artery until it becomes lodged in a terminal artery (Chapman and Wideman, 2000). In broilers susceptible to idiopathic pulmonary arterial hypertension, wedge pressures higher than the right atrial pressures but lower than the pulmonary arterial pressure are suggestive of excessive precapillary resistance (Chapman and Wideman, 2000; Lorenzoni et al., 2008; Wideman and Hamal, 2011).

Within the larger scope of this project, wedge pressures will be taken in the broilers of both the high and low tryptophan groups in order to confirm their susceptibility to idiopathic pulmonary arterial hypertension. This data along with wire myography data (which measures vasoconstriction as a function of tension) of pulmonary artery, 1A and 2A pulmonary arteriole responses to ATP, and ATPase data will be useful in determining whether or not ATP plays an important role in the development of idiopathic pulmonary arterial hypertension in broilers. The overall aim of this study is to learn more about the mechanisms leading to increased pulmonary arterial pressure, plexogenic arteriopathy, and ultimately idiopathic pulmonary arterial hypertension in broilers and humans.

# Chapter 3:

# METHODOLOGY

The role of ATPase activity in the vascular wall was investigated through the use of male broilers susceptible to idiopathic pulmonary arterial hypertension as a model. Broilers were reared at the Poultry Environmental Research Laboratory at the University of Arkansas Poultry Research Farm. They were kept in environmental chambers with wood shavings for litter, a constant thermoneutral chamber, rapid exchange of ambient air, etc. The broilers were separated into two groups, differing only in diet. The diets differed in the levels of tryptophan, a necessary precursor and rate-limiting step for the production of serotonin. In the control group, broilers were provided with a commercial diet (Cobb-Vantress Chick Starter, Siloam Springs, Arkansas) with approximately 0.22% tryptophan by weight with 2.2 tryptophan/kg feed (Corzo et al., 2005). The experimental group was fed the same base diet (Cobb-Vantress Chick Starter, Siloam Springs, Arkansas) with four times the recommended total tryptophan need supplemented in (0.88% by weight). These diets were continued from day one of hatching through eight weeks of age (or until when euthanized).

At four to five weeks of age, the broilers were euthanized in order to remove the lungs and examine the pulmonary vasculature. After euthanization at the University of Arkansas Poultry Research Farm, the lungs were put on ice and transported to the laboratory in the HPER, where the experiments took place. The lungs were dissected to remove a portion of the pulmonary artery, 1A arteriole, and 2A arteriole (making sure to remove blood within the vessels). These vessels were assessed for the activity of the enzymes that break down ATP (ectoATPase, ATPase). Specifically, the amount of

ATPase activity was quantified through the use of a QuantiChrom ATPase Assay (BioAssay Systems, Hayward, California). This ATPase Assay detected amounts of free phosphate ion ranging from 2 pmoles of phosphate to 2,000 pmoles of phosphate. Because extracellular ATP was either translocated into the endothelium or broken down into ADP + $P_i$ or AMP + $P_iP_i$, the amount of phosphate ion was related to the ATPase activity.

**QuantiChrom ATPase Assay**

In a clear bottom 96-well plate, phosphate standards were prepared by using a premix solution of 50 μM phosphate diluted down to create eight 40 μL standard duplicates, ranging from 0 μM to 50μM phosphate. A series dilution of enzyme was performed by setting up reaction wells with 20 μL assay buffer, 10μL 4mM ATP, and half of a vessel (both to create a duplicate and because the whole vessel had too much phosphate for the assay to quantify accurately). Duplicates of the reaction wells were set up next to control wells of 20 μL assay buffer, 10 μL distilled water, and 10 μL 4mM ATP. This ATP was added in order to assess the amount of ATPase activity of the vessels in response to the same amount of ATP (4mM ATP) per reaction well. After the standard wells, reaction wells, and control wells were prepared, 200 μL QuantiChrom reagent was added to each well. After a one minute of mixing on a plate shaker and 5 minute incubation at 37°C to allow for color development, the vessels were removed with tweezers, labeled, and frozen in excess NaCl solution (pH 7.4). Finally, using a spectrophotometer (Bio-Tek, Winooski, Vermont) set at 620nm, the absorbance was measured and recorded. Using the linear regression equation from the standard curve, the

μM concentration of phosphate, representative of the amount of ATPase activity in each pulmonary artery, 1A arteriole, and 2A arteriole was calculated.

After the assessment of the μM concentration of phosphate ion in each of the three vessels for each one of 20 broiler chickens, the vessels were prepared for quantification of the amount of protein in the vessels. The protein assays were necessary to normalize the data for the differing sizes of the vessels.

**Preparation of Vessels for Protein Assays**

The stored frozen vessels from the ATPase assay were thawed in a water bath at 37°C for 5 minutes. Then, each vessel was transferred into a grinding tube (one at a time) in 375μL NaCl solution (7.4 pH). Using a power drill with a textured glass rod flared at the end to fit tightly inside of the grinding test tube, the vessels were ground down until the NaCl solution had no large aggregates (i.e. the vessels were ground down to a cellular level). The ground vessel solution was transferred 100 μL at a time into a clean tube. After three minutes of centrifugation, the vessels were frozen for further the protein analysis.

Due to the size differences between the pulmonary artery and the arterioles, two assays had to be used to quantify the protein concentrations of each. The Coomassie Protein Assay (Thermo Scientific, Waltham, Massachusetts) was used to determine the protein concentrations of the pulmonary arteries because of its accuracy in detecting higher amounts of protein (accurately measures 1 μg/mL to 1500 μg/mL protein concentration range), and the Micro BCA Protein Assay (Thermo Scientific, Waltham, Massachusetts) was used to determine the protein content in the 1A and 2A arterioles due to its accuracy in detecting the lower ranges of protein (accurately measures 0.5 μg/mL to

20 μg/mL protein concentration range). Different assays were run on the different vessels because the protein concentrations of the 1A and 2A arterioles were too small to be detected by the Coomassie Assay, whereas the protein concentrations of the pulmonary arteries were too large to be detected by the Micro BCA Assay (because the pulmonary arteries were larger than the arterioles). Before either assay was run on the vessel solution, the samples were thawed in a 37 °C water bath for 5 minutes. Afterwards, the samples were centrifuged for 3 minutes to allow the larger precipitants fall to the bottom of the vial. It was important to only use the top supernatant as the sample for the protein assays.

**Coomassie Protein Assay—Pulmonary Artery**

In a clear bottom 96-well plate, nine duplicate 5 μL diluted albumin (BSA) standards, between 0 μg/mL and 2,000 μg/mL protein concentration were prepared using an albumin standard (2.0 mg/mL in a solution of 0.9% saline and 0.05% sodium azide). Then, 5 μL of sample (from the ground pulmonary vessel in excess NaCl solution) was transferred to the sample wells. Once each standard and sample well had 5 μL of either standard or sample respectively, 250 μL of Coomassie Reagent was added to each well. After 60 seconds of mixing on a plate shaker, the absorbance was measured using a spectrophotometer (Bio-Tek, Winooski, Vermont) at 595 nm. Using the regression equation of the standard curve, the protein concentrations of the pulmonary vessels were determined (μg/mL).

**MicroBCA Protein Assay—1A and 2A arterioles**

In a clear bottom 96-well plate, nine duplicate 150 μL diluted albumin (BSA) standards, between 0 and 200 μg/mL protein concentration were prepared using an

albumin standard (2.0 mg/mL in a solution of 0.9% saline and 0.05% sodium azide). Then, 150 μL of sample (the ground 1A arteriole or 2A arteriole solution in excess NaCl solution) was transferred into the sample wells. After, 150 μL Working Reagent Concentrate (25:24:1, Working Reagent MA: MB: MC) was added to each well. After one minute of mixing on a plate shaker, two hours of incubation at 37 °C for the color development, and five minutes of cool down to room temperature, the absorbance was measured with a spectrophotometer (Bio-Tek, Winooski, Vermont) at 562 nm. Using the regression equation of the standard curve, the protein concentrations of the 1A arteriole and 2A arteriole vessels were determined (μg/mL).

After the concentrations of phosphate ion (μM) and protein (μg/mL) were collected for each vessel of the 20 broilers, the data was normalized for vessel size (giving μM of phosphate/μg of protein per mL). Once the data was normalized, statistical analyses were run (e.g. one way repeated measures tests).

# Chapter 4:

# RESULTS

## Effect of Diet on ATPase Activity in Vessels by Type

There are no statistically significant differences between the two diet groups in any of the vessel types. Results did not show an effect between the diets and ATPase activity in the vessels (overall p=0.0797). Upon visual inspection of Figure 1, it appears that high tryptophan had greater ATPase activity in the pulmonary artery; however this relationship was opposite in the 1A and 2A arterioles. When analyzed alone, this flip flop trend has a p>0.05, suggesting that it is not a statistically supported relationship. On its own, although the 2A arteriole appears to have a difference between the diets upon looking at Figure 1, when a pairwise comparison was run to look only at the 2A activity between high and low tryptophan diets, there was no statistical difference with a p value >0.05 (=0.0830).

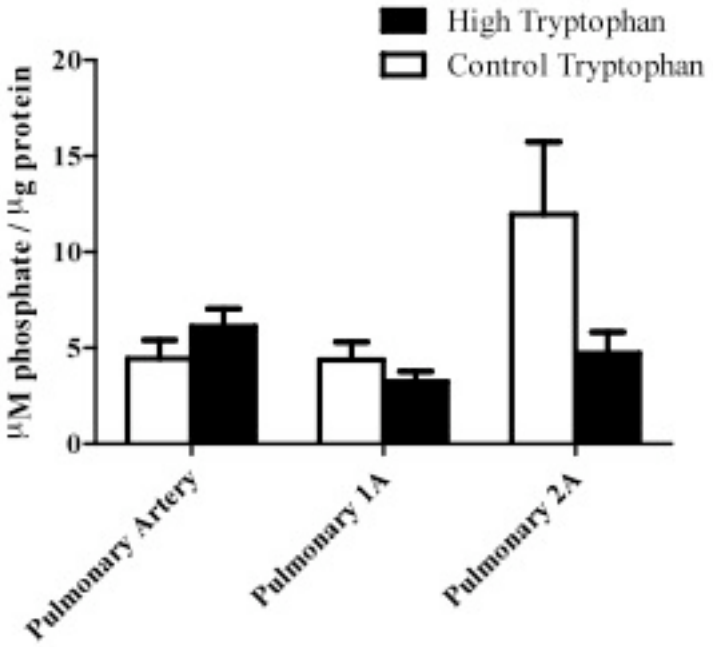


**Figure 1: ATPase Activity of Vessels by Diet**
There is no statistical difference in ATPase activity by vessel type (pulmonary artery, pulmonary 1A arteriole, or pulmonary 2A arteriole) for either the high tryptophan diet group or the control diet group (n=20 for all vessel types). Bars indicate S.E.M.

**ATPase Activity Vessel Comparison**

Overall, the comparison of ATPase activity between vessel types was a statistically sound one ($p=0.0181$ for the overall test), meaning that the overall test was statistically significant ($p<0.05$). Between vessel types, only the comparison between the 2A arteriole and the 1A arteriole revealed a statistical difference where the 2A arteriole had a greater ATPase activity than the 1A arteriole ($p<0.05$; $p=0.0165$; Figure 2; *). There were no statistical differences between the pulmonary artery with either the 1A arteriole ($p=0.0746$) or with the 2A arteriole ($p=0.1249$).

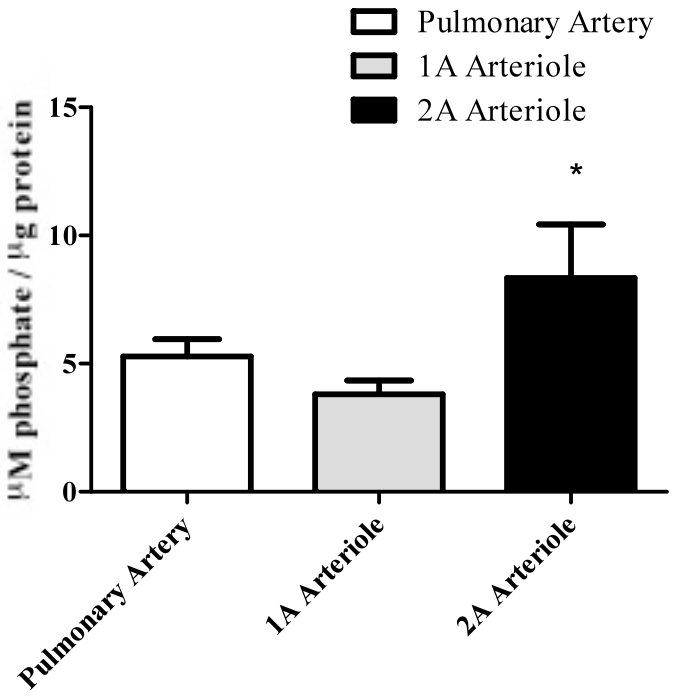


**Figure 2: ATPase Activity by Vessel Type**
There is no statistical difference in ATPase activity between the pulmonary arteries and either the 1A arterioles or the 2A arterioles (n=20 for all vessel types). However, there is a difference (*) between the 1A arterioles and 2A arterioles. Bars indicate S.E.M.

**Phosphate Variability in Vessel Types by Diet**

The amount of variability was examined in of the amounts of phosphate measured in the pulmonary arteries, 1A arterioles, and 2A arterioles in both the control and high tryptophan diets. Looking at Figure 3—showing the means and outliers of each group of vessels by diet—it appears that the 2A arterioles of the control diet have an extremely

varied range. Table 1, however, shows that the 1A arterioles of both diets have the most outliers statistically. Both show the trend that the pulmonary arteries have the most phosphate measured, followed by the 1A arterioles and then the 2A arterioles. Figure 3 also shows that the outliers of the vessel types overlap into ranges indicative of different vessel types.

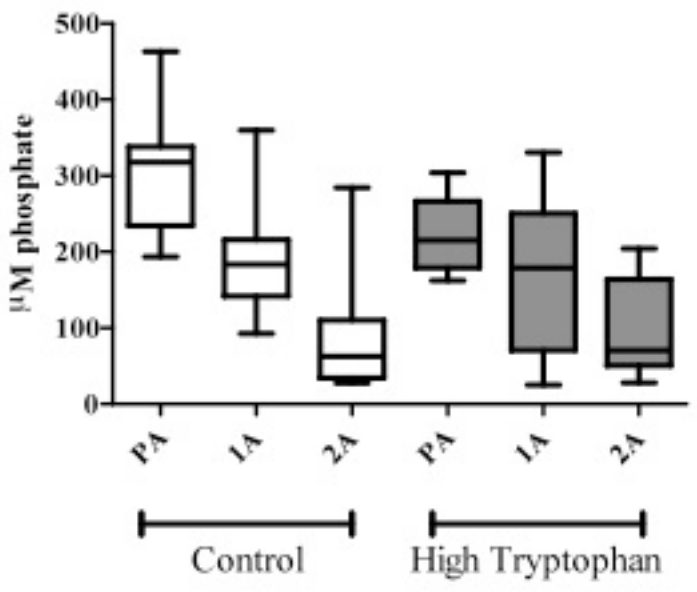


**Figure 3: Variability in Phosphate Measured by Diet and Vessel Type** The variability of the 2A arterioles appears to be the highest, though all groups show some outliers. (PA = Pulmonary Artery; 1A = 1A arteriole; 2A = 2A arteriole).

**Table 1: Phosphate Mean, Standard Deviation of Mean, Outliers for Vessel Types by Diet**

| | | **Pulmonary Artery** | **1A Arteriole** | **2A Arteriole** |
|---|---|---|---|---|
| **Control Diet** | | | | |
| | **Mean** | 301.8 µM | 188.3 µM | 88.6 µM |
| | **Std. Dev. Of Mean** | 25.3 | 23.4 | 25.4 |
| | **Number of Outliers** | 2 | 3 | 2 |
| **High Tryptophan Diet** | | | | |
| | **Mean** | 223.0 µM | 172.2 µM | 101.2 µM |
| | **Std. Dev. Of Mean** | 15.7 | 31.9 | 20.7 |
| | **Number of Outliers** | 3 | 8 | 2 |

**Protein Variability in Vessel Types by Diet**

The amount of variability in the protein measured in each vessel type by diet is shown in both Figure 4 and Table 2. Figure 4 shows that the range of protein measured in the control pulmonary arteries is the largest. Although Table 2 shows that both the 1A and 2A arterioles in the control diets have the most outliers, they are shown in the box-whisper figure to have a smaller range than the pulmonary arteries. Both the table and figure show the trend of decreasing protein concentration from the pulmonary arteries to the 1A arterioles and then the 2A arterioles. This suggests that the pulmonary arteries are the largest and the 2A arterioles are the smallest.

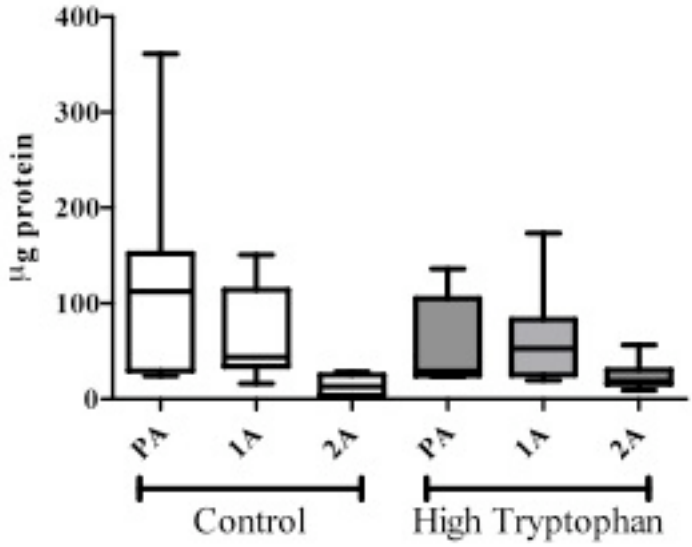


**Figure 4: Variability in Protein Measured by Diet and Vessel Type**
The variability of pulmonary arteries appears to be the highest in the control diet of all the groups, followed by the 1A arterioles in the high tryptophan diet group. (PA = Pulmonary Artery; 1A = 1A arteriole; 2A = 2A arteriole).

**Table 2: Protein Mean, Standard Deviation of Mean, Outliers for Vessel Types by Diet**

| | | **Pulmonary Artery** | **1A Arteriole** | **2A Arteriole** |
|---|---|---|---|---|
| **Control Diet** | | | | |
| | **Mean** | 116.3 µg | 63.4 µg | 13.7 µg |
| | **Std. Dev. Of Mean** | 31.8 | 14.7 | 3.3 |
| | **Number of Outliers** | 1 | 4 | 4 |
| **High Tryptophan Diet** | | | | |
| | **Mean** | 53.8 µg | 62.9 µg | 24.2 µg |
| | **Std. Dev. Of Mean** | 13.9 | 14.4 | 4.3 |
| | **Number of Outliers** | 3 | 2 | 2 |

# Chapter 5:

# DISCUSSION

The goal of this specific study was to examine the role of ATPase activity in different pulmonary vessels (pulmonary artery, 1A and 2A arterioles) within the lung vasculature of broilers susceptible to idiopathic pulmonary arterial hypertension. Furthermore, the effect of dietary differences was examined in relation to the ATPase activities of these vessels. According to this study's results, although there is no statistical difference in how the diets affected ATPase activity, there was a trend reversal seen between the pulmonary artery and the arterioles. The arterioles in the high tryptophan diet appeared to have less ATPase activity than those in the control diets. This would suggest that diets higher in tryptophan would lead to a worse case of pulmonary arterial hypertension characterized by a lower ATPase activity. Although this trend appeared in the arterioles, it was the opposite in the pulmonary artery. Furthermore, when comparing ATPase activities of the different vessels without regard to the diets, a statistical difference was found between the ATPase activities of the 1A and 2A arterioles, but not between the pulmonary arteries with either of the arterioles. These combined results may suggest that pulmonary arteries perhaps do not have much contribution to the development of idiopathic pulmonary arterial hypertension. The difference found between the 1A and 2A arterioles was interesting because there was more ATPase activity in the 2A arterioles. This may have been due to their smaller anatomy than the 1A arterioles, causing more ATPase activity due to the shear stress caused by the movement of fluid through a smaller diameter. On the other hand, it may also suggest that they were breaking down ATP in greater concentrations than the 1A arterioles.

Perhaps this is an adapted mechanism to prevent increases in pulmonary arterial pressure in the smaller vasculature through a higher metabolism of ATP.

With regard to the larger study, vascular functioning data further suggest that pulmonary arteries perhaps do not have much contribution to the increased pulmonary arterial pressure and pulmonary vascular resistance related to the development of idiopathic pulmonary arterial hypertension. Specifically, their lack of vasoconstriction to stimulation by ATP suggests the lack of purinergic receptors or lack of sensitivity to ATP acting on the vascular walls. In contrast, the wire myography data showed increased vasoconstriction due to ATP in both the 1A and 2A arterioles regardless of feed type, possibly implicating the presence of purinergic receptors. Specific to the 1A arterioles, the higher levels of vasoconstriction along with a decreased ATPase activity compared to the 2A arterioles further implicate the presence of these purinergic receptors. This is because in order to have a high level of vasoconstriction less ATP needs to be broken down by ATPase so it can act on the vascular walls of pulmonary smooth muscle cells. This may also suggest that the 1A arterioles play an important role in the development of increased pulmonary arterial pressure and pulmonary vascular resistance. This is an interesting contrast to the 2A arteriole situation that has more ATPase activity and less contraction than the 1A arterioles. Less contraction caused by higher ATPase activity prevents more ATP from binding and causing vasoconstrictive effects. It may be possible that this increased ATPase activity is a preventative measure used by 2A arterioles to avoid excessive pulmonary arterial pressure and pulmonary vascular resistance leading to pulmonary hypertension. Perhaps because the 2A arterioles are anatomically smaller than the 1A arterioles, they have increased metabolism of

extracellular ATP as a means to prevent vasoconstriction in this smaller vessel. This may be a mechanism acting to prevent excessive constriction in the smaller vessels within the pulmonary vasculature in order to be proactive against the effects of pulmonary hypertension. The differences between the 1A and 2A arterioles may be due to different receptor responsiveness or different mechanisms of the use of ATP. Overall however, the difference between them may implicate that the 1A arterioles play a more important function in the development of pulmonary hypertension than the 2A arterioles.

In order to make clear that these broilers had developed pulmonary hypertension or were susceptible to pulmonary hypertension, wedge pressures were measured as part of the larger project. They were able to detect the earliest changes associated with pulmonary hypertension through the direct measurement of pulmonary arterial pressure (Lorenzi, Anthony, and Wideman, 2008). Data collected indicated that broilers in the high tryptophan group were more susceptible to idiopathic pulmonary arterial hypertension (due to increases in pulmonary arterial pressure) than those in the control group. These results may suggest that the broilers fed higher levels of tryptophan synthesized more serotonin, which in turn, caused more vasoconstriction and higher pulmonary arterial pressures than in the control group. This also supports the hypothesis that higher tryptophan leads to a worse case of idiopathic pulmonary arterial hypertension. However, although all broilers in the high tryptophan diet group had higher pulmonary arterial pressures than those in the control group, not all met the clinical level of >25mmHg indicative of idiopathic pulmonary hypertension. Regardless, broilers with high pulmonary arterial pressures had low wedge pressures, confirming the upstream, precapillary vasoconstrictions characteristic of pulmonary arterial

hypertension, as opposed to pulmonary venous hypertension (Chapman and Wideman, 2000; Wideman and Hamal, 2011). Though not all of the broilers developed high pulmonary arterial pressure, the wedge pressures confirmed that these broilers were susceptible to idiopathic pulmonary arterial hypertension in accordance with previous research. This wedge pressure data further enhances the trend seen in the arterioles of the high tryptophan animals. Less ATPase activity in the arterioles further implies a more severe case of hypertension.

**Limitations and Variations in Data**

Despite revealing a possible important mechanism leading to increased arteriolar vascular resistance and eventual development of idiopathic pulmonary arterial hypertension in broilers, there were some limitations to this research project. In the QuantiChrom ATPase Assay, the vessels had to be split in half due to overdevelopment of the reagent in a short period of time, possibly leading to the release of excess ATP from the endothelia. Furthermore, although the goal was to identify role of ATPase activity on the surface of pulmonary arterial smooth muscle cells, because the endothelia of the vessels was not removed, its role in the production of ATP cannot be ruled out. This excess ATP would have added to the amount of ATP in the assay, which when broken down and measured by its phosphate, would inaccurately represent the amount of ATPase activity present. However, previous research suggests that these amounts of ATP by the endothelia would be minimal (Sprague *et al.*, 2003). Furthermore, there were challenges imposed by the vessels themselves. For example, there was much variability among the vessels, especially the 1A arterioles, possibly leading to decreased accuracy in statistical tests (many $p>0.05$). Some vessels also had blood still attached, despite

attempts to remove it all before assays were run. This blood could have led to increases in the amount of ATP being broken down by ATPase activity, thus increasing the measured phosphate levels.

All three vessel types were initially analyzed for protein content using the Coomassie Assay, however, the protein concentrations of some 1A arterioles and all of the 2A arterioles were too small to be detected by this assay. Therefore, the arterioles were analyzed for protein using the Micro BCA Protein Assay, which is more sensitive to lower levels of protein. The reason that the pulmonary arteries were not assessed using the Micro BCA Assay, was that their sample ran out due to the larger required amounts of sample to run the Coomassie Assay in duplicates. Unfortunately, adequate samples of the pulmonary artery solutions were not available to run the MicroBCA protein assay. The problem with using different assays is that there is not continuity in the means of measurement, reducing result credibility. Finally, excess blood remaining in or on the vessels could have increased the amount of protein, thus misrepresenting phosphate/protein levels and therefore ATPase activity within the vessels. An attempt was made to remove all of the excess blood from the vessels before any assays occurred.

There was a lot of variation between the vessels themselves. Such variation likely took away from statistical validity of many of the tests. Some possible sources of variation in the vessels, especially in the 1A and 2A arterioles, occurred anatomically. For example, as the broilers aged their vessels gained fat pockets that caused the vessels to float and retain more blood—thus altering the amount of protein and possibly phosphate measured in the assays leading to outliers and a large range of data for each

vessel type. It was likely a combination of both variation in the phosphate and protein data that affected the lack of statistical validity to differences between vessel type.

**Conclusion**

Overall, this research implicates the roles of the 1A and 2A arterioles and ATPase activity in the pathology of idiopathic pulmonary arterial hypertension in the broiler model. Unfortunately, there is no previous research to compare this to; however, these results suggest an exciting new area that may be related to the development of increased pulmonary arterial pressure. In the future, research should look into the mechanism behind ATP in the 1A and 2A arterioles, as it may be an important element in the development of idiopathic pulmonary arterial hypertension. Such research is needed to determine whether or not ATP is acting on purinergic receptors on the walls of broiler pulmonary arterial smooth muscle cells or due to other mechanisms entirely.

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