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OXIDATIVE STRESS AND THE DEVELOPMENT OF ENDOTHELIAL DYSFUNCTION IN CONGENITAL HEART DISEASE WITH INCREASED PULMONARY BLOOD FLOW: LESSONS FROM THE NEONATAL LAMB

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Abstract

Congenital heart diseases associated with increased pulmonary blood flow commonly lead to the development of pulmonary hypertension. However, most patients who undergo histological evaluation have advanced pulmonary hypertension, and therefore it has been difficult to investigate aberrations in signaling cascades that precede the development of overt vascular remodeling. The purpose of this review is to discuss the role played by oxidative and nitrosative stress in the lung and their impact on the signaling pathways that regulate vasodilation, vessel growth and vascular remodeling in the neonatal lung exposed to increased pulmonary blood flow.

A lamb model of post-natal increased pulmonary blood flow

The presence of a large systemic-to-pulmonary communication, such as that seen in infants with truncus arteriosus, an aortopulmonary window, or ventricular septal defects, results in increased pulmonary blood flow (PBF). The outcome of this anomalous postnatal hemodynamic state is a progressive structural and functional disruption of the normal development of the pulmonary vascular bed (Heath & Edwards 1958). If not corrected, these vascular changes result in obliteration of the pulmonary vascular bed and death secondary to severe cyanosis and right heart failure. Early surgical intervention can prevent the development of pulmonary vascular disease; however, children still suffer significant morbidity and mortality in the perioperative period due to acute and sustained elevations in PBF. Although the chronic changes in vascular morphology are well described, the early determinants of the increased vascular reactivity that occurs prior to overt vascular remodeling remain incompletely understood.

In children with CHD with left-to-right shunts, several alterations in the pulmonary vasculature occur, including a delay in the normal fall in pulmonary vascular resistance

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(PVR). However, attempts at producing an animal model of increased PBF in the postnatal animal including monocrotaline injection followed by the creation of an abdominal aortacaval shunt in rats failed to simulate the conditions of CHD as the systemic-to-pulmonary graft was not present during the transition from fetal to neonatal PBF (Fasules et al 1994). This was a crucial omission given the dramatic changes in vascular tone, vascular function, and gene expression that occur during this transitional circulation. To alleviate these issues over a decade ago we developed a lamb model of CHD with increased PBF (Shunt) by surgically introducing an aorto-pulmonary anastomosis in the **fetal** lamb at approximately 135–140 days of gestation (term = 145–150 days). With the use of fetal surgical techniques and side biting vascular clamps, an 8 mm aorto-pulmonary shunt is placed between the ascending aorta and the main pulmonary artery (Reddy et al 1995). This does not change fetal hemodynamics (Reddy et al 1995). However, in the postnatal period, these lambs display morphological and physiological features that mimic the human disease. At 1 month of age, these lambs fail to thrive, have elevated pulmonary artery pressure (PAP) associated with an increase in PBF ($Q_p:Q_s$, ~2.5–3:1), and increased left and right atrial pressures (Reddy et al 1995). Within a few days after birth a number of signaling pathways are altered in the lung due to the increased PBF. Importantly, this model has enabled us to evaluate the early signaling abnormalities that lead to the development of the endothelial dysfunction that precedes overt vascular remodeling. This has proven to be a major strength of this model compared to the rodent models of PH induced by monocrotaline injection or exposure of chronic hypoxia in the presence or absence of VEGF receptor antagonism where studies have focused on the later stages of the disease process. Interestingly, the disruption of redox environment appears to be the common mechanism underlying the early derangements in signaling pathways we have elucidated in our model. The following sections will discuss the factors that generate oxidative and nitrosative stress in the lung and their impact on the signaling pathways that regulate vasodilation, vessel growth and vascular remodeling.

1. Endothelial nitric oxide synthase

A. Overview

Nitric oxide (NO) is mostly produced by endothelial nitric oxide synthase (eNOS) and is critical for maintaining vessel wall homeostasis, blood flow, and smooth muscle cell (SMC) relaxation. Endothelial NOS is a homodimer with each monomer containing a C-terminal reductase domain, with binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN), linked to an N-terminal oxygenase domain, containing binding sites for Ca^{2+} /Calmodulin, a heme prosthetic group, molecular oxygen, L-arginine, and (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4). Endothelial NOS catalyzes the oxidation of the guanidino nitrogen of L-arginine to yield L-citrulline and NO and during the catalytic cycle, the redox equivalents from NADPH can become “uncoupled” from the generation of NO and instead generate superoxide (O_2^-). This uncoupling of eNOS is a significant contributor to the vascular dysfunction in pulmonary hypertension (PH) as uncoupling not only decreases NO generation, the O_2^- formed can also decrease the bioavailability of NO by binding rapidly to form the reactive nitrogen species (RNS), peroxynitrite ($ONOO^-$). There is a developmental increase in eNOS uncoupling and $ONOO^-$ generation in the pulmonary arteries of Shunt lambs over the first two months of life (Oishi et al 2008) correlating with blunted vasodilation in response to the calcium ionophore, A23187 (Steinhorn et al 2001).

B. Arginine metabolism

Endothelial NOS derived superoxide can be formed under conditions in which the substrate, L-arginine becomes limited and L-arginine levels are decreased in Shunt lambs as early as

two weeks after birth (Figure 1) (Sharma et al 2009). There appears to be two mechanisms underlying the decreases in L-arginine: An increase in L-arginine degradation and a reduction in L-arginine generation. The manganese metalloenzyme, arginase, which catalyzes the hydrolysis of L-arginine to L-ornithine and urea is increased in Shunt lambs. While the activity of the caveolar enzymes involved in the recycling of L-citrulline and other L-arginine byproducts back to L-arginine: ornithine transcarbamylase, argininosuccinate synthetase (ASS), and argininosuccinate lyase (ASL) are attenuated (Sharma et al 2009). Further, the increased arginase activity is associated with increased formation of polyamines and L-proline, which has been shown to promote SMC growth and collagen synthesis (Maarsingh et al). Therefore, the up-regulation of arginase activity could promote aberrant pulmonary vascular remodeling and neointima formation in later stages of PH.

In addition, to reductions in L-arginine, asymmetric dimethyl-L-arginine (ADMA), an endogenous, L-arginine analogue levels are increased in Shunt lambs (Sun et al). ADMA is the product of asymmetric dimethylation of arginine moieties within proteins through the action of the protein arginine methyltransferases (PRMTs) while ADMA is metabolized to L-citrulline and dimethylamine by the dimethylarginine dimethylaminohydrolases (DDAH). ADMA is a competitive inhibitor of eNOS, competing with L-arginine for the substrate binding site. In the presence of ADMA, the ferrous-dioxy species dissociates from the heme group of eNOS causing the production of superoxide rather than NO. However, the uncoupling effect appears to be dependent on the type of methylated arginine used as L-NAME blocks O_2^- production from eNOS while L-NMMA does not (Pou et al 1999, Xia et al 1996, Xia & Zweier 1997). The importance of L-arginine: ADMA ratio in the development of endothelial function is demonstrated by the fact that the supplementation of Shunt lambs with L-arginine increases NO production and prevents the formation of $ONOO^-$ (Aggarwal et al 2011, Sun et al, 2011).

C. GTP Cyclohydrolase & Tetrahydrobiopterin

Endothelial NOs uncoupling can also occur when the levels of the cofactor, BH_4 are reduced. BH_4 levels are regulated by its *de novo* synthesis and its oxidative degradation. The first and rate-limiting step in the *de novo* biosynthesis of BH_4 involves GTP cyclohydrolase I (GCH1), which converts GTP into 7, 8-dihydroneopterin triphosphate. The subsequent steps are catalyzed by 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) with 6-pyruvoyl-5,6,7,8-tetrahydropterin being formed as an intermediate followed by BH_4 . In the salvage pathway, 6-pyruvoyl-5,6,7,8-tetrahydrobiopterin is converted into sepiapterin by sepiapterin synthase, which is further reduced by SR and dihydrofolate reductase (DHFR) to form BH_4 . BH_4 acts as a 1-electron donor during the reductive oxygen activation at the eNOS active site forming the trihydrobiopterin radical ($BH_3\cdot$). This can be recycled to BH_4 by eNOS itself using an electron from the reductase domain. The direct role of BH_4 depletion in the progression of PH is seen in the hyperphenylalaninemic mutant mice which display a 90% reduction in GCH1 activity, decreased BH_4 and NO levels, and a pulmonary hypertensive phenotype (Nandi et al 2005) while the BH_4 precursor, sepiapterin improves NO signaling in PAEC isolated from fetal lambs with persistent pulmonary hypertension of the newborn (Teng et al, 2011). Similarly, sapropterin dihydrochloride improves the oxygenation in patients with PH (Robbins et al, 2011). In Shunt lambs, there is a decrease in BH_4 levels by 2-weeks of age (Sun et al, 2011). This decrease appears to be due to both oxidative depletion of BH_4 to BH_2 and decreased biosynthesis (Sun et al, 2011), as L-arginine supplementation prevents the attenuation of BH_4 levels, presumably by decreasing peroxynitrite generation and the subsequent oxidation of BH_4 . Furthermore, reduced BH_4 levels correlate with decreased GCH1 protein levels (Sun et al, 2011).

D. Heat Shock Protein 90 & Caveolin-1

Recent investigations have identified a new molecular pathway regulating GCH1 protein through its interaction with heat shock protein 90 (Hsp90). Hsp90 is a member of a molecular chaperone family of proteins that are upregulated in response to stress. Hsp90 is ATP dependent and the ATPase site of the chaperone is responsible for the auto-phosphorylation required to enable Hsp90 to interact with client proteins. Hsp90 increases eNOS activity by facilitating the Ca^{2+} /calmodulin induced displacement of the negative regulator, caveolin-1 from eNOS. Disruption of the Hsp90-eNOS interaction in PH attenuates the NO production and increases eNOS uncoupling and endothelial dysfunction (Pritchard et al 2001). However, the molecular mechanism involved in this process is still unclear. It maybe that upon hsp90 binding there is a change in the structural conformation of eNOS that reduces the likelihood of activated molecular oxygen to escape from the heme. But this is not supported by published data. Further, it is unclear whether decreased eNOS-hsp90 interactions drive the association of eNOS with caveolin-1. Indeed the role of caveolin-1 in regulating NO signaling is complex. Our recent data indicate that caveolin-1 has both a positive compartmentation affect on eNOS that enhances plasma membrane targeting (Tian et al 2010) and appears to be independent of the classic negative regulation caveolin-1 exerts by reducing calcium/calmodulin binding.

E. GTP Cyclohydrolase I & hsp90

In addition to eNOS, Hsp90 also chaperones GCH1 (Sun et al). In Shunt lambs, the interaction of Hsp90 and GCH1 is decreased while the association of GCH1 with Hsp70 and the C-terminus of Hsp70-interacting protein (CHIP) is increased (Sun et al). Hsp70 is involved in both the folding and degradation of Hsp90 client proteins by recruiting CHIP, an E3 ubiquitin ligase (Jiang et al 2003). Once ubiquitinated, proteins are then targeted for proteasomal degradation. GCH1 is poly-ubiquitinated and its proteasomal degradation is increased in Shunt lambs resulting in decreased levels of BH_4 (Sun et al). The mechanism by which Hsp90-GCH1 interactions are attenuated appears to be due to ADMA-mediated increase in mitochondrial dysfunction (Sun et al) and L-arginine supplementation preserves Hsp90-GCH1 interactions and BH_4 and NO levels in Shunt lambs (Sun et al).

2. The mitochondrion

Mitochondrial dysfunction has been documented in a number of diseases including pulmonary hypertension, Alzheimer's disease, cystic fibrosis, ageing, and diabetes, and is characterized by an altered mitochondrial membrane potential ($\Delta\Psi_m$), a shift towards glycolysis (increased lactate, decreased cellular pH), decreased ATP generation, and increased ROS generation. Mitochondria are also a major source of ROS production in the cardiovascular system. Approximately 2–3% of the molecular oxygen utilized by the mitochondria as the terminal electron acceptor for the electron transport chain (ETC) is incompletely reduced to superoxide instead of H_2O (Boveris & Chance 1973). The main sites of mitochondrial derived ROS are complexes 1, 2, 3 of the ETC. Within complex 1, ubiquinone is reduced to ubiquinol, this process is thought to be a major source of ROS and inhibition of this process by rotenone block superoxide production. ROS are also produced by the auto-oxidation of the semiquinone radical formed at complex 3 with O_2 . NO can also inhibit O_2 binding to complex 4 of the ETC via nitrosylation of the heme or by blocking the active site. The result is inhibition of mitochondrial respiration and increased superoxide production. Shunt lambs display several markers of mitochondrial dysfunction, including increased levels of uncoupling protein-2 (UCP-2), decreased levels of the mitochondrial antioxidant, SOD2, and an increased lactate:pyruvate ratio (Figure 1) (Sharma et al 2008) suggesting a shift to a glycolytic phenotype.

In, the presence of excess calories and low cytoplasmic ADP, complex V prevents H^+ from entering the matrix increasing $\Delta\Psi_m$. Ultimately, the increased negative potential prevents further H^+ pumping, stalling the ETC. Upstream of complex V, complexes I–IV continue to leak electrons and produce superoxide. With oxidative phosphorylation attenuated, the cell shifts ATP production to a glycolytic pathway in the cytoplasm. This is known as the Warburg effect (Warburg 1956). This shift from oxidative phosphorylation to glycolysis induces metabolic remodeling and hyperpolarized mitochondria that cannot decrease $\Delta\Psi_m$, an obligate requirement for the induction of apoptosis. Intriguingly, the same mitochondrial hyperpolarization is also present in PASMC isolated from humans with PH (Bonnet et al 2007), which may in part explain the resistance to apoptosis that drives the proliferative vascular remodeling. Treatment of PASMC with antioxidants results in depolarization of the $\Delta\Psi_m$ and apoptosis, suggesting a key role of ROS in the mitochondrial membrane hyperpolarization (Wedgwood & Black 2003).

Mitochondrial dysfunction has also been demonstrated in other models of PAH. In the fawn-hooded rat (FHR) that spontaneously develop PH, mitochondrial dysfunction and hyperpolarization is associated with reduction in ROS production. This decrease in ROS results in a more reduced cellular environment leading to the inhibition of a superoxide sensitive K^+ channel (Kv1.5) and pulmonary vascular constriction (Bonnet et al 2006). However, the redox regulation of Kv1.5 is far from resolved, as oxidation by hydrogen peroxide has been shown to enhance Kv1.5 channel activity in isolated pulmonary artery rings (Schach et al 2007) while in PASMC, oxidation was shown to inhibit the Kv current (Cogolludo et al 2006). In addition, increased levels of ADMA leads to mitochondrial dysfunction and increased mitochondrial derived ROS in PAEC (Sud et al 2008). These findings suggest that mitochondrial dysfunction can result in increased or decreased ROS generation and it is possible that both these events, occurring in different cells within the vessel, is required to promote vascular dysfunction.

3. NADPH Oxidase

The transmembrane NADPH oxidases (Nox) are major sources of ROS in the vasculature. The Nox family consists of seven catalytic homologues: Nox1-5 and Duox1-2, however, only Nox1, Nox2, Nox4, and Nox5 are found in the vasculature. Each Nox isoform requires various regulatory proteins that are essential to its activity such as Rac1, p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox}, NOXO-1, NOXA-1. As their name suggests, these enzymes oxidize NADPH to obtain their source of electrons. Subsequently, the two electrons reduce FAD to FADH₂ and are then transferred one at a time to the inner and outer heme groups. Finally, molecular O₂ acts as the terminal electron acceptor and is reduced by 1 electron to O₂⁻. Nox derived O₂⁻ has been shown to play a key role in vascular dysfunction in many different models of PH. In Shunt lambs, the Nox system is robustly upregulated (Figure 1) (Sharma et al). These lambs exhibit increased levels of the Nox1 and 2 regulatory proteins: p47^{phox} and Rac1 (Sharma et al). In addition, these regulatory proteins are increasingly localized to both the endothelial and SMC layers of pulmonary arteries (Sharma et al). There is also an overall increase in Nox derived O₂⁻ in Shunts at 2- and 4-weeks although this is lost by 8-weeks of age (Sharma et al). Similarly, in a lamb model of persistent pulmonary hypertension of the newborn (PPHN) generated by surgical ligation of the ductus arteriosus, increased O₂ levels correlate with an increase in p67^{phox} at both 2- and 9-days post ligation in both total lung homogenate and isolated pulmonary arteries (Brennan et al 2003). In this model, increased levels of p67^{phox} localize to the adventitia and smooth muscle layers (Brennan et al 2003).

Nox derived O₂⁻ is associated with medial thickening, disordered proliferation and migration, impaired angiogenesis, and disturbed fibrinolysis. For example, PASMC isolated

from both sheep and humans exhibit increased proliferation in response Nox derived O_2^- (Diebold et al, Wedgwood et al 2001). Thrombin treatment increases PASMC proliferation in a Nox dependent manner and increases the expression of plasminogen activator inhibitor-1 (PAI-1) (Diebold et al). PAI-1 inhibits fibrin clearance and promotes PASMC proliferation (Diebold et al). TGF β -1 treatment also increases Nox4 expression and Nox4 derived O_2^- (Sturrock et al 2006). In PAEC isolated from fetal lambs with PPHN there is increased Nox2 and Nox4 mRNA expression and increased p67^{phox} and Rac1 protein levels (Teng et al 2009). These PAEC exhibited decreased tube formation, cell proliferation, scratch recovery, cell invasion, and increased cell apoptosis, all of which were reversed upon treatment with the Nox inhibitor, apocynin (Teng et al 2009). Using *ex vivo* angiogenesis assays, the pulmonary arteries isolated from these lambs were found to have attenuated sprouting, which was again improved by apocynin (Teng et al 2009). Taken together these data suggest that the impaired angiogenesis associated with PPHN is due in part to oxidative stress derived from Nox. However, in Shunt lambs there is an angiogenic burst between 1–4-weeks of age associated with increased TGF- β 1-VEGF signaling (Mata-Greenwood et al 2003b) resulting in a two-fold increase in blood vessel number in the Shunt lambs at 4-weeks of age. Interestingly microarray analysis indicates that there is a pro-angiogenic phenotype induced in Shunt lambs as early as three days after birth (Tian et al) and the angiogenic burst is lost by 8-weeks of age (Mata-Greenwood et al 2003a). Thus, increases in ROS can have both pro- and anti-angiogenic effects with respect to the pulmonary system.

4. Xanthine oxidase

Another source of vascular ROS is xanthine oxidoreductase (XOR). XOR exists in two forms, as xanthine dehydrogenase (XD), which is the primary gene product of XOR, and as xanthine oxidase (XO), which is formed through post-translational modifications of XD. In the vascular wall, XD can be converted to XO by irreversible proteolysis or by reversible sulfhydryl oxidation. The cofactor, NAD⁺ is the primary electron acceptor for XD while, XO is unable to bind NAD⁺ and therefore uses O_2 as its electron acceptor. Both forms of XOR can produce ROS, but XO forms the majority of the O_2^- and H_2O_2 as a by-product of hypoxanthine metabolism. Although XD is the predominant form found in normal cells and tissues, XO plays an important role in cell and tissue injuries. Various forms of stimuli such as cytokines induce the conversion of the XD to the XO, resulting in increased generation of ROS and RNS. In PH, XO activity tends to dominate over XD activity and is a significant source of ROS production. XO activity is increased in IPAHA patients compared with healthy controls (Spiekermann et al 2009). Similarly, in Shunt lambs increased XO protein levels are observed in the endothelial and smooth muscle layers of pulmonary arteries at 2- and 4-weeks of age (Figure 1) (Sharma et al). Coincidentally, there is also a significant increase in XO derived O_2^- during the same period (Sharma et al). However, at 8-weeks of age there is a significant decrease in XO protein levels, suggesting that increases in XO activity may play a role in the early phases of PH prior to the development of endothelial dysfunction (Sharma et al). This possibility is supported in a rat model of PH induced by chronic hypoxia (Hoshikawa et al 2001). In this model, lung XO activity was predominantly enhanced during the initial 3 days of hypoxia exposure and inhibition of XO activity with allopurinol limited to the initial 3-days of hypoxia was sufficient to reduce the right ventricular hypertrophy and the pulmonary vascular thickening that develops during the 3 weeks of chronic hypoxia (Hoshikawa et al 2001). Together these data have led to the idea that the XO induced oxidative stress occurs early during an “induction phase” and is necessary and sufficient to induce an “adaptation phase” marked by pulmonary vascular remodeling and resulting in PH (Hoshikawa et al 2001). Interestingly, XD can also reduce circulating nitrite molecules into NO and inhibit SMC proliferation (Zuckerbraun et al 2010) suggesting a potential therapy to prevent PH progression. However, XO activity appears to

predominate over XD in PH, which would favor oxidation and O_2^- production rather than the reduction of nitrite and NO formation. Thus, although nitrite may have therapeutic potential in PH, XD may not be the predominant enzyme involved in the reduction of nitrite. Indeed, nitrite can also be reduced by other heme containing and copper containing enzymes, such as hemoglobin, myoglobin, and cytochrome c.

5. Endothelin-1

Endothelin-1 (ET-1) is a small 21 polypeptide, produced predominately by the endothelium and to a lesser extent, vascular smooth muscle. ET-1 is a potent vasoconstrictor and plays an important role in responding to hemodynamic stresses. Increased ET-1 is an early event in Shunt lambs occurring by 1-week of age (Ovadia et al 2003). Several studies have also demonstrated increased ET-1 plasma concentrations in children with CHD and PH (Yoshibayashi et al 1991). ET-1 levels are also significantly elevated in patients with PAH, and the severity and prognosis of the disease correlating with the concentration of ET-1 (Rubens et al 2001). There are two ET-1 receptors derived from separate genes: an ETA and an ETB receptor. ETA receptors are particularly predominant in vascular smooth muscle and myocytes, while ETB is the major ET receptor found in EC. ETA and ETB have opposing actions in the vasculature, although, many exceptions abound wherein both receptors elicit similar biological responses. ETA activation causes vasoconstriction mediated by phospholipase C activation and a rise in PASM C Ca^{2+} , while ETB activation, at least initially, causes vasodilation due to the release of NO from PAEC. At 4-weeks of age, the enhanced circulating levels of ET-1 (Ovadia et al 2003) lead to increased vasoconstriction mediated via an increase in the expression of ETA receptor and a decrease in ETB receptor (Figure 1) (Black et al 2000). Interestingly by 8-weeks of age, the expression of the ETB receptor returns to control levels, but its localization is altered, being predominantly on PASM C, where it mediates vasoconstriction (Black et al 2003). ETA stimulation tends to promote cell proliferation and fibrosis, while ETB opposes it. In PASM C, the proliferative effect of ET-1 is mediated by O_2^- generation via ETA receptor activation (Wedgwood et al 2001) and the mitogenic effect of ET-1 on PASM C can be blocked by antioxidants (Wedgwood et al 2001).

6. Growth factors

A. Overview

The excessive muscularization of small pulmonary arteries in children with PH secondary to CHD suggests that the abnormal expression of growth factors, mitogenic for vascular SMC, is involved in vascular remodeling. Moreover, angiogenesis-related molecules have been detected in the pulmonary vasculature of patients with severe PAH (Tuder et al 2001). In Shunt lambs, an early transient angiogenic burst is observed between 1- and 4-weeks of age (Tian et al). Three days after birth, microarray analysis has revealed an up-regulation of 89 and a down-regulation of 26 angiogenesis-related genes in Shunts compared to control lambs (Tian et al). Several growth factors such as transforming growth factor β 1 (TGF- β 1), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), have been implicated in the process of angiogenesis and muscularization in PH (Figure 1).

B. TGF- β 1

Members of the TGF- β superfamily play a critical role in the regulation of cellular growth and differentiation. Clinical studies have demonstrated an association between the increased or decreased expression of TGF- β 1 in adult patients suffering from various forms of PH (Tuder et al 2001). In Shunt lambs, TGF- β 1 levels are increased by 1-week of age when arterial morphology is relatively normal. At 4-weeks of age, the TGF- β 1 expression reaches its peak, which corresponds to increased vessel number, muscularity of the intra-acinar

arteries, and a significant increase in medial thickness of small pulmonary arteries (Mata-Greenwood et al 2003b). Shunts also have a profound imbalance between the expression of TGF- β 1 receptors, ALK-5 and ALK-1 in EC of small pulmonary arteries. As early as 1-week of age, there is a down regulation of the anti-angiogenic receptor, ALK-5 and an up-regulation of the pro-angiogenic receptor, ALK-1 (Mata-Greenwood et al 2003b). Further, mutations involving TGF- β receptor II genes have been observed within the endothelial cells of plexiform lesions from patients with severe PH (Yeager et al 2002). Plexiform lesions are tufts of cellular capillary formations within the lumen of dilated aneurysmal thin-walled arteries. Plexiform lesions most commonly develop immediately distal to bifurcation sites in small pulmonary arteries (~50–300 μ m), which are particularly susceptible to injury due to the high levels of fluid shear stress distal to the bifurcation of the vessel.

C. VEGF

TGF- β 1 induces pro-angiogenic effects also by indirectly up-regulating VEGF. Reports also show a parallel increase in expression of TGF- β 1 and VEGF in models of atrial fibrillation and adult PH (Seko et al 2000). *In vitro* studies have shown that cyclic stretch-induced VEGF expression is dependent on previous activation of SMC by TGF- β 1 (Zheng et al 2001). VEGF mediates its pro-angiogenic effects via two transmembrane tyrosine kinase endothelial-specific receptors: fms-like tyrosine kinase-1 (Flt-1) and kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/Flk-1). Increased VEGF expression has been observed in the tracheal aspirates and type II pneumocytes of neonates with PPHN (Lassus et al 2001). Similarly, VEGF levels are increased in the lungs of newborns with congenital diaphragmatic hernia and PH and in the lungs of adults with advanced pulmonary vascular disease secondary to CHD (Shehata et al 1999). In Shunt lambs, VEGF and its receptors are elevated at 4- and 8-weeks of age, correlating with increased angiogenesis and blood vessel number (Mata-Greenwood et al 2003a).

D. FGF-2

Studies suggest that VEGF contributes to the first steps of angiogenesis by inducing endothelial proliferation and migration. However, in later stages of vascular remodeling, VEGF has been shown to synergize with FGF-2 (Cavallaro et al 2001). For example, in patients with COPD, enhanced bronchial expression of VEGF and its receptors Flk-1 and Flt-1 and increased levels of FGF-1, FGF-2 and its receptor, FGFR-1 (Kranenburg et al 2005a, Kranenburg et al 2005b) jointly contribute to extensive muscularization of precapillary pulmonary vessels. FGF-2 is primarily a SMC mitogen involved in the early proliferation of SMC and in neointimal thickening following vascular injury. The progressive increase in FGF-2 protein has been observed within the smooth muscle layer of pulmonary arteries in a rat model of monocrotaline-induced PH (Arcot et al 1995). In addition, elevated FGF-2 protein levels were detected in the urine and plasma of adults with PAH (Benisty et al 2004). Elevated FGF-2 levels have also been observed in shunt lambs, suggesting the involvement of FGF-2 in the SMC proliferation observed in these lambs (Wedgwood et al 2007).

E. ROS & Growth Factors

The role of ROS in the regulation of growth factors in PH is complex. For instance, in PASMC, cyclic stretch induces a significant increase in VEGF expression both at the mRNA and protein level (Mata-Greenwood et al 2005). The increased VEGF mRNA is preceded by both an increased expression and secretion of TGF- β 1 and an increase in ROS generation by the activation of NADPH oxidases (Mata-Greenwood et al 2005). Neutralizing TGF- β 1 abolishes the cyclic stretch-dependent increase in both O₂⁻ generation and VEGF expression (Mata-Greenwood et al 2005). Further, blocking ROS production using NADPH oxidase inhibitors, the cyclic stretch-dependent increase in VEGF expression

is attenuated (Mata-Greenwood et al 2005). In addition, it has been shown that ROS, via NADPH activation, can increase FGF-2 expression in PASMC (Black et al 2008). FGF-2 expression can also be stimulated by other factors known to increase ROS signaling in PASMC (ET-1 and TGF- β 1), and this can also be attenuated by antioxidants (Black et al 2008).

7. Nitrosative stress and post-translational modifications

Peroxynitrite is a powerful oxidant that can modify protein structure and function through the nitration of accessible tyrosine residues. Early nitrosative stress is an early contributor to the development of PH in Shunt lambs. At 2-weeks of age, Shunts have elevated levels of nitrated eNOS, correlating with increased eNOS uncoupling (Oishi et al 2008). Several nitration sites on eNOS have been identified (Zickus et al 2008). Although, the exact role of each nitration event is not known, the nitration of Tyr 331, 357, and 373 located in the Hsp90-binding domain of eNOS are of particular interest (Zickus et al 2008) since the nitration of these tyrosines may hinder the binding of Hsp90. Alternatively, it is possible that sustained nitrosative stress in the shunts may also nitrate Hsp90. The negative charge conferred to both eNOS and Hsp90 by nitration could potentially repel their interaction which may explain the attenuation of the eNOS:Hsp90 interactions observed in the Shunt lambs (Zickus et al 2008). The nitration of Hsp90 has been reported in the ductal ligation lamb model of PH (Konduri et al 2007). Animal and human studies have shown that the early down-regulation of eNOS signaling is accompanied by a compensatory release of vasodilatory compounds, such as prostacyclin and natriuretic peptides. At 4-weeks of age, Shunts have elevated B-type natriuretic (BNP) peptide and cGMP levels (Oishi et al 2007). The vasodilatory effects of cGMP are mediated through protein kinase G (PKG). However, recent data suggest that the nitration of PKG in these lambs attenuates the kinetic activity of the enzyme and results in impaired vasodilation and increased smooth muscle proliferation (Figure 1) (Aggarwal et al). The nitration dependent decrease in PKG activity has also been demonstrated in the lungs of the patients with idiopathic PAH (Zhao et al 2009).

The nitration of carnitine acetyltransferase (CrAT) is another indicator of early nitrosative stress in the Shunt lambs (Sharma et al 2008). CrAT belongs to the carnitine acyltransferase pathway, which maintains normal mitochondrial function. This pathway consists of carnitine palmitoyltransferases (CPT1 and CPT2) and carnitine acetyltransferase (CrAT), where the CPTs transesterify medium and long chain fatty acyl chains and CrAT transesterifies short chain acyl chains. A decrease in CrAT activity elevates acyl-CoA, which inhibit multiple enzymes involved in β -oxidation. In Shunt lambs, there is a 2-fold decrease in CrAT expression, but 20-fold attenuation in CrAT activity, suggesting a posttranslational modification (Sharma et al 2008). In fact, *in vitro* studies using purified CrAT enzyme have demonstrated that the nitration of CrAT impairs its enzymatic activity (Sharma et al 2008). Thus, protein nitration is an important modification, regulating the activity of various proteins in Shunt lambs. Whether nitration would serve as the basis of the vascular pathology in PH or only be recognized as an early biomarker is yet to be determined.

Conclusion

This review has highlighted the results obtained from a decade and a half of work focused using a lamb model with an *in utero* placement of an aorto-pulmonary vascular graft, which most closely represents the altered hemodynamic state of children who have CHD with increased PBF. Our studies have shown that early derangements in the redox environment in the pulmonary vasculature are instrumental for the development of the endothelial dysfunction and precede overt vascular remodeling. In the past, significant attention has been focused on the use of antioxidants, L-arginine, BH₄, and ET-1 receptor blockers for the

management of PH. However, the results highlighted in this review (Figure 1) may help to identify novel targets for therapeutic intervention in these children. Although clinical trials involving antioxidant therapies have not been very promising, an understanding of the temporal regulation of ROS and RNS that appears to occur in CHD with increased PBF could assist to regulate the dose, the time and the duration of the treatment resulting in better outcomes. It is possible that commencing the antioxidant therapy at early stages of disease could result in improved resolution.

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Fig. 1. Summary of ROS/RNS generation and the downstream targets involved in vascular remodeling in a neonatal lamb model (Shunt) of congenital heart disease (CHD) with pulmonary hypertension (PH) secondary to increased pulmonary blood flow

There are several sources of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that contribute to the development of oxidative and nitrosative stress and subsequent vascular dysfunction in Shunt lambs with PH. Endothelial NOS (eNOS) uncoupling (1), which significantly generates both ROS and RNS, is mediated by limited L-arginine and tetrahydrobiopterin (BH₄) availability. L-arginine levels are diminished due to increased degradation by arginase up-regulation and attenuated synthesis by argininosuccinate lyase (ASL) and argininosuccinate synthetase (ASS) down-regulation. Moreover, a sustained

increase in asymmetric dimethylarginine (ADMA) levels prohibits L-arginine binding to eNOS. In addition, eNOS function is impaired by decreased GTP cyclohydrolase-1 (GCH1) enzyme. Low GCH1 levels limit the bioavailability of BH₄, an essential co-factor for NO generation. Finally, the disruption of Hsp90-eNOS interaction potentiates the uncoupling of the enzyme. In Shunt lambs, ADMA also appears to promote mitochondrial dysfunction (2). ADMA increases mitochondrial ROS and decreases ATP levels. Several markers of mitochondrial dysfunction are observed, including increased levels of uncoupling protein-2 (UCP-2), decreased levels of the mitochondrial superoxide dismutase-2 (SOD2), and an increased lactate:pyruvate ratio. In addition, several subunits of NADPH oxidase (3), including p47^{phox} and Rac1, are up-regulated, inducing ROS production in the pulmonary vasculature. In Shunts, there is also a significant increase in xanthine oxidase (XO) protein levels and XO derived O₂⁻ in the pulmonary vasculature (4). Further, altered endothelin-1 (ET-1) (5) signaling contributes to oxidative stress and vascular dysfunction in PH. Shunt lambs have elevated levels of ET converting enzyme-1 (ECE-1) and subsequent increased ET-1 in their peripheral lung tissue. The protein levels of the ET-1 receptors: ETA and ETB are altered. ETA, which predominantly mediates vasoconstriction in smooth muscle cells (SMC), is increased. However, ETB, which provokes a vasoconstrictive response in SMC and a vasodilatory response in endothelial cells (EC), is increased in pulmonary SMC and decreased in pulmonary EC. The role of ROS in mediating endothelial and smooth muscle proliferation is complex. Oxidative stress induces the expression of several growth factors, such as transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), and fibroblast growth factor-2 (FGF-2) (6). In Shunts, there is a profound dysregulation of TGF-β1 receptors, ALK-5 and ALK-1. There is a down-regulation of the anti-angiogenic receptor, ALK-5, and an up-regulation of the pro-angiogenic receptor, ALK-1 (6). In addition, ROS influences the over-expression of VEGF and its receptors, Flt-1 and Flk-1, thereby adding to endothelial proliferation and migration (6). The up-regulation of FGF-2 by oxidative stress contributes to extracellular matrix deposition and smooth muscle wall thickening in Shunts (6). The down-regulation of NO signaling in PH is followed by a compensatory increase in vasodilatory molecules, such as B-type natriuretic peptide (BNP) and cGMP (7). However, vasodilation in PH is attenuated due to a nitration induced decrease in protein kinase G-1α (PKG-1α) activity (7).