Right Ventricular Nitric Oxide Signaling in an Ovine Model of Congenital Heart Disease: A Preserved Fetal Phenotype

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Running Title: RV NO Signaling in CHD
Abstract

We recently reported superior right ventricle (RV) performance in response to acute afterload challenge in lambs with a model of congenital heart disease (CHD) with chronic left-to-right cardiac shunts. Compared to control animals, shunt lambs demonstrated increased contractility, due to an enhanced Anrep effect – the slow increase in contractility following myocyte stretch. This advantageous physiologic response may reflect preservation of a fetal phenotype as the RV of shunt lambs remains exposed to increased pressure postnatally. Nitric oxide (NO) production by NO synthase (NOS) is activated by myocyte stretch and is a necessary intermediary of the Anrep response. The purpose of this study was to test the hypothesis that NO signaling is increased in the RV of fetal lambs compared to controls, and shunt lambs have persistence of this fetal pattern. An 8mm graft was placed between the pulmonary artery and aorta in fetal lambs (shunt). NOS isoform expression, activity, and association with activating cofactors were determined in fetal tissue obtained during late-gestation and in 4-week old juvenile shunt and control lambs. We demonstrated increased RNA and protein expression of NOS isoforms and increased total NOS activity in the RV of both shunt and fetal lambs, compared to control. We also found increased NOS activation and association with cofactors in shunt and fetal RV, compared to control. These data demonstrate preserved fetal NOS phenotype and NO signaling in shunt RV, which may partially explain the mechanism underlying the adaptive response to increased afterload seen in the RV of shunt lambs.

Key Words: Right ventricle, congenital heart disease, nitric oxide signaling, Anrep effect
Introduction

Patients with pulmonary hypertension (PH) secondary to congenital heart disease (CHD) have improved functional ability and prolonged survival compared to patients with PH due to other causes.\(^{(15, 22)}\) One possible explanation for these observations is that the right ventricle (RV) in patients with PH associated with CHD is uniquely able to respond to the increased afterload created by pulmonary vascular disease.\(^{(17)}\) For example, the RV in CHD is continually exposed to either increased pressure, volume, or both, potentially altering the normal perinatal transition of the RV from the dominant ventricle in fetal circulation to a low-resistance, high capacity chamber in postnatal circulation. Given these physiologic perturbations, we hypothesized that the RV in CHD has a preserved fetal phenotype, which confers an enhanced functional ability to respond to increased afterload.

In our model of CHD with chronic left-to-right shunting, we previously demonstrated that the RV of shunt lambs has an adaptive response compared to control RV when challenged with an acute afterload increase (imposed by pulmonary artery banding,) with increased contractility and preservation of mechanical efficiency and ventriculo-vascular coupling.\(^{(19)}\) Our observation in the shunt RV of a sustained increase in contractility following afterload challenge is consistent with the Anrep effect, the second slow increase in contractility in response to myocyte stretch.\(^{(10)}\) Before our report, this critical physiologic mechanism had only been observed in the left ventricle (LV), as the normal RV is exquisitely sensitive to increased afterload.
Nitric oxide (NO) signaling has been demonstrated as a critical mediator of excitation-contraction coupling and the slow force response (the in vitro equivalent of the Anrep effect.) For example, in single myocyte experiments, Petroff and colleagues demonstrated that NO produced by endothelial NO synthase (eNOS) is a necessary mediator of increased calcium release in response to stretch.(26) In contrast, Jian and colleagues found neuronal NOS (nNOS), rather than eNOS, to be the critical NOS isoform for intracellular calcium release after myocyte stretch.(18) The theoretical link between myocyte stretch and intracellular calcium release, or mechanochemotransduction, is due to subcellular compartmentalization of NO production at the sarcoplasmic reticulum (SR) which allows for NO-mediated regulation of calcium handling.(29)

Therefore, the purpose of the present study was to test the hypothesis that NOS activity and its associated signaling is increased in the RV of shunt lambs compared to controls, and this increased NOS signaling in the shunt RV mirrors RV fetal expression and activity. To this end, we used our established, clinically relevant lamb model of a congenital cardiac defect with a large left-to-right shunt, created by the in utero placement of an 8 mm aorta-to-pulmonary shunt (shunt lambs.) We examined NOS isoform expression and activity, association with important co-factors, and activating signals in 4-week old control and shunt lamb LV and RV tissue, and compared these elements to expression in fetal LV and RV.
Materials and Methods

Surgical Preparation and Hemodynamics

A total of 6 pregnant mixed-breed Western ewes (137-141 d gestation, term = 145 d) were anesthetized. Fetal exposure was obtained through the horn of the uterus; a left lateral thoracotomy was performed on the fetal lamb. With the use of side biting vascular clamps, an 8.0-mm vascular graft was anastomosed between the ascending aorta and main pulmonary artery of the fetal lambs. This procedure was previously described in detail.(27) Control lambs were either provided by twin gestation (n=3) or age-matched (n=2.) Control lambs did not undergo a lateral thoracotomy. LV and RV tissue was harvested and hemodynamic measurements were performed when control and shunt lambs were 4- to 5-weeks old. Fetal tissue was harvested from late gestation fetal lambs at the same gestation age as fetal surgery, 137-141 d gestation.

At the time of hemodynamic study, lambs were anesthetized and catheters were placed into the right and left atrium, main pulmonary artery, and femoral artery (as previously described.)(27) An ultrasonic flow probe (Transonics Sytems, Ithaca, NY) was placed around the left pulmonary artery to measure pulmonary blood flow.

At the end of the protocol, all lambs were euthanized with a lethal injection of sodium pentobarbital (150 mg/kg) followed by bilateral thoracotomy as described in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The Committee on Animal Research of the University of California, San Francisco, approved all protocols and procedures.

Quantitative Real Time PCR
RNA extraction from sheep hearts was performed using Qiagen RNeasy Fibrous Tissue Mini Kit (Qiagen, Toronto, ON) with manufacturer’s protocols. Identical amounts of purified total RNA was used as starting material. First strand cDNA was synthesized from each sample and subjected to reverse transcription using the Clontech RNA to cDNA ecoDry Premix (Oligo dT) Kit (Clontech Laboratories, Inc. CA) with manufacturer’s protocol.

cDNA templates were mixed with gene specific primers and FastStart Universal SYBR Green Master with Rox reference dye (Roche Applied Science). Primers (Table 1) were designed with public OligoPerfect Designer software (Life Technologies, NY). GAPDH was used as the internal control. The qPCR reactions were completed with an iCycler iQ Real-Time PCR Detection System (Bio-Rad) and always in triplicate. Relative expression levels of genes were analyzed. Differences in cycle threshold number (C(t)) were calculated by normalizing the sample cycle threshold of the targeted gene with that of the internal control reference gene 18S. The deltadeltaCT (calculated as CT[target]-CT[reference]) method was utilized to determine relative abundance of expression, as described previously.(21)

Immunohistochemistry

Sections from RV and LV of control and shunt juvenile lambs were fixed and prepared for immunohistochemistry staining as previously described.(3) Then, tissue sections were incubated with anti-myosin heavy chain (MF-20) (Developmental Studies Hybridoma Bank) antibody and anti-eNOS, -nNOS, or -iNOS (all Santa Cruz Biotech) antibody in blocking solution at 4°C overnight. After 3 washes with PBS for 5 minutes,
samples were hybridized with fluorescent Green 488 goat anti-rabbit polyclonal and
fluorescent Red 555 goat anti-mouse monoclonal secondary antibodies (Molecular
Probes) at a concentration of 1:500 in blocking solution for 60 minutes at room
temperature. After 3 further washes with PBS, the slide were mounted with mounting
solution with 4’,6-diamidino-2-phenylindole (DAPI.)

Images were taken with a Hamamatsu c10600 ORCA-R2 Digital Camera on a Zeiss
Axio Imager Z2 using a ×10 DIC objective and the X-cite 120 Mercury/Halide system
and then analyzed using ZEN pro 2012 software (Carl Zeiss Microimaging, Thornwood,
NY.) All images were subsequently processed using Adobe Photoshop CS5 software
(Adobe, San Jose, CA.)

Western Blot and Densitometry Analysis

Protein determinations were performed by Western Blot analysis as previously
described.(2) Primary antibodies against eNOS (Santa Cruz Biotech,) phospho-eNOS-
serine1177 (Cell Signaling,) nNOS (Santa Cruz Biotech,) inducible NOS (iNOS) (Santa
Cruz Biotech,) Calmodulin (Santa Cruz Biotech,) 90-kDa heat shock protein (hsp90)
(polyclonal, BD Transduction laboratories,) Akt (Santa Cruz Biotech,) phospho-Akt
(Santa Cruz Biotech,) ryanodine receptor 2 (RyR2) (Affinity Bioreagents,) and S-
nitrosocysteine (Abcam) were utilized. Anti-β-Actin was used as the reference protein
for loading controls. Quantification of protein band density in X-ray films from ECL
Western Blots was performed by a public domain Java image processing program
Image J (NIH Image.)

Immunoprecipitation
RV and LV protein was isolated from control, shunt, and fetal hearts, and an equal amount of protein was used in each experiment. Specific antibodies were cross-linked with Protein A/G magnetic beads based on manufacturer’s instructions (Pierce crosslink Immunoprecipitation Kit, Thermo Scientific, Rockford Ill.) Protein extracts were incubated with IP-antibody/protein A/G magnetic beads overnight 4°C. The antigen-antibody beads were next washed twice with IP Lysis/Wash Buffer. Finally, the antigen/antibody complexes were eluted and used for Western blot.

Isolation of Sarcoplasmic Reticulum (SR) Membrane Vesicles

Cardiac SR vesicles were isolated from heart according to Buck et al. (8) Briefly, LV and RV tissue were homogenized in five volumes of homogenization buffer (1 M KCl, 10 mM Tris-maleate, pH 7.0) and a cocktail of protease inhibitors (Roche Applied Science.) The homogenate was centrifuged for 20 min at 10,000 × g at 4 °C. The supernatant was discarded, and the remaining pellet was homogenized in an ice-cold homogenization buffer and then centrifuged for 20 min at 6000 × g at 4 °C. The supernatant from this step was again centrifuged for 25 min at 24,000 × g at 4 °C, and the resulting supernatant was further centrifuged for 120 min at 41,000 × g at 4 °C. The final pellet was resuspended in buffer with 10% sucrose, 10 mM Tris-maleate, 0.9% NaCl, pH 6.8. Aliquots were snap frozen in liquid N2 and stored at −80 °C until used.

NOS Activity Assay

RV and LV tissues were homogenized, and NOS activity was determined using the conversion of [3H]L-arginine to [3H]L-citrulline as previously described.(25) All activities were normalized to the amount of protein in each lysate. To determine the potential
contribution of iNOS to total NOS activity, assays were repeated without calcium supplementation.

**Statistical Analysis**

For Western blot protein analysis and NOS activity data are shown as means ± standard deviation. Quantitative PCR data are shown as means ± standard error of the mean. Analysis of variance (ANOVA) was used to compare differences in these parameters between the three groups with Bonferroni correction for multiple comparisons. A $p<0.05$ was considered significant.
Results:

Characteristics of Juvenile Shunt and Control Lambs

There were no differences in gestational age at delivery or total body weight between shunt and control lambs (11.1±1.6 vs. 12.8±2.6 kg, respectively, \( p=0.24 \)) in shunt lambs compared to controls, both RV and LV and interventricular septum (LV+S) chamber weights were increased, relative to body weight (BW) (RV/BW 2.5x10^{-3}±4.9x10^{-4} vs 1.3x10^{-3}±9.6x10^{-5}, respectively, \( p<0.001 \); LV+S/BW 6.1x10^{-3}±6.5x10^{-4} vs. 3.3x10^{-3}±2.1x10^{-4}, respectively, \( p<0.001 \)). Thus, there is gross morphologic evidence of global, biventricular hypertrophy in shunt lambs. At the conclusion of each study, the vasculature of each lamb was examined; there were no patent ductus arteriosi or foramina ovalia.

Hemodynamics of Juvenile Shunt and Control Lambs

As expected, shunt lambs had significantly higher pulmonary blood flow compared to control lambs (indexed left pulmonary artery blood flow: 175±28.6 vs. 40.1±16.4 ml/min/kg body weight, respectively; \( p<0.0001 \)) The ratio of pulmonary to systemic blood flow (Qp:Qs) was 2.76±0.7 in shunt lambs. Mean pulmonary artery pressures were also significantly elevated in shunt lambs, compared to control lambs (24.7±1.4 vs. 11.6±3.0 mmHg, respectively, \( p<0.0001 \)) There were no significant differences between shunt and control lambs in heart rate or mean systemic systolic blood pressure (62±5.6 vs. 68±6.7 mmHg, respectively, \( p=0.8 \)) As expected, systemic diastolic blood pressure was significantly lower in shunt lambs, compared to controls (30.3±3.7 vs. 53.6±4.1, respectively, \( p<0.001 \)).
NOS Isoform Expression:

In examining NO-mediated signaling in the RV of shunt animals, we first investigated the expression of various NOS isoforms. Using qPCR, we quantified mRNA levels of eNOS, nNOS, and iNOS. The transcription of all three isoforms was greater in the RV of shunt and fetal lambs, compared to control lambs (eNOS, 3.0±1.0- and 4.9±0.7-fold control, respectively; nNOS 10.2±4.9- and 25.3±7.2-fold control, respectively; iNOS 2.0±0.7- and 3.2±0.8-fold control, respectively; \( p < 0.05 \), Figure 1A-C.) The transcription of eNOS and nNOS was greater in the LV of fetal lambs, compared to shunt and control lambs. Similarly, when we investigated protein expression of the various NOS isoforms, we found that protein expression of all three isoforms was greater in the RV of shunt and fetal lambs, compared to control lambs (eNOS, 1.3-fold and 1.6-fold control, respectively; nNOS 1.4±0.1- and 1.6±0.2-fold control, respectively; iNOS 1.4±0.1- and 1.7±0.2-fold control, respectively; \( p < 0.05 \), Figure 1D-F.) The protein expression of all three isoforms was greater in the LV of fetal lambs, compared to shunt and control lambs.

Immunohistochemistry

As increased NOS isoform expression may be due to multiple cell types within the ventricle, we then performed immunohistochemistry on serial RV sections obtained from shunt lambs to determine colocalization of NOS isoforms within cardiac tissue (Figure 2.) As expected, there was prominent localization of eNOS within endothelial cells found in myocardial capillaries in both shunt and control RV tissue. However, in shunt RV tissue, eNOS localizes to both the cardiomyocyte nucleus and cytoplasm; in control RV
tissue, eNOS localization within the cardiomyocyte is confined to the nucleus (Figure 2A.) In control RV tissue, nNOS does not localize within the cardiomyocytes while in shunt RV tissue nNOS localizes within cardiomyocyte cytoplasm (Figure 2B.) Finally, iNOS appears to have some localization within the cardiomyocyte of both control and shunt RV tissue (Figure 2C.)

NOS activity:

Having shown increased RNA transcription and protein expression of all three NOS isoforms in fetal and shunt RV, compared to control, we next quantified NOS activity in these tissues. The assay distinguishes between total NOS activity and Ca$^{2+}$-independent activity, which is due to iNOS alone. Total NOS activity was increased in the RV of shunt and fetal lambs, compared to control lambs (2.5±0.56-fold control, 3.5±0.37-fold control, respectively; $p<0.05$, Figure 3B.) In contrast, total NOS activity in the LV was not different between groups (Figure 3A.) Ca$^{2+}$-independent NOS activity in the RV and LV was not different between groups.

NOS Regulation and Signaling Activity:

After establishing increased NOS expression and activity in shunt and fetal RV, we next sought to determine the relationship between NOS associated signaling proteins and activating pathways. Using immunoprecipitation, we found increased association of eNOS with calmodulin in the RV of shunt and fetal lambs, compared to controls (1.8±0.2-fold control, 1.5±0.1-fold control, respectively; $p<0.05$, Figure 4A.) Although this association was increased in the LV of fetal lambs ($p<0.05,$) there were no differences between control and shunt lambs. We next examined the association of
eNOS with hsp90. We found hsp90 to be more associated with eNOS in the RV of shunt and fetal lambs, compared to controls (1.4±0.1- and 1.4±0.2-fold control, respectively; \( p<0.05 \), Figure 4B.) Although this association was also increased in the LV of fetal lambs (\( p<0.05 \)), there were no differences between control and shunt lambs.

Akt is a serine-threonine kinase that associates with the eNOS complex through interaction with hsp90, increasing eNOS activity by phosphorylating serine residue 1177.\(^{(1, 23)}\) To better understand the importance of this pathway in the RV of shunt lambs, we first investigated Akt expression. We found both increased transcription of Akt mRNA (2.9±0.3- and 4.1±0.2-fold control, respectively; \( p<0.05 \), Figure 5A) and Akt protein expression (2.1±0.2- and 2.3±0.2-fold control, respectively; \( p<0.05 \), Figure 5B) in the RV of shunt and fetal lambs, compared to controls. Although Akt expression was increased in the LV of fetal lambs (\( p<0.05 \)), there were no differences between control and shunt lambs (Figure 5B.) Next, we found greater Akt phosphorylation in the RV of shunt and fetal lambs, compared to controls; however, the p-Akt proportion was even greater in fetal RV tissue, compared to shunt (1.3±0.1- and 1.5±0.1-fold control, respectively; \( p<0.05 \), Figure 5C.) We found greater Akt phosphorylation in the LV of fetal lambs, compared to shunt and control lambs (with no differences between shunt and control). Lastly, we examined Akt activation of eNOS by quantifying the proportion of eNOS phosphorylated at the serine 1177 residue (Ser1177-p-eNOS.) We found greater Ser1177-p-eNOS in the RV of shunt and fetal lambs, compared to controls; however the proportion was even greater in fetal than shunt lambs (1.9±0.2- and 1.5±0.2-fold control, respectively; \( p<0.05 \), Figure 5D.) In the LV, Ser1177-p-eNOS was greater in fetal lambs compared to shunt and control lambs (\( p<0.05 \).)
RV NO Signaling in CHD

RyR2 is a calcium release channel located at the SR and vital part of excitation-contraction coupling. (30) Post-translational modification of RyR2 via S-nitrosylation (−SNO) of cysteine residues increases the open probability of RyR2 channels, thus increasing calcium transient in cardiomyocytes and increasing contractility. Using immunoprecipitation for RyR2, we then examined the portion of RyR2 with −SNO modification. We found greater RyR2-SNO proportion in the RV of shunt and fetal lambs, compared to controls; however, the proportion was even greater in fetal than shunt lambs (1.4±0.16- and 1.9±0.08-fold control, respectively, \( p<0.05 \), Figure 6.) In the LV, the RyR2-SNO proportion was greater in fetal lambs compared to shunt and control lambs (\( p<0.05 \).)
Discussion

In fetal circulation, the RV accounts for the majority of cardiac output, and the fetal RV is more resilient to physiologic stress—such as acidosis and hypoxia—compared to the fetal LV. During the transition to postnatal circulation, the RV remodels to a thin-walled, compliant ventricle coupled to a low impedance pulmonary vascular system. After this transition is complete, the RV is unable to effectively or efficiently respond to increases in afterload, which may be due to loss of the protective structural and molecular fetal phenotype. Conversely, in CHD with a post-tricuspid level shunt, this normal postnatal transition is delayed and abrogated; for example, the attenuated fall in pulmonary vascular resistance and pulmonary artery pressure after birth in patients with a large VSD is well described. Although the effects of this altered transition in CHD on RV function and remodeling are unclear, patient case series suggest that normal regression of RV wall thickness does not occur in patients with CHD who ultimately develop Eisenmenger’s syndrome. Consequently, these patients with PH associated with CHD have better functional ability and improved survival compared to patients with primary PH. Interestingly, among patients with primary PH, there is considerable variability in indices of RV function (cardiac output and right atrial pressure) relative to pulmonary artery pressure elevation. There is a subpopulation of patients who retain an adaptive phenotype with concentric hypertrophy and preserved mechanical efficiency; accordingly, these patients have improved survival compared to patients with a “maladaptive” RV phenotype. The importance of RV function in both PH and CHD is gaining recognition, but the
mechanisms underlying the adaptive RV phenotype are unclear, and yet may represent targets for novel therapies.

To this end, the current study utilized a clinically relevant lamb model of CHD with a large, unrestrictive aortopulmonary shunt placed during fetal life. Importantly such a shunt does not change fetal hemodynamics, but mimics the physiology of patients with CHD (with systemic-to-pulmonary shunts) during the transition from fetal to postnatal circulation. We demonstrated previously that shunt lambs have superior cardiac performance in response to acute increases in RV afterload due to the Anrep effect.(19) Thus, this fetal model is ideal for investigations into underlying mechanisms for RV adaptation. The signaling mechanisms of the LV Anrep effect are well described and implicate a pivotal role for LV NO signaling.(5, 10) Therefore, we focused this investigation on potential alterations in cardiac NO signaling.

Increased cardiac NO signaling has a variety of paracrine and autocrine effects which ultimately maximize cardiac function: dilation of coronary arteries with enhanced oxygen delivery, increased angiogenesis in hypertrophy, increased lusitropy with optimization of diastolic reserve, and improved excitation-contraction coupling which is crucial for the Anrep effect.(5) To our knowledge, the studies presented herein are the first to describe a unique RV NO signaling phenotype. In these investigations, we found a distinct RV shunt phenotype (compared to the shunt LV) with consistently increased NOS expression, activity, and association of important cofactors for NOS activation.

We first demonstrated increased expression of all NOS isoforms in the RV of shunt and fetal lambs; eNOS and nNOS are the most important isoforms, as they are constitutively
expressed in cardiomyocytes. Both eNOS and nNOS isoforms have been implicated in single myocyte experiments as critical mediators of the Anrep effect.(18, 31) Although we also found increased iNOS expression, the importance of this finding is less clear. The NOS activity assay demonstrated low levels of calcium-independent NOS activity, attributable to iNOS; further, there were no differences in calcium-independent activity between control, shunt, and fetal lambs in either ventricle. However, total NOS activity was significantly increased in the RV of both shunt and fetal animals, reflecting important functional consequences of increased protein expression of eNOS and nNOS. Although iNOS expression is increased in the RV of shunt and fetal lambs, compared to control lambs, we found no increase in iNOS-derived calcium-independent NOS activity. iNOS-derived NO production is known to contribute to increase reactive oxygen species (ROS) and inhibit calcium-independent NOS activity;(11) conversely, maintenance of eNOS/iNOS expression ratio is known to suppress iNOS-derived NO production.(14) Given that protein expression of all three NOS isoforms are increased to a similar extent, there are no significant changes in eNOS/iNOS ratio between groups; this may account for the low level of calcium-independent NOS activity seen in all three groups, as iNOS-derived NO production is inhibited under basal conditions. Beyond increased expression and total activity, the shunt and fetal RV had a global picture of increased NOS activation. In each case, there was greater eNOS association with critical cofactors calmodulin and hsp90, which increase NOS activity.(2, 7) Ca\(^{2+}\)-Calmodulin binding to the eNOS complex is critical for disruption of inhibiting interactions with eNOS and also promotes allosteric modification of eNOS to de-repress reductase catalytic activity to proceed.(13) Hsp90 likely increases NOS activity through
two mechanisms, increasing the affinity of NOS for Ca\(^{2+}\)-calmodulin,(7) and recruiting Akt to the NOS complex and preventing its inactivation via dephosphorylation.(12, 31) Further, Akt expression, activation, and phosphorylation of eNOS on serine residue 1177 were similarly upregulated. These findings may be due to stretch mediated mechanisms of progressive NOS activation which were recently reviewed.(5) The RV of shunts is likely also subject to these stretch-mediated mechanisms given significantly increased end-diastolic, end-systolic and overall stroke volumes seen in the RV of shunt lambs compared to control.(19)

nNOS has also been implicated as a critical mediator of excitation-contraction coupling, partly through reversible nitrosylation of cysteine residues on RyR2, a crucial calcium channel on the SR. RyR2 exists as a tetramer and is progressively activated by nitrosylation of thiol groups with maximal activation (2- to 3-fold baseline) achieved when ~3 thiol groups per subunit are nitrosylated.(32) nNOS localizes to the SR, making it the most likely NOS isoform responsible for RyR2 nitrosylation.(6) With further, myocytes derived from nNOS knockout mice have decreased RyR2 activity, a phenotype that can be rescued through addition of SNAP, an NO donor and nitrosylating agent.(30) In the current study, we have demonstrated increased expression of nNOS RNA and protein and increased nitrosylation of RyR2 in SR vesicles isolated from shunt and fetal RV tissue; this provides a possible mechanism underlying our earlier observation of superior cardiac performance in the RV of shunt lambs.(19)

In this study, we demonstrated increased NO signaling from increased isoform expression and total NOS activity as well as signaling associated with both eNOS and
nNOS. However, NO signaling in CV physiology and pathology is complex, with NO production having differing and even opposing effects depending on subcellular localization and production. The precise physiologic effects of these observations, as well as the effects of increased NO activity on physical properties such as angiogenesis and the development of ventricular hypertrophy are crucial matters for further investigation.

Perhaps the most intriguing finding of the current study was the consistent pattern of NOS expression and activation between the RV of shunt and fetal lambs. Although not identical, the alterations in shunt and fetal RV expression – compared to control lambs – were remarkably similar. The current investigation into shunt and fetal RV expression was necessarily limited, but these data strongly support our hypothesis that the hemodynamic stimulus of an unrestricted systemic-to-pulmonary shunt at the time of birth attenuates the normal transition from a fetal RV phenotype and that these preserved fetal characteristics confer a physiologic advantage. A more global investigation of expression similarities and dissimilarities between shunt and fetal RV is necessary to further explore this hypothesis.

The most important limitation of this study was that we did not test the functional implications of the observed increases in NO signaling. An important next area of investigation will be pharmacologic modulation of the NO pathway in both isolated cardiac and whole animal preparations to recapitulate the observed physiologic adaptations we found in the RV of shunted animals. However, the current findings provide important foundational work for these studies.
In summary, our findings of increased NOS expression and activity in the RV of shunt and fetal lambs, taken together with our physiologic observations, suggest a potentially crucial role for NO signaling in the adaptive RV response to increased afterload under conditions of chronic (since birth) left-to-right shunting. In almost all other animal models of either PH or CHD, the RV suffers maladaptive hypertrophy with progression to RV failure. In contrast, insights gained from this model with an adaptive physiologic response may ultimately point to innovative therapeutic strategies. For example, the current finding of increased RV NO signaling, if validated to have advantageous physiologic consequences, has clear potential therapeutic implications as many existing PH therapies target the NO pathway. Further, these findings lend further support to the hypothesis that patients with certain types of CHD have a preserved, advantageous fetal phenotype, which may ultimately lead to previously overlooked strategies in treating all patients with RV failure in PH and CHD.
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DISCLOSURES
The authors have no conflict of interests to disclose.


FIGURE LEGENDS

**Figure 1:** NOS Isoform Expression

A-C: Transcription of eNOS, nNOS, and iNOS RNA are upregulated in shunt and fetal RV, relative to control. There is no significant difference between shunt and fetal NOS isoform RNA expression. RNA expression quantified by qPCR, data are shown as mean ± SEM. A: eNOS RNA, B: nNOS RNA, C: iNOS RNA.

D-F: Protein expression of eNOS, nNOS, and iNOS are upregulated in shunt and fetal RV, relative to control. There are no differences in RV eNOS and nNOS expression between shunt and fetal groups, fetal RV iNOS expression is significantly greater than shunt RV iNOS expression. D: eNOS protein, E: nNOS protein, F: iNOS protein. Protein expression quantified by Western Blot. For each isoform, immunoblots were performed different membranes in parallel using identical aliquots of the same protein homogenate. n=5 in all groups. * p < 0.05, compared to control, δ p < 0.05, compared to shunt. Immunoblots shown performed using pooled samples for publication purposes only. The bar graphs and stated results represent densitometry performed on individual samples.

**Figure 2:** Localization of NOS isoforms within control and shunt RV tissue. For each panel, control RV is on the left, shunt RV is on the right. Stains are arranged vertically: Top Panel: DAPI (blue), 2nd Panel: Myosin (red), 3rd Panel: NOS isoform (green), Bottom Panel: Merge. A: eNOS is localized within shunt RV cardiomyocyte cytoplasm
and nuclei while eNOS localizes only within cardiomyocyte nuclei in control RV. B: nNOS does not localize within cardiomyocytes in control RV tissue but does in shunt RV. C: iNOS localizes within cardiomyocytes in both control and shunt RV tissue.

Figure 3: Total NOS activity and calcium-independent NOS activity in LV and RV tissue from control, shunt, and fetal lambs. A: In LV tissue, there were no differences in either total NOS activity or calcium-independent NOS activity (iNOS activity.) B: In RV tissue, both shunt and fetal lambs had increased total NOS activity, compared to controls, but there were no differences between groups in calcium-independent NOS activity. n=5 in all groups. Values are mean ± standard deviation. * p < 0.05, compared to control.

Figure 4: eNOS association with allosteric modulator calmodulin and chaperone protein hsp90. A: After immunoprecipitation (IP) using anti-calmodulin (CaM) antibody, immunoblot was performed for eNOS. eNOS is significantly more associated with CaM in both shunt and fetal RV tissue, compared to controls. eNOS levels were normalized to CaM protein after IP. B: After IP using anti-eNOS antibody, immunoblot was performed for hsp90. hsp90 is significantly more associated with eNOS in both shunt and fetal RV, compared to controls. Hsp90 levels were normalized to eNOS protein after IP. n=5 in all groups. Values are mean ± standard deviation. * p < 0.05, compared to control. Immunoblots shown performed using pooled samples for publication purposes only. The bar graphs and stated results represent densitometry performed on individual samples.
**Figure 5:** Akt expression and phosphorylation, and eNOS phosphorylation. 

**A:** Transcription of Akt RNA is upregulated in shunt and fetal RV, compared to control. Fetal LV has increased Akt RNA transcription, but there is no increase in shunt LV Akt RNA expression, compared to control. Values are mean ± SEM. 

**B:** Akt protein expression is significantly increased in shunt and fetal RV tissue, relative to control. While fetal LV Akt protein expression is significantly increased, there is no change in shunt LV expression, relative to control. Values are mean ± standard deviation. 

**C:** Akt phosphorylation is increased in shunt RV tissue, relative to control, and an even greater proportion of fetal RV Akt is phosphorylated, compared to both shunt and control. Fetal LV tissue also had increased Akt phosphorylation. IP was performed with Akt antibody, and then p-Akt protein was quantified and normalized to total Akt protein. Values are mean ± standard deviation. 

**D:** eNOS phosphorylation by Akt at serine residue 1177 is increased in shunt RV tissue, relative to controls; Ser1177-p-eNOS is further increased in fetal RV tissue, compared to both shunt and control. Fetal LV tissue also has significantly greater Ser1177-p-eNOS, relatively to control, but there are no differences in shunt and control LV. Values are mean ± standard deviation. n=5 in all groups. *p < 0.05, compared to control, δp < 0.05, compared to shunt. Immunoblots shown performed using pooled samples for publication purposes only. The bar graphs and stated results represent densitometry performed on individual samples.

**Figure 6:** Ryanodine receptor 2 (RyR2) modification by S-nitrosylation (SNO). After isolation of sarcoplasmic reticulum vesicles, IP was performed for RyR2, then IB was performed for SNO at the appropriate molecular weight for RyR2. A more significant
proportion of RyR2 exists in nitrosylated form in both shunt and fetal RV, compared to controls. n=5 in all groups. Values are mean ± standard deviation. * p < 0.05, compared to control, δ p < 0.05, compared to shunt. Immunoblots shown performed using pooled samples for publication purposes only. The bar graphs and stated results represent densitometry performed on individual samples.

Table 1: Quantitative PCR Primers

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<th>Target Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>gtctcctctctctcctt</td>
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<tr>
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A

**IP:** CaM

**IB:** eNOS

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<tbody>
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<tr>
<td>RV</td>
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MW (kDa) 23

B

**IP:** eNOS

**IB:** hsp90

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MW (kDa) 140

**IB:** CaM

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<tr>
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**MW (kDa) 23**

**hsp90/eNOS Ratio**

**Relative hsp90/eNOS Expression (Fold Control)**

- Control
- Shunt
- Fetal

**Relative eNOS/CaM Ratio**

**Relative eNOS/CaM Expression (Fold Control)**

- LV
- RV

- Control
- Shunt
- Fetal