## Title

Simvastatin restores pulmonary endothelial function in the setting of pulmonary over-circulation.



## Authors

Boehme, Jason
Sun, Xutong
Lu, Qing
et al.

## Publication Date

2024

## DOI

10.1016/j.niox.2023.11.007

Peer reviewed

HHS Public Access

Author manuscript

*Nitric Oxide*. Author manuscript; available in PMC 2024 April 25.

Published in final edited form as:

*Nitric Oxide*. 2024 January 01; 142: 58–68. doi:10.1016/j.niox.2023.11.007.

# Simvastatin restores pulmonary endothelial function in the setting of pulmonary over-circulation

**Jason T. Boehme**[a], **Xutong Sun**[b,c], **Qing Lu**[b,c], **Jubilee Barton**[a], **Xiaomin Wu**[d], **Wenhui Gong**[a], **Gary W. Raff**[e], **Sanjeev A. Datar**[a], **Ting Wang**[b,c], **Jeffrey R. Fineman**[a,f], **Stephen M. Black**[b,c,g,*]

[a]Department of Pediatrics, University of California San Francisco, San Francisco, CA, 94143, USA

[b]Center for Translational Science, Florida International University, 11350 SW Village Parkway, Port St Lucie, FL, 34987-2352, USA

[c]Department of Environmental Health Sciences, Robert Stempel College of Public Health and Social Work, Florida International University, Miami, FL, 33199, USA

[d]Department of Medicine, University of Arizona Health Sciences, Tucson, AZ, 85719, USA

[e]Department of Surgery, University of California Davis, Davis, CA, 95817, USA

[f]Cardiovascular Research Institute, University of California San Francisco, San Francisco, CA, 94143, USA

[g]Department of Cellular Biology & Pharmacology, Howard Wertheim College of Medicine, Florida International University, Miami, FL, 33199, USA

## Abstract

Statin therapy is a cornerstone in the treatment of systemic vascular diseases. However, statins have failed to translate as therapeutics for pulmonary vascular disease. Early pulmonary



*Corresponding author. Florida International University's Center for Translational Science 11350 SW Village Parkway Port St. Lucie, FL, 34987-2352, USA. stblack@fiu.edu (S.M. Black).

Generative AI

Generative AI technology was not used in the composition of the manuscript or any supporting content.

CRediT authorship contribution statement

**Jason T. Boehine:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Xutong Sun:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Qing Lu:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Jubilee Barton:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Xiaomin Wu:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Wenhui Gong:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Gary W. Raff:** Investigation, Methodology, Writing – review & editing. **Sanjeev A. Datar:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Ting Wang:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Jeffrey R. Fineman:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing. **Stephen M. Black:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing, Project administration.

Conflicts of interest

The authors declare they have no conflicts of interest with the contents of this article.

vascular disease in the setting of congenital heart disease (CHD) is characterized by endothelial dysfunction, which precedes the more advanced stages of vascular remodeling. These features make CHD an ideal cohort in which to re-evaluate the potential pulmonary vascular benefits of statins, with a focus on endothelial biology. However, it is critical that the full gamut of the pleiotropic effects of statins in the endothelium are uncovered. The purpose of this investigation was to evaluate the therapeutic potential of simvastatin for children with CHD and pulmonary over-circulation, and examine mechanisms of simvastatin action on the endothelium. Our data demonstrate that daily simvastatin treatment preserves endothelial function in our shunt lamb model of pulmonary over-circulation. Further, using pulmonary arterial endothelial cells (PAECs) isolated from Shunt and control lambs, we identified a new mechanism of statin action mediated by increased expression of the endogenous Akt1 inhibitor, C-terminal modifying protein (CTMP). Increases in CTMP were able to decrease the Akt1-mediated mitochondrial redistribution of endothelial nitric oxide synthase (eNOS) which correlated with increased enzymatic coupling, identified by increases in NO generation and decreases in NOS-derived superoxide. Together our data identify a new mechanism by which simvastatin enhances NO signaling in the pulmonary endothelium and identify CTMP as a potential therapeutic target to prevent the endothelial dysfunction that occurs in children born with CHD resulting in pulmonary over-circulation.

**Keywords**

CTMP; Akt1; Nitric oxide; Endothelial dysfunction; Endothelial nitric oxide synthase; Pulmonary vascular disease; Pulmonary hypertension; Congenital heart disease

## 1. Introduction

Statin therapy is a cornerstone in the treatment of systemic vascular diseases. While some of their therapeutic benefits are attributable to lowering serum cholesterol, research has clearly shown that their impacts on vascular diseases are mediated through diverse and wide-reaching biological mechanisms [1]. Despite, this, statins have failed to translate as therapeutics for pulmonary vascular disease. In experimental pre-clinical models of pulmonary arterial hypertension (PAH), statins have been shown to exert potent biologic effects, particularly on the pulmonary artery smooth muscle cells (PASMCs), attenuating and reversing vascular medial wall thickening, and inhibiting proliferation and promoting apoptosis of PASMCs within the vasculature [2]. Despite these promising pre-clinical results, the clinical efficacy of statin therapy in pulmonary hypertension (PH) remains controversial [3]. Existing randomized clinical trials have been disappointing, failing to show significant improvements in progression and physiologic disease outcomes [4–7].

Congenital heart disease (CHD) is an important cause of PAH in children and adults [8]. Heart defects that expose the pulmonary arteries to increased pressure and flow provoke biologic alterations and eventual pathologic remodeling of the vasculature. Early in the course of these vascular changes, restoration of normal blood flow patterns will reverse the progression of disease. However, with prolonged exposure to abnormal hemodynamics, the vascular changes become irreversible and progressive, resulting in severe PAH [9]. CHD is one of the few etiologies of PAH in which a natural progression is understood, and in

which early disease states can be well characterized [9]. Of particular note, early pulmonary vascular disease in the setting of CHD is characterized primarily by endothelial dysfunction, which precedes the more advanced stages of proliferative vascular remodeling [9]. These features make CHD an ideal cohort in which to re-evaluate the potential pulmonary vascular benefits of statins, with a focus on endothelial biology. Outside their effects on circulating lipids and atherogenesis, statins are known to exert protective and beneficial effects directly on the vascular endothelium in a variety of pathologic states. This includes amelioration of ischemia/reperfusion injury, attenuation of sepsis-induced systemic hypotension [10] and pulmonary vascular permeability [11], and protection against fetal/placental vascular sequelae of hypertension in pregnancy [12]. Notably, many of these effects are shown to be mediated, at least in part, through modulation of the nitric oxide (NO) signaling pathway [10–12]. NO signaling and endothelial NO synthase (eNOS), the predominant source of endothelial NO, play a central role in the pathology of PAH and constitute one of the major therapeutic targets in clinical care [13]. Disturbances of endothelial NO production are of particular importance to the early stages of vascular dysfunction observed in CHD [9,14]. While CHD patients have been included to some extent in clinical trials of statins in PAH, these are exclusively adult patients with clinical metrics suggesting advanced disease phenotypes, likely affected by significant vascular remodeling beyond just endothelial dysfunction. In the Atorvastatin in Pulmonary Arterial Hypertension APATH study, the largest of these trials, ~45 % of enrolled patients had a history of CHD. On subgroup analysis these patients, like the cohort as a whole, did not show any benefit to therapy. However, the average age of these patients is ~35 years and the mean pulmonary vascular resistance is quite high (~1500 dyn-sec/cm^5), with nearly all patients exhibiting symptoms of heart failure (WHO class II-III) [6].

We have previously explored the effects of altered pulmonary hemodynamics on eNOS function and NO signaling in CHD utilizing a large animal model of pulmonary overcirculation [15]. The hallmark of this model is the presence of endothelial dysfunction with impaired vasodilation and vascular tone, with only early signs of medial hypertrophy and vascular remodeling [15–17]. This endothelial dysfunction is heavily mediated by post-translational regulatory mechanisms that disrupt the physiologic function of eNOS, leading to decreased production of NO and increased production of reactive oxygen species. Of note, we have shown previously that eNOS activity in this model is specifically regulated via phosphorylation events at serine (S) residues 617 and 1177 by the regulatory kinase Akt1 [18]. Canonically, such phosphorylation events are thought to activate eNOS based on in-vitro evaluations of enzyme kinetics [19,20]. However, we have previously shown in-vivo that under nitrative stress conditions, such as those present in our model of pulmonary overcirculation, Akt-mediated phosphorylation of eNOS at $S^{617}$ results in a conformational change and exposure of a peptide sequence targeting enzymatic redistribution from the plasma membrane to the mitochondria, where it impacts mitochondrial function [18]. These particular signaling pathways, dysregulated in our shunt model, are notable for their significant overlap with pathways shown to be modulated by statin therapy in other evaluations of endothelial function. This leads us to hypothesize that earlier stages of pulmonary vascular disease progression in CHD might be more responsive to the endothelial-based, “pleotropic” effect of statins than the patients represented in existing

clinical trials. In this study, we sought to better understand the potential impacts of this therapy in such a patient cohort.

## 2. Materials and methods

### 2.1. Lamb model, hemodynamic assessment and vasoreactivity testing

As described in detail previously [21], an 8.0 mm Gore-tex® vascular graft was used to create an anastomosis between the ascending aorta and main pulmonary artery in anesthetized late gestation male and female fetal lambs (137–141 days gestation) from mixed-breed Western ewes. Following spontaneous delivery, shunt lambs received daily treatment with oral simvastatin (n = 7, 2 mg/kg/day) or gelatin capsule (vehicle lambs, n = 6). Simvastatin dosing was extrapolated based on a literature review of effective doses reported in a number of animal models, with additional weight given to dosing regimens utilized in cardiopulmonary and vascular diseases (range 0.5–20 mg/kg), as well as scant data available from pediatric patients (0.15–0.4 mg/kg) [22–24]. Four-weeks after spontaneous delivery, statin and vehicle treated shunt lambs were anesthetized, mechanically ventilated, and instrumented to continuously measure hemodynamics and harvest tissue. Vasoreactivity testing was performed as described previously using acetylcholine (Ach) chloride (1 μg/kg) [16]. Vital signs were monitored continuously during the study, and animals were given intravenous fluids and prophylactic antibiotics per protocol. At the end of each study, all lambs were euthanized with a lethal injection of sodium pentobarbital followed by bilateral thoracotomy as described in the NIH Guidelines for the Care and Use of Laboratory Animals. All protocols and procedures were approved by the Committees on Animal Research of the University of California, San Francisco and University of California, Davis. Lung tissue was obtained for eNOS protein levels, NOS activity (conversion of $^{3}$H-L-arginine to $^{3}$H-L-citrulline), and superoxide (EPR) determinations, and blood was obtained for NOx (indirect determinant of bioavailable NO via chemiluminescence) determinations.

### 2.2. Cell isolation and culture

Primary pulmonary artery endothelial cells from shunt lambs were isolated from sections of the proximal (main, left and right) pulmonary arteries by explant method as previously described [25] PAEC identity was confirmed for each unique cell line by immunostaining (>99 % positive) with established vascular endothelial cell markers. Cells were maintained in culture with a medium of DMEM (Glucose 1 g/L) with sodium pyruvate (110 mg/L) and glutamine supplemented with 10 % Fetal Bovine Serum (Lonza), and standard antimicrobial agents. They were grown in an incubator at 5 % $CO_2$ and 37 °C and passaged between 70 and 90 % confluence. All experiments were performed on passage-matched (within ± 1 passage) primary cells between passages 4–8.

### 2.3. Measurement of NOS activity

NOS activity was determined in the peripheral lung tissue from statin and vehicle treated shunt lambs using the conversion of [$^{3}$H] arginine to [$^{3}$H] citrulline as previously described [26].

### 2.4. Measurement of superoxide levels in peripheral lung tissue

EPR measurements were performed in the peripheral lung tissue from statin- and vehicle-treated shunt lambs as described previously, utilizing the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) [15,27]. CMH was chosen based on its demonstrated high sensitivity in biologic specimens. Lower bounds on quantification are based on spin probe characteristics and experimental strategy, though vary highly based on these factors and normalization parameters, and are poorly defined in general [28]. NOS-derived superoxide levels were determined by incubating duplicate samples with the NOS inhibitor, ethylisothiourea (ETU, 100 μM) and calculating the reduction from paired uninhibited samples.

### 2.5. Measurement of bioavailable NO (NOx) from plasma, cells, and cell culture media

Bioavailable NO (NOx) was measured as previously described [17] using a Sievers 280i Nitric Oxide Analyzer (GE Analytical, Boulder, CO). Results were analyzed by measuring the area under the curve of the chemiluminescence signal from cell culture media and from plasma following protein removal by precipitation with cold ethanol and centrifugation. *Per manufacturer documentation, the Sievers Nitric Oxide Analyzer NOA 280i* supports *the construction of calibration curves down to limits of 0.2 μM for 10 μl of sample injection*.

### 2.6. Preparation of protein extracts and Western blot analysis

Lung and PAEC protein extracts were prepared and used for Western blot analysis as previously described [29]. Mitochondrial fractions were purified using a commercially available kit (Qiagen Inc) per manufacturers specifications. Briefly, protein extracts (50 μg) were separated on 4–20 % Tris-SDS gels. All gels were electrophoretically transferred to Immuno-Blot PVDF membrane (Bio-Rad Laboratories) and blocked with 5 % nonfat dry milk in Tris-buffered saline containing 0.1 % Tween-20 (TBST). Membranes were then probed at room temperature with primary antibodies to Akt1 (Cell Signaling, Danvers, MA), pS473 Akt1 (Cell Signaling, Danvers, MA), eNOS (BD Transduction, Franklin Lakes, NJ), pS1177 eNOS (BD Transduction, Franklin Lakes, NJ), pS617 eNOS (Millipore, Burlington, MA and Invitrogen, Waltham, MA), and CTMP (Cell Signaling, Danvers, MA). Membrane were washed and then incubated with appropriate secondary IgG conjugated to horseradish peroxidase. Protein bands were then visualized with chemiluminescence (SuperSignal West Femto Substrate Kit, Pierce Laboratories, Rockford, IL) on a Kodak 440CF Image Station (Kodak, Rochester, NY) or a LI-COR Odyssey image station (Lincoln, NE). Bands were quantified using the LI-COR Image Station software. To normalize for protein loading, blots were re-probed with the housekeeping protein, β-actin for tissue and whole-cell lysates, and VDAC for mitochondrial protein fractions.

### 2.7. DAF-FM

NO levels in cultured PAECS were determined by using DAF-FM diacetate (ThermoFisher, Waltham, MA), a cell-permeable fluorescent dye, according to the product's instruction. Briefly, cells were aspirated, rinsed in PBS and cultured in media with DAF-FM diacetate (5 μM) for 30 min in the dark at 37 °C, then subjected to fluorescence microscopy. Fluorescent images were taken by using an Olympus IX51 microscope equipped with a

CCD camera (Hamamatsu Photonics). We quantified the average fluorescent intensities by using ImagePro Plus version 5.0 imaging software (Media Cybernetics).

### 2.8. Immunohistochemistry and eNOS/mitochondrial co-localization

Endothelial cells were grown on cover glass to 100 % confluent. Cells were then fixed with 4 % paraformaldehyde for 30 min, at room temperature. The permeabilization was done with 100 % cold methanol at −20 °C for 5 min. Cells were then blocked with 1 % BSA for 1 h, at room temperature, then incubated with primary antibody at 4 °C overnight. Slides were washed then incubated at room temperature for 1 h with secondary antibody. Cells were mounted on microscope slides using Prolong Gold Anti Fade Reagent (Cell Signaling). Nikon Eclipse TE2000-U microscope, with Hamamatsu digital camera C11440 was used to obtain immunofluorescent images. For each independent cell line 10 HPF per treatment group were randomly selected for image analysis. The images were analyzed with NIS-Elements (Nikon) to evaluate the Pearson's correlation for fluorescent colocalization for each HPF.

### 2.9. Adenoviral overexpression of CTMP

Ovine Shunt PAEC (100,000 cells/well) were plated into 6-well plate, allowed to adhere overnight, then transduced with the adenoviral CTMP overexpression construct AdCTMP (Vector Biolabs) according to the manufacturer's specifications at a multiplicity of infection (MOI) of 10:1 in 1 ml of growth medium for 48 h as described above.

## 3. Results

### 3.1. Statin therapy preserves pulmonary vascular endothelial nitric oxide signaling

Following spontaneous delivery, shunt lambs received daily treatment with oral simvastatin (2 mg/kg/day) or gelatin capsule vehicle. At 4–6 weeks of age, all shunt animals were subjected to terminal physiologic evaluation and *in vivo* vascular reactivity analysis and tissues were collected for biochemical analyses. Baseline hemodynamic parameters between groups (6 statin treated shunt and 7 vehicle treated shunt animals) were measured and found to be similar (Table 1). We examined eNOS, the principal source of vascular NO and mediator of endothelium-dependent vasodilation, and found no difference in eNOS protein expression in the lung tissue of statin compared to vehicle-treated shunt lambs by Western blot analysis (Fig. 1A). We have previously shown that the endothelial dysfunction in shunt animals is related to a progressive enzymatic uncoupling of eNOS over time, leading to a reduction in NO production and increasing NOS-derived superoxide [15]. Turning our attention to enzyme activity, we identified a four-fold increase in NOS activity in statin-treated compared to vehicle-treated shunt lambs (Fig. 1B). This corresponded to significantly diminished levels of lung tissue superoxide levels and NOS-derived superoxide levels in statin treated compared to vehicle-treated shunt animals (Fig. 1C & D). We have previously demonstrated uncoupled eNOS as a source of significant superoxide/ROS production in the vascular endothelium and, taken with the increase in NOS activity, this result is consistent with restoration of eNOS coupling [15]. However, it is worth noting that statins have been shown to ameliorate oxidative stress in the cardiovascular system through various mechanisms which may also be contributing to these observations.

Particularly, impacts of statin therapy on PPARγ signaling and the activation of small GTPases such as Rac1 are known to modulate vascular ROS production and intersect with previously described alterations in the pulmonary vasculature of the shunt animals [17]. Evaluation of plasma samples revealed an ~2-fold increase in the level of bioavailable NOx in plasma samples of statin-treated, compared to vehicle-treated shunt lambs, which would be consistent with increased NO production (Fig. 1E) [30,31]. We have previously shown that shunt lambs have an impaired response to the vasodilator acetylcholine (Ach), which requires endothelial stimulation and the acute production of eNOS-derived NO to induce vasodilation [16]. Vasoreactivity testing performed in the intact animals demonstrated that statin therapy significantly improved the endothelial response to Ach, with treated animals exhibiting a 21 % reduction in mean pulmonary artery pressure (mPAP) compared to a 13 % reduction in vehicle-treated shunt lambs ($p < 0.05$), as well as a 15 % decrease in pulmonary vascular resistance (PVR) compared to 4 % in the vehicle-treated group ($p < 0.05$) (Fig. 1F) signifying a substantial improvement in vasorelaxation. Taken together these data indicates a significant improvement in physiologic endothelial function with statin therapy associated with evidence of enhanced eNOS activity and improved enzymatic coupling.

### 3.2. Statin therapy increases NO generation and decreases NOS-derived superoxide in pulmonary artery endothelial cells

In order to better understand the results observed in our animal model, we turned to primary cell culture in order to characterize the *in vitro* effects of statin therapy in pulmonary artery endothelial cells (PAECs). Statin therapy has been shown to modulate eNOS activity and endothelial NO production *in vitro* through several potential mechanisms, with variability, described based on treatment duration, underlying pathologic conditions, and the vascular bed examined [32–35]. Specific evaluations of the pulmonary vascular endothelium in this regard are scarce, and have largely been limited to models of hypoxic injury [36,37]. Thus, we first evaluated control PAECs. Upon exposure serum free media for 7 h followed by 1 h of simvastatin (5 μM), control PAECs demonstrated significantly increased production of NO by both DAF-FM fluorescence and $NO_x$ determination (Fig. 2A and B). This was accompanied by a significant decrease in the production of NOS-derived superoxide (Fig. 2C) in statin-treated cells, suggesting improved enzymatic coupling. eNOS activity is subject to multiple regulatory mechanisms, including expression changes, phosphorylation events and other post-translational modifications, and protein-protein interactions [38]. We have previously shown from work in our shunt model that eNOS activity and function in the PAECs are affected by Akt-mediated phosphorylation at serine (S) residues 617 and 1177 [18]. Examining these phosphorylation events in control PAECs, we found evidence of diminished $S^{1177}$ and $S^{617}$ phosphorylation of eNOS in the statin-treated cells (Fig. 2D and E). Akt1 itself is activated by phosphorylation at $S^{473}$, and we observe a corresponding decrease in relative $S^{473}$ phosphorylation of Akt (Fig. 2F) with statin treatment. In enzymatic studies, phosphorylation at $S^{1177}$ and $S^{617}$ tends to activate eNOS activity by increasing its sensitivity to $Ca^{2+}$ calmodulin [19,20]. However, we have previously shown *in vivo* that under nitrative stress conditions, Akt-mediated phosphorylation of eNOS at these residues, particularly $S^{617}$, promotes enzymatic redistribution from the plasma membrane to the mitochondria limiting NO generation [18]. Evaluating this phenomenon in our control PAECs, we see by immunofluorescence that statin therapy significantly diminishes

mitochondrial co-localization of eNOS (Fig. 2G) and decreases levels of eNOS in the mitochondrial protein fraction as determined by Western blot analysis (Fig. 2H).

### 3.3. Pulmonary artery endothelial cells from shunt animals exhibit Akt induced mitochondrial translocation of eNOS and enzymatic uncoupling

We next evaluated the effects of prolonged exposure to increased pressure and flow on this mechanism by evaluating PAECs derived from shunt animals compared to physiologically normal age matched controls under baseline cell culture conditions. We see that shunt PAECs exhibit significantly increased Akt phosphorylation at $S^{473}$ (Fig. 3A) along with increased eNOS phosphorylation at $S^{1177}$ (Fig. 3B) and $S^{617}$ (Fig. 3C), consistent with increased Akt-mediated phosphorylation of eNOS. Correspondingly, shunt PAECs exhibit significantly greater mitochondrial distribution of eNOS by immunofluorescent colocalization (Fig. 3D) as well as evidence of enzymatic uncoupling, demonstrated by diminished production of NO (Fig. 3E and F) along with increased production of NOS-derived superoxide (Fig. 3G).

### 3.4. Statin therapy in shunt pulmonary artery endothelial cells inhibits mitochondrial translocation of eNOS and restores enzymatic coupling

We next investigated statin therapy using primary shunt PAECs, to determine if the treatment effects observed in control PAECs could potentially offset the abnormalities induced by chronic exposure to pulmonary overcirculation. We see again in shunt PAECs that statin therapy diminishes Akt1 phosphorylation at $S^{473}$ (Fig. 4A) and decreases mitochondrial trafficking of eNOS (Fig. 4B), as in treated control PAECs. Importantly, we find that in the shunt PAECs, this is associated with improvements in NO production (Fig. 4C and D), and evidence of improved enzymatic coupling with a significant decrease in the production of NOS-derived superoxide (Fig. 4E).

### 3.5. Statin therapy in shunt lambs inhibits Akt1 mediated phosphorylation and mitochondrial redistribution of eNOS

In order to validate our findings from the cell culture models, we turned to the biochemical evaluation of whole lung tissue from statin- and vehicle-treated shunt lambs. As observed with the treatment of PAECs, statin therapy *in vivo* was associated with a significant reduction in $S^{473}$-phosphorylated Akt1 (Fig. 5A) in lung tissue. Correspondingly, we found relative reductions in both $pS^{1177}$ and $pS^{617}$ eNOS respectively (Fig. 5B & C), compared to vehicle-treated shunt lambs. Also, as seen in the PAECs, these changes in Akt1 and eNOS phosphorylation were associated with decreases in relative mitochondrial eNOS levels in the lung of statin-treated shunt lambs (Fig. 5D).

### 3.6. Statin therapy increases PAEC and whole lung CTMP levels

We have previously shown in our shunt model that increases in Akt1 $S^{473}$ phosphorylation, as well as eNOS S617 phosphorylation in PAECs, correlate with reduced levels of carboxy-terminal modulator protein (CTMP) and decreased interaction of CTMP with Akt1 [39]. CTMP is an endogenous negative regulator of Akt1, which acts through interaction at the C-terminal domain of Akt to prevent its activation by phosphorylation at $S^{473}$ and $T^{308}$

[40]. We confirmed that shunt PAECs have significantly decreased CTMP levels relative to control cells (Fig. 6A), and sought to determine if CTMP was contributing to the observed decrease in Akt1 activity with statin therapy. First evaluating the effects in our cell culture model, we see that statin treatment increases cellular CTMP levels relative to untreated cells in both control (Fig. 6B) and shunt (Fig. 6C) PAECs. This is reflected upon examination of whole lung tissue, with statin-treated shunt lambs exhibiting increased CTMP compared to vehicle treated (Fig. 6D).

### 3.7. CTMP overexpression inhibits mitochondrial translocation of eNOS and restores enzymatic coupling

To confirm that the observed increase in CTMP with statin therapy is relevant to the endothelial improvements in eNOS function and NO signaling, we used an adenoviral vector to overexpress (OE) CTMP in untreated shunt PAECs. The adenoviral vector was able to potently induce CTMP expression with a clear dose-dependent response based on multiplicity of infection (Fig. 7A). Evaluating the effects on eNOS function, we found that adenoviral OE of CTMP enhanced cellular NO production as determined by both DFA-FM fluorescence (Fig. 7B) and increased $NO_x$ in cell culture media (Fig. 7C) compared to non-transduced shunt PAECs. As with statin therapy, this CTMP mediated improvement in NO production was coupled with a decrease in mitochondrial localized eNOS (Fig. 7D) and improved enzymatic coupling with diminished NOS-derived superoxide (Fig. 7E).

## 4. Discussion

eNOS is subject to complex regulatory mechanisms, both transcriptional and post-transcriptional, though these rapid changes are believed to be facilitated by phosphorylation. Phosphorylation events at specific serine, threonine, and tyrosine residues stimulate and, at some loci, inhibit eNOS activity. These phosphorylation events are mediated by multiple kinases, notably Akt1, PKA, and AMPK, in response to various stimuli [38]. Statins have been previously demonstrated to enhance eNOS function, stimulating increased NO production by endothelial cells within minutes of exposure [33] due to their ability to impact eNOS phosphorylation through modulation of Akt1 activity, although PKA and AMPK can also be involved [33,34,41]. Akt1 itself is canonically activated by mobilization to the plasma membrane and its phosphorylation at Serine 473 ($S^{473}$) by the mTOR complex 2 (mTORC2) and at Threonine 308 ($T^{308}$) by pyruvate dehydrogenase kinase 1 (PDK1). Akt1 activity is also modulated heavily through other mechanisms, including protein-protein interactions [42]. eNOS $S^{1177}$ is a consistent target of Akt1-mediated phosphorylation, which is widely shown to stimulate increased NO production by enhancing $Ca^{2+}$/Calmodulin sensitivity and enhancing electron flow through the reductase domain [38]. Akt1 has also been shown to phosphorylate eNOS at $S^{617}$, though the role of this modification is less straightforward. $S^{617}$ phosphorylation is often identified simply as an activating event, and phosphomimetic substitutions of this residue do indeed show increased $Ca^{2+}$ sensitivity and NO generation. However, de-phosphorylated mimetic substitutions similarly show increased NO production, with discrepant findings in enzyme kinetic assays compared to transfected cell lines. These alterations have further been shown to significantly impact various protein-protein interactions of eNOS [43]. Previous work from our group

has demonstrated a unique role for phosphorylation at this eNOS $S^{617}$ residue, specifically facilitated by Akt1 that has undergone nitration at the Tyrosine 350 ($Y^{350}$) residue in response to increased cellular nitrative stress [18]. Structural simulations indicate that this $S^{617}$ phosphorylation displaces an autoinhibitory domain, exposing the nearby pentabasic eNOS mitochondrial targeting sequence (residues 628–632) [18,44], leading to increased trafficking and localization of eNOS to the mitochondrial membrane.

In this study, we expand on previously described mechanisms of eNOS dysfunction in the pulmonary vascular endothelium induced by prolonged exposure to abnormal hemodynamic forces. Here, and in prior work, we have shown that this exposure is associated with Akt1 activation via phosphorylation at $S^{473}$ and $T^{308}$ [39], while others have also demonstrated the effects of shear forces on Akt1 activation [45,46]. The activation of Akt1 has also been previously associated with Akt1 nitration at $Y^{350}$, itself shown to increase Akt1 activity overall, and specifically facilitating eNOS phosphorylation at $S^{617}$ [39]. Based on previous work, this nitration of Akt1 can be induced by increased levels of ADMA, while the increased $S^{473}$ phosphorylation is due, at least in part, to loss of CTMP in Shunt lambs [39]. We show here that the activation of Akt1 and phosphorylation of eNOS at both $S^{617}$ and $S^{1177}$ in these circumstances is associated with mitochondrial translocation of eNOS and decreased NO production, with prominent evidence of enzymatic uncoupling. Though $pS^{1177}$ is consistently shown to activate eNOS, here we see an overall decrease in cellular NO production. This leads us to conclude that other regulatory mechanisms supersede the activation by $pS^{1177}$, resulting in the observed net decrease. We know that important eNOS regulatory mechanisms in addition to Akt1 are deranged in the shunt endothelial cells, including abnormalities of enzymatic substrates such as arginine [47], and binding partners such as Hsp90 [27]. Hsp90 chaperones eNOS and promotes NO production, as well as maintaining eNOS in a coupled, dimerized configuration, and facilitating interactions with kinases, including Akt1 [35]. Disruption of Hsp90/eNOS interactions could contribute to the evidence of enzymatic uncoupling we see in our data. Though not examined directly in this study, shear stress is also an important stimulus that tends to increase both expression and luminal distribution of the protein caveolin 1 [48], an important binding partner and negative regulator of eNOS activity at the plasma membrane [38]. Caveolar dynamics are further implicated in having direct effects on Akt1 activation and function in response to mechanical stimuli [49].

Importantly, it is unclear exactly how the phosphorylation of eNOS at S617 is impacting NO production and enzymatic coupling. This event is strongly implicated by previous work to initiate increased mitochondrial translocation of eNOS, as observed in this study. Still, it remains unclear exactly what role this mitochondrial eNOS plays. Data is sparse, but suggests that under physiologic conditions, mitochondrial eNOS decreases mitochondrial oxygen consumption and superoxide production, without impairing cellular ATP or NO production [18,44]. Notably, eNOS targeted to the mitochondrial membrane via fusion to a cytochrome c oxidase targeting sequence failed to produce NO [50]. The relevance of such a fusion construct to the mitochondrial eNOS described here is uncertain, but if the mitochondrial eNOS in this setting fails to produce NO efficiently at the expense of a more productive localization at the plasma membrane, it is likely contributing to the impaired cellular NO generation. Perhaps more interesting, we have previously demonstrated

that mitochondrial translocation of eNOS induced by non-physiologic stressors, such as exogenous ADMA, leads to nitrosative stress and nitration of mitochondrial proteins such as carnitine acetyltransferase (CrAT) [17]. The associated disruptions of carnitine metabolism, in turn, contribute to mitochondrial dysfunction, compromised cellular ATP generation, and perpetuation of oxidative and nitrosative stress [51]. This suggests possible interactions, and even potential feed-forward mechanisms, by which nitrosative stress contributes to the Akt1-mediated mitochondrial targeting of eNOS, which perpetuates nitrosative stress by suppressing carnitine metabolism. This could lead to compounding disruptions of mitochondrial function, cellular energy generation and cellular redox balance, which might contribute to diminished NO production and help account for the prominent enzymatic uncoupling we observe.

Interestingly, we find that statin therapy in PAECs is associated with diminished activation of Akt1-mediated phosphorylation of eNOS in both control and shunt PAECs. Other models of endothelial statin exposure demonstrate acute activation of Akt1 and increased phosphorylation of eNOS [33,52]. Notably, these studies often focus on relatively acute time frames, ranging from minutes to hours following exposure. Results from animal models shed some insight on longer-term and *in vivo* exposures. Still, they are also more variable, with many showing increased phosphorylation of eNOS at $S^{1177}$ with statin treatment, but often implicating mechanisms other than canonical Akt1 activation. Furthermore, these are typically injury models, evaluating the responses to statins only in the setting of concurrent pathologic derangements [34,37]. There is also evidence that even within models, the endothelial responses to statins are dependent on the specificities of distinct vascular beds [35]. In this study, we identify rescue of CTMP as a novel mechanism by which statins mediate eNOS activity and endothelial NO production in a model of pressure/flow induced endothelial dysfunction. The importance of CTMP as an endogenous negative regulator of Akt1 signaling is well established [53], and we have previously shown that this regulatory mechanism is impaired in shunt lambs [39]. The regulatory control of CTMP remains poorly understood overall, though previous work from our group has shown that heat shock protein 70 (Hsp70) coordinates proteasomal degradation of CTMP, which is induced by the presence of ADMA and disruption of CTMP-Akt1 interaction [39]. Statins have complex interactions with the ER stress response, and it is possible they may rescue CTMP through inhibition of stress-induced proteasomal degradation [54]. Statins have also been shown to lower ADMA levels in the serum of patients [55] and may be rescuing CTMP, and also directly modulating Akt1 nitration, by impacting circulating and cellular production of ADMA. We have previously identified CTMP as a potential therapeutic target in pulmonary vascular disease, and it has similarly garnered interest in malignancies characterized by dysregulated Akt1 signaling [56,57]. In this study we associate CTMP levels with eNOS enzymatic performance both *in vitro* and *in vivo*, and also identify statins as a potential pharmacologic modulator of this regulatory mechanism. Additional work is needed to understand these effects and elaborate on the impacts of statin therapy on other relevant pathways such as Hsp90 and caveolin 1. However, the physiologic improvements we see in the animal model are encouraging, and further study of statins in this model may help us identify novel therapeutic targets relevant to children with CHD and pulmonary vascular dysfunction.

## Funding

This research was supported in part by HL60190 (SMB), HL137282 (SMB/JRF), HL134610 (SMB/TW/EZ), HL142212 (SMB/EZ), HL146369 (SMB/TW/JRF), HL133034 (SD), HL148512 (JB) and the Interdisciplinary Training in Cardiovascular Research T32 HL007249 (to XW) all from the National Institutes of Health.

## Data availability

Data will be made available on request.

## References

[1]. Oesterle A, Laufs U, Liao JK, Pleiotropic effects of statins on the cardiovascular system, Circ. Res 120 (1) (2017.Jan 6) 229–243. [PubMed: 28057795]

[2]. Nishimura T, Vaszar LT, Faul JL, Zhao G, Berry GJ, Shi L, Qiu D, Benson G, Pearl RG, Kao PN, Simvastatin rescues rats from fatal pulmonary hypertension by inducing apoptosis of neointimal smooth muscle cells, Circulation (2003).

[3]. Wang L, Yang T, Wang C, Are statins beneficial for the treatment of pulmonary hypertension? Chronic Dis Transl Med 3 (2017) 213–220. [PubMed: 29354804]

[4]. Wilkins MR, Ali O, Bradlow W, Wharton J, Taegtmeyer A, Rhodes CJ, Ghofrani HA, Howard L, Nihoyannopoulos P, Mohiaddin RH, Gibbs JSR, Simvastatin as a treatment for pulmonary hypertension trial, Am. J. Respir. Crit. Care Med 181 (2010) 1106–1113. [PubMed: 20460548]

[5]. Kawut SM, Bagiella E, Lederer DJ, Shimbo D, Horn EM, Roberts KE, Hill NS, Barr RG, Rosenzweig EB, Post W, Tracy RP, Palevsky HI, Hassoun PM, Girgis RE, Randomized clinical trial of aspirin and simvastatin for pulmonary arterial hypertension: ASA-STAT, Circulation 123 (2011) 2985–2993. [PubMed: 21593252]

[6]. Zeng WJ, Xiong CM, Zhao L, Shan GL, Liu ZH, Xue F, Gu Q, Ni XH, Zhao ZH, Cheng XS, Wilkins MR, He JG, Atorvastatin in pulmonary arterial hypertension (APATH) study, Eur. Respir. J 40 (2012) 67–74. [PubMed: 22362846]

[7]. Anand V, Garg S, Duval S, Thenappan T, A systematic review and meta-analysis of trials using statins in pulmonary arterial hypertension, Pulm. Circ 6 (2016) 295–301. [PubMed: 27683606]

[8]. Badlam JB, Badesch DB, Austin ED, Benza RL, Chung WK, Farber HW, Feldkircher K, Frost AE, Poms AD, Lutz KA, Pauciulo MW, Yu C, Nichols WC, Elliott CG, Simms R, Fortin T, Safdar Z, Burger CD, Frantz RP, Hill NS, Airhart S, Elwing J, Simon M, White RJ, Robbins IM, Chakinala MM, United States pulmonary hypertension scientific registry: baseline characteristics, Chest 159 (2021) 311–327. [PubMed: 32858008]

[9]. Van Der Feen DE, Bartelds B, De Boer RA, Berger RMF, Pulmonary arterial hypertension in congenital heart disease: translational opportunities to study the reversibility of pulmonary vascular disease, Eur. Heart J 38 (2017) 2034–2040. [PubMed: 28369399]

[10]. McGown CC, Brown NJ, Hellewell PG, Reilly CS, Brookes ZLS, Beneficial microvascular and anti-inflammatory effects of pravastatin during sepsis involve nitric oxide synthase III, Br. J. Anaesth 104 (2010) 183–190. [PubMed: 20086063]

[11]. Ren Y, Li L, Wang MM, Cao LP, Sun ZR, Yang ZZ, Zhang W, Zhang P, Nie SN, Pravastatin attenuates sepsis-induced acute lung injury through decreasing pulmonary microvascular permeability via inhibition of Cav-1/eNOS pathway, Int. Immunopharm 100 (2021), 108077.

[12]. Chimini JS, Possomato-Vieira JS, da Silva MLS, Dias-Junior CA, Placental nitric oxide formation and endothelium-dependent vasodilation underlie pravastatin effects against angiogenic imbalance, hypertension in pregnancy and intrauterine growth restriction, Basic Clin. Pharmacol. Toxicol 124 (2019) 385–393. [PubMed: 30318719]

[13]. Rosenzweig EB, Abman SH, Adatia I, Beghetti M, Bonnet D, Haworth S, Ivy DD, Berger RMF, Paediatric pulmonary arterial hypertension: updates on definition, classification, diagnostics and management, Eur. Respir. J 53 (2019).

[14]. Dickinson MG, Bartelds B, Borgdorff MAJ, Berger RMF, The role of disturbed blood flow in the development of pulmonary arterial hypertension: lessons from preclinical animal models, Am J Physiol Cell Mol Physiol 305 (2013). L1–L14.

[15]. Oishi PE, Wiseman DA, Sharma S, Kumar S, Hou Y, Datar SA, Azakie A, Johengen MJ, Harmon C, Fratz S, Fineman JR, Black SM, Progressive dysfunction of nitric oxide synthase in a lamb model of chronically increased pulmonary blood flow: a role for oxidative stress, Am. J. Physiol. Lung Cell Mol. Physiol 295 (2008) 756–766.

[16]. Mohan Reddy V, Wong J, Liddicoat JR, Johengen M, Chang R, Fineman JR, Altered endothelium-dependent responses in lambs with pulmonary hypertension and increased pulmonary blood flow, Am. J. Physiol 271 (1996).

[17]. Sun X, Sharma S, Fratz S, Kumar S, Rafikov R, Aggarwal S, Rafikova O, Lu Q, Burns T, Dasarathy S, Wright J, Schreiber C, Radman M, Fineman JR, Black SM, Disruption of endothelial cell mitochondrial bioenergetics in lambs with increased pulmonary blood flow, Antioxidants Redox Signal 18 (2013) 1739–1752.

[18]. Rafikov R, Rafikova O, Aggarwal S, Gross C, Sun X, Desai J, Fulton D, Black SM, Asymmetric dimethylarginine induces endothelial nitric-oxide synthase mitochondrial redistribution through the nitration-mediated activation of Akt1, J. Biol. Chem 288 (2013) 6212–6226. [PubMed: 23255608]

[19]. Tran QK, Leonard J, Black DJ, Nadeau OW, Boulatnikov IG, Persechini A, Effects of combined phosphorylation at Ser-617 and Ser-1179 in endothelial nitric-oxide synthase on EC50(Ca2+) values for calmodulin binding and enzyme activation, J. Biol. Chem 284 (2009) 11892–11899. [PubMed: 19251696]

[20]. Haque MM, Ray SS, Stuehr DJ, Phosphorylation controls endothelial nitric-oxide synthase by regulating its conformational dynamics, J. Biol. Chem 291 (2016) 23047–23057. [PubMed: 27613870]

[21]. Reddy VM, Meyrick B, Wong J, Khoor A, Liddicoat JR, Hanley FL, Fineman JR, In utero placement of aortopulmonary shunts: a model of postnatal pulmonary hypertension with increased pulmonary blood flow in lambs, Circulation 92 (1995) 606–613. [PubMed: 7634475]

[22]. Alfon J, Guasch JF, Berrozpe M, Badimon L, Nitric oxide synthase II (NOS II) gene expression correlates with atherosclerotic intimal thickening. Preventive effects of HMG-CoA reductase inhibitors, Atherosclerosis 145 (1999) 325–331. [PubMed: 10488960]

[23]. Ropponen JO, Syrjälä SO, Hollmén M, Tuuminen R, Krebs R, Keränen MA, Vaali K, Nykänen AI, Lemström KB, Tikkanen JM, Effect of simvastatin on development of obliterative airway disease: an experimental study, J. Heart Lung Transplant 31 (2012) 194–203. [PubMed: 22305382]

[24]. García-de-la-Puente S, Luis Arredondo-García J, Gutiérrez-Castrellón P, Bojorquez-Ochoa A, Reyna Maya E, Del Pilar Pérez-Martínez M, Efficacy of simvastatin in children with hyperlipidemia secondary to kidney disorders, Pediatr. Nephrol 24 (2009) 1205–1210. [PubMed: 19238452]

[25]. Hoage T, Ding Y, Xu X, Cardiovascular development, Cardiovasc Dev Methods Protoc 843 (2012) 11–21.

[26]. Black SM, Heidersbach RS, McMullan DM, Fineman JR, Inhaled nitric oxide inhibits nos activity in lambs: a potential mechanism for rebound pulmonary hypertension, Crit. Care Med 27 (1999) 1849–1856.

[27]. Sud N, Sharma S, Wiseman DA, Harmon C, Kumar S, Venema RC, Fineman JR, Black SM, Nitric oxide and superoxide generation from endothelial NOS: modulation by HSP90, Am. J. Physiol. Lung Cell Mol. Physiol 293 (2007) 1444–1453.

[28]. Eaton GR, Eaton SS, Quantitation of spin probe-detectable oxidants in cells using electron paramagnetic resonance spectroscopy: to probe or to trap? Compr Coord Chem III (1–9) (2021) 44–59.

[29]. Sharma S, Oishi PE, Datar SA, Jr F, Black SM, L-carnitine preserves endothelial function in a lamb model of increased pulmonary blood flow shruti, Pediatr. Res 74 (2013) 39–47. [PubMed: 23628882]

[30]. Kelm M, Nitric oxide metabolism and breakdown, Biochim. Biophys. Acta Bioenerg 1411 (1999) 273–289.

[31]. Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Gödecke A, Schrader J, Schulz R, Heusch G, Schaub GA, Bryan NS, Feelisch M, Kelm M, Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals, Free Radic. Biol. Med 35 (2003) 790–796. [PubMed: 14583343]

[32]. Laufs U, La Fata V, Plutzky J, Liao JK, Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors, Circulation 97 (1998) 1129–1135. [PubMed: 9537338]

[33]. Harris MB, Blackstone MA, Sood SG, Li C, Goolsby JM, Venema VJ, Kemp BE, Venema RC, Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors, Am. J. Physiol. Heart Circ. Physiol 287 (2004) 560–566.

[34]. Rossoni LV, Wareing M, Wenceslau CF, Al-Abri M, Cobb C, Austin C, Acute simvastatin increases endothelial nitric oxide synthase phosphorylation via AMP-activated protein kinase and reduces contractility of isolated rat mesenteric resistance arteries, Clin. Sci 121 (2011) 449–458.

[35]. Brouet A, Sonveaux P, Dessy C, Moniotte S, Balligand JL, Feron O, Hsp90 and caveolin are key targets for the proangiogenic nitric oxide-mediated effects of statins, Circ. Res 89 (2001) 866–873. [PubMed: 11701613]

[36]. Wu X, Lin D, Li G, Zuo Z, Statin post-treatment provides protection against simulated ischemia in bovine pulmonary arterial endothelial cells, Eur. J. Pharmacol 25 (2010) 114–120.

[37]. Murata T, Kinoshita K, Hori M, Kuwahara M, Tsubone H, Karaki H, Ozaki H, Statin protects endothelial nitric oxide synthase activity in hypoxia-induced pulmonary hypertension, Arterioscler. Thromb. Vasc. Biol 25 (2005) 2335–2342. [PubMed: 16166567]

[38]. Fulton DJR, Transcriptional and posttranslational regulation of eNOS in the endothelium, Adv. Pharmacol 77 (2016) 29–64. [PubMed: 27451094]

[39]. Sun X, Kellner M, Desai AA, Wang T, Lu Q, Kangath A, Qu N, Klinger C, Fratz S, Yuan JXJ, Jacobson JR, Garcia JGN, Rafikov R, Fineman JR, Black SM, Asymmetric dimethylarginine stimulates Akt1 phosphorylation via heat shock protein 70-facilitated carboxyl-terminal modulator protein degradation in pulmonary arterial endothelial cells, Am. J. Respir. Cell Mol. Biol 55 (2016) 275–287. [PubMed: 26959555]

[40]. Maira SM, Galetic I, Brazil DP, Kaech S, Ingley E, Thelen M, Hemmings BA, Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane, Science (80-) 294 (2001) 374–380.

[41]. Pei Y, Ma P, Wang X, Zhang W, Zhang X, Zheng P, Yan L, Xu Q, Dai G, Rosuvastatin attenuates monocrotaline-induced pulmonary hypertension via regulation of Akt/eNOS signaling and asymmetric dimethylarginine metabolism, Eur. J. Pharmacol 666 (2011) 165–172. [PubMed: 21641341]

[42]. Mayer IA, Arteaga CL, The PI3K/AKT pathway as a target for cancer treatment, Annu. Rev. Med 67 (2016) 11–28. [PubMed: 26473415]

[43]. Mount PF, Kemp BE, Power DA, Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation, J. Mol. Cell. Cardiol 42 (2007) 271–279. [PubMed: 16839566]

[44]. Gao S, Chen J, Brodsky SV, Huang H, Adler S, Lee JH, Dhadwal N, Cohen-Gould L, Gross SS, Goligorsky MS, Docking of endothelial nitric oxide synthase (eNOS) to the mitochondrial outer membrane: a pentabasic amino acid sequence in the autoinhibitory domain of eNOS targets a proteinase K-cleavable peptide on the cytoplasmic face of mitochondria, J. Biol. Chem 279 (2004) 15968–15974. [PubMed: 14761967]

[45]. Dimmeler S, Assmus B, Hermann C, Haendeler J, Zeiher AM, Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells, Circ. Res (1998) 334–341. [PubMed: 9710127]

[46]. Wang SP, Iring A, Strilic B, Juárez JA, Kaur H, Troidl K, Tonack S, Burbiel JC, Müller CE, Fleming I, Lundberg JO, Wettschureck N, Offermanns S, P2Y2and Gq/G11 control blood pressure by mediating endothelial mechanotransduction, J. Clin. Invest 125 (2015) 3077–3086. [PubMed: 26168216]

[47]. Sharma S, Kumar S, Wiseman DA, Tian J, Rehmani I, Datar SA, Oishi PE, Fratz S, Venema RC, Fineman JR, Black SM, Alterations in lung arginine metabolism in lambs with pulmonary

hypertension associated with increased pulmonary blood flow, Vasc. Pharmacol 51 (2009) 359–364.

[48]. Boyd NL, Park H, Yi H, Boo YC, Sorescu GP, Sykes M, Jo H, Chronic shear induces caveolae formation and alters ERK and Akt responses in endothelial cells, Am. J. Physiol. Heart Circ. Physiol 285 (2003) 1113–1122.

[49]. Sedding DG, Hermsen J, Seay U, Eickelberg O, Kummer W, Schwencke C, Strasser RH, Tillmanns H, Braun-Dullaeus RC, Caveolin-1 facilitates mechanosensitive protein kinase B (Akt) signaling in vitro and in vivo, Circ. Res 96 (2005) 635–642. [PubMed: 15731459]

[50]. Jagnandan D, Sessa WC, Fulton D, Intracellular location regulates calcium-calmodulin-dependent activation of organelle-restricted eNOS, Am. J. Physiol. Cell Physiol 289 (2005) 1024–1033.

[51]. Sharma S, Sun X, Agarwal S, Rafikov R, Dasarathy S, Kumar S, Black SM, Role of carnitine acetyl transferase in regulation of nitric oxide signaling in pulmonary arterial endothelial cells, Int. J. Mol. Sci 14 (2013) 255–272.

[52]. Wang J, Tokoro T, Matsui K, Higa S, Kitajima I, Pitavastatin at low dose activates endothelial nitric oxide synthase through PI3K-AKT pathway in endothelial cells, Life Sci. 76 (2005) 2257–2268. [PubMed: 15733940]

[53]. Liu X, Yang Q, Zhu LH, Liu J, Deng KQ, Zhu XY, Liu Y, Gong J, Zhang P, Li S, Xia H, She ZG, Carboxyl-terminal modulator protein ameliorates pathological cardiac hypertrophy by suppressing the protein kinase B signaling pathway, J. Am. Heart Assoc 7 (2018).

[54]. Mollazadeh H, Atkin SL, Butler AE, Ruscica M, Sirtori CR, Sahebkar A, The effect of statin therapy on endoplasmic reticulum stress, Pharmacol. Res 137 (2018) 150–158. [PubMed: 30312664]

[55]. Serban C, Sahebkar A, Ursoniu S, Mikhailidis DP, Rizzo M, Lip GYH, Kees Hovingh G, Kastelein JJP, Kalinowski L, Rysz J, Banach M, A systematic review and meta-analysis of the effect of statins on plasma asymmetric dimethylarginine concentrations, Sci. Rep 5 (2015) 1–8.

[56]. Niu F, Duan Y, Man Y, Liu W, Dai T, Zhang H, Li C, Wei D, Mitochondrial protein LETM1 and its-mediated CTMP are potential therapeutic targets for endometrial cancer, Anti Cancer Drugs (2022). Publish Ah:1–10.

[57]. Simon PO, McDunn JE, Kashiwagi H, Chang K, Goedegebuure PS, Hotchkiss RS, Hawkins WG, Targeting AKT with the proapoptotic peptide, TAT-CTMP: a novel strategy for the treatment of human pancreatic adenocarcinoma, Int. J. Cancer 125 (2009) 942–951. [PubMed: 19405118]

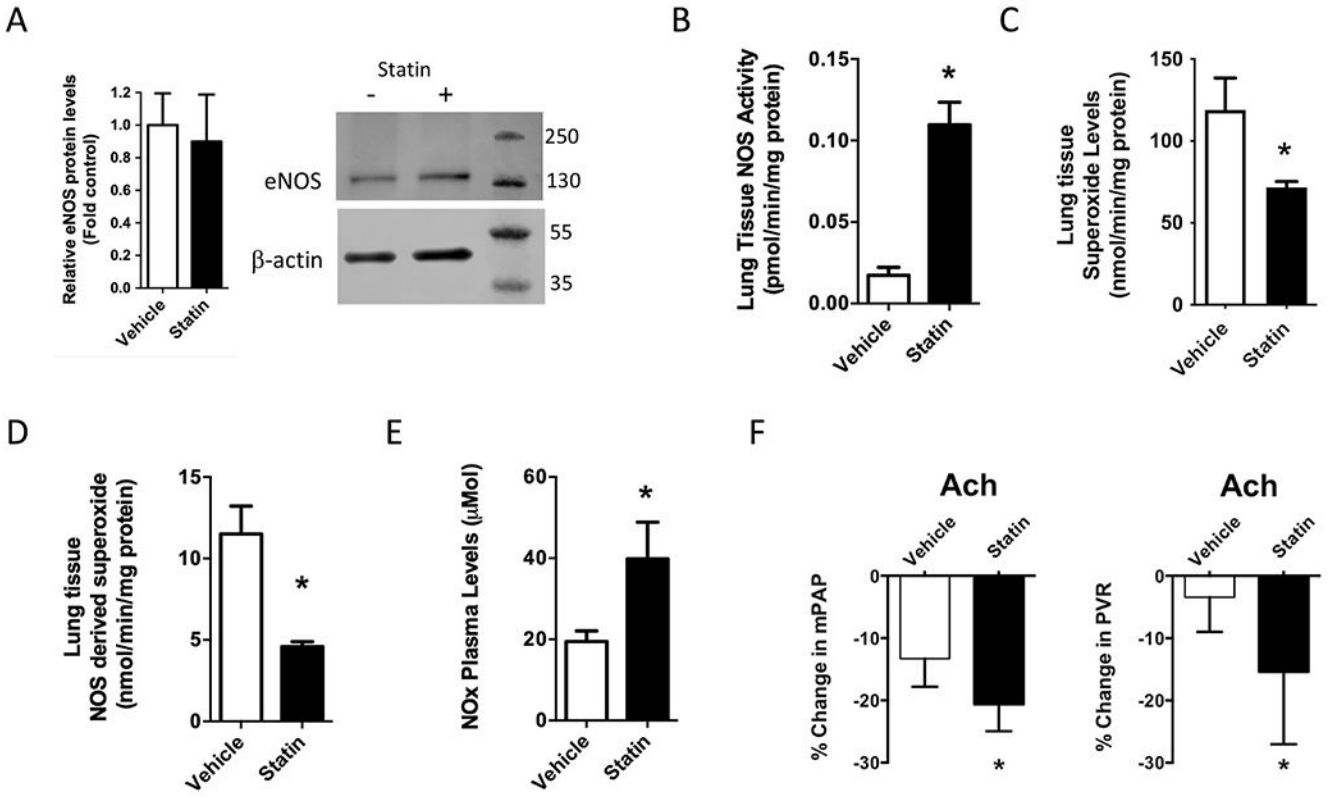


**Fig. 1. Simvastatin enhances endothelial function in Shunt lambs.**

Changes in Western blot analysis shows that total eNOS protein levels are unchanged between vehicle- and simvastatin-treated shunt lambs (N = 4) (A). A representative image is shown, β-actin was used to normalize for protein loading. NOS activity in lung tissue is higher in simvastatin-treated than vehicle-treated shunt lambs (B). Conversely, both total lung superoxide production (C), as well as NOS-derived superoxide (D), a measure of eNOS uncoupling, are decreased in simvastatin-treated shunt lambs (N = 6). $NO_x$ levels from plasma are significantly higher in simvastatin-treated than vehicle-treated shunt lambs (D). PAP and PVR are analyzed as percent change from immediate pre-treatment baseline, in response to acetylcholine (ACh, 1 μg/kg), an endothelium-dependent vasodilator, in vehicle- (N = 7) and simvastatin-treated (N = 6) shunt lambs (F). Values are mean ± SD or SEM. *P < 0.05 by two tailed *t*-test comparing percent reduction in physiologic parameters from baseline (A). *P < 0.05 by two tailed *t*-test comparing samples from vehicle-treated to statin-treated Shunt lambs (B–F).

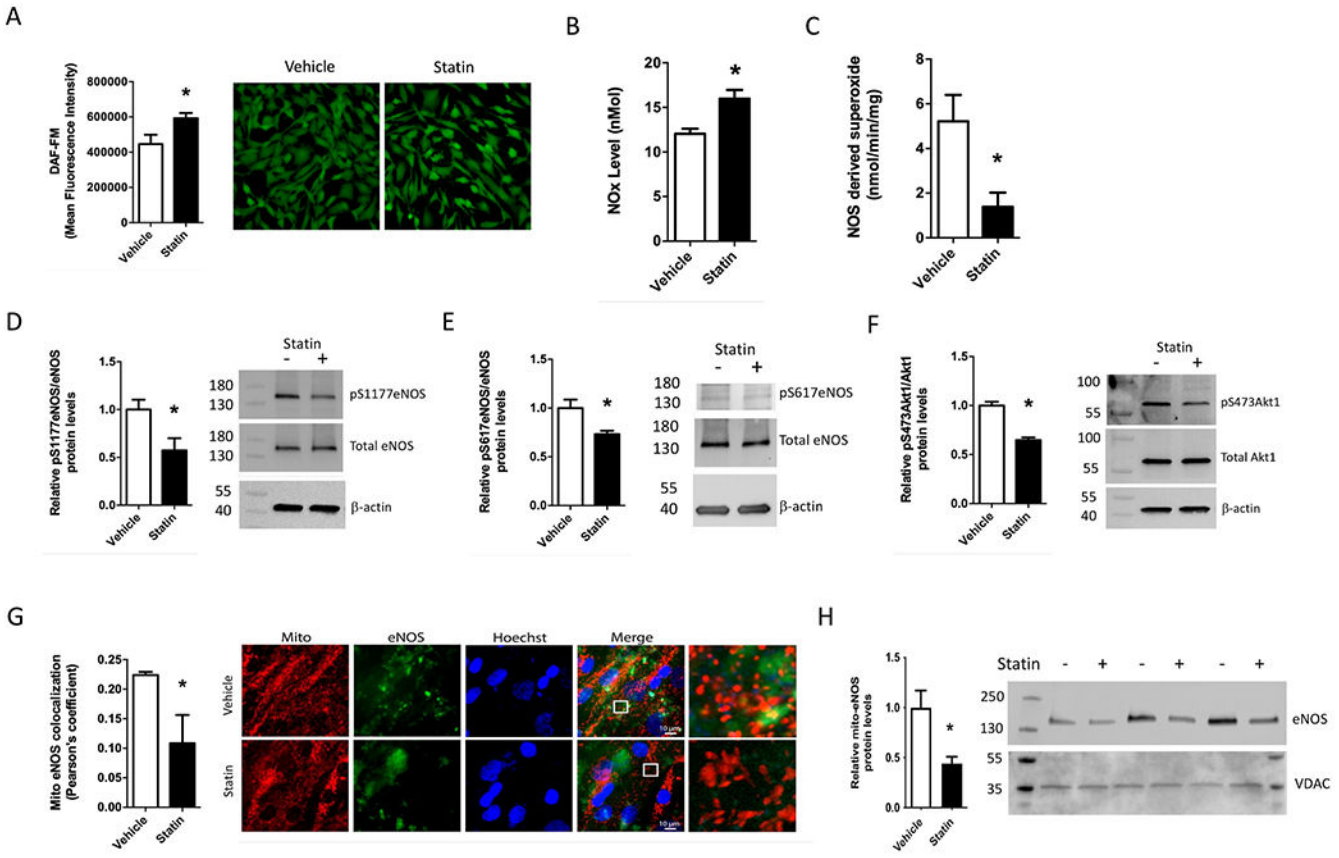


**Fig. 2. Simvastatin enhances eNOS coupling in control pulmonary arterial endothelial cells.**

Simvastatin (5 μM for 1 h) increases NO levels in PAECs derived from control lambs by both DAF fluorescence intensity and bioavailable NOx in cell media (A, B) and decreases NOS-derived superoxide levels from treated PAECs (C). Western blot analysis shows that simvastatin decreases eNOS phosphorylation at $S^{1177}$ (D) and $S^{617}$ (E) relative to total eNOS. This corresponds to a decrease in Akt1 activity as determined by reductions in $pS^{473}$ levels relative to total Akt (F). Simvastatin decreases the levels of eNOS localized to the mitochondria as determined by colocalization of eNOS and mitochondria by comparing mean Pearson coefficient derived from fluorescence microscopy (G) or presence of eNOS in mitochondrial protein fractions by Western blotting (H). Representative images are shown for each Western blot and for the fluorescence microscopy, β-actin (D–F) or VDAC (H) was used to normalize for protein loading. Duplicate blots were run or re-probed with eNOS or Akt1 to allow data to be presented as a ratio of phospho to total protein. Values are mean ± SEM. N = 6 apart from Fig. 2G (N = 3). *P < 0.05 by two tailed *t*-test comparing statin treated to vehicle treated samples.

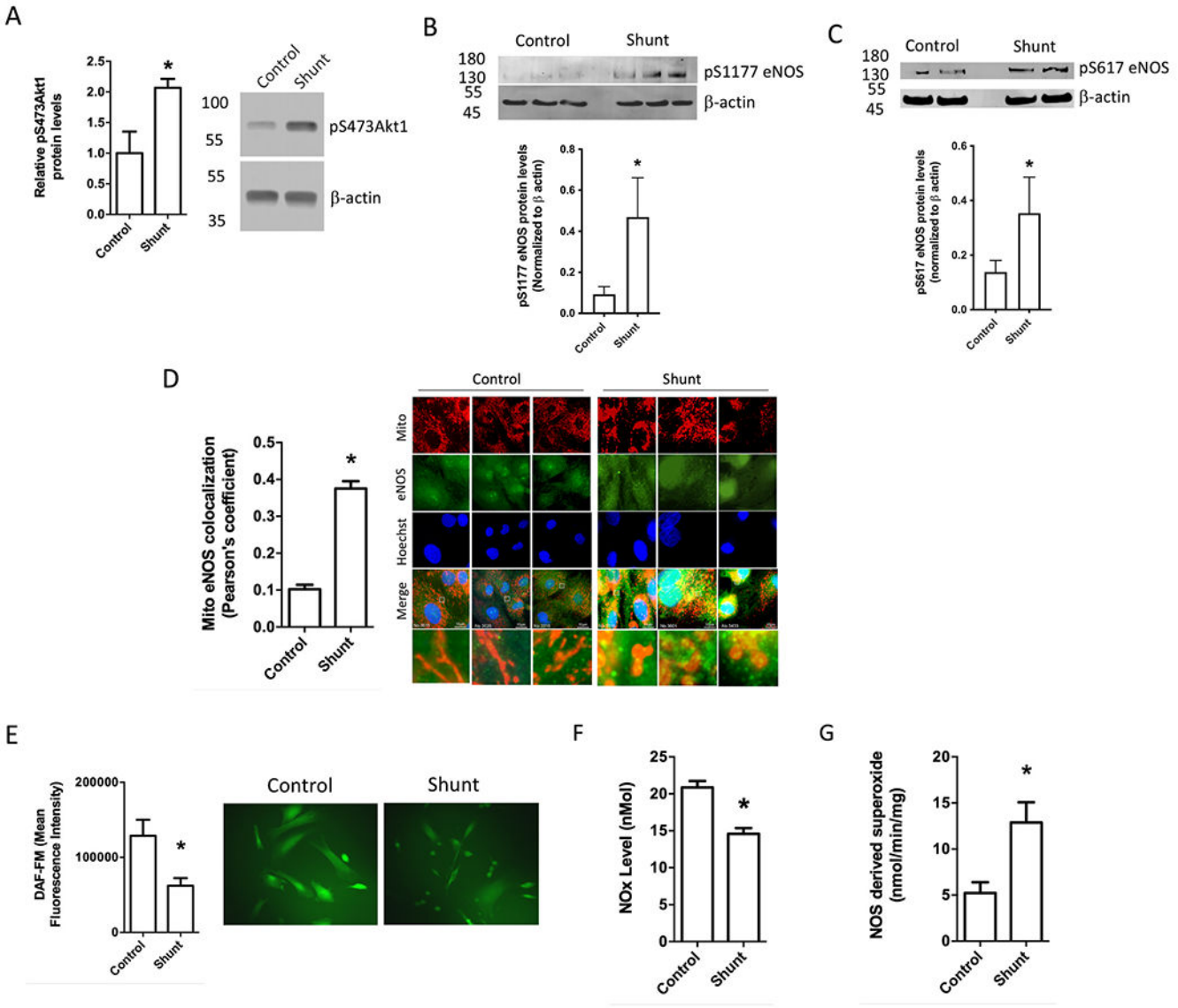


**Fig. 3. Increases in eNOS phosphorylation correlate with increased Akt1 activity in pulmonary arterial endothelial cells isolated from shunt lambs.**

Western blot analysis shows that Akt1 activity is increased in Shunt PAECs as determined by increased total $pS^{473}$ levels (A). This corresponds to increased eNOS phosphorylation at $S^{1177}$ (B) and $S^{617}$ (C). Representative images are shown for each Western blot, β-actin was used to normalize protein loading. The levels of eNOS localized to the mitochondria is increased in Shunt PAECs as determined by colocalization of eNOS and mitochondria comparing mean Pearson coefficients derived from fluorescence microscopy (D). Representative images are shown. NO levels are decreased in shunt PAECs by both DAF fluorescence intensity and bioavailable NOx in cell media (E, F) and NOS-derived superoxide from PAECs are enhanced (G). Values are mean ± SEM. N = 3 Control and N = 3 Shunt PAEC. *P < 0.05 by two tailed *t*-test comparing shunt to control samples.

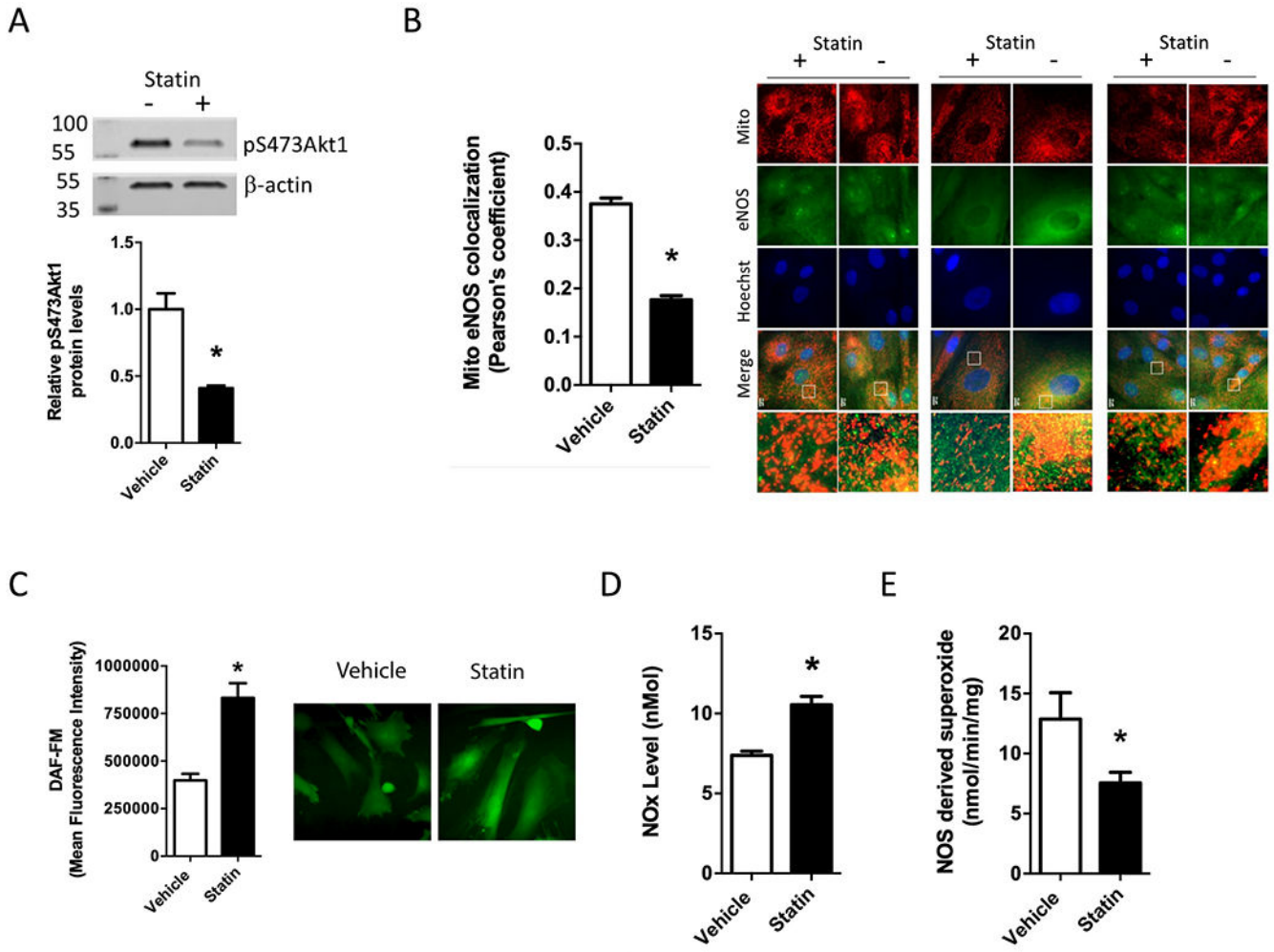


**Fig. 4. Simvastatin reduces mitochondrial eNOS levels and enhances coupling in pulmonary arterial endothelial cells isolated from shunt lambs.**

Western blot analysis shows that simvastatin (5 μM treatment for 24 h) decreases Akt1 activity in Shunt PAECs as determined by decreased $pS^{473}$ levels (N = 3) (A). Simvastatin also decreases the levels of eNOS localized to the mitochondria as determined by colocalization of eNOS and mitochondria comparing mean Pearson coefficients derived from fluorescence microscopy (B) (N = 3). Simvastatin also increases NO levels in shunt PAECs by both DAF fluorescence intensity and bioavailable NOx in cell media (C, D) (N = 6) and decreases NOS-derived superoxide levels from PAECs (E) (N = 6). Values are mean ± SEM. *P < 0.05 by two tailed *t*-test comparing statin treated to vehicle treated samples.

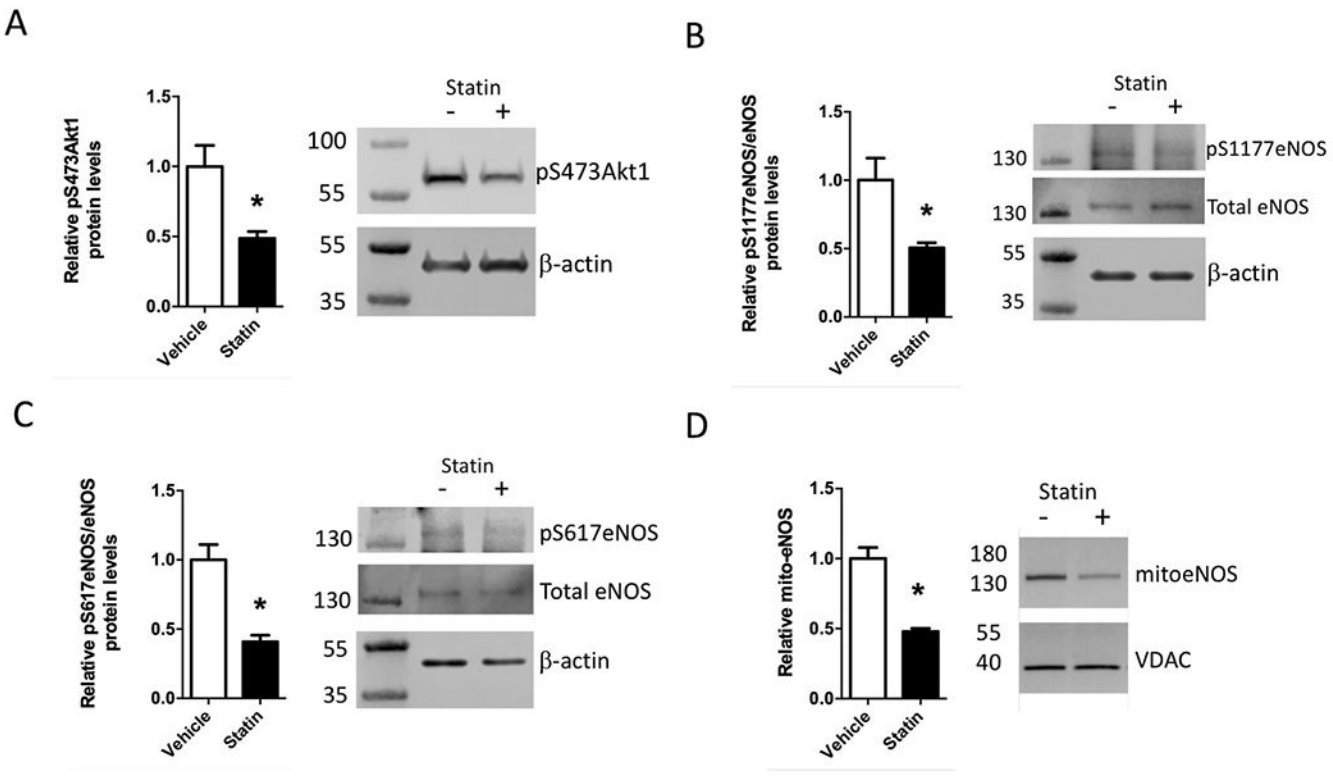


**Fig. 5. Simvastatin decreases mitochondrial eNOS levels in shunt lambs.**

Western blot analysis of peripheral lung tissue shows that Akt1 activity is decreased in simvastatin-compared to vehicle-treated Shunt lambs as determined by decreased $pS^{473}$ levels (A). Western blot analysis also shows that eNOS phosphorylation at $S^{1177}$ (B) and $S^{617}$ (C) as well as the levels of eNOS localized to the mitochondria is decreased in simvastatin-compared to vehicle-treated shunt lambs. Representative images are shown for each Western blot. β-actin (A–C) or VDAC (D) was used to normalize for protein loading. Duplicate blots were run or re-probed with eNOS or Akt1 to allow data to be presented as a ratio of phospho to total protein. N = 4 or the simvastatin-treated group. N = 4 for vehicle-treated group. Values are mean ± SEM. *P < 0.05 by two tailed t-test comparing statin treated to vehicle treated samples.

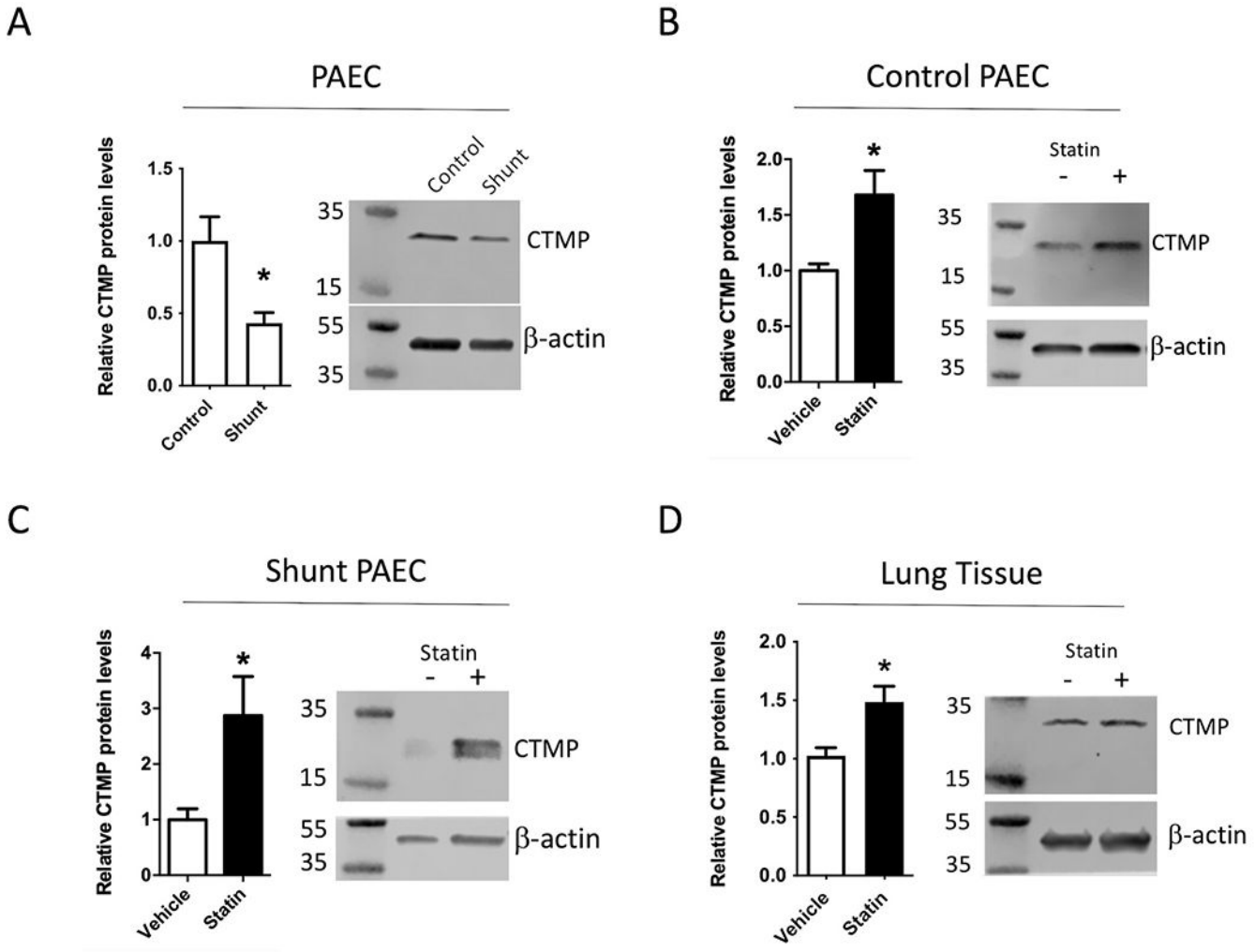


**Fig. 6. Simvastatin increases the level of the endogenous Akt1 inhibitor, CTMP.**

Western blot analysis shows that CTMP levels are decreased in Shunt PAECs compared to control PAECs (A). Simvastatin (5 μm) increases CTMP levels in both Control- (B) and shunt-PAECs (C) relative to their respective matched vehicle treated PAECs, and in peripheral lung tissue of statin compared to vehicle treated shunt lambs (D). Values are mean ± SEM. *P < 0.05 by two tailed *t*-test comparing shunt to control samples (A) or statin treated to vehicle treated samples (B–D). N = 6 for simvastatin-treated PAEC. N = 6 for the simvastatin and vehicle-treated lambs.

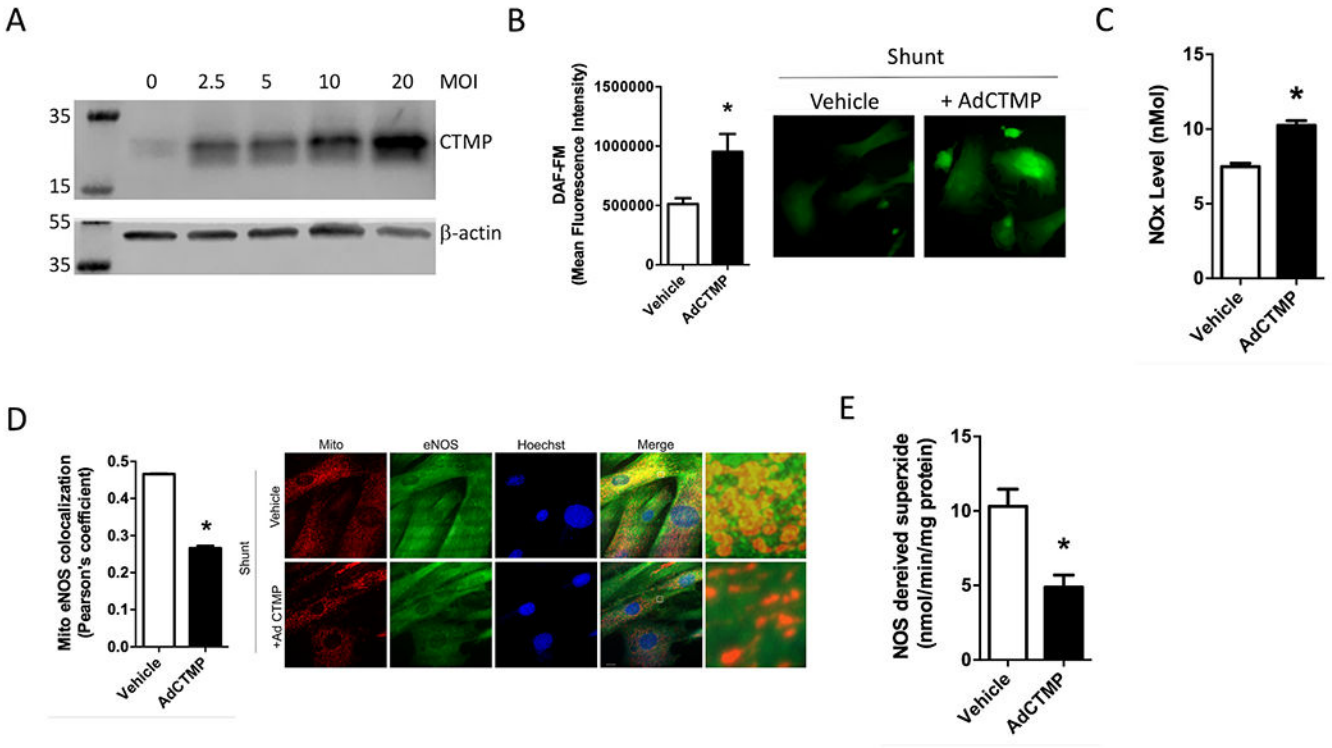


**Fig. 7. CTMP over-expression decreases mitochondrial eNOS and restores NO signaling in pulmonary arterial endothelial cells isolated from shunt lambs.**

PAECs were transduced with increasing multiplicity of infection (MOI) from 1 to 20 viral particles/cell exposure of an adenovirus expressing CTMP and Western blot analysis was used to determine the increase in CTMP protein levels (A). An MOI of 10 was then used to transduce shunt PAECs for 48 h. CTMP over-expression increases NO generation in shunt PAECs by both DAF fluorescence intensity and bioavailable NOx in cell media (B, C). The increase in NO generation correlates with a decrease in the levels of eNOS localized to the mitochondria as determined by colocalization of eNOS and mitochondria comparing mean Pearson coefficients derived from fluorescence microscopy (D) and reduced NOS-derived superoxide from PAECs (E). Values are mean ± SEM. N = 6 *P < 0.05 by two tailed *t*-test comparing AdCTMP to vehicle treated controls.

## Table 1

**Baseline hemodynamic measurements of Shunt lambs.**

Baseline hemodynamic measurements from vehicle compared to statin-treated shunt animals. HR = heart rate, mPAP = mean Pulmonary Arterial Pressure, mSAP = mean Systemic Arterial Pressure, Q LPA = flow in left pulmonary artery, RAP = right atrial pressure, LAP = left atrial pressure, iLPAVR = indexed (to body weight in kg) LPA vascular resistance, Qp:Qs = ratio of pulmonary to systemic blood flow. N = 6 statin treated shunt and N = 7 vehicle treated shunt animals. P-value by two tailed *t*-test for each parameter is also reported.

| Parameter | Vehicle-treated (N = 7) | Statin-treated (N = 6) | P value |
|---|---|---|---|
| HR (bpm) | 139.0 ± 20.7 | 151.3 ± 31.7 | .42 |
| mPAP (mm Hg) | 25.8 ± 6.7 | 28.6 ± 11.4 | .60 |
| Systolic SAP (mm Hg) | 100.2 ± 13.6 | 121.3 ± 25.1 | .08 |
| Diastolic SAP (mm Hg) | 26.4 ± 4.1 | 20.7 ± 7.7 | .22 |
| mSAP (mm Hg) | 52.1 ± 4.7 | 60.0 ± 9.3 | .07 |
| Q LPA (ml/min/kg) | 2.3 ± 0.4 | 2.22 ± 0.6 | .77 |
| RAP (mm Hg) | 3.0 ± 1.1 | 3.8 ± 2.8 | .51 |
| LAP (mm Hg) | 12.5 ± 6.1 | 9.9 ± 3.6 | .38 |
| iLPAVR (mm Hg *min/ml/kg) | 68.8 ± 22.5 | 86.0 ± 30.2 | .26 |
| Qp:Qs | 2.6 ± 1.3 | 2.9 ± 1.0 | .65 |