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Nicotinamide Phosphoribosyltransferase Promotes Pulmonary Vascular Remodeling and is a Therapeutic Target in Pulmonary Arterial Hypertension

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Conflict of Interest Statement

The authors have declared that no conflict of interest exists.

Author Contributions

JC and RFM designed the research studies. JC, JRS, SS, SZ, DVJ, TA, KMS, AY, SS, VR, AS, HG, JT and HT performed the experiments. JC, JRS and RFM analyzed the data. SMC, SQY, SC, RD, PH, JXJY and JGNG provided specimens and/or reagents. JXJY and JGNG provided input on the design of portions of the research studies. JC and RFM wrote the manuscript.

Abstract

Background—Pulmonary arterial hypertension (PAH) is a severe and progressive disease, a hallmark of which is pulmonary vascular remodeling. Nicotinamide phosphoribosyltransferase (NAMPT), is a cytozyme which regulates intracellular NAD levels and cellular redox state, regulates histone deacetylases, promotes cell proliferation and inhibits apoptosis. We hypothesized that NAMPT promotes pulmonary vascular remodeling, and that inhibition of NAMPT could attenuate pulmonary hypertension.

Methods—Plasma and mRNA and protein levels of NAMPT were measured in the lungs and isolated pulmonary artery endothelial cells (PAECs) from PAH patients, as well as in lungs of rodent models of pulmonary hypertension (PH). *Nampt*^{+/-} mice were exposed 10% hypoxia and room air for 4 weeks and the preventive and therapeutic effects of NAMPT inhibition were tested in the monocrotaline and Sugen-hypoxia models of PH. The effects on NAMPT activity on proliferation, migration, apoptosis and calcium signaling were tested in human pulmonary artery smooth muscle cell (hPASMC).

Results—Plasma and mRNA and protein levels of NAMPT were increased in the lungs and isolated pulmonary artery endothelial cells (PAECs) from PAH patients, as well as in lungs of rodent models of pulmonary hypertension (PH). *Nampt*^{+/-} mice were protected from hypoxia-mediated PH. NAMPT activity promoted human pulmonary artery smooth muscle cell (hPASMC) proliferation via a paracrine effect. In addition, recombinant NAMPT stimulated hPASMC proliferation via enhancement of store-operated calcium entry by enhancing expression of Orai2 and STIM2. Finally, inhibition of NAMPT activity attenuated monocrotaline and Sugen hypoxia induced PH in rats.

Conclusions—Our data provide evidence that NAMPT plays a role in pulmonary vascular remodeling and its inhibition could be a potential therapeutic target for PAH.

Keywords

Pulmonary hypertension; NAMPT; pulmonary vascular remodeling

Subject Terms

Pulmonary Biology; Vascular Biology; Smooth Muscle Proliferation and Differentiation

Introduction

Pulmonary arterial hypertension (PAH) is a progressive disease caused by functional and structural changes in the pulmonary vasculature, which lead to increased pulmonary vascular resistance and, eventually right ventricular failure and death. PAH is characterized by vasoconstriction, in-situ thrombosis, vascular inflammation and pulmonary artery smooth muscle cell (PASMC) and pulmonary artery endothelial cell (PAEC) proliferation and apoptosis resistance¹. PAEC dysfunction and injury are believed to play a critical role in the pathogenesis of the disease and can trigger PASMC proliferation and migration through activation growth factors or disruption of cell survival mediators^{2, 3}. Although important progress has been made in recent years, the precise cellular and molecular basis of

pulmonary vascular remodeling in PAH continues to evolve and the discovery of therapeutic targets that attenuate vascular remodeling is still ongoing.

Nicotinamide phosphoribosyltransferase (NAMPT), also called visfatin and pre-B cell colony-enhancing factor (PBEF), was first characterized as a cytokine acting on early B-lineage precursor cells, and is a pleiotropic protein with extracellular proinflammatory cytokine-like activity and intracellular enzymatic activity as a phosphoribosyltransferase^{4–10}. It has been demonstrated as the rate-limiting component of the mammalian NAD biosynthesis pathway from nicotinamide¹¹. NAMPT activity has been linked to several processes involving cellular adaptation to stress responses. These include resistance to senescence, apoptosis and increase in cell proliferation and regulation of cellular redox state^{6–8, 12, 13}. At the pathological level NAMPT promotes inflammatory responses by increasing inflammatory cell survival and increasing pro-inflammatory cytokine production^{8, 14–16}. NAMPT is also up regulated in many types of malignancies where it promotes cell survival, proliferation and resistance to chemotherapeutic agents^{7, 17–21}. In vascular cells NAMPT promotes endothelial cell survival and angiogenic activity as well as smooth muscle cell survival^{10, 13, 22–24}. We therefore hypothesized that NAMPT may play an important role in PAH pathobiology by promoting pulmonary vascular remodeling during pulmonary hypertension development, and that inhibition of NAMPT could attenuate experimental pulmonary hypertension.

Methods

Human Lung Tissues, Human PAECs and Human PASMCs (hPASMCs)

Approval for the collection of plasma and the use of human lung tissues and cells was granted by Institutional Review Boards of participating centers. Plasma samples were obtained from 103 patients with PAH defined by the 2013 Nice classification²⁵ and 53 controls including patients with hypothyroidism, osteoporosis, nephrolithiasis, diabetes mellitus, osteoarthritis and hypertension. De-identified human explanted peripheral lung tissues and PAECs used in this study were from four control subjects (2 unsuitable organ donors and 2 chronic obstructive pulmonary disease patients without PH) and patients with idiopathic PAH (IPAH, 4 patients diagnosed on the basis of National Institutes of Health PAH Registry criteria) obtained at the Cleveland Clinic Foundation. PAECs were isolated as previously described²⁶. In addition, a primary hPASMC and hPAEC cell lines from Lonza (CC-2581 and CC-2530) were used for cell transfection and proliferation assays. Cells were cultured at 37°C in their complete medium with 10% fetal bovine serum and studied at passages 5 to 8.

Reagents, Pharmacologic Inhibitors and Antibodies

The information for reagents, pharmacologic inhibitors and antibodies is provided in the supplementary Materials and Methods.

Western Blotting

Solubilized protein lysates isolated from lung tissues and cells were used to detect NAMPT, STIM2, ORAI2 and β -actin. Cells were lysed in a modified radioimmunoprecipitation assay

(mRIPA) lysis buffer with a protease and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO), and protein quantification and Western blot analysis were performed according to standard procedures.

RNA Isolation and Real-time PCR

Total RNA from lung tissues or cells was isolated using the RNeasy Plus Mini Kit (Cat.#74134, Qiagen). Two micrograms of purified RNA was attenuate transcribed to single-stranded cDNA using the Taqman RNA attenuate transcription kit (Cat.#N8080234, Applied Biosystems, Inc. [ABI]). Real-time PCR was performed on an ABI 7900HT machine. Specific Taqman quantitative real-time PCR assays were ordered from ABI (specific assay IDs available upon request). The relative mRNA expression levels were normalized to the expression of a housekeeping gene, glucose-6-phosphate dehydrogenase (G6PDH), and determined by calculating the Ct value, as detailed in the manufacturer's guidelines.

Mouse PASM C isolation

A mixture of 5 ml of medium 199 (M199) growth medium containing 5 g/l low-melting-point agarose type VII (Sigma, St. Louis, MO), 5 g/l iron beads (diameter <10 μ M; Sigma), and antibiotics (penicillin and streptomycin) was slowly injected over a period of 60 seconds through the RV, thereby perfusing the PA. M199 growth medium (1 ml) containing 5 g/l agarose type VII was injected in airways through the trachea. The lungs were plunged in cold PBS to cause the agarose to gel. Because of the rapidly solidifying nature of the agarose and the size of the iron particles, the likelihood of traversing the capillary space is minimized. All the lobes were then isolated and finely minced in a Petri dish. The tissue was further disrupted by passing through a 16-gauge followed by an 18-gauge needle approximately five times. The suspension was then mixed in M199 growth medium containing 80 U/ml type IV collagenase (Sigma) and incubated at 37°C for 90 min. With the use of a magnetic column (Invitrogen), the arteries or arterial tissues containing the iron beads were collected. The supernatant was aspirated and the arteries were washed and suspended in 5 ml M199 containing 20% FBS. Aliquots of the suspension were transferred to T25 culture flasks. Cells from the hypoxic group were incubated at 3% O₂, whereas cells from the normoxic control were cultured in air. Smooth muscle cell purity was determined by immunostaining with smooth muscle specific α -actin antibody.

Cell Proliferation Assays

Cell proliferation was determined using either a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay or cell counting. BrdU assays (QIA58, Calbiochem, San Diego, CA) were performed in a 96-well format according to manufacturer's instructions, using starting cell densities of 4000 cells/well. For cell counting, cells were trypsinized and resuspended after experimental procedures; densities were counted with a TC10™ automated cell counter (Bio-Rad). FBS or PDGF was used as positive controls.

Overexpressing NAMPT in hPASCs and hPAECs

A human wild-type NAMPT expression sequence was cloned downstream of the ubiquitin promoter in a third generation lentiviral plasmid. Separately, a control plasmid was

generated by cloning a GFP expression sequence in the same position. Co-transfection of either control-GFP or NAMPT-expressing constructs with three other packaging plasmids in HEK 293FT cells produced lentivirus capable of expressing either GFP or NAMPT. Viral concentration was determined by qPCR using primers spanning the HIV 5' LTR region. hPASCs and hPAECs were infected with control or NAMPT virus at an MOI of 5 for experiments performed 72 hours later.

Plasma and cell culture medium NAMPT measurement

Plasma NAMPT levels were measured using Bio-Plex Pro Human Diabetes Visfatin Set (Bio-Rad, cat.#171-B7012M). NAMPT secreted from hPAECs during the culture was measured using a Nampt (Visfatin/PBEF) (human) ELISA Kit (Adipogen, cat.#AG-45A-0006YEK-KI01).

[Ca²⁺]_{cyt} Measurement

[Ca²⁺]_{cyt} was measured in PASC loaded with fura-2 (4μM) in a Nikon digital fluorescent imaging system. Cells were loaded with 4 μM fura-2 acetoxymethyl ester (fura-2/AM) for 60 min at 25°C and [Ca²⁺]_{cyt} was measured using a ratiometric method at 32°C. Cyclopiazonic acid (CPA, a specific Ca²⁺-ATPase inhibitor) was used to induce store-operated calcium entry (SOCE).

Transfection of Small Interfering RNA (siRNA) in hPASCs

Using Lipofectamine™ RNAiMAX Reagent (Invitrogen, cat.# 13778-150), hPASCs were transfected with NAMPT siRNA (L-000458-00), STIM2 siRNA (L-013166-01-0005), ORAI2 siRNA (L-015012-00) or scrambled siRNA (D-001810-02), which were purchased from Dharmacon (Thermo Fisher Scientific, Lafayette, CO, USA) according to the manufacturer's protocol.

Animal Models of Pulmonary Hypertension and Hemodynamic Measurements

All experiments were approved by the Ethics and Animal Care Committee of the University of Illinois at Chicago. Three rodent models of PH were used in this study. They included a mouse model of hypoxia-induced PH, a rat model of monocrotaline (MCT)-induced PH and a rat model of Sugen-hypoxia induced severe PH. *Nampt*^{-/-} mice exhibit embryonic lethality thus *Nampt*^{+/-} mice were used in this study. Male mice (8-week old *Nampt*^{+/-} mice in C57BL/6 background and their WT siblings) were exposed to hypoxia (10% O₂) in a ventilated chamber for four weeks. Male Sprague-Dawley rats (190–200 g) were used for the MCT and Sugen-hypoxia induced PH studies. In the MCT-induced PH model, MCT was dissolved in 0.5N HCl to 200 mg/ml, neutralized to pH 7.4 with 0.5N NaOH, and then diluted with sterile water to 60 mg/ml. One dose of MCT (60 mg/kg body weight) was subcutaneously injected to rats. Control rats were injected with the equivalent volume of dissolvent solution according to their weights. Food and water were provided *ad libitum* and the rats are checked once per day. For the prevention experiment, FK866 (2.5 mg/kg) was started in day 2 after the MCT injection and continued for two weeks; for the therapeutic experiment, FK866 (2.5mg/kg every 48 hours) was started day 14 after the MCT injection and continued for two weeks. Our historical data shows that rats in day 14 after MCT

injection develop pulmonary hypertension as demonstrated by significantly increased RVSP and RVH (Supplemental Figure S1). Each group included 6 to 8 rats. In the Sugen-hypoxia PH model, one dose of Sugen (20 mg/kg) was given subcutaneously at the first day of hypoxia exposure (10% O₂). After three-week chronic hypoxia exposure, the rats were placed back to room air. FK866 (2.5 mg/kg every 48 hours) was then started two weeks after reoxygenation and continued for three weeks. Each group included 10 to 12 rats. Hemodynamic data after two-week reoxygenation indicates that rats develop significant PH at this time point (Supplemental Figure S2). Right ventricular systolic pressure (RVSP) was determined by right heart catheterization using a Millar pressure transducer catheter. A weight ratio of the right ventricle divided by the sum of left ventricle and septum (RV/(LV+S)) was measured to determine the extent of right ventricular hypertrophy (RVH). The RV contractility index was calculated as $(dp/dt)_{\max}/\text{instantaneous RV pressure}_{\max}$, as previously described.²⁷ Pulmonary artery remodeling was assessed using Aperio ImageScope software (version 11) after lungs were stained with hematoxylin and eosin (H&E). A minimum of 10 microscopic fields were examined for each slide. Approximately twenty muscular arteries with diameters (D) 50–100 μm or D < 50 μm per lung section were outlined and measured. Vessel remodeling was calculated as $((\text{external vessel area} - \text{internal vessel area}) / \text{external vessel area})$, as previously described²⁸. Occlusive pulmonary arteries were counted from H&E stained lung slides from each group.

Statistical Analysis

Statistical analysis of experimental data was performed using GraphPad Prism 5.1 (GraphPad Software, Inc., La Jolla, CA). Results are expressed as mean ± standard error of mean (SEM), except where noted, from at least three experiments. Student *t*-test and ANOVA were used to compare two and three groups, respectively except for the comparison of NAMPT plasma levels between control and PAH subjects where a Mann-Whitney test was used. A *P* < 0.05 was considered statistically significant.

Results

NAMPT is Up-regulated in Patients with PAH and in Experimental Rodent PH Models

Plasma NAMPT levels were significantly elevated in patients with PAH (n=103, median [IQR] 3197 [1493–7280]) when compared to controls (n=53, median [IQR] 1686 [990–2608]; *p*<0.001 by Mann-Whitney test, Figure 1A, Supplemental Table 1). mRNA and protein levels of NAMPT were significantly elevated in the lungs and PAECs from patients with PAH when compared to control subjects (Figures 1B–1F and supplemental Figure S3). Pulmonary artery smooth muscle cells isolated from PAH patients did not express higher levels of NAMPT, compared to controls (Supplemental Figure S4).

A similar pattern was found in different rodent models of PH including a mouse model of hypoxia-mediated pulmonary hypertension (HPH, 10% hypoxia for 4 weeks, Figure 2A), a rat model of monocrotaline-induced PH (2 weeks after administration of monocrotaline [60 mg/kg bw, SQ], Figure 2B) and a rat model of the VEGF receptor blocker sugen plus hypoxia mediated PH (sugen [20 mg/kg bw, SQ] followed by 10% hypoxia for 3 weeks and normoxia for 3 weeks, Figure 2C). In addition, the NAMPT expression profiles in lung

tissues from these different rodent models of PH were examined using immunofluorescence staining. As shown in Supplemental Figures S5–6, NAMPT expression in experimental PH appears to increase in the pulmonary vasculature mainly in PAECs and the adventitia, but not in the media, as well as in airway and alveolar epithelial cells. Alveolar macrophages also express NAMPT, especially in the rat model of PH mediated by monocrotaline, as demonstrated by CD68 immunostaining (data not shown).

In aggregate, these results suggest that NAMPT may play a role in the development of pulmonary hypertension.

NAMPT Heterozygous (*Nampt*^{+/-}) Mice are Protected from HPH

To examine the effect of NAMPT on the development of pulmonary hypertension, *Nampt*^{+/-} mice and their WT siblings in a C57BL6 background were exposed to 10% hypoxia for four weeks. Compared to WT siblings, *Nampt*^{+/-} mice exhibited significantly lower baseline levels of NAMPT and NAD in lung tissue homogenates (Supplemental Figure S7). After four-week hypoxia exposure, *Nampt*^{+/-} mice displayed significantly lower right ventricular systolic pressure (RVSP, Figure 3A), decreased RVH (right ventricular hypertrophy, assessed by RV/(LV+S) ratio, Figure 3B), and less severe pulmonary vascular remodeling in response to hypoxia when compared to control mice (Figure 3C–E). Furthermore, PASMCs isolated from *Nampt*^{+/-} mice were less proliferative compared to WT mice as demonstrated by BrdU incorporation and cell counting (Figure 3F–3G). Similarly, silencing NAMPT via siRNA attenuated complete medium, hypoxia or PDGF-mediated hPASMC proliferation (Supplemental Figure S8).

NAMPT activity promotes PASMC proliferation

In addition to increased expression, PAECs isolated from patients with PAH secreted more NAMPT in culture media than control cells (Figure 4A). Further, conditioned media from PAECs isolated from PAH patients significantly promoted hPASMC proliferation. This effect was significantly attenuated by the NAMPT enzymatic activity inhibitor FK866²¹ (Figure 4B). Lentivirus-mediated NAMPT overexpression promoted both hPAECs and hPASMC proliferation (Figure 4C–I). When compared to control cells, hPAECs overexpressing NAMPT secreted more NAMPT and conditioned media from these cells stimulated more hPASMC proliferation (Figure 4J). These effects were attenuated by FK866 (Figure 4F, 4I, 4J). Finally, recombinant NAMPT protein (rNAMPT) promoted hPASMC proliferation in a dose-dependent manner (an effect that was attenuated by FK866, Figure 4K) and enhanced hPASMC migration (an effect that was attenuated by FK866, Supplemental Figure S9). Taken together these data suggest that NAMPT promotes hPASMC proliferation and migration in an autocrine and paracrine manner.

Inhibition of NAMPT activity promotes PASMC apoptosis

Since NAMPT promotes resistance to apoptosis^{6–8} and resistance to apoptosis is a major contributor to the pathobiology of PAH, we tested whether inhibition of NAMPT activity would promote PASMC apoptosis. As shown in Supplemental Figure S10, treatment with FK866 induced hPASMCs apoptosis. Further, treatment with rNAMPT protected PASMCs

from H₂O₂ induced apoptosis, an effect that was attenuated by concomitant FK866 treatment.

NAMPT Enhances Store-Operated Calcium Entry (SOCE) in PSMCs

NAMPT activity promotes inflammatory responses including the expression of inflammatory cytokines. For instance, NAMPT derived NAD promotes TNF- α synthesis by activated immune cells²⁹. Since inflammatory pathways, particularly the cytokine TNF- α , have been shown to promote SOCE^{30, 31}, we hypothesized that NAMPT-induced PSMC proliferation would be mediated by enhancement of SOCE. To test this hypothesis, we treated PSMCs with rNAMPT (20 μ g/ml) for 48 hours. [Ca²⁺]_{cyt} was measured in PSMCs loaded with fura-2/AM (4 μ M) using a fluorescence microscope, and cyclopiazonic acid (CPA) was used to induce SOCE. After 48 hours rNAMPT treatment significantly enhanced CPA-mediated SOCE in PSMCs (Figure 5A).

ORAI calcium release-activated calcium modulator 2 (Orai2) and stromal interaction molecule 2 (STIM2) are up regulated in PSMCs from patients with PAH and contribute to enhanced store-operated calcium entry and to the transition of these cells from a contractile to proliferative phenotype³². Given these findings we tested whether NAMPT activity would enhance SOCE via up regulation of Orai2 and STIM2. NAMPT treatment increased the expression of both Orai2 and STIM2 (Figure 5B–E) but did not change the expression of Orai1 and STIM1 (data not shown). Inhibition of NAMPT via FK866 attenuated NAMPT-mediated STIM2 and ORAI2 upregulation (Figure 5B–E) and silencing of either Orai2 or STIM2 attenuated NAMPT-induced hPSMC proliferation (Figure 5F). This data suggests that SOCE is required for the proliferative effect of NAMPT in PSMCs.

Finally, the effect of NAMPT on the L-type calcium channel mediated signaling was also examined. Blockage of L-type channels with verapamil did not affect rNAMPT mediated hPSMC proliferation (Supplemental Figure S11).

Inhibition of NAMPT by FK866 prevents and attenuates monocrotaline-induced PH in rats

To examine the potential therapeutic role of NAMPT inhibition in PH, we evaluated the effect of FK866 in monocrotaline (MCT)-induced PH model in rats. In PH prevention experiments FK866 (2.5mg/kg every 48 hours IP) or vehicle were given to rats (n = 6–8 animals per group), starting one day after subcutaneous injection of MCT (60 mg/kg bw) and in PH reversal experiments FK866 (2.5mg/kg every 48 hours IP) or vehicle were given to rats (n = 6–8 animals per group), starting fourteen days after MCT injection (60 mg/kg bw). When compared to vehicle-treated rats, treatment with FK866 prevented (Figure 6) and also attenuated (Figure 7) the development of PH. In both experiments, FK866 treatment significantly reduced the PH assessed by the RVSP as well as RVH, RV contractility index and the degree of pulmonary artery muscularization. Furthermore, FK866 treatment decreased PSMCs proliferation, as demonstrated by PCNA and smooth muscle actin double immunostaining of lung tissues (Supplemental Figure S12).

Inhibition of NAMPT by FK866 attenuates Sugen-hypoxia mediated PH in rats

The rat model of PH mediated by Sugen and hypoxia induces severe pulmonary vascular remodeling characterized by obliterative pulmonary vascular lesions with neointima formation in distal pulmonary arteries. We assessed if NAMPT inhibition by FK866 (2.5 mg/kg every 48 for three weeks) after rats (n= 8–12 per treatment group) were given sugen (20 mg/kg, SQ, once), exposed to chronic hypoxia for 3 weeks, and then kept in normoxia for another two weeks. When compared to controls, FK866 treatment attenuated the development of PH assessed by changes in RVSP, RVH and RV contractility index as well as the development of medial hypertrophy and neointima formation (Figure 8). FK866 treatment also decreased cell proliferation in obliterative lesions (Supplemental Figure S13). Taken together with the results from the MCT studies, these data suggest that NAMPT inhibition is a novel therapeutic target in PAH.

Discussion

Here we show that NAMPT is upregulated in the plasma, lungs and PAECs isolated from patients with PAH, and in different rodent models of PH. PAECs from patients with PAH are more proliferative and secrete more NAMPT which promotes PASMCM proliferation in a paracrine fashion via calcium signaling. We also show that NAMPT contributes to pulmonary vascular remodeling in vivo and that pharmacologic inhibition of NAMPT activity attenuates experimental PH. NAMPT is a multifunctional protein with extracellular proinflammatory cytokine-like activity as well as intracellular enzymatic activity as a phosphoribosyltransferase, which regulates NAD levels⁴. As such, NAMPT regulates the activity of NAD-dependent enzymes, such as sirtuins and poly(ADP-ribose) polymerases, and functions as a master regulator of cellular metabolism, mitochondrial biogenesis and adaptive and maladaptive responses to cellular stress¹⁰.

There are several potential mechanisms for the upregulation of NAMPT in patients with IPAH and in experimental models of PH. As major mediators contributing to PAH pathobiology, hypoxia, upregulation of growth factors and inflammatory pathways can also influence NAMPT expression. The NAMPT promoter has hypoxia-inducible factor responsive elements that affect the transcriptional regulation of NAMPT^{33, 34}. The NAMPT promoter also contains binding sites for NF-1, AP-1, and NF- κ B and tumor necrosis factor α (TNF α), IL-1 β and IL-6 increase NAMPT expression⁹. Epigenetic regulation of NAMPT expression via micro RNAs has been demonstrated in PAECs³⁵, colorectal cancer cell lines³⁶, hepatocytes³⁷ and TZM-bl HeLa cell lines³⁸. It is possible that similar effects could occur in the context of PAH. Further studies in this area are warranted. NAMPT levels are also increased and correlate with disease severity in metabolic syndrome and diabetes^{39–41} as well as coronary artery disease⁴². This suggests that NAMPT should be studied as a prognostic biomarker in PAH.

Uncontrolled PASMCM proliferation and resistance to apoptosis are major contributors to the pathobiology of PAH. Our results demonstrate that NAMPT promotes PASMCM proliferation in a dose-dependent manner, suggesting that it plays a significant role in the pulmonary vascular remodeling associated with PAH. Ca²⁺ signaling in PASMCMs promotes vascular remodeling through its stimulatory effect on PASMCM proliferation and migration. Previous

studies have shown that PASMC from idiopathic PAH patients exhibit enhanced SOCE when compared to control PASMCs and that SOCE contributes to PASMC proliferation^{32, 43–47}. Specifically, STIM2 and Orai2 both contribute to SOCE and are upregulated in PASMCs from patients with idiopathic PAH and from animals with experimental pulmonary hypertension in comparison to controls ultimately contributing to the transition of these cells from a contractile to proliferative phenotype^{32, 47}. We show that NAMPT enhances SOCE in PASMCs by increasing the expression of STIM2 and Orai2 and that inhibition of NAMPT activity decreases the expression of these two proteins. In line with these observations, Ye and colleagues have shown that NAMPT knockdown blunted thrombin-mediated increases in Ca²⁺ entry in human PAEC⁴⁸. We speculate that NAMPT activity could enhance SOCE by upregulating inflammatory pathways as shown in human aortic smooth muscle cells⁴⁹. Inflammatory cytokines such as TNF- α have been known to increase agonist-induced [Ca²⁺]_i response and contractility in airway smooth muscle cells^{31, 50–52} as well as to increase SOCE in these cells^{30, 31, 53}. Furthermore, LPS-induced activation of both the NF- κ B and p38 MAPK signaling pathways induce STIM1 expression in PAECs⁵⁴. Interestingly, by increasing NAD levels, NAMPT promotes TNF synthesis via regulation of SIRT6 activity^{29, 55}. The extracellular cytokine-like activity of NAMPT could also potentially activate NF- κ B signaling and further enhance SOCE^{56, 57}.

The paracrine effects of NAMPT have been demonstrated in other studies. Pilai and colleagues⁵⁸ showed that cardiac-specific overexpressing NAMPT transgenic mice spontaneously developed cardiac hypertrophy and that treatment of cardiomyocytes with recombinant NAMPT induced cardiomyocyte hypertrophy. Yoon and colleagues⁵⁹ demonstrated that deacetylation of lysine 53 in intracellular NAMPT by SIRT1 predisposes the protein to secretion by adipocytes and increases secreted extracellular NAMPT activity. Adipose tissue-specific NAMPT knockout and knockin mice showed reciprocal changes in circulating NAMPT levels which affected hypothalamic NAD⁺ and SIRT1 signaling as well as physical activity. Finally, administration of a NAMPT-neutralizing antibody suppressed NAMPT enzymatic activity by 90% for recombinant NAMPT in vitro and by 40% for extracellular NAMPT in plasma and also decreased hypothalamic NAD⁺ production in vivo.

It is clear that given its pleiotropic effects, NAMPT can exert other effects that promote pulmonary vascular remodeling beyond inducing PASMC proliferation in a paracrine manner. For instance, our results and the work of others suggest that NAMPT activity directly promotes endothelial and smooth muscle cell proliferation^{12, 13}. Our data from *Nampt*^{+/-} mice and from NAMPT loss of function studies in hPASMCs also suggest that normal NAMPT levels are required to promote cell proliferation both in physiologic and pathologic conditions. By regulating cellular NAD levels and the activity of NAD dependent enzymes such as sirtuins and ADP-ribosyltransferases, NAMPT plays an important role in the regulation of programmed cell death pathways. In models of acute inflammation NAMPT activity prevents the apoptosis of neutrophils⁶⁰. NAMPT also protects cells from apoptosis by inhibiting the activity of p53^{61–63}. SIRT3 has also been found to prevent loss of mitochondrial membrane potential and cell death in response to hypoxia and staurosporine treatment⁶⁴. Current and future studies by our group are seeking to elucidate the molecular mechanisms associated with increased proliferation and cell survival of PAECs and PASMCs in the context of PAH. Finally, T and B lymphocytes and macrophages are present

in increased numbers in and around the pulmonary arteries of patients with PAH and these cells could be another source of NAMPT within the PAH lung microenvironment.⁶⁵

Our results show that NAMPT inhibition with FK866 has therapeutic effects in pre-clinical models of PH by attenuating pulmonary vascular remodeling characterized by medial hypertrophy and neointima formation. FK866 specifically inhibits NAMPT activity likely by functioning as a substrate (NAM) mimetic which leads to a decrease in NAD synthesis.²¹ NAMPT inhibitors have been shown to have therapeutic effects in preclinical studies by inhibiting cell proliferation and promoting apoptosis in cancer models²¹, inhibiting T-cell proliferation and survival in autoimmune encephalomyelitis⁵⁵, inhibiting inflammatory cytokine production in collagen-induced arthritis⁶⁶ and inducing neutrophil apoptosis in LPS-induced acute lung injury⁶⁰. NAMPT inhibitors have also been studied in phase I-II human clinical trials in patients with cancer⁶⁷⁻⁷⁰. In these studies, the main dose limiting toxicities included hematologic abnormalities (thrombocytopenia, leukopenia and anemia) as well as several gastrointestinal symptoms such as nausea, vomiting, anorexia and abdominal pain.⁶⁷⁻⁷⁰

Our hemodynamic studies are limited by an indirect assessment of PA pressures via measurement of RVSP and lack of a direct assessment of cardiac output or left ventricular function. Further animal and human studies are needed to completely characterize the effects of NAMPT inhibition on the pulmonary and systemic vasculatures as well as its effects on right and left ventricular function. The results of our pre-clinical studies also highlight the relative limitations of the existing experimental PH models. NAMPT inhibition had a much more pronounced therapeutic effect in the monocrotaline than in the Sugen-hypoxia PH model. This is consistent with the notion that the Sugen-hypoxia PH model more closely mimics the severe pulmonary vascular remodeling and pulmonary hypertension associated with human PAH, suggesting that this model is likely more suitable to test the translational potential of novel therapeutic strategies for the disease.

In summary, we have demonstrated that NAMPT promotes pulmonary vascular remodeling and contributes to PAH pathobiology. Most importantly, our results suggest that NAMPT is a novel potential therapeutic target for PAH that should be explored in future preclinical and clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Perspective

What is new?

- Nicotinamide phosphoribosyltransferase (NAMPT), a pleiotropic protein with extracellular proinflammatory cytokine-like activity and intracellular enzymatic activity as a phosphoribosyltransferase, is upregulated in human and experimental pulmonary hypertension and promotes pulmonary vascular remodeling in vivo and in vitro.

What are the clinical implications?

- Inhibition of NAMPT enzymatic activity is a potential novel therapeutic strategy for pulmonary arterial hypertension.

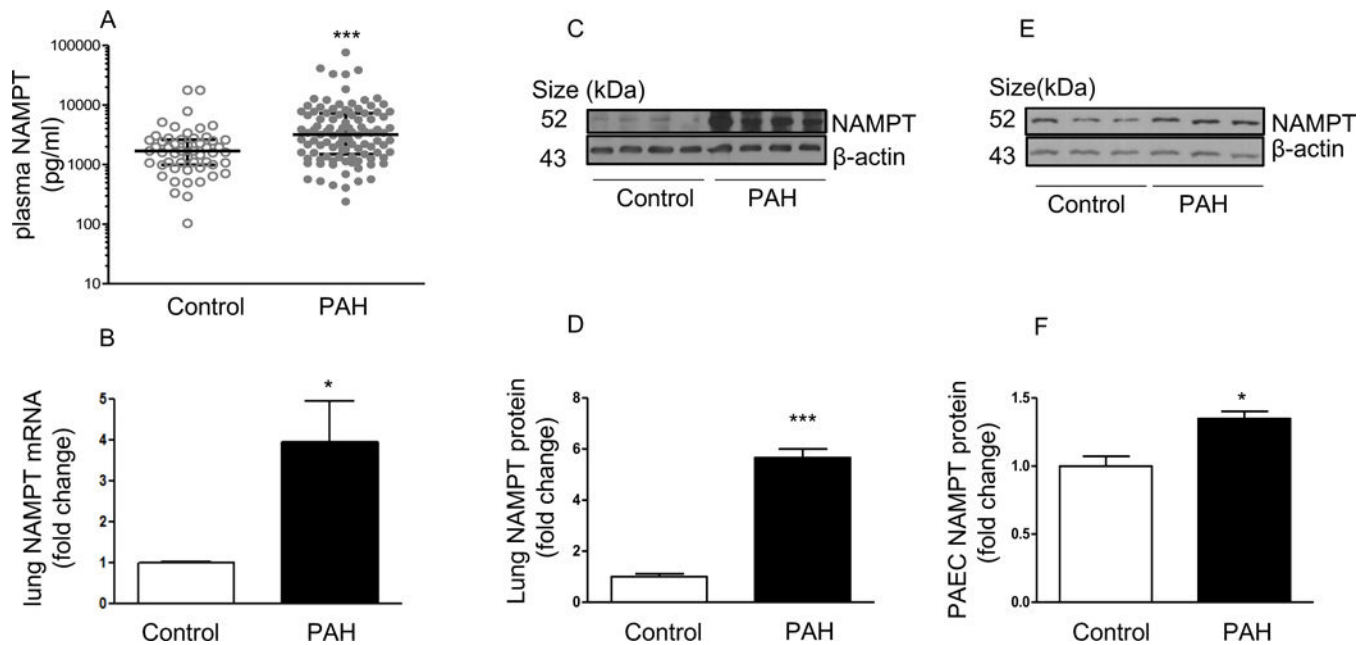


Figure 1. Plasma NAMPT levels, mRNA and protein levels of NAMPT in the lungs and PAECs are up regulated in patients with PAH

(A) Plasma NAMPT levels are higher in patients with PAH (n = 103) when compared to non-PAH controls (n = 53); (B) NAMPT mRNA levels are upregulated in the lungs of the patients with PAH (n = 4), compared to non-PAH controls (n = 4); (C–D) Representative Western blotting images and β -actin-normalized quantification of protein demonstrate that NAMPT expression is significantly increased in lungs from PAH patients when compared to non-PAH controls; (E–F) Representative Western blotting images and β -actin-normalized quantification of protein demonstrate that NAMPT protein levels were upregulated in PAECs isolated from PAH patients (n = 3) when compared to non-PAH controls (n = 3). Results are expressed as mean \pm SEM. *, p < 0.05; ***, p < 0.001 versus control.

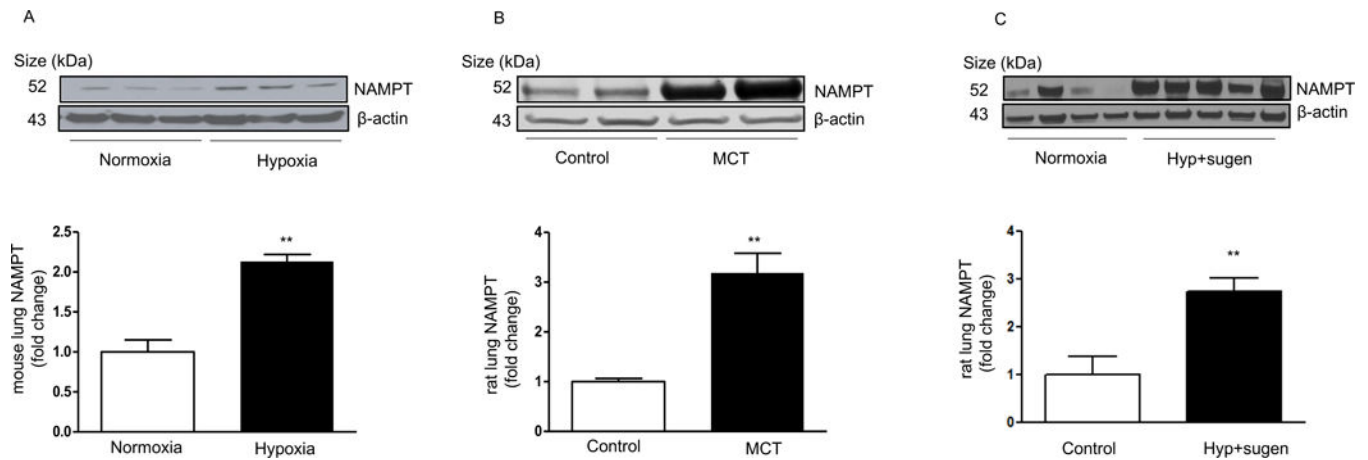


Figure 2. NAMPT protein levels are significantly increased in lungs from three different rodent models of pulmonary hypertension

(A) Representative Western blotting images and β -actin-normalized quantification of protein demonstrate that NAMPT expression is significantly increased in mouse lungs after 4-week 10% hypoxia exposure. (B) Representative Western blotting images and β -actin-normalized quantification of protein demonstrate that NAMPT expression is significantly increased in the lungs from a rat model of PH mediated by monocrotaline. (C) Representative Western blotting images and β -actin-normalized quantification of protein demonstrate that NAMPT expression is significantly increased in the lungs from hypoxia plus sugen mediated PH in rats. **, $p < 0.01$ versus controls.

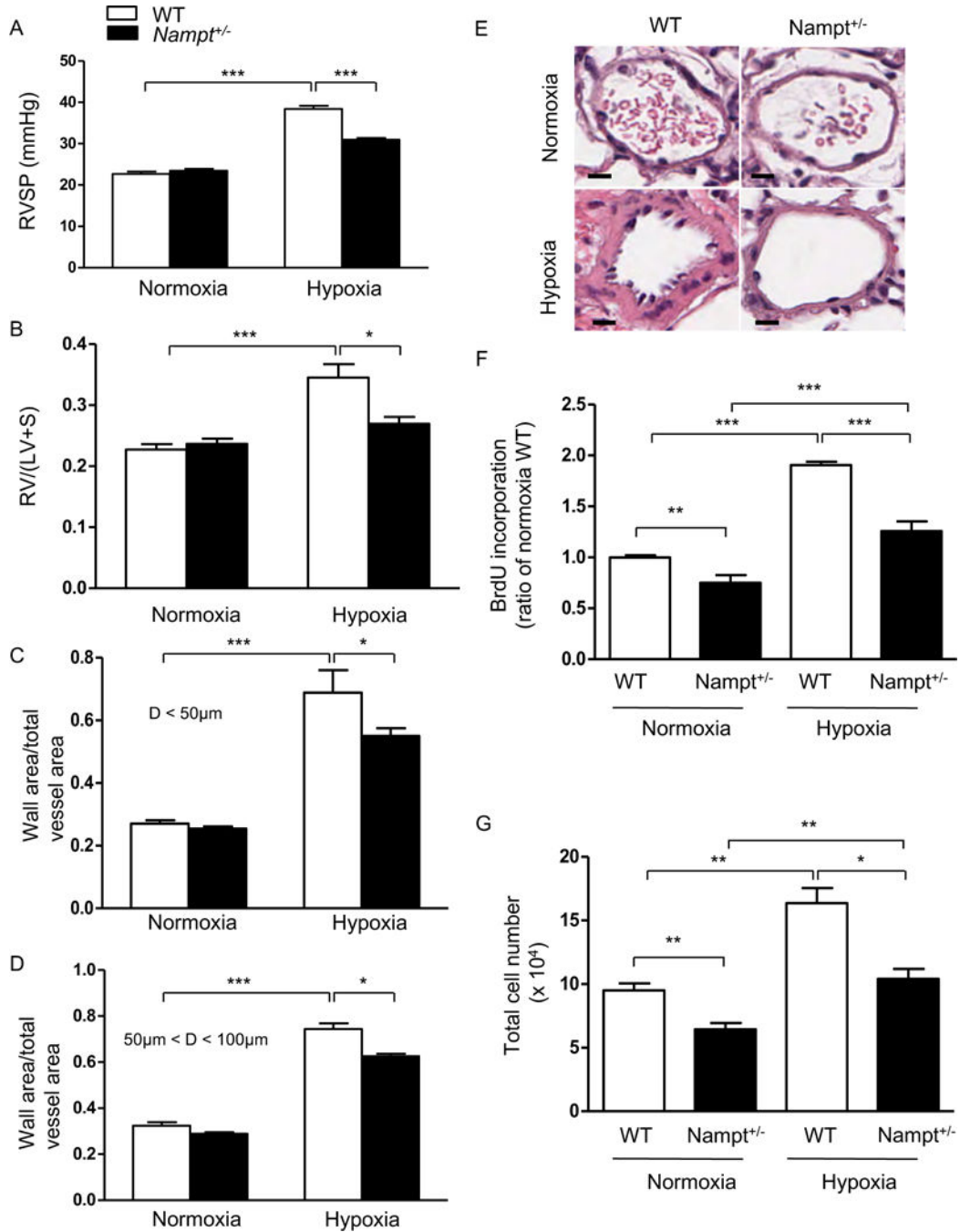


Figure 3. *Nampt*^{+/-} mice are protected against hypoxia-mediated pulmonary hypertension and PSMCs isolated from *Nampt*^{+/-} mice are less proliferative

When compared to WT controls, *Nampt*^{+/-} mice exposed to hypoxia developed (A) less severe elevations in RVSP, (B) less right ventricular hypertrophy (RV/(LV+S)) and lower increases in ratios of wall area to total vessel area of pulmonary arteries less than 50 μm (C) and 50–100 μm in diameter (D). (E) Representative pulmonary artery images in the lung sections of control and *Nampt*^{+/-} mice exposed to normoxia or hypoxia. BrdU proliferation assays (F) and cell counting data (G) demonstrate that PSMCs isolated from *Nampt*^{+/-} mice are less proliferative compared to WT mice under either normoxia or hypoxia. Bar

size: 20 μm . Results are expressed as mean \pm SEM; n = 10 per group. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus hypoxia WT group.

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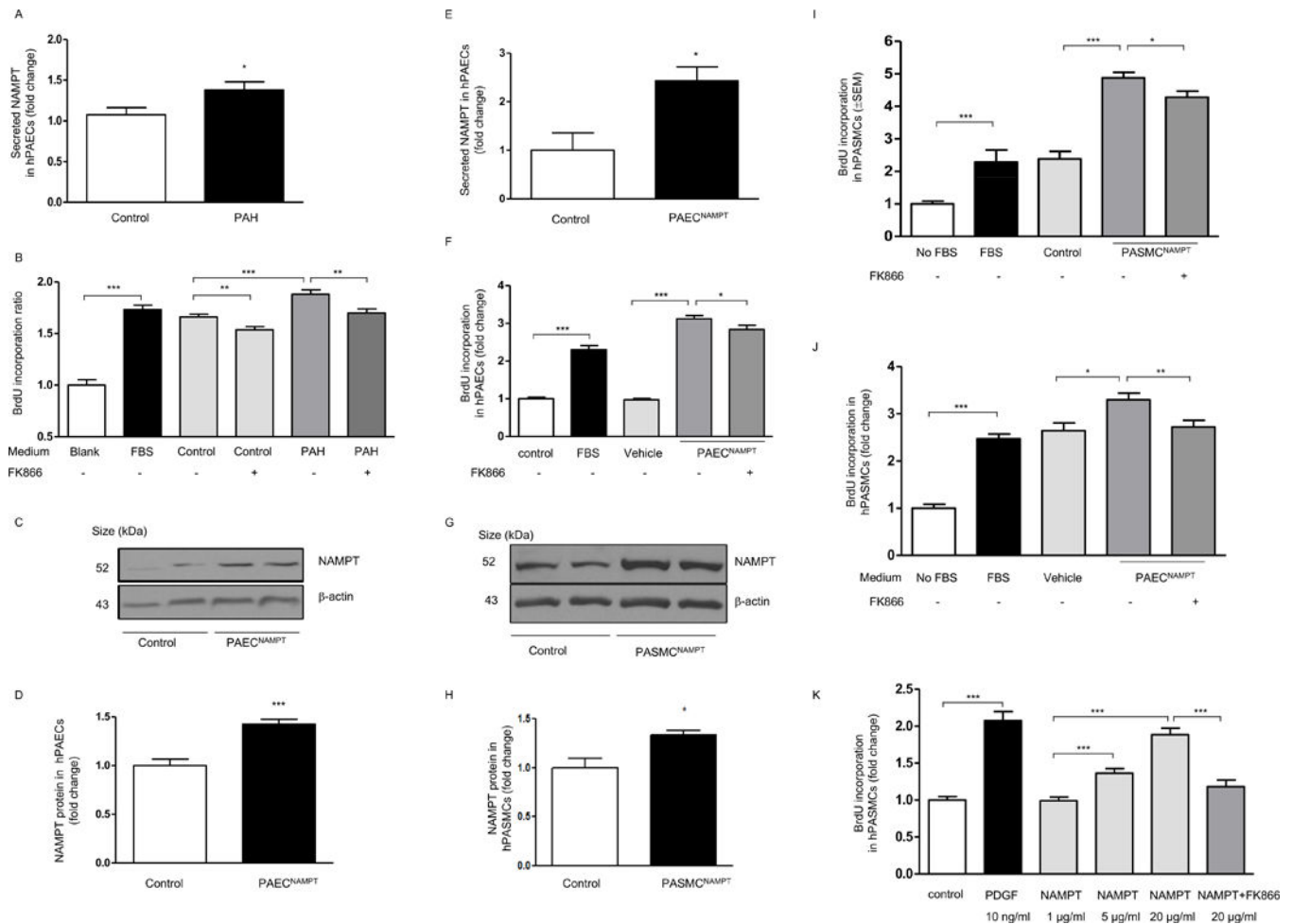


Figure 4. NAMPT promotes proliferation of PSMCs or PAECs in a paracrine and autocrine manner

(A) PAECs isolated from PAH patients secrete more NAMPT compared to non-PAH controls (n=3 per group); (B) BrdU incorporation assays demonstrate that cell culture medium of PAECs isolated from PAH patients stimulates more PSMC proliferation, and this effect can be attenuated by the specific NAMPT inhibitor, FK866 (10 μM) (ANOVA $P < 0.05$ for the control to PAH comparison). Overexpressing NAMPT in hPAECs increases NAMPT expression and secretion (C–E). NAMPT overexpression promotes cell proliferation and its proliferative effect is attenuated by FK866 (10 μM) (F, ANOVA $P < 0.05$ for the vehicle to NAMPT and NAMPT+FK-866 comparison). Overexpressing NAMPT in hPASMCs (G–H) promotes cell proliferation and its proliferative effect is attenuated by FK866 (I, ANOVA $P < 0.05$ for the control to NAMPT and NAMPT+FK-866 comparison). Cell culture medium of PAECs overexpressing NAMPT stimulates more PSMC proliferation, and this effect is attenuated by FK866 (10 μM) (J, ANOVA $P < 0.05$ for the vehicle to NAMPT and NAMPT +FK-866 comparison). (K) recombinant NAMPT protein stimulates human PSMC proliferation in a dose dependent manner and this effect is attenuated by FK866 (10 μM, ANOVA $P < 0.05$ for the different NAMPT doses and NAMPT+FK-866 comparison). Results are expressed as mean \pm SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $P < 0.001$ versus the

pairwise comparison indicated by brackets. FBS or PDGF were used as positive controls for BrdU proliferation assays as stated in the specific figures.

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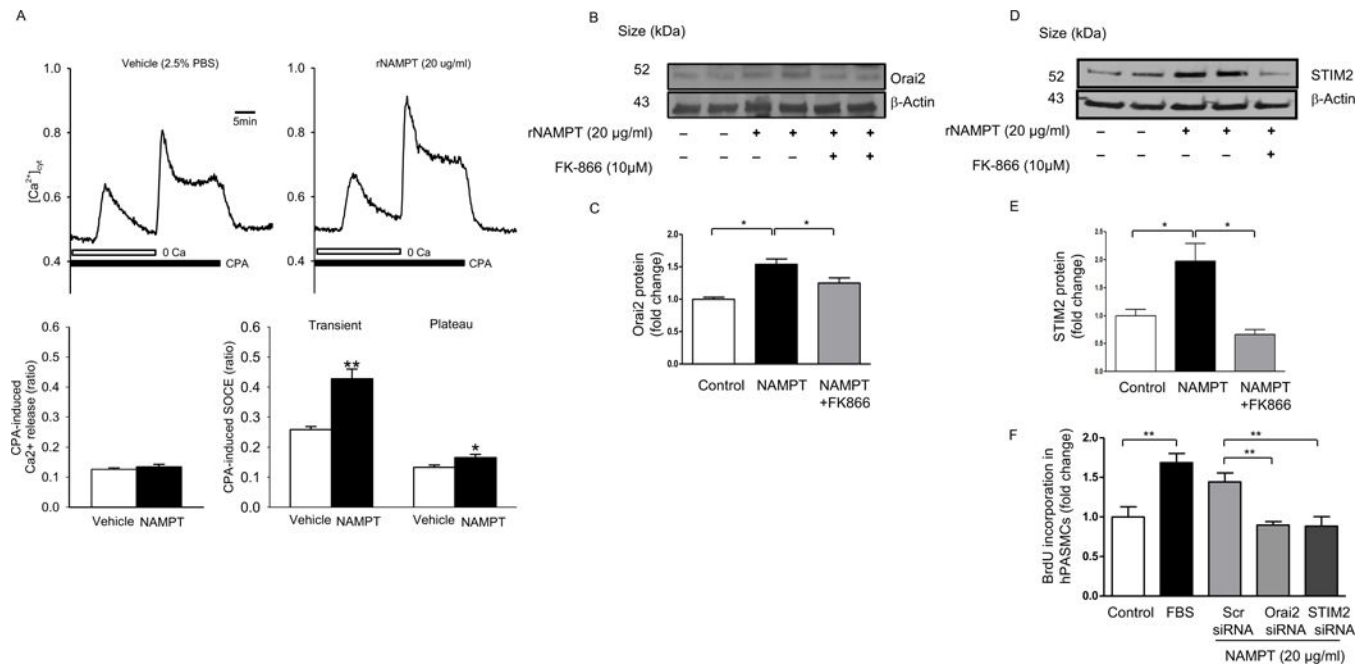


Figure 5. NAMPT Enhances Store-Operated Calcium Entry (SOCE) in PSMCs

Incubation of hPASMCs with rNAMPT protein (20 μg/ml, 48 hrs) enhances cpA-mediated store operated calcium entry (SOCE) (A), and increases ORAI2/STIM2 expression which is attenuated by FK866 (10 μM) (B–E). Silencing of STIM2 or ORAI2 attenuates rNAMPT-mediated hPASMC proliferation (F). Results are expressed as mean ± SEM, experiments were repeated at least three times. *, $p < 0.05$; **, $p < 0.01$.

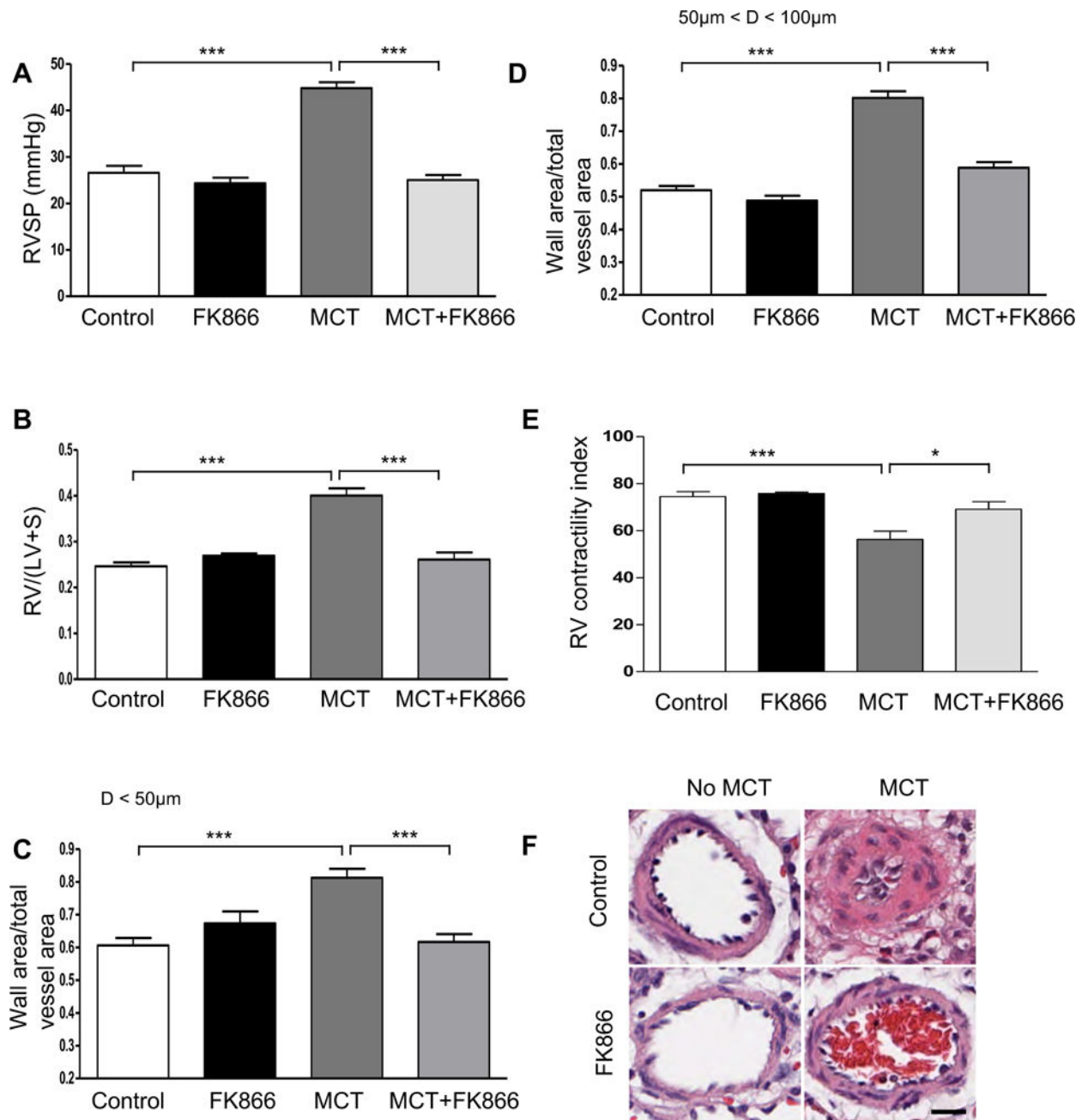


Figure 6. NAMPT inhibition prevents monocrotaline-induced pulmonary hypertension in rats (A) Changes in RVSP. (B) Changes in RV/(LV+S). (C) Changes in ratios of wall area to total vessel area of pulmonary arteries less than 50 μm in diameter in the lung sections of control and FK866 treated (2.5mg/kg every 48 hours IP) groups with or without one dose of monocrotaline (MCT, 60 mg/kg bw, SQ). (D) Changes in ratios of wall area to total vessel area of pulmonary arteries 50–100 μm in diameter in the lung sections of control and FK866 treated groups with or without one dose of MCT (60 mg/kg bw, SQ). (E) Changes of RV contractility index after FK866 treatment. The RV contractility index was calculated as $(dp/dt)_{\text{max}}/\text{instantaneous RV pressure}_{\text{max}}$, as demonstrated previously.²⁷ (F) Representative pulmonary artery images in the lung sections of control and FK866 treated (2.5mg/kg every

48 hours IP) groups with or without one dose of MCT (60 mg/kg bw, SQ). Bar size: 20 μ m. Results are expressed as mean \pm SEM; n = 6–8 per group. *, p < 0.05; ***, p < 0.001.

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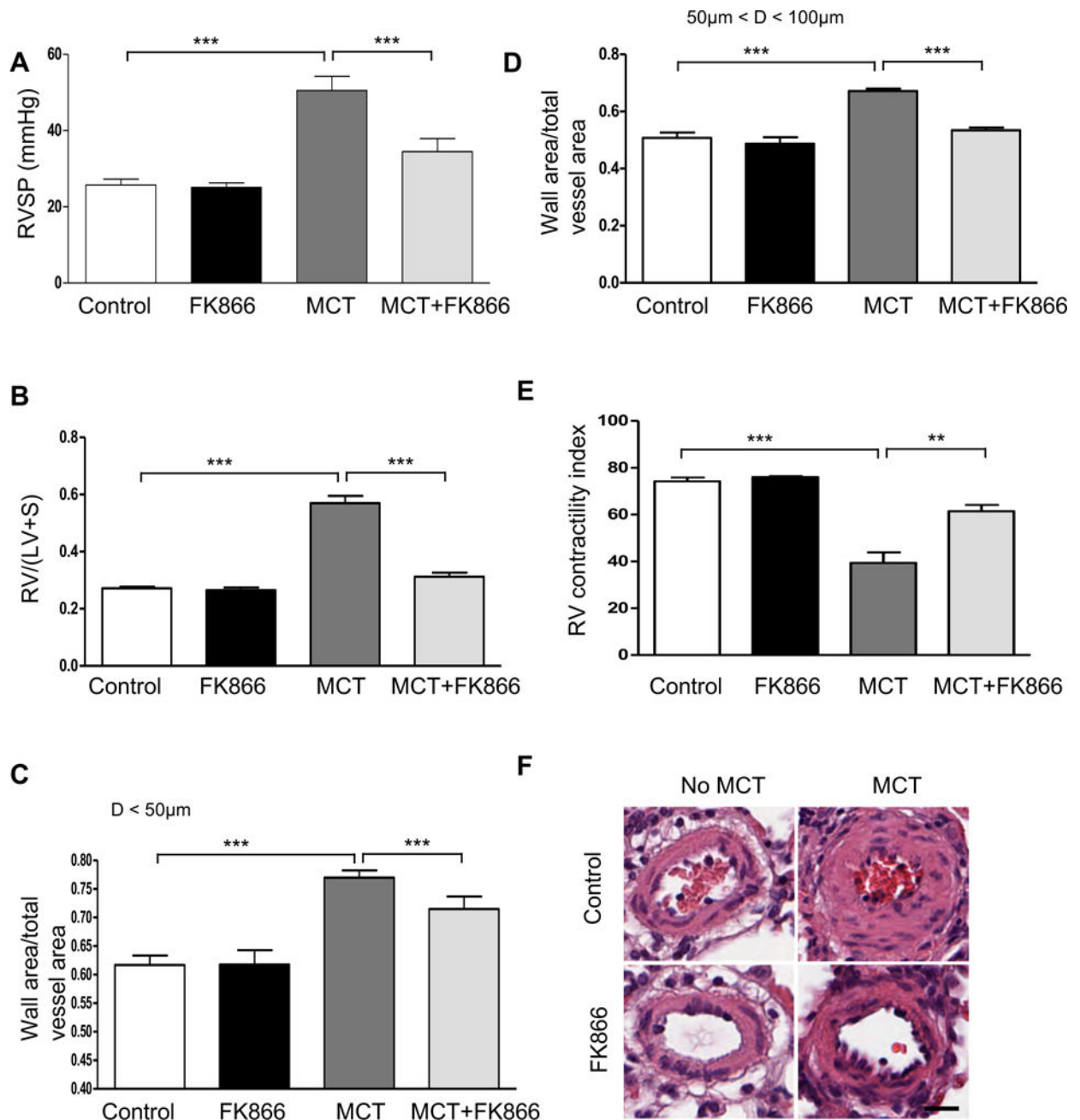


Figure 7. NAMPT inhibition attenuates monocrotaline-induced pulmonary hypertension in rats (A) Changes in RVSP. (B) Changes in RV/(LV+S). (C) Changes in ratios of wall area to total vessel area of pulmonary arteries less than 50 μ m in diameter in the lung sections of control and FK866 treated (2.5mg/kg every 48 hours IP) groups with or without one dose of monocrotaline (MCT, 60 mg/kg bw, SQ). (D) Changes in ratios of wall area to total vessel area of pulmonary arteries 50–100 μ m in diameter in the lung sections of control and FK866 treated (2.5mg/kg every 48 hours IP) groups with or without one dose of MCT (60 mg/kg bw, SQ). (E) Changes of RV contractility index after FK866 treatment. The RV contractility index was calculated as $(dp/dt)_{\max}/\text{instantaneous RV pressure}_{\max}$, as demonstrated previously.²⁷ (F) Representative pulmonary artery images in the lung sections of control and

FK866 treated (2.5mg/kg every 48 hours IP) groups with or without one dose of MCT (60 mg/kg bw, SQ). Bar size: 20 μ m. Results are expressed as mean \pm SEM; n = 6–8 per group. **, p < 0.01; ***, p < 0.001.

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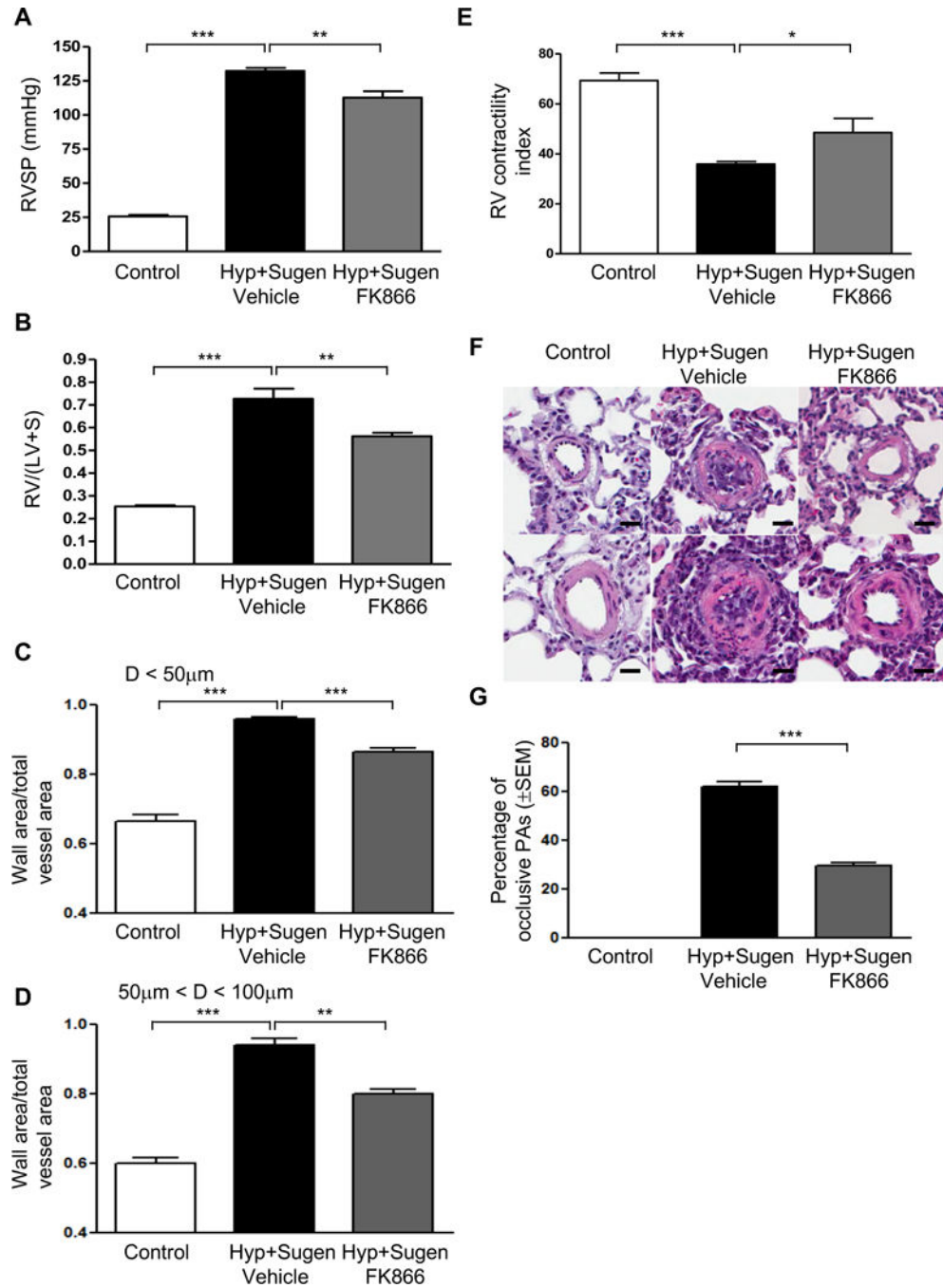


Figure 8. NAMPT inhibition attenuates hypoxia plus sugen-mediated pulmonary hypertension in rats

(A) Changes in RVSP. (B) Changes in RV/(LV+S). (C) Changes in ratios of wall area to total vessel area of pulmonary arteries less than 50 μm in diameter in the lung sections of control and FK866 treated (2.5mg/kg every 48 hours IP) groups; (D) Changes in ratios of wall area to total vessel area of pulmonary arteries 50–100 μm in diameter in the lung sections of control and FK866 treated (2.5mg/kg every 48 hours IP) groups; (E) Changes of RV contractility index after FK866 treatment. The RV contractility index was calculated as $(dp/dt)_{\text{max}}/\text{instantaneous RV pressure}_{\text{max}}$, as demonstrated previously.²⁷ (F) Representative

pulmonary artery images in the lung sections of control and FK866 treated (2.5mg/kg every 48 hours IP) groups; (G) Percentage of occlusive PAs significantly decreases with FK866 therapy (2.5mg/kg every 48 hours IP). Bar size: 20 μ m. Results are expressed as mean \pm SEM; n = 8–12 per group. *, p < 0.05; **, p < 0.01; ***, p < 0.001

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