의학박사 학위논문

Fatty acid-dependent mitochondrial activity and its regulation by neuronal nitric oxide synthase in hypertensive rat hearts

고혈압 쥐 심장에서 지방산에 의한 미토콘드리아 활성의 신경 산화 질소 합성 효소에 의한 조절

2021 년 02 월

서울대학교 대학원

의과학과 의과학전공

오우나

A Thesis of the Degree of Doctor of Philosophy

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February 2021

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Fatty acid-dependent of mitochondrial activity and its regulation by neuronal nitric oxide synthase in hypertensive rat hearts

by

Yu Na Wu M.D.

A thesis submitted to the Department of Biomedical Science in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science at Seoul National University College of Medicine

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ABSTRACT

Fatty acid (FA)-dependent metabolism is important in maintaining cardiac contractile function. Under disease conditions, FA metabolism shifts from FA to glucose-dependent pathways. Recent research has shown that FA-dependent metabolism is dysregulated in hypertensive (HTN) myocardium. Neuronal nitric oxide synthase (nNOS) regulates cardiac physiology and pathology and is involved in mitochondrial activity through its interactions with mitochondrial complexes. Until recently, the mechanisms of the regulation of mitochondrial complexes by nNOS with FA in HTN remain unclear. Therefore, we aimed to investigate mitochondrial activity with FA supplementation in sham and angiotensin II (Ang II)-induced HTN rat hearts and nNOS regulation of complex-mediated mitochondrial activity under these conditions.

Our results showed that oxygen consumption rate (OCR) and intracellular ATP were increased by palmitoyl-carnitine (PC) or palmitic acid (PA). Furthermore, mitochondrial complex I and complex II (C-I and C-II) activity were increased by PA or PC in sham rat hearts. In HTH, C-I activity was increased but C-II activity was reduced by PC, result in reduced mitochondrial OCR. In the presence of C-II inhibitor (malonate, 30 mM) or C-I inhibitor (rotenone, 5μM), OCR was decreased with PA or PC supplementation both in cardiomyocytes and mitochondrial fraction from sham rat hearts. In HTN, however, malonate did not affect mitochondrial OCR in the presence of PC but OCR was increased with rotenone. Therefore, FA increased mitochondrial activity through enhancing of C-I and C-II activity in sham. By contrast, FA-dependent

mitochondrial activity was reduced by C-II downregulation in HTN, despite of the fact that C-I activity was increased by PC. nNOS protein was expressed similarly in sham and HTN LV mitochondrial fraction. Inhibition of nNOS with S-methyl-l-thiocitrulline (SMTC) did not affect OCR or cellular ATP in the presence of PC or PA in sham, but increased OCR in HTN without changing myocardial ATP level. SMTC increased C-I activity only in sham (with C-II activity unaffected), but both C-I and C-II activity were increased by SMTC in HTN. In addition, OCR was increased by SMTC+PC or PA with malonate in sham mitochondrial fraction and cardiomyocyte, but such effects were not observed in the presence of rotenone, indicating that nNOS attenuates C-I-mediated OCR. In contrast, SMTC increased OCR with rotenone pretreatment but not with malonate in HTN, suggesting that nNOS modulates C-II-mediated OCR in HTN. Furthermore, nNOS-derived NO was increased by rotenone in LV myocytes with PA in sham. nNOS-derived NO was partially reduced by malonate with PA in HTN.

In parallel, I went on and investigated the effects of nNOS on OCR in atrial myocardium. In atria, OCR was greater in HTN-LA compared to those in sham-LA and PA increased OCR further in sham-LA but reduced it in HTN-LA. SMTC or Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) reduced PA-increment of OCR in sham-LA but exerted no effect in HTN-LA. PA reduced $eNOS^{Ser1177}$, $nNOS^{Ser1417}$ and NO level in HTN-LA but exerted no effect in sham-LA, indicating that NO deficiency underlie reduced mitochondrial activity in HTN-LA. *S*-palmitoylation is an important post-transcriptional modification in the

presence of PA. *S*-palmitoylation inhibitor, 2-bromopalmitate (2BP), prevented PA-dependent decrease of $nNOS^{Ser1417}$ and OCR only in HTN-LA, suggesting *S*-palmitoylation and nNOS inhibition mediate PA-dependent OCR reduction in HTN-LA.

Taken together, my research revealed novel mechanisms of FA-dependent mitochondrial activities and complex regulation by nNOS in healthy and HTN rat hearts. Metabolic dysregulation by nNOS underlie atrial and ventricular remodeling in HTN.

Some of the work are published in Pflugers Archive European Journal of Physiology (Wu YN et al., 2020 Jul 23 and Wu YN et al., 2020 Sep 17).

Keywords: cardiac myocytes, mitochondria, mitochondrial activity, complex I, nNOS, hypertension.

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CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

NO: nitric oxide

nNOS: Neuronal nitric oxide synthase

eNOS: Endothelial nitric oxide synthase

L-NAME: n(omega)-nitro-L-arginine methylester

SMTC: S-methyl-L-thiocitrulline

Ang-II: Angiotensin II

SNP: Sodium Nitroprusside

LV: Left Ventricle

LA: Left atrial myocardium

HTN: hypertension

ATP: adenosine-5'-triphosphate

FA: fatty acid

FCCP: Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone

PA: palmitic acid

PC: palmitoyl carnitine

2BP: 2-bromo palmitate

$Na_2S_2O_4$: sodium dithionite

Malonate: Malonic acid

INTRODUCTION

Cardiac excitation-contraction coupling.

Myocardial contraction is triggered by cytosol Ca^{2+}. Ca^{2+} ions enter cardiomyocyte via voltage-gated L-type calcium channels (LTCC) in the t-tubular system and diffuse into dyadic cleft to stimulate Ca^{2+} release from ryanodine receptors on sarcoplasmic reticulum (SR). Increased Ca^{2+} binds to troponin C (TnC) in thin filament to initiate conformational changes of troponin I and tropomyosin, to remove the latter from blocking actin-myosin binding. The process turns on contractile machinery and Ca^{2+}-TnC dissociation reverses the events for relaxation (Palmiter and Solaro 1997, Eisner, Choi et al. 2000). Ca^{2+} is uptaken into SR via SR Ca^{2+} -ATPase and is extruded out of the cell via sarcolemmal Na^{+}/Ca^{2+} exchanger (NCX) and Ca^{2+}-ATPase. ATP generation from mitochondria maintains energy supply for cardiac work, i.e. ATP-binding to myosin and its hydrolysis fuels the force generation of the myofibril. Cardiac metabolism is well recognized to be a limiting factor for contractile dysfunction in heart failure.

Cardiac metabolism and contraction

In healthy adult heart, majority of ATP production is derived through oxidative phosphorylation in the mitochondria. To sustain sufficient ATP supply, the heart utilizes several kinds of energy substrates including carbohydrates, lipids, amino acids and ketone bodies (Neely and Morgan 1987, Opie 1992). Carbohydrates and FAs are essential in

cardiac metabolism through glucose oxidation or β-oxidation, respectively.

Approximately 70% to 90% of cardiac ATP is produced by the FA oxidation. The remaining 10% to 30% comes from the oxidation of glucose and lactate, as well as small amounts of ketone bodies and amino acids (Doenst, Nguyen et al. 2013). Glucose is phosphorylated and becomes a substrate for the glycolytic pathway, glycogen synthesis, ultimately broken down to pyruvate and enters TCA cycle in the mitochondria. On the other hand, FAs enter cardiac myocytes through FA transporters such as FAT/CD36 (Fig.1), then esterified to long chain fatty acyl CoA and shuttled into mitochondrial matrix after the formation of long chain acylcarnitine by carnitine palmitoyltransferase 1 (CPT-1), a key enzyme that determines the fate of FA for β-oxidation (Lopaschuk, Ussher et al. 2010). Accordingly, CPT-1 inhibitors, such as etomoxir, perhexiline, and oxfenicine, have been associated with reduced cardiac FA oxidation and elevated glucose oxidation in both animal models and humans (Kolwicz, Purohit et al. 2013). In this process, glucose oxidation and β-oxidation produce acetyl CoA to activate TCA cycle to generate electron donor-NADH and $FADH_2$.

Electrons from NADH and $FADH_2$ are transported to O_2 by electron carriers organized in four respiratory complexes (complex I-V, Acin-Perez, Fernandez-Silva et al. 2008). In particular, electrons are transferred to O_2 via complex I to complex IV and proton transportation across mitochondrial inner membrane establishes electrochemical gradient, which becomes the drive to the synthesis of ATP by complex

V, a process known as oxidative phosphorylation.

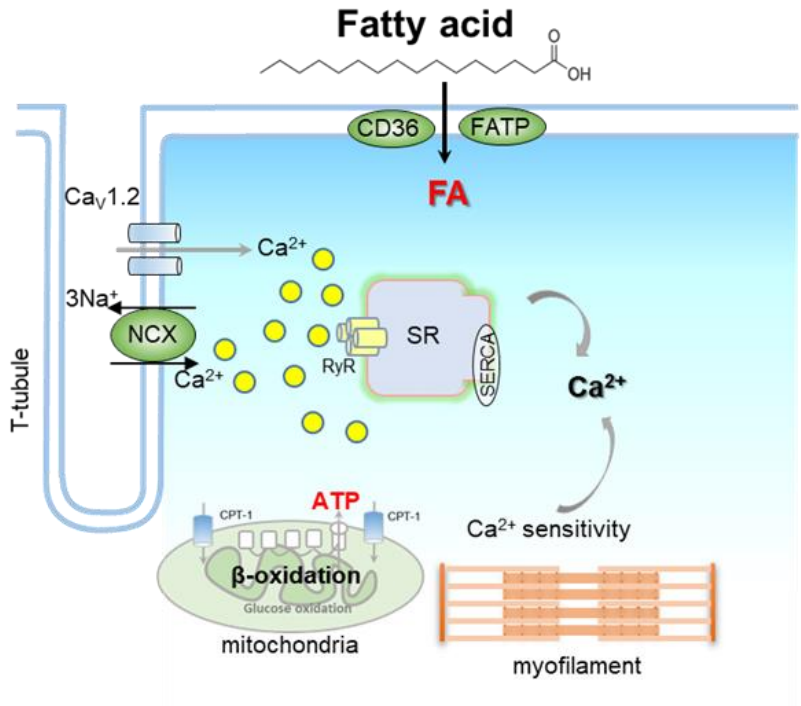

Figure 1. Schematic diagram of FA metabolism in the heart. FA enters cardiac myocytes through FA transporters such as FATP (fatty acid transport protein)/CD36, generate ATP through β-oxidation to maintain cardiac contractile function.

FA metabolism and cardiac mitochondrial activity in diseased heart

Modification of respiration and metabolism has been suggested to underlie the contractile dysfunction in failing hearts (Rosca and Hoppel 2010). As shown in Fig.2, during disease progression, β-oxidation is down-regulated and glycolysis becomes the main means for energy (metabolic shift) for the preservation of the pumping function of the heart (Lopaschuk, Ussher et al. 2010). Indeed, it has been indicated that

patients with HTN exhibited reduced FA oxidation in left ventricular (LV) (de las Fuentes, Herrero et al. 2003, de las Fuentes, Soto et al. 2006) and this phenomenon precedes an increase in the LV mass and may be responsible for decreased myocardial efficiency (de las Fuentes, Soto et al. 2006). In the failing myocardium, both glucose- and FA-dependent metabolism are limited (Fig.2), and energy deficiency becomes an important precursor for impaired myocardial contraction. The basis of FA metabolic imbalance is complex, and almost half of the hypertensive patients are with the comorbidities of hyperlipidemia (Couet, Li et al. 1997). Until recently, FA metabolism and cardiac dysfunction in HTN is still a matter of discussion.

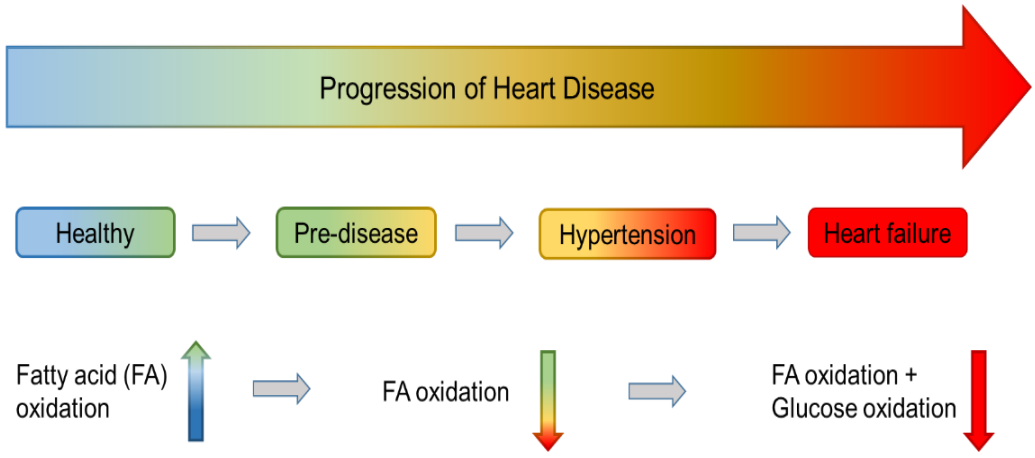

Figure 2. FA metabolism and mitochondrial activity in healthy and diseased heart. FA oxidation and metabolism is the predominant source of myocyte ATP in healthy heart. During disease progression, FA oxidation becomes down-regulated and both glucose- and FA-oxidation are limited in heart failure.

Nitric oxide synthases (NOS) in cardiac metabolism and function.

Nitric oxide (NO) plays crucial roles in maintaining cardiac function, including cardiac contractility, Ca^{2+} handling, oxidative stress and cardiac metabolism (Jin, Yin et al. 2017). NO regulation of mitochondrial function is essential in regulating these phenomena, and is implicated to be one of the major influences of cardiac mitochondrial activity in both healthy and diseased hearts (Fillmore, Mori et al. 2014).

It has been known that NO is produced by NO synthases (NOS), neuronal NOS (nNOS or NOS1); inducible NOS (iNOS or NOS2); endothelial NOS (eNOS or NOS3). Until now, eNOS is generally considered the major NOS isoform in the myocardium to regulate NO-mediated cardiac functions. We and others have shown that nNOS is constitutively expressed in the SR of cardiac myocytes and regulates sarcoplasmic Ca^{2+}-ATPase (SERCA) reuptake of intracellular Ca^{2+} (Jin, Jang et al. 2012, Jin, Jang et al. 2013, Zhang, Jin et al. 2014). NO regulation of mitochondrial function has been implicated to be one of the major influences of cardiac metabolism in healthy and diseased hearts (Litvinova, Atochin et al. 2015). In previous study, we have shown that nNOS-derived NO exerts different functions with PA supplementation in cardiac mitochondria from healthy and HTN rat hearts (Jin, Yin et al. 2017). nNOS has been regarded as one of the major NOS isoforms in diseased heart (Brown and Borutaite 2007) and Ca^{2+}-induced NO release from murine heart mitochondria (Kanai, Pearce et al. 2001), presumably through nNOS. Mitochondrial NOS (mtNOS) in the inner membrane of cardiac mitochondria is known to be an α-splice variant of the nNOS

transcript, close to the energy transduction and O_2^- production machinery (Kanai, Pearce et al. 2001). Following these findings, NO in cardiac mitochondria has been of intensive research (Boveris, Arnaiz et al. 2002, Zaobornyj, Valdez et al. 2009). So far, the interactions between nNOS and mitochondrial complex activities in the presence of FA in normal and HTN hearts are unclear.

nNOS and mitochondrial complexes

nNOS has been reported to be associated with mitochondrial C-I (NADH-dehydrogenase) activity (Parihar, Nazarewicz et al. 2008), by forming a strong protein-protein interaction (Schafer, Seelert et al. 2006, Genova and Lenaz 2014). Mitochondrial C-I catalyzes the transfer of two electrons from NADH, via flavin mononucleotide (FMN) and a series of iron-sulfer centers (Fe-S) to ubiquinone (UQ) due to proton translocation (Walker 1992, Walker, Arizmendi et al. 1992). However, electrons can also be transferred backwards by C-II, in the presence of succinate, via C-I to nNOS; and nNOS activity can be activated under these conditions (Bombicino, Iglesias et al. 2016). Evidences also show that NO exerts inhibitory effect on the respiratory chain at C-II to cytochrome C electron transport, in a reversible manner (Poderoso, Carreras et al. 1996). The interactions between complexes and nNOS may exert reciprocal regulation of mitochondrial function. So far, such an effect has not been studied, especially in diseased hearts with PA supplementation. HTN is a risk factor for developing left ventricular (LV) hypertrophy and heart failure due to mechanical and metabolic disturbances (Jin, Jang et al.

2012, Jin, Jang et al. 2013). Accordingly, we aimed to investigate the functional associations between nNOS and mitochondrial complexes and investigated the mechanisms in HTN rat hearts.

Metabolism in atrial myocardium

Atrial myocardium is highly dynamic and responds to stretch (Blume, Mcleod et al. 2011) for higher glycolytic capacity compare to those in ventricles (Savabi and Kirsch 1991), but FA metabolism and its regulations in atria are remained unknown. Recently, evidences relating to altered metabolism and its associations with atrial dysfunction are emerging (Karam, Chavez-Moreno et al. 2017). In atrial myocardium, HTN and pressure-overload predisposes atrial contractile dysfunction (Lip, Coca et al. 2017), conditions those affect cardiac metabolism. The presence of LV hypertrophy due to arterial HTN impair atrial function (Abhayaratna, Seward et al. 2006).

In atria, HTN and hyperlipidemia are known to be the major risk factors of abnormal function and adverse remodeling (Homan, Reyes et al. 2019). Due to the prevalence of HTN in atrial myocardial disorders, it is important to understand atrial metabolism with FA in HTN. During atrial arrhythmia progression, glucose oxidation is upregulated compare to FA oxidation in LA (Jie, Li et al. 2019), indicating that abnormal metabolism under pathological condition. Previously, FA-dependent metabolism in atrial myocardium has shown to be decreased in AF, but glucose metabolism is upregulated (Jie, Li et al. 2019). FA-dependent mitochondrial activity in HTN remains to be determined. eNOS-derived

NO has been known to play a critical role in FA-dependent energy production in muscle (Le Gouill, Jimenez et al. 2007). Indeed, research from our group has shown that FA increased mitochondrial activity in normal rat LV myocytes which is mediated by eNOS activity (Jin, Wu et al. 2017). In HTN, eNOS protein expression is reduced in Ang II-induced LV cardiomyocytes and FA-dependent mitochondrial activity is mediated by nNOS, which is upregulated in HTN LV myocytes (Jin, Jang et al. 2013, Jin, Yin et al. 2017). NO is well known to modulate myocardial contractility and mitochondrial activity in healthy and diseased hearts (Jin, Jang et al. 2012, Jin, Jang et al. 2013, Jin, Yin et al. 2017); it functions as a key regulator of cardiac metabolism under pathological stimuli (Niu, Watts et al. 2012, Zhang, Jin et al. 2014).

S-palmitoylation has been established to be an important post-translational modification that affects the translocation and the activity of target proteins in the presence of PA (one of the common saturated FAs) in a variety of cell types including cardiomyocytes (Linder and Deschenes 2007). The reversible nature of *S*-palmitoylation provides a potential mechanism for protein shuttling between intracellular compartments (Linder and Deschenes 2007, Charollais and Van Der Goot 2009, Fukata and Fukata 2010), e.g. eNOS palmitoylation is known to be relocated at caveolae (Shaul 2002, Sullivan and Pollock 2003) to activate or inactivate this protein (Couet, Li et al. 1997, Anderson 1998, Frank, Woodman et al. 2003). So far, the involvement of *S*-palmitoylation in regulating eNOS and nNOS activities in atrial myocardium in HTN has not been studied.

Taken together, the main aim of the research is to study the effects of FA supplementation on cardiac mitochondrial activity in sham and HTN and explore the mechanisms of mitochondrial regulation by nNOS in healthy and hypertensive rat hearts.

Outline of the thesis

The work presented in this thesis investigates "Cardiac mitochondrial activity with FA supplementation and the regulation by neuronal nitric oxide synthase in sham and HTN rat hearts".

In Part I and II, I investigated FA regulation of cardiac mitochondrial activity and functional associations between nNOS and mitochondrial complexes in sham and angiotensin II-induced HTN rat hearts. In essence, this part demonstrates that nNOS attenuates C-I-mediated mitochondrial OCR in the presence of FA in sham and this is mediated through mutual modulations between C-I and nNOS activity. In HTN, nNOS attenuates C-I and C-II activity whereas interactions between nNOS and C-II are important in FA-dependent mitochondrial activity.

In Part III, I investigated PA-regulation of mitochondrial OCR in left atrial myocardium (LA) of sham and HTN rats and their regulations by eNOS and nNOS. The core of the findings are that PA reduced mitochondrial OCR, nNOS activity and NO bioavailability in HTN-LA. *S*-palmitoylation and nNOS^{Ser1417} reduction by PA are responsible for mitochondrial dysfunction in HTN-LA.

The schematic diagram illustrates of the present study (Figure 3).

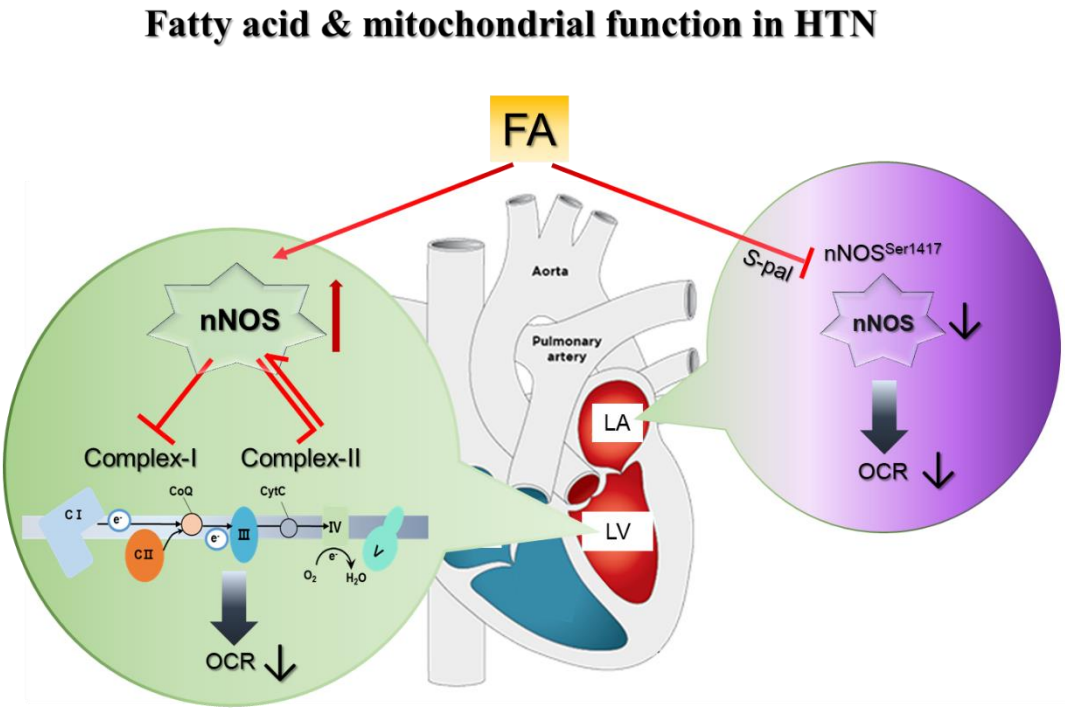

Figure 3. Schematic diagram illustrating FA regulation of mitochondrial activity and its regulation by nNOS in sham and HTN rat LV and LA.

MATERIALS AND METHODS

1. Animals

Left ventricular (LV) myocardium and left atrium (LA) was obtained from Sprague-Dawley (SD) rats (10-12 weeks old, male) in the study. Hypertension was induced by infusing angiotensin II (Ang II) subcutaneously in rats using osmotic minipumps, and they were paired with sham-operated group, as reported before (Jin, Jang et al. 2013, Jin, Yin et al. 2017). Briefly, rats (of 8weeks old, male) were anesthetized with isoflurane (2.5%). An osmotic minipump (Alzet model 2004, DURECT corporation, San Francisco, CA, USA) containing Ang II (200ul, 6mM, infusion rate 125ng/min/kg) was implanted in the mid-scapular region for 4weeks. Sham-operated animals underwent the same surgical procedure, except for no pump insertion. Blood pressures were measured every 3 days (from 2 days before the operation) for 4weeks by Non-Incasive Blood Pressure System, tail-cuff method (CODA, Torrington, CT, USA). The study protocol was in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 196), and also conforms to the Institutional Animal Care and Use Committee(IACUC) in Seoul National University (IACUC approval No.: SNU-101213-1; SNU-160119-4-6).

2. Measurement of blood pressure in sham and HTN rats

Both systolic and diastolic blood pressures (SBP and DBP) were increased from 1 week following Ang II infusion (125 ng/min/kg) and continuously increased, and heart rate were progressively slower up to the period studied. At 4 weeks, SBP, DBP were significantly different between sham and HTN rats, and lose of body weight was observed in HTN compared to sham (Table 1).

Table 1 Heart rate, blood pressure and body weight in sham and hypertensive rats

Characteristics	Sham		HTN		
	Means ±SEM	Hearts (n)	Means ±SEM	Hearts (n)	*p* value
Heart rate (bpm)	470.1±9.2	16	433.6±13.5***	16	0.0004
SBP (mmHg)	124.4±4.71	16	157.9±4.11***	16	<0.001
DPB (mmHg)	90.14±2.03	16	114.5±3.46***	16	<0.001
Body Weight (8w,*g*)	244.6±3.27	16	240.4±1.50	16	0.243
Body Weight (12w,*g*)	381.6±7.53	16	363.7±7.28*	16	0.024

SBP is systolic blood pressure, DBP is diastolic blood pressure

* $p<0.05$ compared with sham; ** $p<0.01$ compared with sham; *** $p<0.001$ compared with sham

Both systolic and diastolic blood pressure were increased 1 week after Ang II infusion (125ng/min/kg) and continuously increased up to the period studied (Table1). In addition, heart rate was significantly lower in hypertensive rats.

3. Isolation of Left ventricular myocytes

LV myocytes were isolated enzymatically by Langendorff perfusion system as recently described (Jin, Yin et al. 2017). Briefly, the rats were anesthetized with pentobarbital sodium (30mg/kg,i.p) and the hearts

were extracted and rapidly mounted onto the Langendorff perfusion system. The isolated heart was perfused with a nominally Ca^{2+} -free solution for 10 min (in mM, NaCl 135, KCL 5.4, $MgCl_2$ 3.5, glucose 5, HEPES 5, Na_2HPO_4 0.4, taurine 20; pH titrated to 7.40 using NaOH), followed by a further 8 min with the same solution with enzymes added (collagenase type 2, 1mg/ml, Worthington Biochemical Corporation, Lakewood, NJ, USA; protease 0.1mg/ml, bovine serum albumin (BSA) 1.67mg/ml; Ca^{2+} 0.05mM). The LV-free wall was isolated and placed in a separate flask containing fresh collagenase-only solution for 8min (oxygenated and maintained at 37℃). Myocytes were harvested following a further 10-min digestion period, washed, and re-suspended in storage solution (in mM,NaCl 120, KCl 5.4, $MgSO_4$ 5, $CaCl_2$ 0.2. Na-pyruvate 5, glucose 5.5, Taurine 20, HEPES 10, D-manitol 29; pH titrated to 7.40 using NaOH). The myocyte suspension was stored at room temperature and cells were used within 8h of isolation.

4. Mitochondrial fraction enrichment

Mitochondria of rat heart were isolated with the Mitochondria Isolation Kit for Tissue (Abcam ab110168, UK) according to the manufacturer's instruction. Briefly, heart was washed with washing buffer in the kit, homogenized with isolation buffer, and centrifuged at 1000 *g* for 10 min. The supernatant was centrifuged again at 12 000 *g* for 15 min. Pellets (mitochondrial fraction) were washed with isolation buffer with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) twice and resuspended with isolation buffer with protease inhibitor cocktail.

Mitochondria were quantified by bradford assay.

5. Measurement of oxygen consumption rate from LV myocytes and cardiac LA

According to the manufacturer's instructions, oxygen consumption rate (OCR) was measured using a fluorescence-based oxygen sensor (NeoFox, Ocean Optics, Dunedin, FL, USA) connected to a phase measurement system (Instech Laboratories, Inc., Plymouth Meeting, Philadelphia, USA). The sensor was calibrated regularly (every week) according to the manufacturer's instructions. Briefly, isolated LV myocytes (density $2x10^4$/ml) and chopped LA (normalized with protein quantification) were suspended in normal Tyrode's solution and was placed in a sealed chamber (300μl). Record oxygen level in the chamber over 30-min period (37℃) followed by about 10-min recordings with carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; 20μM) to evaluate maximum oxygen consumption of myocytes and LA in the chamber. Changes in the OCR with palmitic acid (PA, 100μM, 30min-1hr) pre-treatment with and without nNOS inhibitor SMTC (100nM, 30min-1hr) or eNOS/nNOS inhibitor L-NAME (10mM, 30min-1hr) incubation, Rotenone (5μM), Malonate (30mM) were calculated over 30-min period in sham and HTN LV and LA.

6. Measurement of mitochondrial C-I activity

LV mitochondrial C-I activity was detected by measuring NADH level

and NADH decline time, then calculate the ratio of NADH consumption and NADH decline time. The NADH consumption was measured using a fluorescence spectrophotometer (Photon Technology International, Birmingham, NJ, USA) at 37℃. Briefly, 0.1mg mitochondria was dissolved in mitochondrial buffer (in mM, D-mannitol 210, sucrose 70, potassium dihydrogen phosphate 5, MOPS 10; pH=7.2) containing metabolic substrates (in mM, Pyruvate 5, Malate 5, Glutamate 5, Succinate 5, ADP 2), monitored in a quartz microcuvete (1mL) with stirring at excitation wave length of 340nm, and emission wavelength of 445nm. PA mimetic, palmitoyl-carnitine (PC, 20μM, 30min-1hr) was used to promote FA metabolism in isolated mitochondria.

7. Immunoblotting

LV myocytes and LA that were lysed in lysis buffer contained 50mM Tris-HCl (pH 7.4), 100mM NaCl, 1%Triton X-100, and 5mM EDTA with protease inhibitor cocktail (Roche Diagnostics, Mannhaim, Germany). Cell and tissue lysates were then centrifuged at 15000g for 30min at 4℃, and the supernatants were acquired. The protein concentration was determined by the Bradford assay. The protein sampled were mixed with Laemmli sample buffer, resolved by 6 and 10% SDS-PAGE, and transferred to polyvinylidine difluoride membranes in 25mM Tris, 192mM glycine, and 20% methanol. Membranes were blocked in 1x TBS containing 1% Tween-20 and 5% skim milk (blocking solution) for 1h at room temperature with gentle rocking, and incubated overnight at 4℃ with anti-nNOS (BD Transduction Laboratories),

nNOS^{Ser1417} (Abcam, ab90443), Cytochrome C (Abcam, ab13575), eNOS (BD Transduction Laboratories), eNOS^{Ser1177} (Cell Signaling), eNOS^{Thr495} (Cell Signaling), AKT (Cell Signaling), AKT^{Ser473} (Cell Signaling), AMPK (Cell Signaling), AMPK^{Thr172} (Cell Signaling), GAPDH (Santa Cruz, Dallas, TX, USA) primary antibodies, followed by relevant secondary antibodies after washing. Blots were developed by ECL Plus Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ, USA). Membranes were stripped using Pierce restore western blot stripping buffer (Thermo Scientific, Waltham, MA, USA) for 30 min, and the relative densities were calculated after normalizing the intensity of each sample band to that of GAPDH or the relative densities were calculated with loading protein quantification.

8. Measurement of NO production

NO production was detected indirectly by measuring nitrite content in LV and LA using NO assay kit (Griess Reagent System). Briefly, cell and tissue were homogenized in Tris-cl buffer (in mM NaCl 150, Tris-cl 50, EDTA 1, 1% Triton X-100, mixtures of protease inhibitors; pH titrated to 7.40 using NaOH). After assaying the protein content with Bradford protocol, 50 μl of the supernatant of lysates was added to the wells and mixed with 50 μl of sulfanilamide solution and incubated for 5–10 min at room temperature in the dark. The same volume of naphthylethylenediamine dihydrochloride (NED, Sigma) solution was added to the wells and incubated for another 5–10 min at room temperature, protected from light. Absorbance of themixture

wasmeasured with amicroplate plate reader (at 540 nm, PowerWave™ XS Microplate Spectrophotometer, BioTek Instruments, USA). Sodium nitrite (Sigma) was used as a standard. NO production was detected in control, PA (100μM, 1hr), Rotenone(5μM), in both sham and HTN LV myocytes. PA (100μM, 1hr), SMTC (100nM, 1hr) and L-NAME (10mM, 1hr) were treated in LA sham and HTN groups. Sodium nitroprusside (SNP, 10μM, 1hr) was used as a positive control in both two samples.

9. Quantification of mitochondrial respiratory complex II activity by ELISA system

The LV myocytes mitochondrial activity of complex II (C-II) was measured using C-II Enzyme Activity Microplate Assay Kit (Colorimetric) (ab109908; Abacam, USA) according to the manufacturer's protocol. Harvest cells as described above then wash cells twice with PBS. Extract the proteins from the cells by adding 10X Detergent solution to sample to a final dilution of 1X and incubate on ice for 30minutes to allow solubilization. After centrifuge the samples for 20min, collect the supernatant and measured the protein concentration use Bradford Protein Assay Reagent (BIO-RAD, Hercules, CA, USA), and all samples were diluted to the manufacturer-recommended concentration. Equal amounts of each sample were loaded onto the plate and incubated for each response time at room temperature. After incubation, all assays were performed following the manufacturer's instructions. In addition, all assays were validated to ensure that the signal was in the linear range of the detection system. Mitochondrial C-

II activity was detected in control, PA (100μM, 30min) and SMTC (100nM, 30min) in both sham and HTN groups. Malonic acid (malonate, 30mM) was used as a negative control.

10. Measurement of LV myocytes ATP level

Intracellular ATP level of LV myocytes was determined using ATP Assay Kit (ab83355; Abcam, Colorimetric/Fluorometric,USA) according to the manufacturer's instructions. Prepared cells ($5x10^4$/ml) were incubated in normal tyrode solution (in mM NaCl 141.4, KCl 4, NaH2PO4 0.33, MgCl2 1, HEPES 10, glucose 5.5, CaCl2 1.8, mannitol 14.5, pH 7.4 NaOH). Wash cells with cold PBS solution followed by lysis with 100μl of ATP Assay buffer as protocol described. Then 50μl of cell lysate and 50μl ATP Assay reaction mix solution was added to the 96-well microplate in the dark at room temperature for 30min, and the absorbance at 570nm in each well was measured using a microplate plate reader (Tecan Microplate Reader: Multi-Mode Infinite M200, San Jose, CA).

11. Statistical analysis

The results are expressed as the means ± standard error of mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's HSD post-hoc tests and two-way ANOVA with Bonferroni post-hoc test for multiple comparisons. $P < 0.05$ was considered statistically significant.

Chemicals

Angiotensin II (Ang II; 6mM, Sigma) was injected to the osmotic minipumps. *S*-methyl-$_L$-thiocitrulline (SMTC; 100nM, Sigma) were used to target nNOS activities. Sodium nitroferricyanide (III) dehydrate (SNP; 10μM, Sigma) was an exogenous NO donor. Palmitic acid (PA; 100μM, Sigma, dissolved in a solution with 0.1M NAOH containing 10%BSA) was used to stimulate FA oxidation. Rotenone (5μM, Sigma) was used to blocks electron transfer from iron-sulfur centers in complex I to ubiquinone. Malonic acid (malonate; 30mM, Sigma) was used as mitochondrial complex II inhibitor. 2-Bromopalmitate (2BP; 100μM, Sigma) was an inhibitor of *S*-palmitoylation. Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; 20-40μM, Sigma) was an uncoupling agent-induced maximal oxygen consumption rate. Sodium hydrosulfite or hyposulfite ($Na_2S_2O_4$; Sigma-Aldrich) was used in calibration for OCR by minimizing O_2 level in the solution.

Results

Part I: FA regulation of mitochondrial activity in sham and HTN rat hearts

First, we detected the parameters of mitochondrial activity in sham and HTN rat. HTN is the most common risk factor for developing LV hypertrophy and heart failure, in our previous study found that cardiac contractile function and mitochondrial activity was dysregulated in HTN (Jin, Jang et al. 2013, Jin, Yin et al. 2017). Here, we aimed to investigate FA-dependent mitochondrial activity in sham and HTN rat cardiomyocytes and isolated cardiac mitochondria., whether mitochondrial C-I or C-II activity was involved in these conditions. LV myocytes and mitochondria fraction were pre-incubated with PA or PC in the experiment.

FA-dependent mitochondrial activity was different in sham and HTN

PC supplementation increased mitochondrial OCR (p<0.0001, n=6; Fig.4A) and PA increased intracellular ATP in LV myocytes (p=0.001, n=4; Fig.4C), indicating increased mitochondrial activity by FA in sham. However, PC reduced mitochondrial OCR (p=0.004, n=6 and n=10; Fig.4B), but did not affect intracellular ATP in HTN (p=0.63, n=6; Fig.4D).

To investigate FA responses to mitochondrial C-I and C-II activity, we examined C-I, C-II activity in sham and HTN rat hearts. As shown in

Fig.5A-B, PC increased C-I and C-II activity (p=0.03, n=5; p=0.0009, n=3). In addition, PC increased C-I activity (p=0.04, n=5; Fig.5C), but PA reduced C-II activity in HTN (p=0.0003, n=4, Fig.5D). These results indicated that there were different effects on CI, C-II-mediated mitochondrial activity in sham and HTN.

FA regulation of C-I, C-II-mediated OCR in sham and HTN

Next, the effects of FA on C-I, C-II-mediated OCR were examined in sham and HTN rat hearts. OCR was significantly reduced in the presence of malonate and rotenone in sham mitochondrial fraction with PC supplementation (p=0.0002, n=6 and n=5; p=0.003, n=6 and n=5; Fig.6A&C). Therefore, FA-dependent mitochondrial OCR was increased through C-I, C-II in sham. In HTN, malonate did not affect OCR with PC supplementation, however, in the presence of rotenone OCR was increased in mitochondrial fraction (p=0.62, n=10 and n=5; p=0.001, n=10 and n=8; Fig.6B&D). These results indicating that there were different effects on C-I and C-II-mediated mitochondrial activity in HTN mitochondrial activity with FA supplementation. Similar experiments were conducted in LV myocytes from sham and HTN rats. Both malonate and rotenone significantly decreased OCR in the presence of PA in LV myocytes from sham and HTN rats (p<0.0001, n=8 and n=7; p=0.0001, n=8 and n=9; p=0.002, n=13 and n=4; p=0.0001, n=8 and n=9; Fig.7). Therefore, FA-dependent mitochondrial activity was different in sham and HTN rat hearts (a schematic diagram of FA-induced mitochondrial activity from sham and HTN was shown in Fig.8).

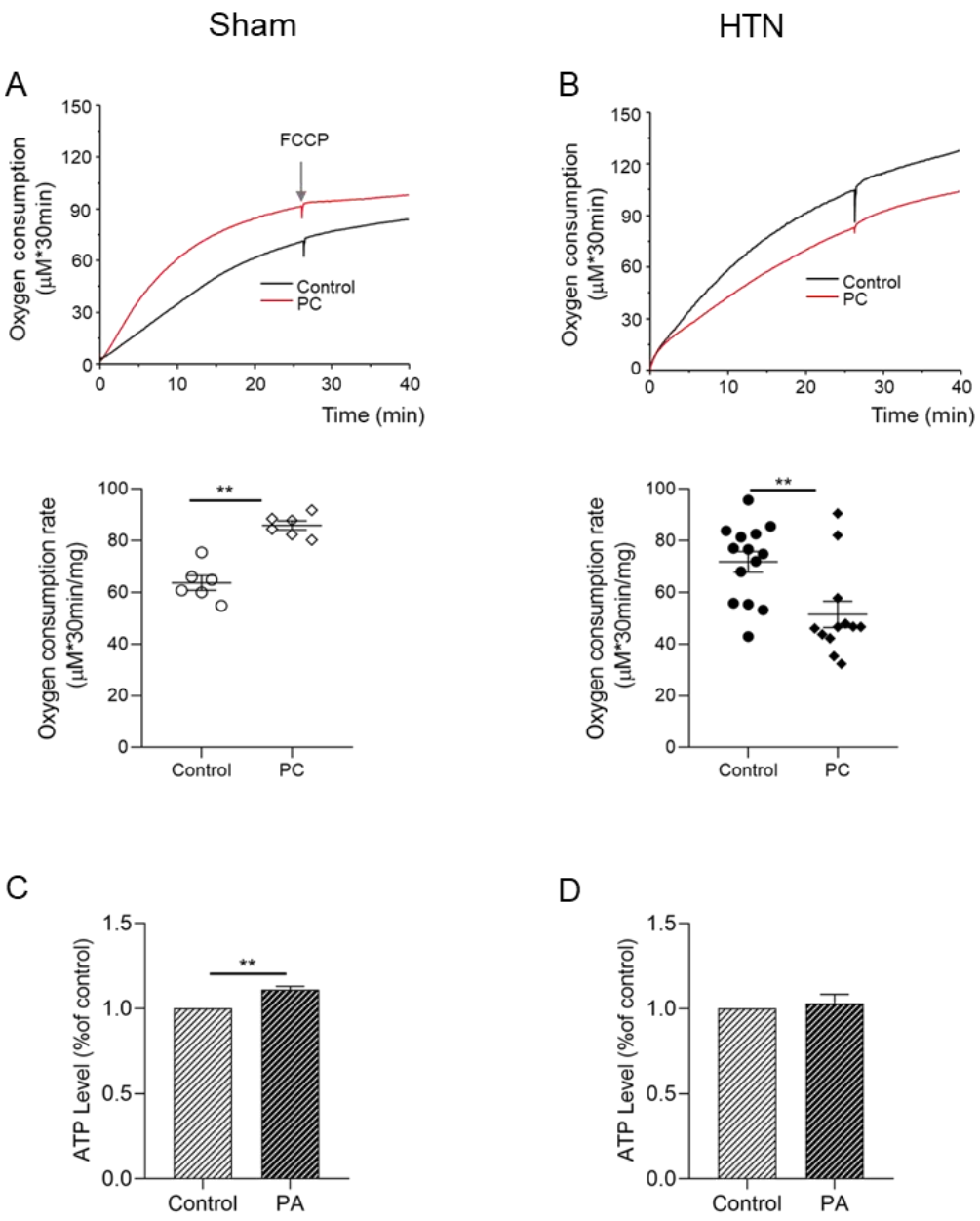

Figure 4. FA regulation of mitochondrial activity in sham and HTN rat hearts. A-B. Representative OCR in sham and HTN rat hearts. PC increased OCR in sham, but reduced OCR in HTN. C-D. Cellular ATP production was increased in sham but did not change in HTN.

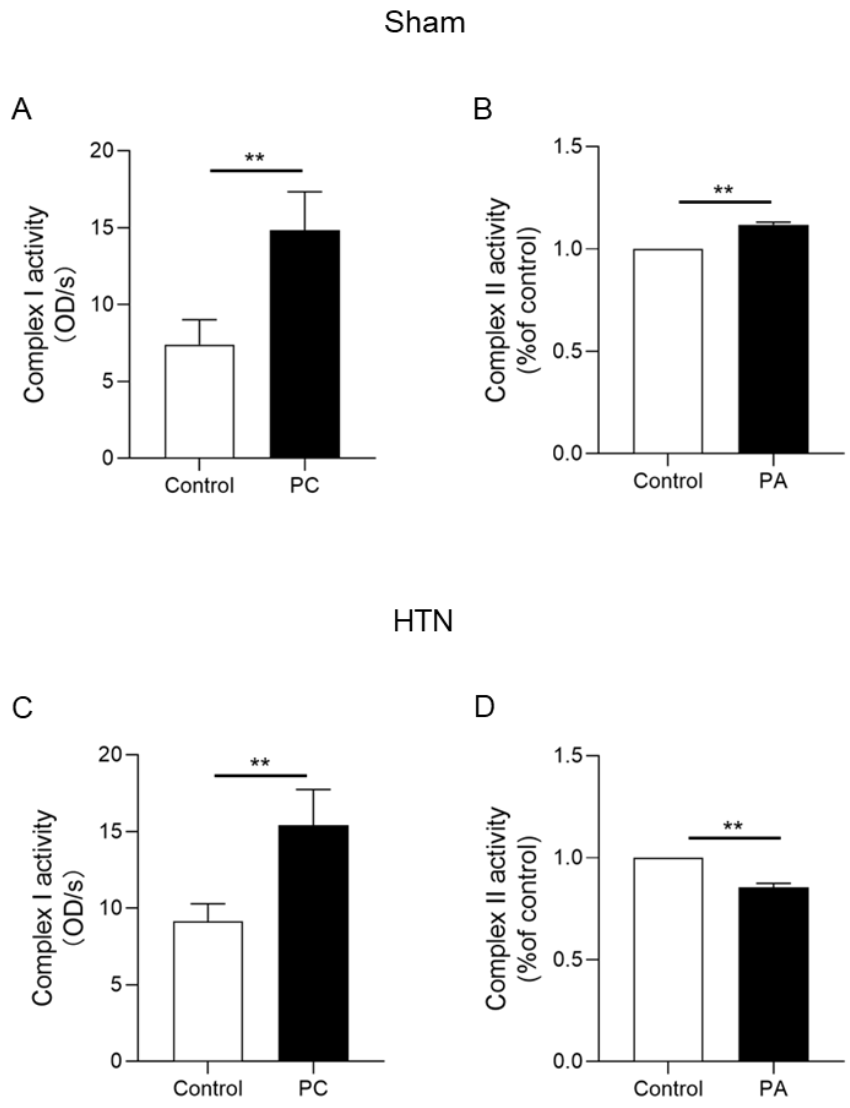

Figure 5. FA regulation of C-I and C-II activity in sham and HTN rat hearts. A-B. C-I and C-II activity were increased by PC or PA. C-D. Mean value of FA-dependent C-I, C-II activity in HTN. FA (PC or PA) increased C-I activity but reduced C-II activity in mitochondrial fraction of HTN LV.

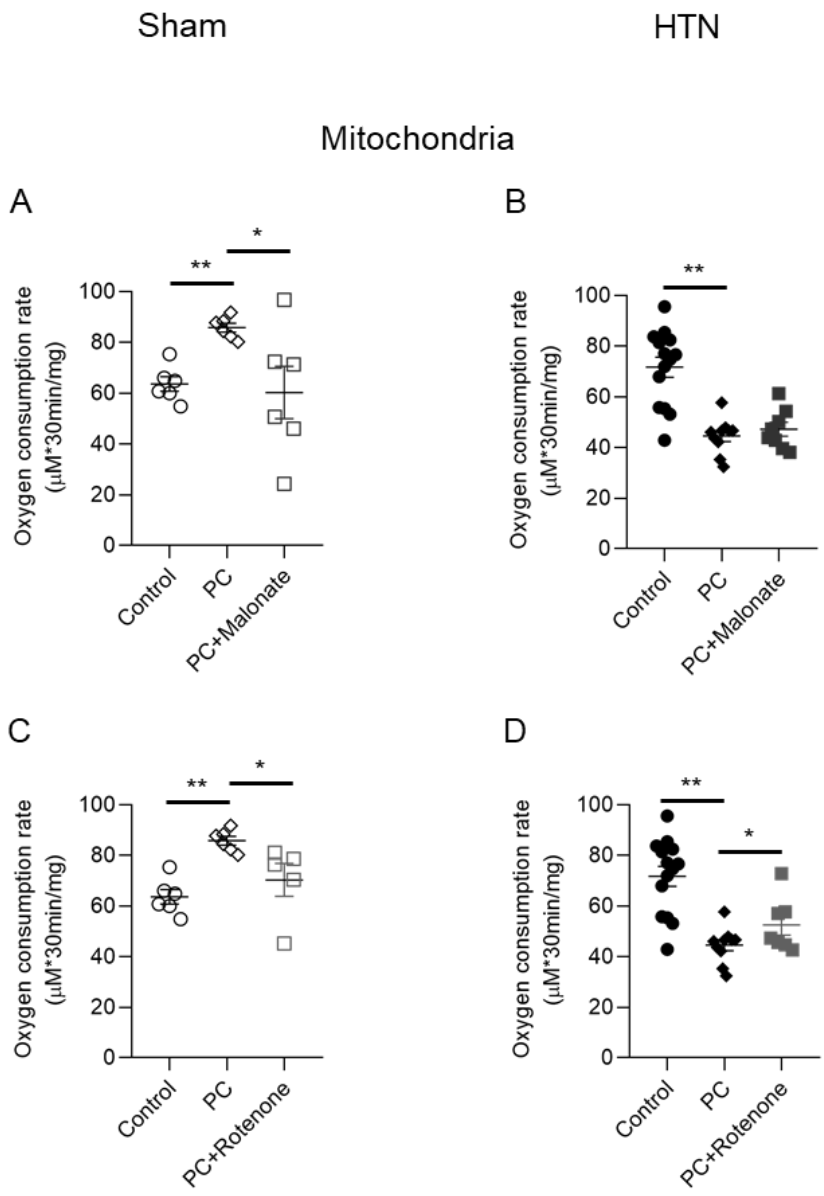

Figure 6. C-I and C-II-mediated OCR with PC supplementation in mitochondrial fraction from sham and HTN rat hearts. A-C. Mean OCR in sham mitochondrial fraction. Malonate and rotenone decreased mitochondrial OCR in sham with PC supplementation. B-D. Mean OCR in HTN mitochondrial fraction. Malonate did not affect OCR, however, rotenone increased OCR.

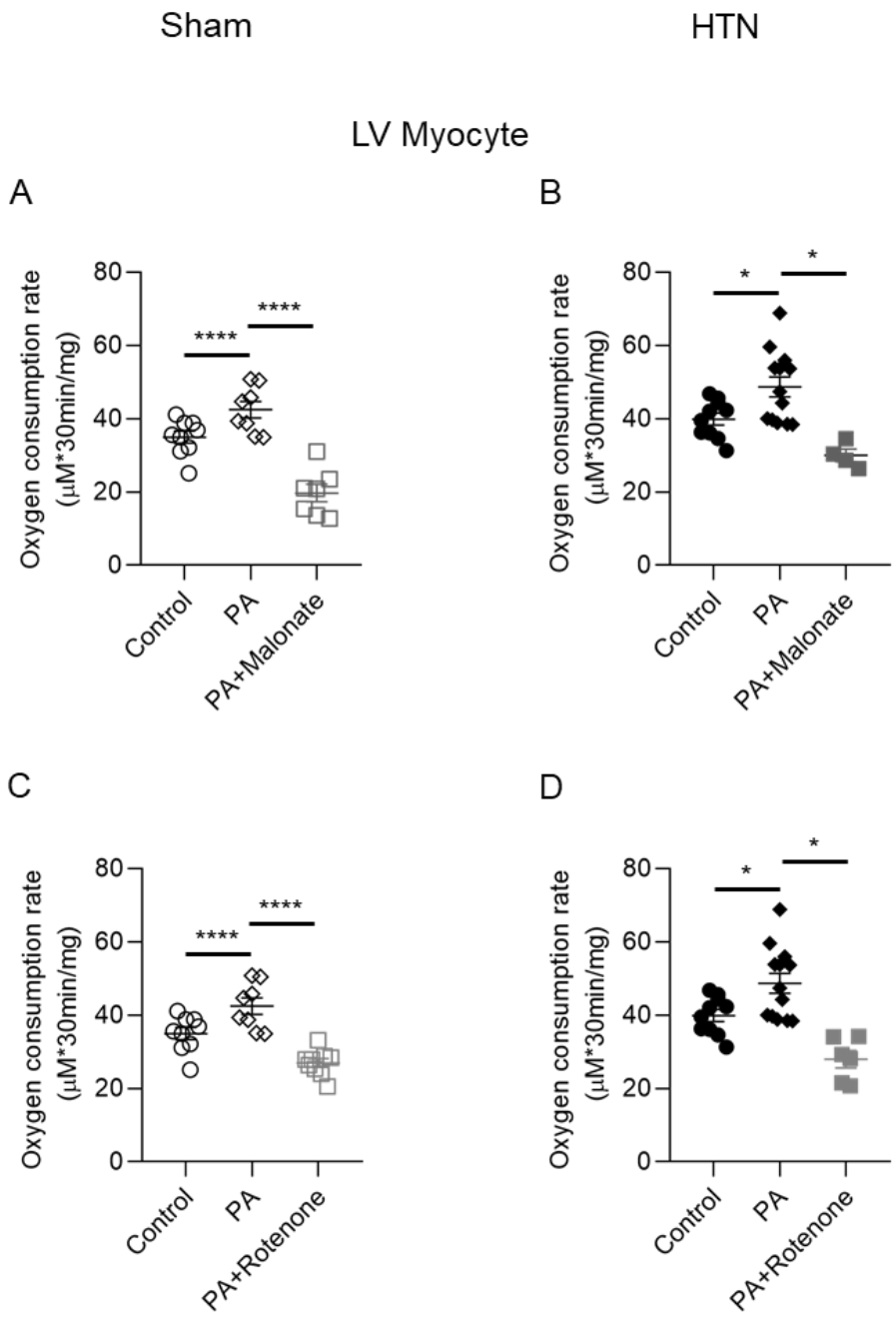

Figure 7. C-I and C-II-mediated OCR with PA supplementation in LV cardiomyocytes from sham and HTN rat hearts. A-C. Mean OCR in the presence of malonate and rotenone in cardiomyocytes from sham. Malonate and rotenone decreased OCR in cardiomyocytes from sham with PA supplementation. B-D. Mean OCR in the presence of malonate and rotenone in cardiomyocytes from HTN. Both malonate and rotenone reduced PA-dependent OCR in HTN cardiomyocytes.

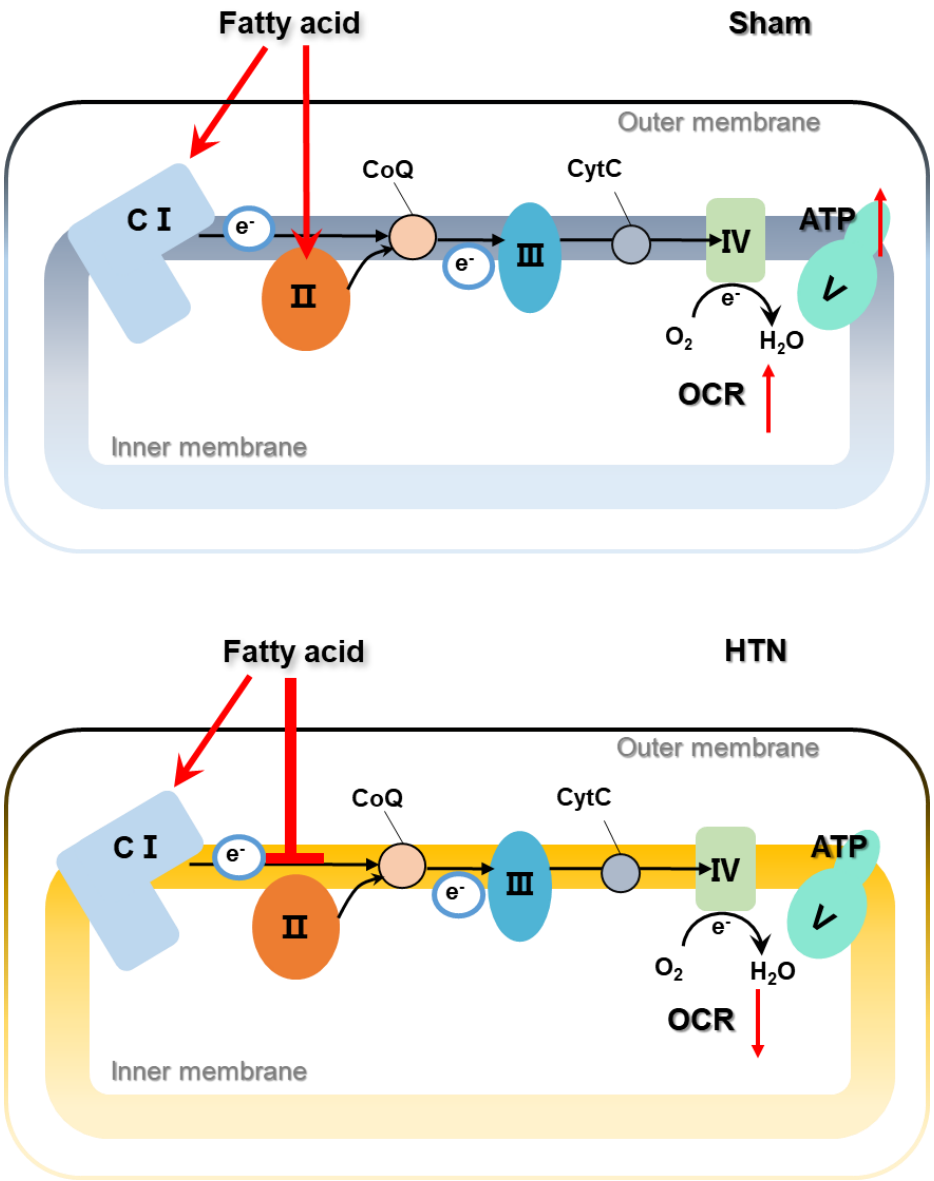

Figure 8. Summary. FA increased mitochondrial OCR and cellular ATP through increasing mitochondrial complex I and II activity in sham. FA reduced mitochondrial OCR, but did not affect cellular ATP in HTN. C-II activity was reduced but C-I activity was increased with FA supplementation in HTN.

Part II: FA-dependent of mitochondrial activity by nNOS in sham and HTN rat hearts

NO is an essential element in regulating cardiac functions. In previously, we found that one of the constitutive NOS-nNOS, have been implicated that involved in regulation of cardiac contractility, Ca^{2+} handling, oxidative stress and cardiac metabolism (Jin, Jang et al. 2013, Jin, Yin et al. 2017). Recently, neuronal nitric oxide synthase (nNOS) has been shown to be co-localized with mitochondrial C-I, whose activity may function as an electron donor through electron transport chain to activate nNOS in the mitochondria. In this part, we aim to investigate nNOS effects on FA-dependent of mitochondrial activity in sham and HTN rat hearts.

Expression of nNOS protein in cardiac mitochondria and its regulation of FA-dependent mitochondrial activities

First, we detected nNOS protein expression in mitochondrial enriched fraction of sham and HTN hearts. As shown in Fig.9, we have observed nNOS protein expression in mitochondrial fraction of both sham and HTN LV and there was no significant difference between two groups (mitochondria: p=0.944, n=4, Fig.9). For comparison, nNOS protein expression was also observed in cytosol and plasmalemmal fractions and the level of expression was greater in HTN cytosol/plasmalemmal compared to that in sham (p=0.004, n=4), in line with our previous study (Jin, Jang et al. 2013). Notably, nNOS protein expression was significantly larger in mitochondrial fraction compared to those in

cytosol/plasmalemmal in both groups (sham: $p<0.0001$, n=4; HTN: $p<0.0001$, n=4, Fig.9).

Next, we examined mitochondrial activities with nNOS inhibition to their responses on FA supplementation in sham and HTN. nNOS inhibition with SMTC did not affect PC-increment of OCR ($p=0.94$, n=6; Fig.10A), or intracellular ATP production in sham ($p=0.99$, n=4; Fig.10B). In HTN, SMTC enhanced OCR in the presence of PC ($p=0.02$, n=10 and n=6; Fig.10C), but did not affect intracellular ATP ($p=0.94$, n=6, Fig.10D). Therefore, these results indicated that nNOS did not affect FA-dependent mitochondrial activity in sham, by contrast, nNOS inhibits mitochondrial OCR in HTN.

nNOS inhibits C-I activity in Sham but inhibits C-I & II in HTN in the presence of PC

Responses of C-I and C-II activity to nNOS inhibition were observed in sham and HTN rat hearts. As shown in Fig.11, nNOS inhibition with SMTC enhanced PC-increment of C-I activity further ($p=0.043$, n=4; Fig.11A) but did not affect C-II activity in sham ($p=0.222$, n=3; Fig.11B). In addition, SMTC enhanced both C-I and C-II activity further in HTN ($p=0.02$, n=5; $p=0.012$, n=4; Fig.11C-D). These results indicating that nNOS inhibits C-I activity in sham, however, in HTN, nNOS inhibits both C-I and C-II activity.

nNOS regulation of mitochondrial activities and its interaction with C-I and C-II in sham

To investigate the effect of nNOS on C-I, C-II mediated mitochondrial activities, we examined OCR response to SMTC when C-II or C-I was inhibited with malonate or rotenone, respectively. As shown in Fig.12, SMTC reversed malonate-inhibition of OCR both in mitochondrial fraction and LV myocytes from sham (p=0.004, n=5; p=0.04, n=6 and n=8; Fig.12A&C). however, SMTC did not affect OCR without PA in the presence of malonate in LV myocytes from sham (Fig.12E).

By contrast, SMTC did not affect OCR in the presence of rotenone with PC supplementation from mitochondrial fraction (p=0.99, n=5; p=0.91, n=9; Fig.12B). Similarly, SMTC or L-NAME did not affect OCR with or without PA in LV myocytes from sham (p=0.85, n=9 and n=7; p=0.86, n=8 and n=7 with PA; Fig.12D&F). Therefore, nNOS attenuates C-I-mediated mitochondrial activity, but not C-II-mediated mitochondrial activity in the presence of FA in sham.

nNOS regulation of mitochondrial activities and its interaction with C-I and C-II in HTN

Similarly, C-I, C-II-meditated nNOS effects of mitochondrial activity were detected in the presence of PA or PC and in the absence of PA before and after SMTC application from HTN. SMTC did not affect OCR in the presence of malonate both in mitochondrial fraction and LV myocytes with FA (p=0.85, PC+malonate vs. SMTC+PC+malonate, n=5; p=0.58, PA+malonate vs. SMTC+PA+malonate, n=6; Fig.13A&C), however, SMTC decreased OCR further without PA in the presence of malonate in HTN LV myocytes (Fig.13E). By contrast, SMTC increased

OCR further in the presence of rotenone+PC (p=0.0005, n=8; Fig.13B), but such an effect was not observed in LV myocytes from HTN with or without PA after SMTC or L-NAME treatment (p=0.07, PA+rotenone vs. SMTC+PA+rotenone, n=6 and n=7; p=0.18, PA+rotenone vs. L-NAME+PA+rRotenone, n=6 and n=8; Fig.13D&F). These results indicating that nNOS inhibits C-II-mediated mitochondrial activity in HTN.

Interactions between nNOS and C-I activity in cardiac mitochondria in the presence of PA in sham

As shown in Fig.12, nNOS modulates C-I-mediated OCR, therefore, to examine the relationship between nNOS and C-I, we investigated nNOS-derived NO in LV myocytes and mitochondria with C-I inhibition. C-I inhibitor, rotenone, did not affect basal NO level (p=0.3, n=6, Fig.14A) or NO response to SMTC or L-NAME (SMTC: p=0.002, n=6; L-NAME: p=0.0009, n=6, Fig.14A), suggesting that rotenone itself does not affect nNOS-derived NO. However, rotenone significantly increased NO in the presence of PA (p<0.0001, n=6, Fig.14B), SMTC or L-NAME decreased rotenone-enhanced NO to the basal level (SMTC: p=0.001, n=6; L-NAME: p=0.007, n=6; Fig.14B). These results suggest that C-I activity controls nNOS-derived NO in cardiac mitochondria in the presence of PA in sham. Therefore, there were mutual modulation between nNOS and C-I in cardiac mitochondria in sham.

Interaction between nNOS and C-II in the presence of PA in HTN

In the absence of PA, malonate reduced NO (p=0.007, n=5; Fig.14C), which was reduced further by SMTC or L-NAME in LV myocytes in HTN (SMTC: p=0.003, n=5; L-NAME: p=0.008, n=5; Fig.14C). Similarly, malonate reduced NO production with PA in HTN LV myocytes (p=0.005, n=5, Fig.14D) and SMTC and L-NAME reduced NO production (SMTC: p=0.003, n=5; L-NAME: p=0.006, n=5; Fig.14D). We suggest that nNOS inhibits C-I and C-II in cardiac mitochondria from HTN rats. C-II seems to maintain nNOS activity under these conditions (a schematic diagram of nNOS interactions with C-I and C-II was shown in Fig.15).

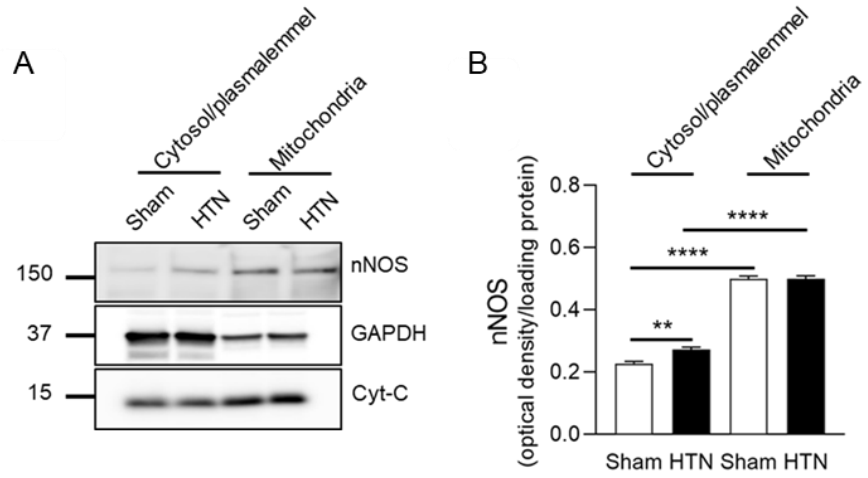

Figure 9. nNOS expression in cytosol/plasmalemmal and mitochondrial fractions in rat myocardium. A. Representative immunoblotting of total nNOS protein, GAPDH and cytochrome C (Cyt-C) in cytosol/plasmalemmel and mitochondrial fraction of sham and HTN LV. B. Mean values of nNOS relative to the loading protein (30 μg).

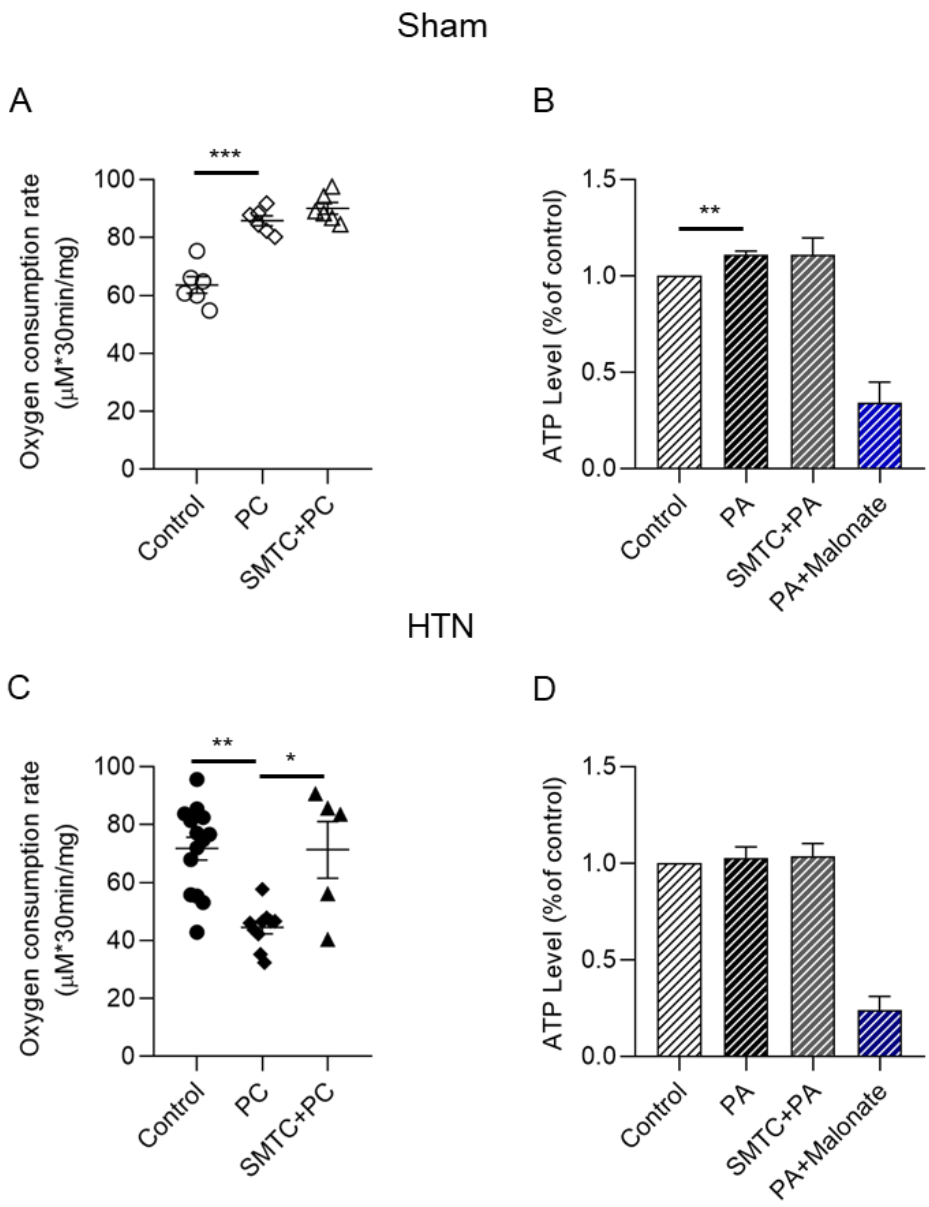

Figure 10. Effects of nNOS on regulating mitochondrial activity with FA supplementation from sham and HTN rat hearts. A-B. Mean OCR and ATP in cardiomyocytes or mitochondria in sham. SMTC did not affect PC-increment of OCR or ATP in sham. C-D. Mean OCR and ATP in cardiomyocytes or mitochondria in HTN. SMTC increased OCR in the presence of PC, but did not affect cellular ATP production.

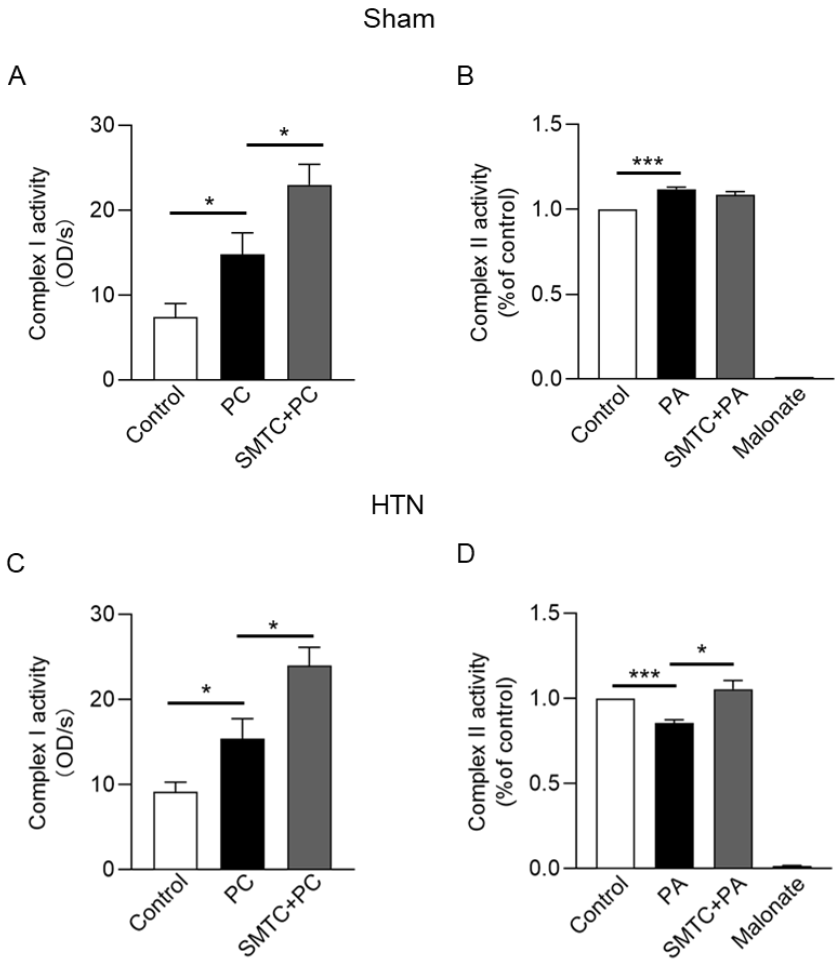

Figure 11. nNOS regulation of C-I and C-II activity in sham and HTN rat hearts. A-B. SMTC enhanced PC-increment C-I activity further but did not affect C-II activity in sham. C-D. SMTC increased both C-I and C-II activities in the presence of PA or PC in HTN.

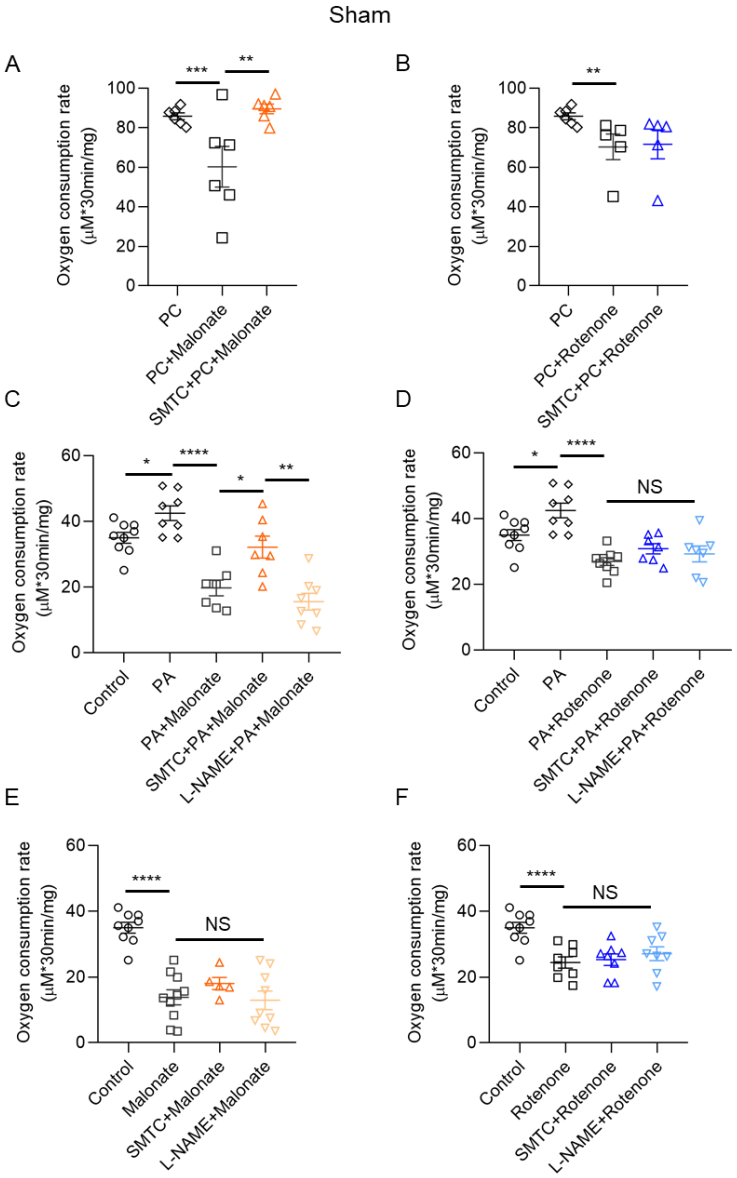

Figure 12. Effects of nNOS on C-I and C-II-dependent OCR with or without FA supplementation from sham rat hearts. A-B. nNOS regulation of OCR with PC in the presence of malonate and rotenone in mitochondrial fraction from sham. SMTC increased OCR in the presence of malonate with PC supplementation, but not in the presence of rotenone. C-D. nNOS regulation of OCR with PA in the presence of malonate and rotenone in cardiomyocytes from sham. SMTC increased OCR in the presence of malonate with PA supplementation, but not in the presence of rotenone. E-F. nNOS regulation of OCR without PA in the presence of malonate and rotenone in cardiomyocytes from sham. SMTC or L-NAME did not affect OCR under these conditions.

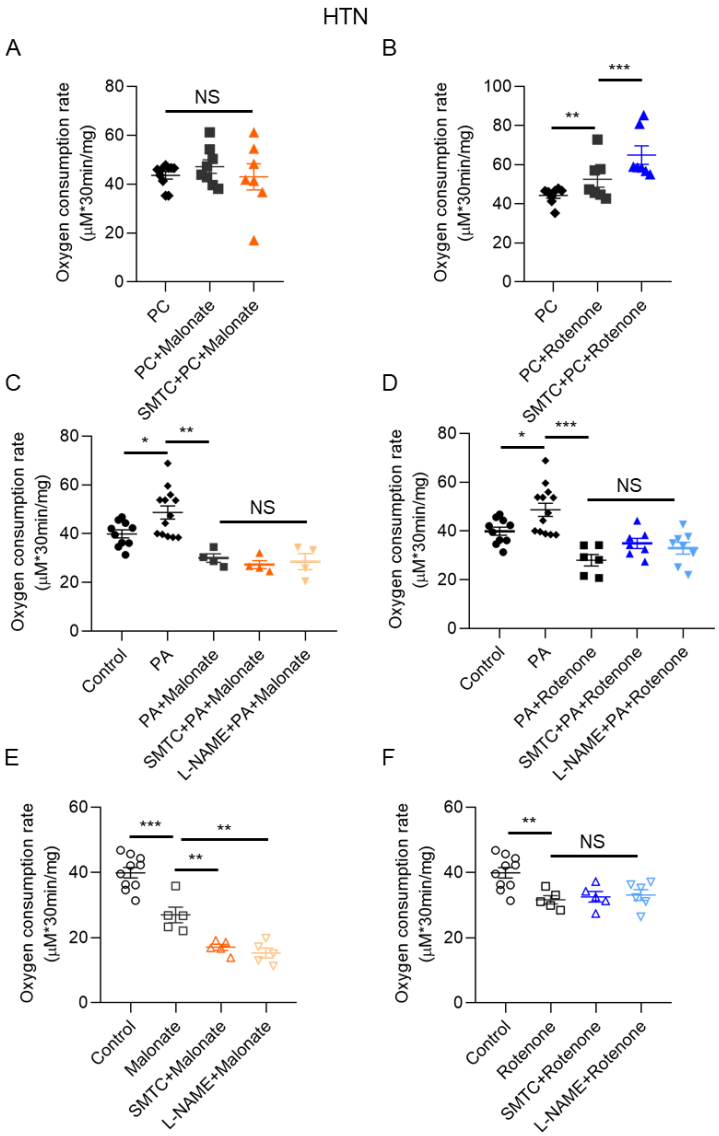

Figure 13. Effects of nNOS on C-I and C-II-dependent OCR with or without FA supplementation from HTN rat hearts. A-B. nNOS regulation of OCR with PC in the presence of malonate and rotenone in mitochondrial fraction from HTN. SMTC increased OCR in the presence of rotenone with PC supplementation, but not in the presence of malonate. C-D. nNOS regulation of OCR with PA in the presence of malonate and rotenone in cardiomyocytes from HTN. Both SMTC and L-NAME did not affect OCR in the presence of malonate and rotenone with PA supplementation. E-F. nNOS regulation of OCR without PA in the presence of malonate and rotenone in cardiomyocytes from HTN. SMTC and L-NAME reduced OCR in the presence of malonate, but exerted no effect with rotenone under these conditions.

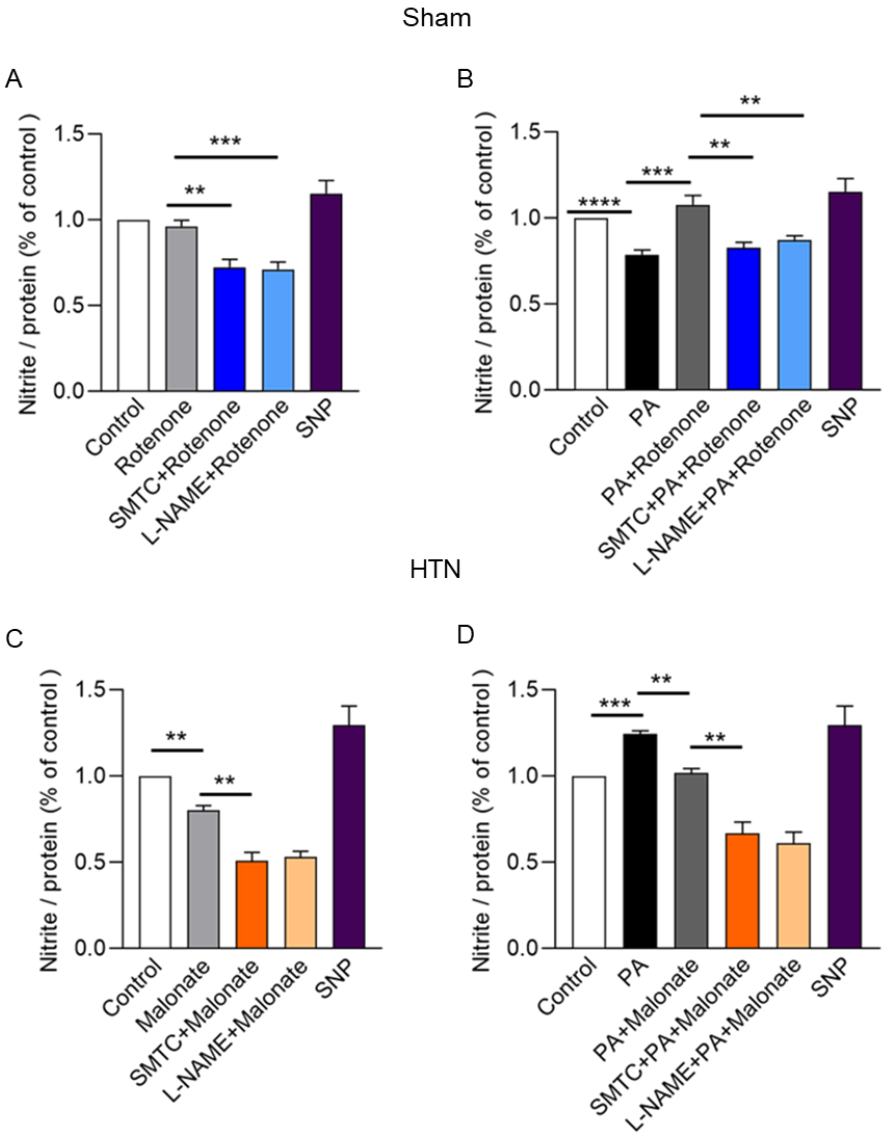

Figure 14. Mutual regulations between nNOS and mitochondrial complexes (C-I or C-II) in the presence of FA in sham and HTN. A-B. nNOS-derived NO was increased by rotenone in the presence of PA (although it was not affected by rotenone without PA). SMTC and L-NAME reduced NO to the basal level under these conditions. C-D. nNOS-derived NO production by C-II in the presence of PA in HTN cardiomyocytes. nNOS-derived NO was reduced by malonate with or without PA in HTN cardiomyocyte. SMTC and L-NAME reduced NO production with or without malonate + PA.

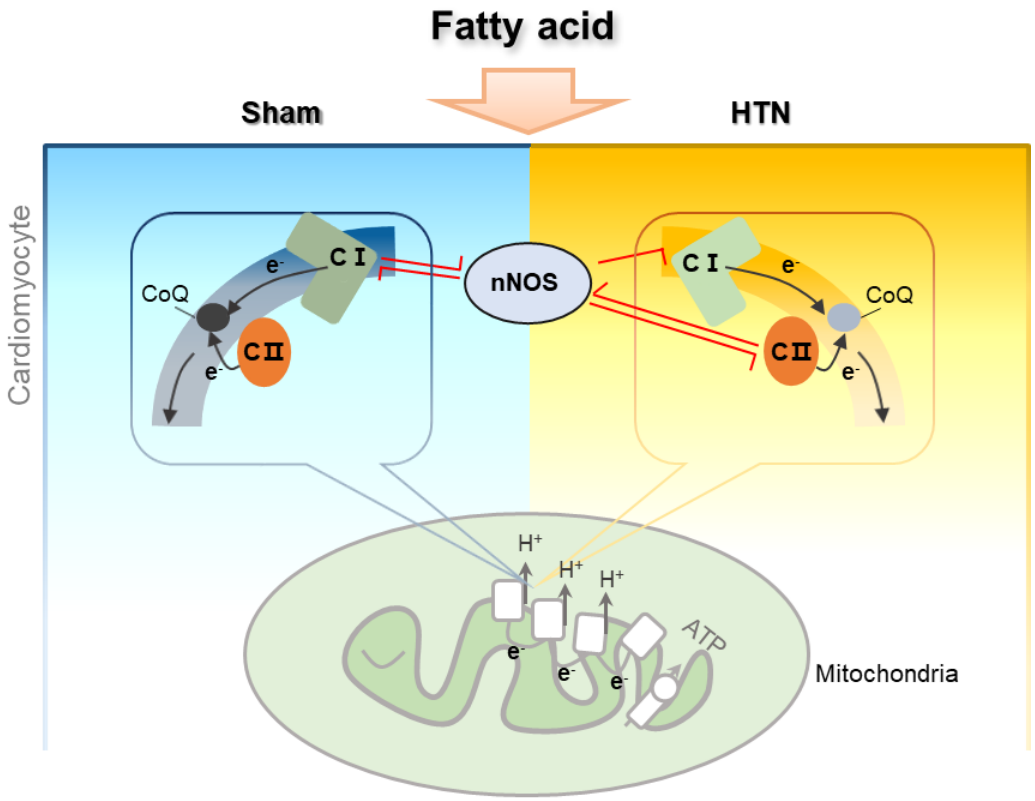

Figure 15. Schematic diagram illustrating the interactions between nNOS and mitochondrial complexes with FA supplementation in sham and HTN rat LV. In sham, there are mutual attenuation between nNOS and C-1; in HTN, nNOS attenuates both C-I and C-II, and is regulated by C-II. nNOS is involved in the regulation of mitochondrial activities through its functional interactions with C-I and C-II in healthy and diseased hearts.

Part III. FA-dependent of mitochondrial activity and its regulation by NOS in sham and HTN atrial myocardium

In atrial myocardium, previous research has shown that atria have higher glycolytic capacity compare to those in ventricles (Savabi and Kirsch 1991). So far, FA metabolism and its regulations by nNOS in atria remain to be determined. In addition, HTN and pressure-overload predisposes arrhythmias (most commonly atrial fibrillation) and diastolic and systolic dysfunction (Lip, Coca et al. 2017), conditions those affect cardiac metabolism. Our previous study have shown that eNOS protein expression is reduced in Ang II-induced LV cardiomyocytes and FA - dependent mitochondrial activity is mediated by nNOS, which has been shown to be upregulated in hypertensive LV myocytes (Jin, Jang et al. 2013, Jin, Yin et al. 2017). **In Part III**, we aim to investigate NOS effects on PA-dependent mitochondrial activity from sham and HTN LA.

OCR in sham-LA, HTN-LA, sham-LV and HTN-LV and their responses to PA supplementation

To evaluate cardiac mitochondrial activity, we measured OCR in sham-LA, HTN-LA with and without PA pre-incubation. As shown in Fig.16, we found that OCR was greater in HTN-LA compared to that in sham-LA (LA: p=0.040, n=6; Fig.16A) and PA significantly increased OCR in sham-LA but reduced it in HTN-LA (sham: p=0.015, n=6; HTN: p=0.013, n=6; Fig.16A).

To compare, we also investigated similar experiment in LV from sham and HTN. By contrast, in LV, OCR was not different between sham and

HTN but PA significantly increased OCR in both groups (LV: sham: p=0.005, n=8 and n=9; HTN: p<0.0001, n=15 and n=23; Fig.16B). These results suggest that PA-induced OCR in LA was different between sham and HTN. And PA response was different between LA and LV.

The effect of eNOS and nNOS on regulating mitochondrial activity in LA and LV of sham and HTN rats

NO is well known to modulate myocardial contractility and mitochondrial activity in healthy and diseased hearts (Jin, Jang et al. 2012, Jin, Jang et al. 2013, Jin, Yin et al. 2017), it functions as a key regulator of cardiac metabolism under pathological stimuli (Niu, Watts et al. 2012, Zhang, Jin et al. 2014). Since eNOS mediates FA-dependent oxidation in LV and nNOS is the main source of NO in LV, which has been shown to be involved in PA-dependent OCR in HTN-LV (Jin, Yin et al. 2017), we have detected the involvement of eNOS and nNOS in sham and HTN-LA. In sham LA, both SMTC and L-NAME decreased PA-induced OCR (SMTC: p=0.005, n=6 and n=5; L-NAME: p=0.004, n=6 and n=7; Fig.17A).

Similarly, we also investigated such an effect in HTN LA. As shown in Fig.17, SMTC or L-NAME exerted no effect on PA-induced OCR in HTN-LA (SMTC: p=0.87, n=6; L-NAME: p=0.93, n=6; Fig.17B). This is because in the presence of PA, OCR was already reduced in HTN-LA.

The effects on LV was also tested for comparison. Our results showed that L-NAME reduced OCR in sham-LV whereas SMTC exerted no

effect (SMTC: p=0.95, n=10 and n=7; L-NAME: p=0.005, n=10 and n=6; Fig.18A).

However, both SMTC and L-NAME reduced PA-induced OCR in HTN-LV (SMTC: p=0.0001, n=24 and n=19; L-NAME: p<0.0001, n=24 and n=15; Fig.18B). These results indicated that nNOS is responsible for the maintenance of OCR in HTN-LV.

PA regulation of nNOS^{Ser1417} and eNOS^{Ser1177} and NOS-derived NO production in LA and LV of sham and HTN rats

To elucidate whether nNOS^{Ser1417} and eNOS^{Ser1177} mediate PA-induced changes in mitochondrial activity, we have investigated PA regulation of eNOS and nNOS activity in LA and LV from sham and HTN rats. As shown in Fig.19A, eNOS^{Ser1177} was increased in HTN-LA (p=0.002, n=8), such an effect was abolished by PA in HTN-LA (p=0.005, n=8, Fig.19A). In the presence of PA, eNOS^{Ser1177} was not changed in HTN-LA compare to sham (p=0.205, n=8). PA did not affect eNOS^{Ser1177} in sham-LA (p=0.17, n=8). eNOS^{Thr495} was not different between sham-LA and HTN-LA (p=0.75, n=4) and PA did not change its phosphorylation in sham or in HTN (Sham: p=0.15, n=4; HTN: p=0.24, n=4, Fig.19). In Fig.19B, nNOS^{Ser1417} tended to be increased in HTN-LA compared to sham-LA (p=0.21, n=7); however, PA significantly reduced nNOS^{Ser1417} in HTN-LA (p=0.02, n=7; Fig.19B).

In LV, eNOS^{Ser1177} was not affected by PA in sham or HTN (p=0.61, n=9; p=0.63, n=4; Fig.20A-C). Conversely, PA increased nNOS^{Ser1417} in

HTN-LV (p=0.03, n=8; Fig.20D) but did not affect nNOS^{Ser1417} in sham-LV (p=0.58, n=4; Fig.20B).

In LA, NO level was significantly greater in HTN compared to that in sham (p=0.01, n=3 and n=4; Fig.21A). SMTC and L-NAME reduced NO production in sham-LA and HTN-LA (in sham-LA, SMTC: p=0.04, n=3; L-NAME: p=0.03, n=3; HTN-LA, SMTC: p=0.003, n=4; L-NAME: p=0.004, n=4; Fig.21A). In LA, PA supplementation reduced NO production in HTN (p=0.003, n=4; Fig.21B) but did not affect in sham-LA (p=0.74, n=11; Fig.21B). Therefore, SMTC and L-NAME significantly reduced NO production in sham-LA (SMTC: p=0.03, n=11 and n=8; L-NAME: p=0.03, n=11 and n=4; Fig.21B). SMTC or L-NAME did not affect NO production further with PA in HTN-LA (SMTC: p=0.64, n=4 and n=6; L-NAME: p=0.45, n=4 and n=5; Fig.21B).

Similarly, NO level was greater in HTN-LV (p=0.0003, n=6 and n=3; Fig.22A). SMTC and L-NAME reduced NO production in sham-LV and HTN-LV (sham-LV, SMTC: p=0.009, n=6; L-NAME: p=0.007, n=6; HTN-LV, SMTC: p=0.04, n=3; L-NAME: p=0.011, n=3; Fig.22A). In LV, PA supplementation increased NO level in HTN-LV, but reduced NO production in sham-LV (p=0.0006, n=3; p=0.0005, n=5; Fig.22B). SMTC and L-NAME reduced PA-increment of NO level in HTN-LV, but failed to affect NO production in sham-LV (HTN-LV, SMTC: p=0.0006, n=3; L-NAME: p=0.0004, n=3; sham-LV, SMTC: p=0.5, n=5; L-NAME: p=0.84, n=3; Fig.22B). These results show clear evidence that nNOS is a key regulator of OCR with PA in HTN-LA.

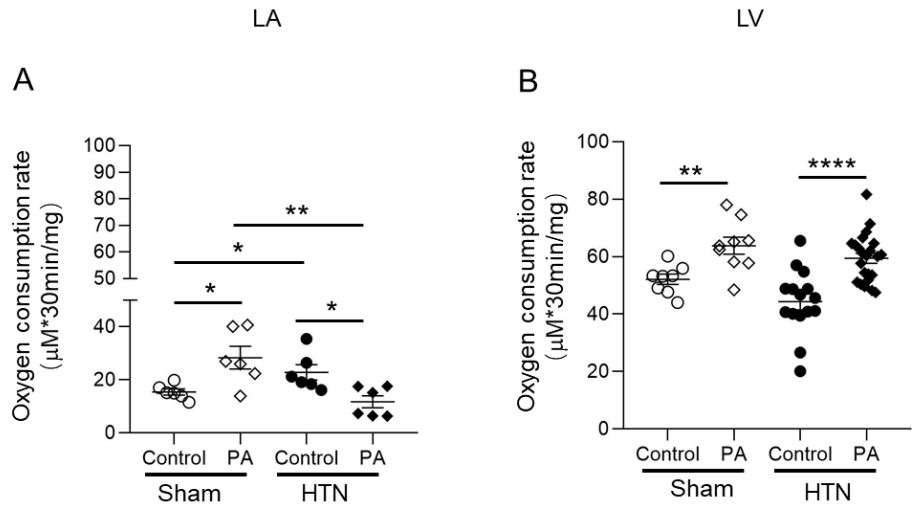

Figure 16. PA-dependent OCR in sham and HTN rat LA and LV. A. OCR was greater in HTN LA compared to that in sham LA and PA significantly increased OCR in sham-LA but reduced it in HTN-LA. B. OCR was not different between sham and HTN but PA significantly increased OCR in both groups.

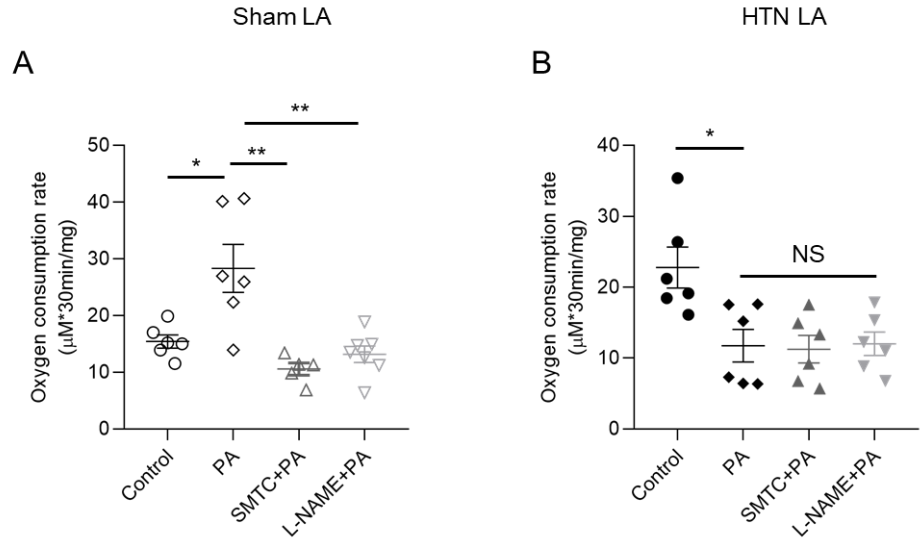

Figure 17. Effects of nNOS and eNOS on PA regulation of OCR in sham and HTN LA. A-B. Both SMTC and L-NAME decreased PA-induced OCR in sham-LA. However, SMTC or L-NAME exerted no effect on OCR in HTN-LA.

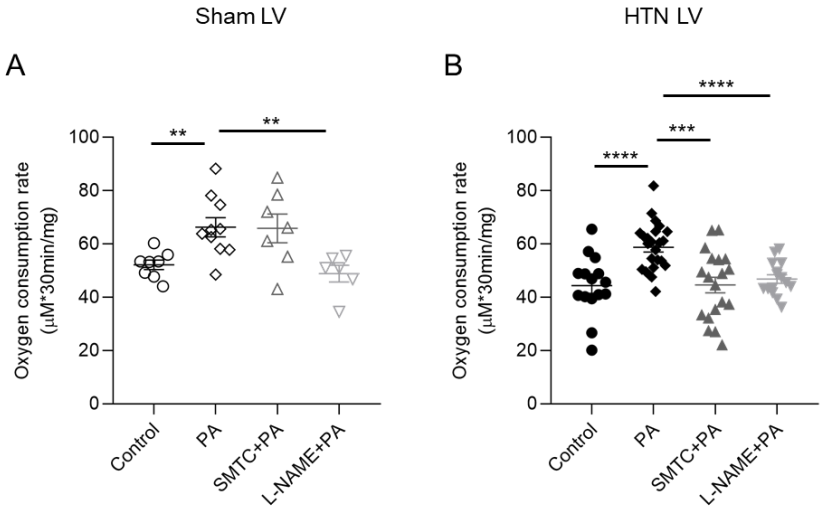

Figure 18. Effects of nNOS and eNOS on PA regulation of OCR in sham and HTN LV. A-B. L-NAME reduced OCR in sham-LV whereas SMTC exerted no effect. However, both SMTC and L-NAME reduced OCR in HTN-LV.

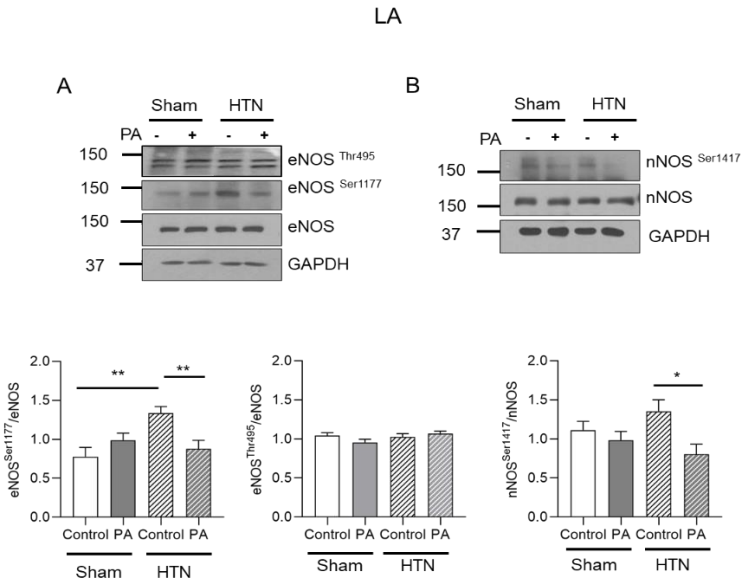

Figure 19. PA regulation of nNOS and eNOS phosphorylations in sham and HTN LA. A&B. Representative immunoblotting of eNOS and nNOS phosphorylation, GAPDH in sham and HTN rat LA homogenates. eNOS^{Ser1177} was increased in HTN in the absent of PA, but in the presence of PA, there was no difference between sham and HTN. However, PA didn't affect eNOS^{Thr495} either in sham or HTN. By contrast, PA decreased eNOS^{Ser1177} and nNOS^{Ser1417} in HTN with no changes on total proteins, such an effect was not observed in sham.

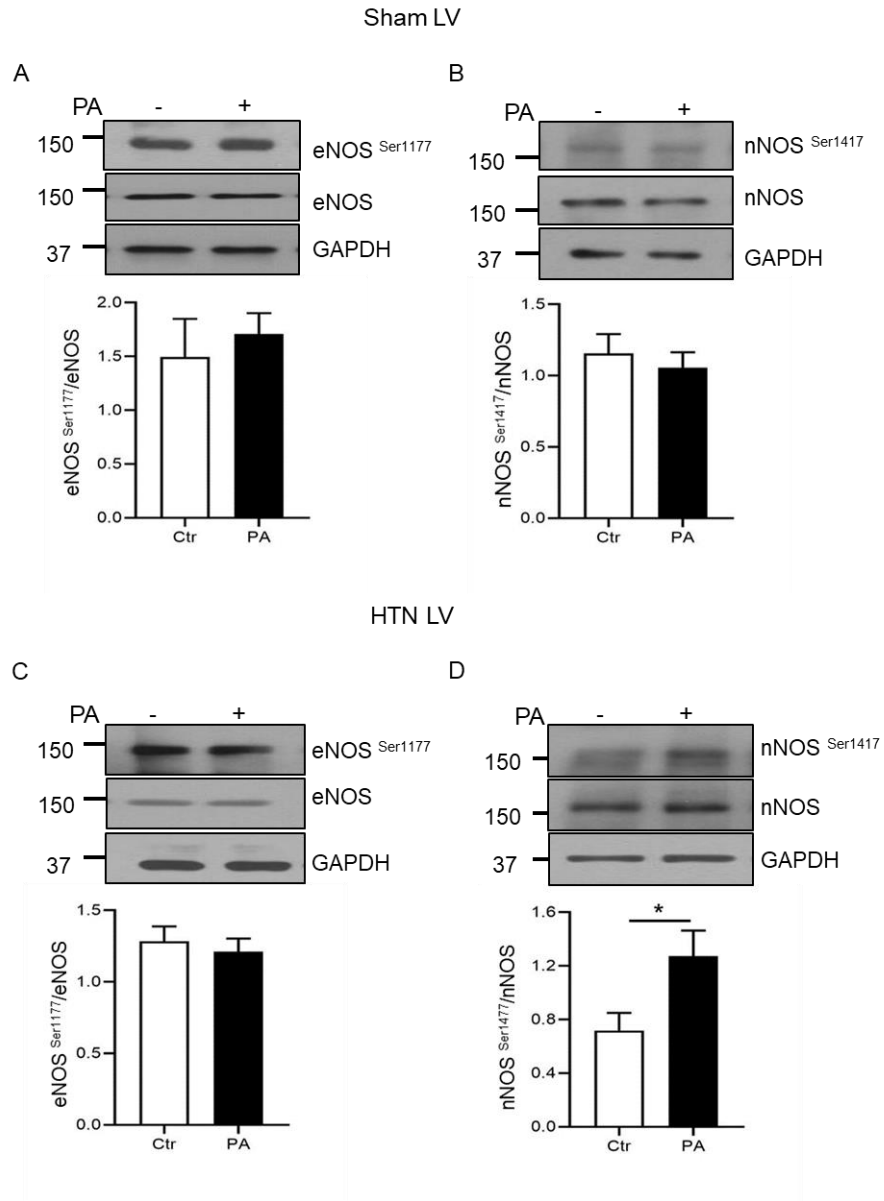

Figure 20. PA regulation of eNOS and nNOS activity in LV from sham and HTN rats. A-B. Representative immunoblotting of the effect of PA on eNOS and nNOS phosphorylation on ser1177 and ser1417 in sham LV. sham LV. PA failed to affect eNOS^{Ser1177} both in sham and HTN LV. C-D. Representative immunoblotting of the effect of PA on eNOS and nNOS phosphorylation on ser1177 and ser1417 in HTN LV. nNOS^{Ser1417} was increased by PA in HTN LV, but eNOS^{Ser1177} was not affected by PA.

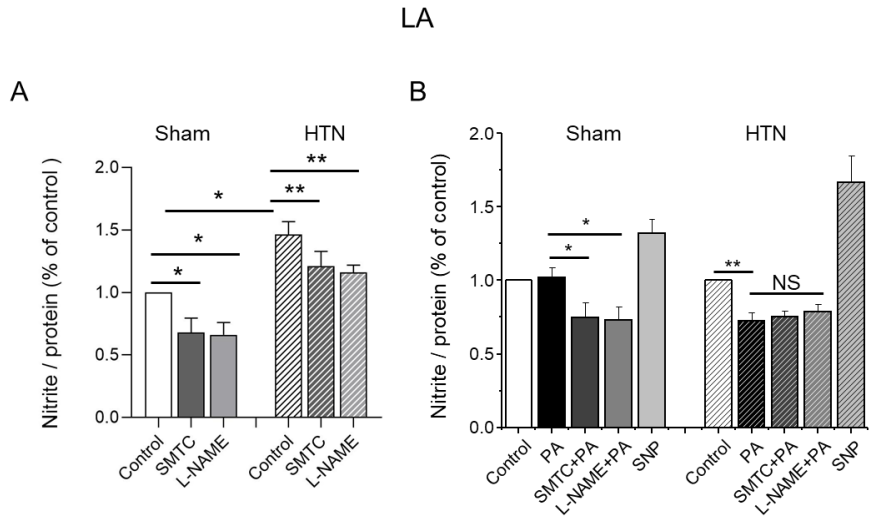

Figure 21. nNOS-derived NO production in LA from sham and HTN, with or without PA. A. NO production was significantly increased both in HTN LA, both SMTC and L-NAME reduced NO production in LA from sham and HTN. B. SMTC and L-NAME significantly reduced PA-induced NO production in sham-LA, despite PA didn't affect NO in sham-LA. By contrast, PA reduced NO production in HTN-LA, SMTC or L-NAME failed to affect PA-induced NO production further both in HTN-LA.

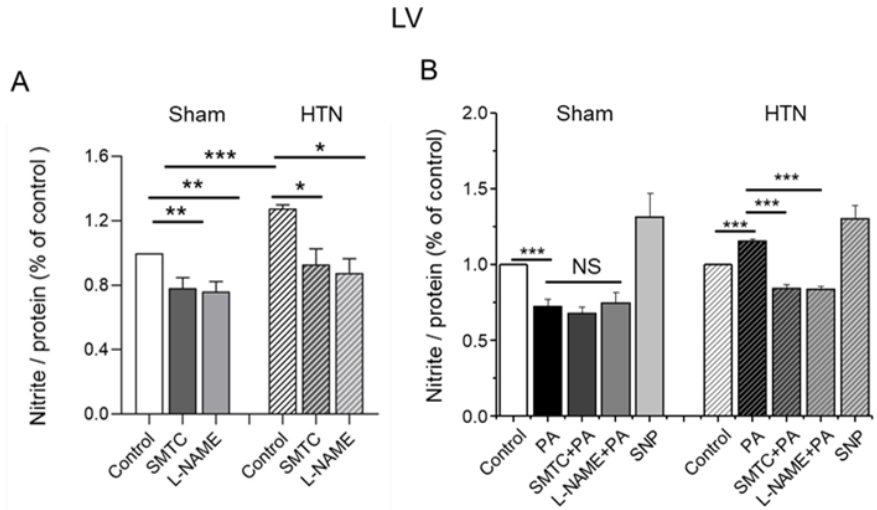

Figure 22. nNOS-derived NO production in LV from sham and HTN, with or without PA. A. NO production was significantly increased both in HTN LV, both SMTC and L-NAME reduced NO production in LV from sham and HTN. B. SMTC and L-NAME significantly reduced PA-induced NO production in HTN-LV, however, PA increased NO in HTN-LV. By contrast, PA reduced NO production in sham-LV, SMTC or L-NAME failed to affect PA-induced NO production further in sham-LV.

2BP effects on eNOS and nNOS phosphorylation on regulating mitochondrial activity from sham and HTN rat hearts

S-palmitoylation is an important post-translational modification (PTM) downstream of PA. Protein *S*-palmitoylation is involved in the covalent attachment of long-chain FAs to thiol groups of cysteine (Cys) residues through thioester linkage. Cys *S*-palmitoylation is a reversible PTM that can dynamically regulate protein stability, trafficking, and activity. 2-bromopalmitate (2BP) is an irreversible inhibitor of many membrane-associated enzymes. Over the last decade, 2BP has become a selective inhibitor of protein *S*-palmitoylation. Indeed, many studies have used 2BP-induced phenotypes as evidence of the importance of palmitoylation in parasitic infection, differentiation, and various other cellular phenotypes. Therefore, we used 2BP and evaluated whether *S*-palmitoylation is involved in atrial mitochondrial activity and regulate $nNOS^{Ser1417}$ and $eNOS^{Ser1177}$, $eNOS^{Thr495}$ in LA and LV from sham and HTN rats.

Our results showed that a potent inhibitor of *S*-palmitoylation, 2BP, did not affect basal OCR in sham-LA (p=0.13, n=10 and n=5; Fig.23), but decreased PA-increment of OCR (p=0.04, n=10 and n=5; Fig.23). Furthermore, 2BP did not affect $nNOS^{Ser1417}$ and $eNOS^{Ser1177}$ and $eNOS^{Thr495}$ before and after PA supplementation (Fig.23).

Since the eNOS and nNOS activity was regulated by the upstream protein AMPK and AKT, we detected the protein level of AMPK and AKT and their phosphorylation site, $AMPK^{Thr172}$ and AKT^{Ser473}. As

shown in Fig.24, we found that 2BP did not affect AMPK^{Thr172} with or without PA supplementation in sham (2BP: p=0.63, n=6; 2BP+PA: p=0.7, n=62; Fig.24), and PA failed to affect AMPK^{Thr172} in sham-LA. However, PA decreased AKT^{Ser473} in sham-LA (PA: p=0.03, n=6, Fig.24), 2BP prevented PA-induced AKT^{Ser473} in sham-LA (2BP+PA: p=0.04, n=6, Fig.24). Therefore, AKT^{Ser473} may be involved in 2BP effects on regulating mitochondrial activity.

2BP regulation of nNOS^{Ser1417}, eNOS^{Ser1177} and eNOS^{Thr495} and OCR in HTN-LA

In HTN-LA, 2BP prevented PA-induced reduction of OCR therefore PA+2BP is significantly greater than PA (p=0.04, n=4; Fig.25). Importantly, in the presence of 2BP, PA increased nNOS^{Ser1417} in HTN (p=0.002, n=8; Fig.25), suggesting 2BP prevented PA-dependent decrease of nNOS activity in HTN-LA. By contrast, PA-induced reduction of eNOS^{Ser1177} was not affected by 2BP pretreatment (p=0.62, n=4; Fig.25). Interestingly, eNOS^{Thr495}, which was unaffected by PA in HTN-LA, was significantly decreased by PA in the presence of 2BP (p=0.002, n=4; Fig.25).

Similar to those in sham-LA, we found that 2BP did not affect AMPK^{Thr172} with or without PA supplementation in HTN-LA (2BP: p=0.81, n=6; 2BP+PA: p=0.74, n=6; Fig.26), and PA failed to affect AMPK^{Thr172}. However, PA decreased AKT^{Ser473} in HTN-LA (PA: p=0.02,

n=6, Fig.26), since 2BP prevented PA-induced AKT^{Ser473} in sham-LA, but failed to affect it in HTN-LA (2BP+PA: p=0.85, n=6, Fig.26). These results suggest that nNOS may be responsible for PA-induced OCR through *S*-palmitoylation in HTN-LA.

2BP regulation of nNOS^{Ser1417} and OCR in sham and HTN LV

In previous study, our own group have demonstrated that 2BP decreased eNOS^{Ser1177} with or without PA in sham and HTN-LV (Jin, Wu et al. 2017) and reduced OCR in HTN-LV, indicating that eNOS regulation by *S*-palmitoylation plays a crucial role in mediating mitochondrial activity in the presence of PA.

Similarly, we found that 2BP decreased PA-induced OCR in HTN-LV (p=0.0006, n=19 and n=9; Fig.27). But 2BP did not affect nNOS^{Ser1417} in the presence of PA (p=0.7, n=7 and n=4; Fig.27), despite PA increased nNOS^{Ser1417}. 2BP didn't affect OCR (p=0.38, n=10 and n=13; Fig.27) and nNOS^{Ser1417} (p=0.27, n=4; Fig.27) in the presence of PA in sham-LV. These results indicated that nNOS^{Ser1417} was not involved in 2BP-dependent of mitochondrial activity. We suggest s-palmitoylation was involved in PA-dependent mitochondrial activity, modulates nNOSSer1417 to regulate mitochondrial function in HTN. (a schematic diagram of the FA-dependent mitochondrial activity and its regulation by NOS in sham and HTN was shown in Fig.28).

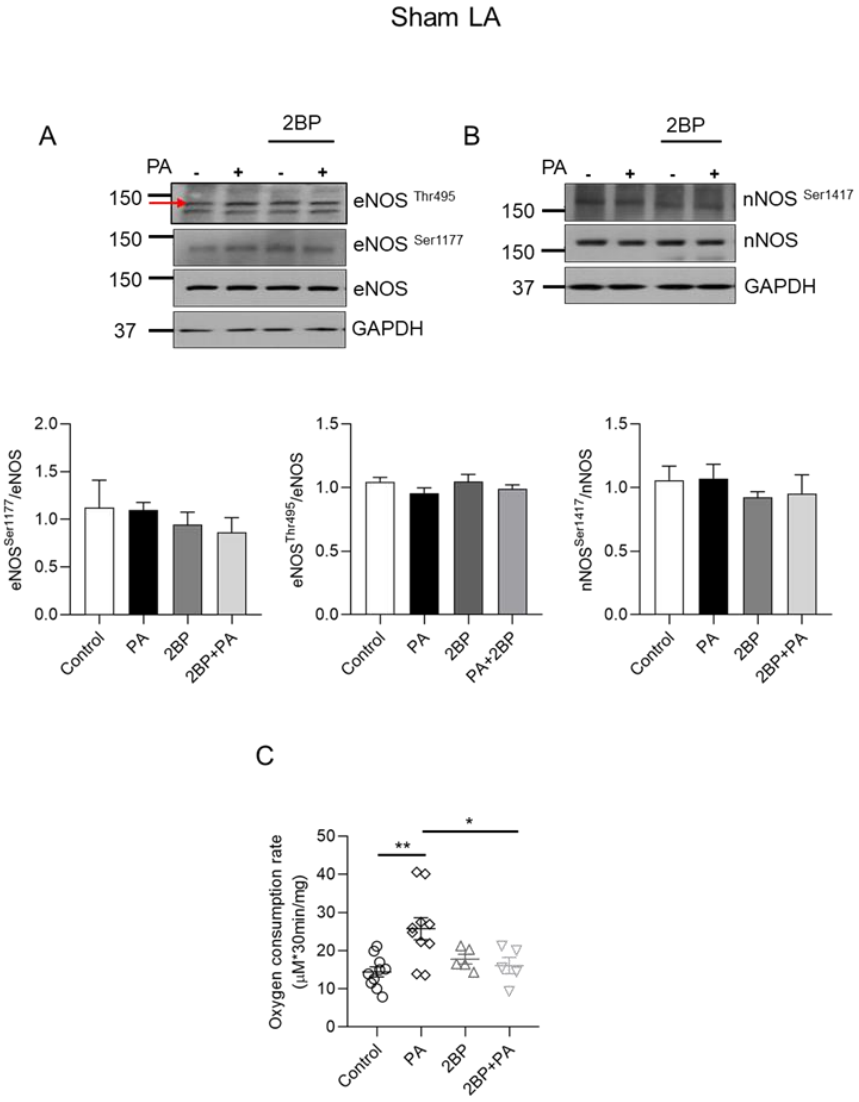

Figure 23. Effects of 2BP on eNOS and nNOS phosphorylations, mitochondrial activity in sham LA. A-B. Representative immunoblotting of the effect of 2BP on eNOS^{Thr495} and eNOS^{Ser1177}, nNOS^{Ser1417} in sham rat LA. 2BP didn't affect eNOS^{Thr49} or eNOS^{Ser1177}, nNOS^{Ser1417} with or without PA. c. 2BP reduced PA-induced OCR, however, 2BP didn't affect OCR in the absent of PA.

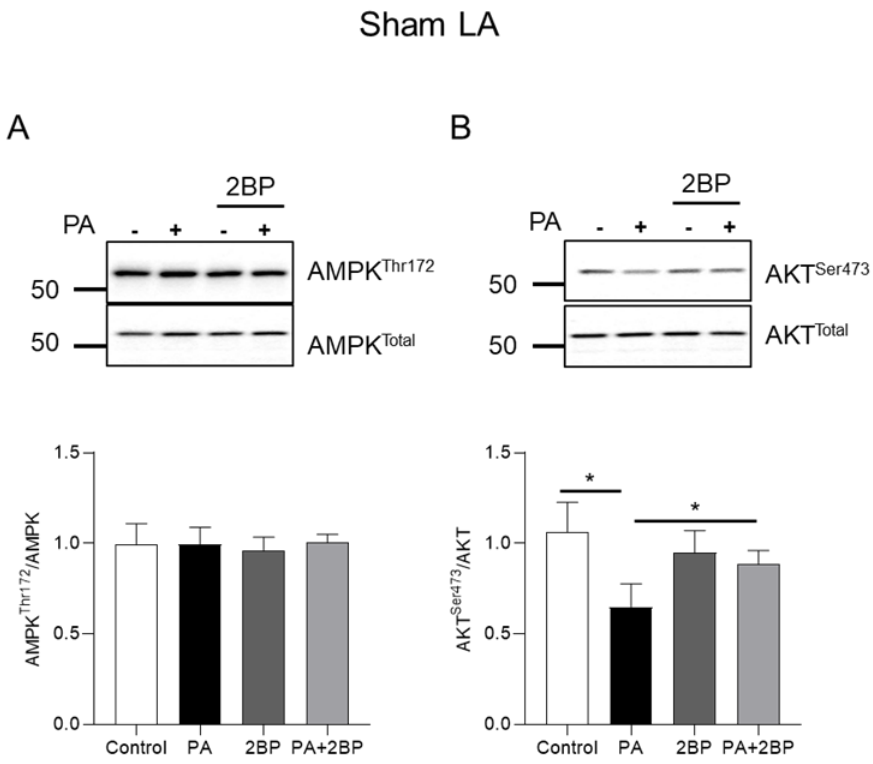

Figure 24. Effects of 2BP on p-AMPK, p-AKT in sham LA. A-B. Representative immunoblotting of the effect of PA and 2BP on $AMPK^{Thr172}$ and AKT^{Ser473} in sham LA. PA or 2BP did not affect $AMPK^{Thr172}$. PA decreased AKT^{Ser473} in sham LA, 2BP prevented PA-induced AKT^{Ser473} in sham LA.

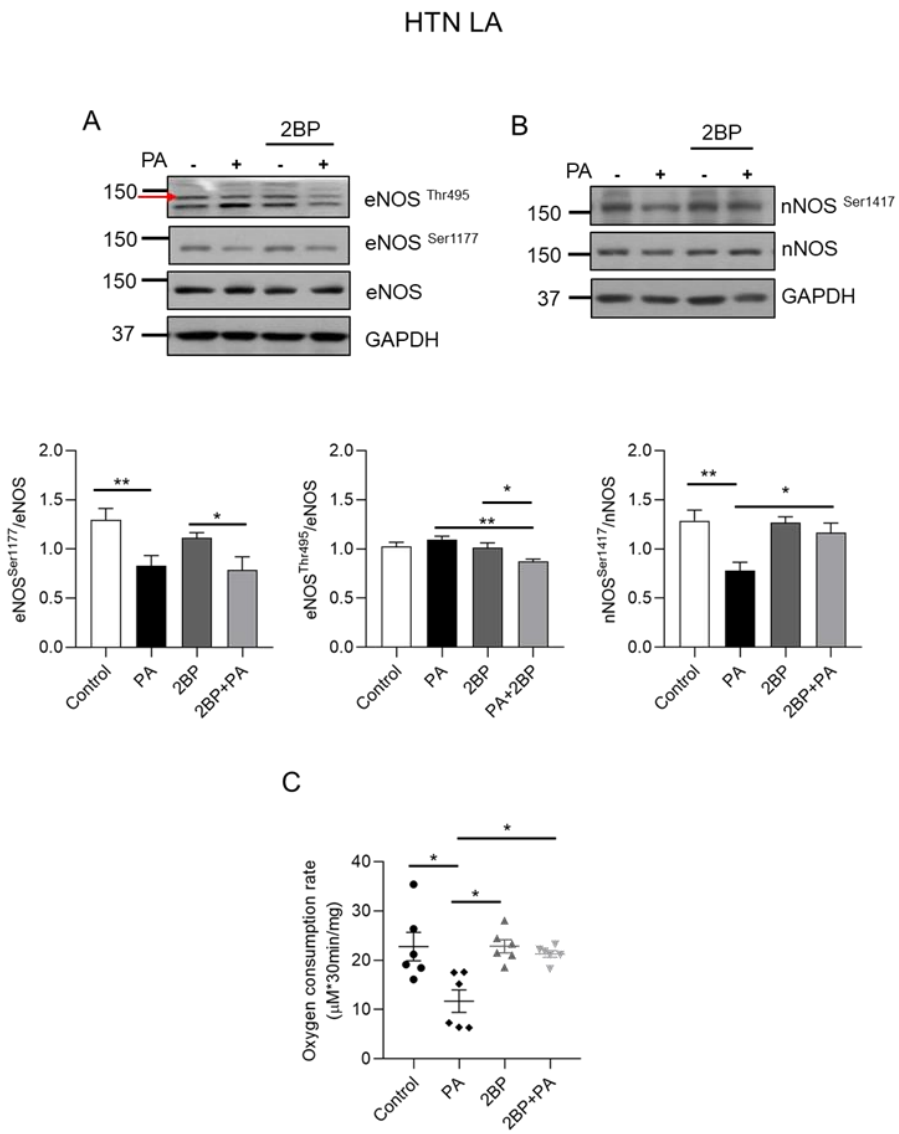

Figure 25. Effects of 2BP on eNOS and nNOS phosphorylations, mitochondrial activity in HTN LA. A-B. Representative immunoblotting of the effect of 2BP on eNOS^{Thr495} and eNOS^{Ser1177}, nNOS^{Ser1417} in HTN rat LA. 2BP increased PA-decreased nNOS^{Ser1417} in HTN rat LA, such an effect was not observed on eNOS^{Ser1177}, however, eNOS^{Thr495} was decreased by 2BP in the presence of PA, but 2BP didn't affect eNOS^{Thr495} without PA. f. 2BP increased PA-decreased OCR, but in the absent of PA, 2BP didn't affect OCR in HTN rat LA.

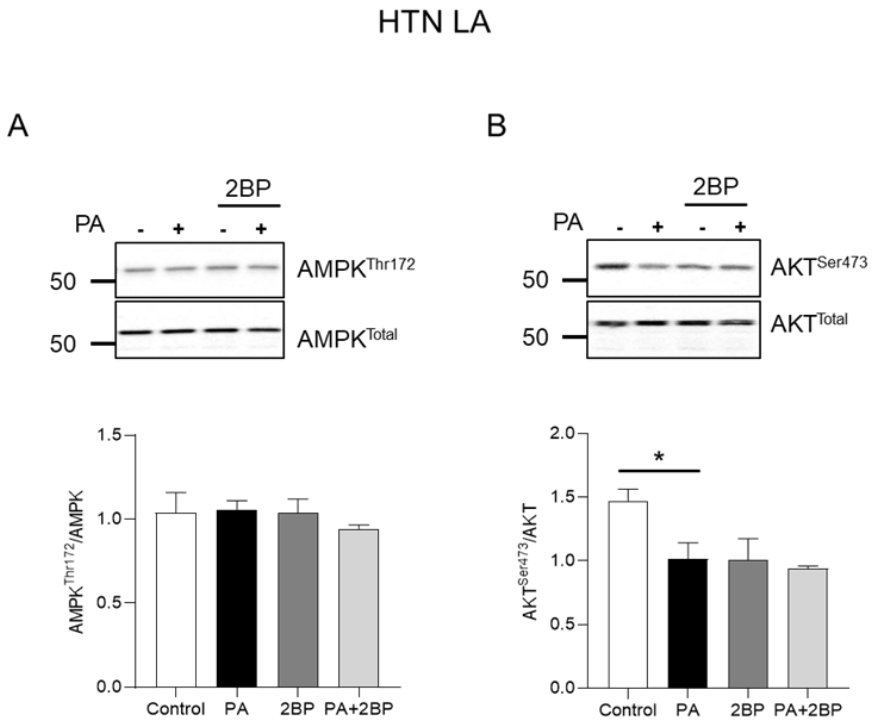

Figure 26. Effects of 2BP on p-AMPK and p-AKT in HTN LA. A-B. Representative immunoblotting of the effect of PA and 2BP on $AMPK^{Thr172}$ and AKT^{Ser473} in HTN LA. PA or 2BP did not affect $AMPK^{Thr172}$. PA decreased AKT^{Ser473} in sham LA, 2BP did not affect PA-induced AKT^{Ser473} in HTN LA**.**

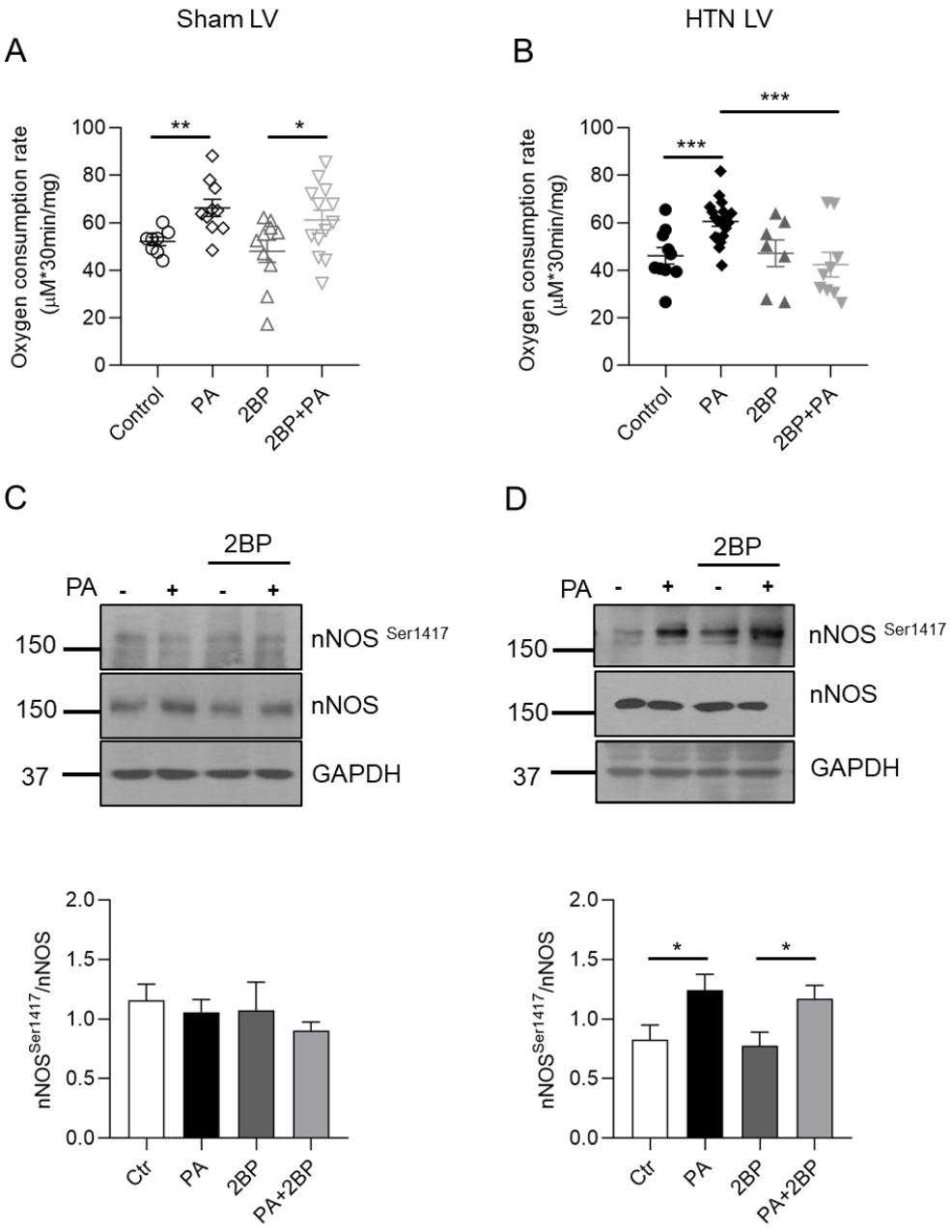

Figure 27. Effects of 2BP on nNOS phosphorylation and mitochondrial activity in sham and HTN rat LV. A&B. 2BP showed negligible effect on PA regulation of OCR in sham, conversely, 2BP reduced PA-induced OCR in HTN, such an effect was disappeared without PA. C&D. Representative immunoblotting of the effect of 2BP on $eNOS^{Ser1177}$ and $nNOS^{Ser1417}$ in sham and HTN rat LV. 2BP didn't affect nNOS phosphorylation on ser1417 with or without PA, either in sham or HTN rat LV.

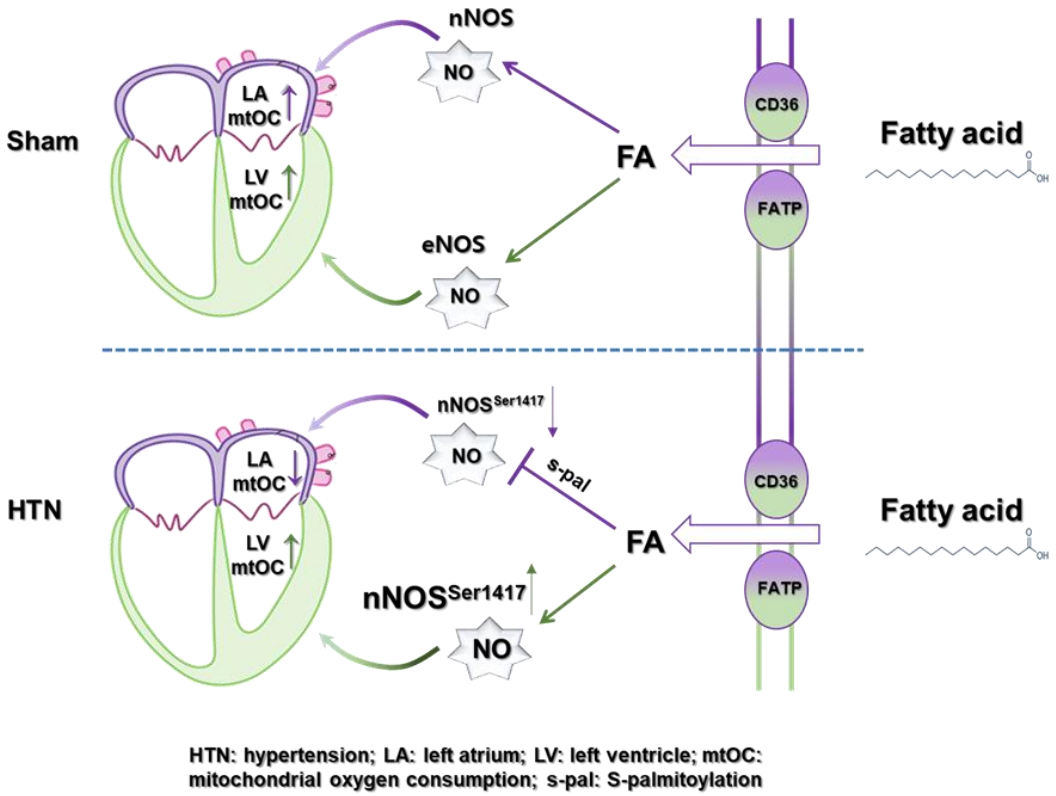

Figure 28. Schematic diagram illustrating FA-regulation of LA and LV by nNOS in sham and HTN. PA increased OCR in sham-LA and LV. eNOS increased OCR in sham-LV without affecting $eNOS^{Ser1177}$ or $nNOS^{Ser1417}$, however, NO production was reduced in sham-LV. By contrast, nNOS promote OCR in sham-LA, despite, NO production was not affected by PA in sham-LA. While, PA-induced OCR was decreased by nNOS-derived NO production reduces in HTN LA. However, PA increased OCR by increasing $nNOS^{Ser1417}$ in HTN-LV, conversely, PA decreased $nNOS^{Ser1417}$ and $eNOS^{Ser1177}$ to attenuate OCR in HTN-LA through protein post-translational modification, *s*-palmitoylation.

Discussion

Part I: FA regulation of mitochondrial activity in sham and HTN rat hearts

FAs are the main energy source for cardiac metabolism. Previously, we have shown that FA increased myocyte contraction in sham rats and PA exerted these effects through affecting key mechanisms in the excitation-contraction coupling (such as increased intracellular Ca^{2+} level). Here, I have investigated FA regulation of mitochondrial activity in sham and HTN rat hearts.

My results clearly showed that FA increased mitochondrial activity (oxygen consumption) and intracellular ATP, suggesting increased metabolism. The effects of PA on myocyte contraction and intracellular ATP were blocked by carnitine palmitol transferase (CPT-1) (Jin, Yin et al. 2017), indicating that PA increased cellular metabolism through FA mediated β-oxidation. I went on and demonstrated that PA increased C-I and C-II activity in cardiac mitochondria in sham. As a result, mitochondrial OCR was greater with PA, which was blocked by rotenone and malonate. In HTN, PA did not increase intracellular ATP and reduced mitochondrial OCR. This is due to the fact that PA inhibited C-II activity, despite that PA increased that of C-I. These results indicated that PA-dependent cellular metabolism is attenuated through C-II inhibition in HTN.

Part II: FA-dependent of mitochondrial activity by nNOS in sham and HTN rat hearts

Our own research and others have shown that nNOS is involved in mitochondrial activity and nNOS co-localized with mitochondrial C-I and C-II activity may function as an electron donor through electron transport chain to activate nNOS in the mitochondria. In line with these results, my results also demonstrated, for the first time, that there are reciprocal interactions between C-I and nNOS activity and such mechanisms exert dynamic regulation of mitochondrial activity in sham and HTN rat hearts. It should be noted that these interactions were only observed in the presence of a strong metabolic substrate, palmitic acid. Evidences support the results are: (1) nNOS protein expression was observed in sham and HTN LV mitochondria; (2) nNOS inhibition with SMTC did not affect PA or PC increment of OCR and ATP; however, SMTC increased OCR in the presence of malonate. This is due to that fact that SMTC increased C-I activity. (3) Inhibition of C-I with rotenone increased nNOS-derived NO in LV myocytes. (4) In HTN, PA or PC reduced C-II and OCR, which was reversed by SMTC; SMTC increased mitochondrial C-I and C-II activity in the presence of PC or PA. SMTC increased OCR in the presence of rotenone but not malonate. (5) Malonate reduced nNOS-derived NO in the presence of PA.

Taken together, our results suggest that there is mutual modulation between nNOS and C-I in normal heart mitochondria and nNOS modulates mitochondrial activity through its interactions with C-I.

However, the activity of nNOS seems dependent on C-II activity; nNOS, in turn, attenuates C-II in HTN. As such, the effects of nNOS on mitochondrial activity are dynamically interacted with mitochondrial complex activities in sham and HTN LV cardiomyocytes.

Cardiac nNOS is the predominant isoform of NOS that is involved in the regulation of cardiac function (Walker 1992, Zhang, Zhang et al. 2008). nNOS has been reported to be associated with C-I, by strong protein-protein interaction between the two proteins (Schafer, Seelert et al. 2006, Genova and Lenaz 2014). It has been demonstrated that mtNOS may produce NO and it is supported by C-I (Bombicino, Iglesias et al. 2016). The interactions between C-I and nNOS suggest their reciprocal regulation of mitochondrial function. Our results clearly showed that nNOS functionally links with C-I, with C-I inhibition increases nNOS-derived NO and nNOS inhibits C-I activity. nNOS inhibition increased C-I-dependent OCR (i.e., in the presence of malonate) and OCR was not increased in the presence of rotenone; these results suggest that nNOS is involved in mitochondrial function through C-I activity. We have known that nNOS plays fundamental roles in regulating cardiac contractility and Ca^{2+} handling. Our result with nNOS and its interaction with C-I and mitochondrial regulation provide novel mechanism of its significance in cardiac metabolism. In diseased heart, it prevents adverse structural and functional remodeling of LV myocardium (Bendall, Damy et al. 2004, Dawson, Lygate et al. 2005, Burkard, Williams et al. 2010); we went on and investigated the role of nNOS in HTN rat heart.

We have shown previously that nNOS protein expression and activity are upregulated in LV myocytes from HTN rat hearts (Jin, Jang et al. 2013). Our results showed that nNOS inhibits C-I and C-II activities in HTN and the activity of nNOS is maintained by C-II activities since nNOS-derived NO is decreased by malonate. With C-I inhibited but not C-II inhibited, SMTC increased mitochondrial OCR in the presence of FA. Since malonate reduced nNOS-derived NO in the presence of PA, we suggest that C-II maintains nNOS activity in HTN. Taken together, these results suggest that nNOS regulates mitochondrial oxygen consumption in HTN through its mutual interactions with C-I and C-II activities. Detailed mechanism of the involvement of nNOS needs to be identified further.

nNOS modulation of C-I and C-II activities may be beneficial to heart protection in HTN. As seen in (Jin, Yin et al. 2017) and in the current study, mitochondrial activity was significantly increased with PA supplementation in HTN and consequently more oxygen is consumed in HTN. This can cause O2 deficiency and the dysfunction of mitochondria (Wilson, Hariri et al. 2004, Bernal-Mizrachi, Gates et al. 2005, Arrell, Elliott et al. 2006), which can be detrimental to cardiac function. Furthermore, abnormal mitochondrial respiration can result in oxidative stress (Arrell, Elliott et al. 2006), uncoupling of the oxidative pathways from mitochondrial ATP synthesis (Beltran, Quintero et al. 2002), or subsequent failure of cellular energetic processes (Weiss, Korge et al. 2003, Arrell, Elliott et al. 2006, Douette and Sluse 2006). For example, Ang-II-induced superoxide production through mitochondria has been

implicated in the cardiac pathogenesis (Jin, Jang et al. 2012, Dikalov, Nazarewicz et al. 2014, Jang, Chun et al. 2015) and mitochondrial reactive oxygen species (mtROS) produced by the respiratory chain during oxidative phosphorylation are the main source of free radicals in most cell types (Zorov, Juhaszova et al. 2014, Scialo, Fernandez-Ayala et al. 2017). Furthermore, reverse electron transfer (RET) in the presence of FAs (and subsequent electron transfer to C-I) increases ROS production; interactions between C-I and nNOS may attenuate C-I and RET, which reduces ROS.

In conclusion, the present study of **Part II**, demonstrates that nNOS mutual links with mitochondrial C-I and nNOS attenuates C-I activity and regulates mitochondrial activity in the presence of FA in healthy rat heart. In HTN, there is functional regulation between nNOS and C-I and C-II in mitochondria and nNOS regulation of mitochondrial activity is dependent on C-I and C-II. This novel finding provides an evidence that is important in adapting nNOS in delaying the progression of pathogenesis in the heart disease.

Part III. FA-dependent of mitochondrial activity and its regulation by NOS in sham and HTN atrial myocardium

It is known that the atrium has distinct structure, gene expression, function and pathology compare to ventricle. Furthermore, energy production and utilization are different between two tissues. Previous research has shown that atrial myocardium is highly dynamic and responds to stretch (Blume, Mcleod et al. 2011) but its FA metabolic

aspect in HTN is not known. We aimed to uncover the role of FA supplementation in mitochondrial activity in atrial myocardium in sham and HTN, explore the mechanistic insights into the regulation. The main findings in this study are that: 1) mitochondrial activity (OCR) is greater in HTN-LA compared to those in sham-LA; PA supplementation increased OCR in sham-LA but reduced it in HTN-LA. This is different from those in LV, where OCR was not different between sham-LV and HTN-LV but PA increased OCR in both groups. 2) Both SMTC and L-NAME prevented PA-induced OCR in sham-LA but did not affect OCR in HTN-LA (since already reduced by PA). SMTC and L-NAME reduced PA-induced OCR in sham-LV and HTN-LV. 3) Compared to Sham-LA, NO production was greater in HTN-LA, $eNOS^{Ser1177}$ was increased and $nNOS^{Ser1417}$ tended to be increased and PA reduced $eNOS^{Ser1177}$, $nNOS^{Ser1417}$ and NO level in HTN-LA. Again, this is different from those in LV, where NO level was greater in HTN-LV and PA increased it further. SMTC and L-NAME inhibited NO production, indicating the predominant role of nNOS in these groups. 4) 2BP blocked PA-induced OCR in sham-LA without affecting $eNOS^{Ser1177}$ and $nNOS^{Ser1417}$. However, PA-induced OCR and reduction of $nNOS^{Ser1417}$ were reversed by 2BP in HTN-LA, suggesting the involvement of *S*-palmitoylation. $AMPK^{Thr172}$ was not affected by PA or 2BP+PA in sham-LA or in HTN-LA. PA decreased AKT^{Ser473} but 2BP did not affect AKT^{Ser473} in the presence of PA in HTN-LA. Collectively, the present study demonstrates that PA potentiates mitochondrial activity mediated by NO in sham-LA. In HTN-LA, PA reduces nNOS activity and NO bioavailability, which is

responsible for impaired mitochondrial activity. Our results show, *for the first time*, that nNOS plays an important part in mediating atrial metabolism and function in normal and hypertensive hearts.

Pressure-overload in HTN predisposes cardiac hypertrophy and reduced LA and LV diastolic function are among the strongest predictors for subsequent development of disease progression (Vaziri, Larson et al. 1994). HTN patients with dyslipidemia possesses the strong risk of atrial fibrillation (Barbier, Alioto et al. 1994, Cioffi, Mureddu et al. 2004). FAs are the predominant metabolic substrates for myocardial ATP in ventricle (Lopaschuk, Ussher et al. 2010, Doenst, Nguyen et al. 2013), atrial myocardium has been acknowledged to have higher glycolytic capacity compare to those in ventricles (Savabi and Kirsch 1991) although it may still prefer FAs for energy production. Recently, evidences relating to altered metabolism and its associations with atrial dysfunction are emerging (Karam, Chavez-Moreno et al. 2017). During atrial arrhythmia progression, glucose oxidation is upregulated compare to FA oxidation in LA (Jie, Li et al. 2019), indicate that abnormal metabolism under pathological condition. Pressure-overload in HTN has a high energy demand and the activation of mitochondrial oxygen consumption in HTN-LA suggest increased metabolic capacity under these conditions. PA reduced OCR in HTN but increased OCR in HTN-LV suggest diverse regulatory mechanisms in two different tissue types.

Reduced NO bioavailability and increased oxidative stress are the common features those are associated with cardiac disorder. Here, our

results showed that L-NAME and SMTC reduced PA-increment of OCR in sham-LA. Intriguingly, NO and OCR are closely associated with each other: NO level was higher in HTN-LA, accordingly, OCR was greater; PA reduced eNOS^{Ser1177} and nNOS^{Ser1417} in HTN-LA and PA reduced NO level as well as OCR. These results indicate that NO bioavailability, which is regulated by FA, plays a critical role in atrial mitochondrial activity in HTN. nNOS is the predominant isoform that is responsible for NO level in atrial myocardium. Our results suggest that the preservation of nNOS-derived NO is important in maintaining normal mitochondrial activity in atrial myocardium. In addition, our results also support that nNOS activity is enhanced in HTN-LV, which is responsible for greater mitochondrial activity in the presence of FA (Jin, Jang et al. 2013, Jin, Yin et al. 2017).

To explore the possible mechanisms of eNOS^{Ser1177} and nNOS^{Ser1417} decrement in HTN-LA, we investigated the effect of *S*-palmitoylation on PA-dependent responses of these NOSs. This is because PA is also the essential substrate for a post-translational modification, *S*-palmitoylation. *S*-almitoylation has been well acknowledged to be associated with eNOS translocation to plasma membrane (caveolae) or lipid domains and increases its activity in endothelial cells (Liu, GarciaCardena et al. 1996). There are evidences showed that inhibition of eNOS palmitoylation with an inhibitor, triacsin C, reduced eNOS phosphorylation at Ser^{1177} in cultured endothelial cells (Blakeman and Weis 2014), suggesting the interplay between eNOS palmitoylation and its phosphorylation. Consistent with these results, recently, we have shown that

depalmitoylation with 2BP reduced eNOS-^{Ser1177} in both sham and HTN-LV (Jin, Wu et al. 2017). However, 2BP reduced PA-increment OCR in sham-LA, eNOS^{Ser1177}, eNOS^{Thr495} and nNOS^{Ser1417} was not affected by 2BP with or without PA, revealing that PA-increment OCR was not associated with eNOS^{Ser1177}, eNOS^{Thr495} and nNOS^{Ser1417} in sham-LA. Furthermore, nNOS is regulated by *S*-palmitoylation indirectly through post-synaptic density protein (PSD-95), a membrane associated guanylate kinase scaffolding protein located in neural postsynaptic densities, represents a major palmitoylated protein in neurons and *S*-palmitoylation of PSD-95 at Cys3 and Cys5 within its N-terminal domain is essential for its postsynaptic targeting (Wan, Roth et al. 2007). In addition, PSD-95 contains PDZ domains that can selectively interacts with the N-terminal PDZ domain of nNOS (Brenman, Chao et al. 1996) so that PSD-95 palmitoylation can activate nNOS activity (Topinka and Bredt 1998). Our results showed that inhibition of *S*-palmitoylation by 2BP prevents PA-inhibition of nNOS^{Ser1417}, as such, OCR was reversed in the presence of PA in HTN-LA. Notably, eNOS^{Thr495} was significantly reduced by PA in the presence of 2BP. Since eNOS^{Thr495} appeared to be unaffected by PA without 2BP, the results indicate that eNOS activity could be modulated by *S*-palmitoylation through maintaining eNOS^{Thr495} in HTN-LA. As a result, OCR was not reduced by PA with 2BP in HTN-LA.

To detect whether upstream regulators of NOS phosphorylation (AMPK and AKT) are involved subsequent to PA, we went on and measured AKT, AMPK and their phosphorylations with and with PA and

PA+2BP. PA or PA+2BP did not affect AMPK and its phosphorylation ($AMPK^{Thr172}$/AMPK) both in sham and HTN-LA. Although PA reduced AKT^{Ser473}, 2BP did not affect its phosphorylation in HTN. Therefore, our new results indicate that AKT or AMPK is not responsible for nNOS phosphorylation subsequent to PA and its post-transcriptional modification, such as *S*-palmitoylation.

In LV, 2BP blocked PA-induced OCR in HTN-LV. $nNOS^{Ser1417}$ was not affected by 2BP in sham and HTN with and without PA, indicating that *S*-palmitoylation does not affect nNOS and its regulation of OCR in LV. We have shown previously that 2BP reduced PA-induced OCR in HTN-LV, by modulating $eNOS^{Ser1177}$. The detailed molecular mechanisms of eNOS and nNOS on mitochondrial activity (oxygen consumption) in atrial myocardium and the involvement of *S*-palmitoylation needs to be explored further.

Atrial metabolism with FA and its regulation in HTN is important in elucidating the pathology of atrium, and there is evidence that abnormal levels of plasma FA are associated with an increased risk of atrial fibrillation and heart failure (Rennison and Van Wagoner 2009, Khawaja, Bartz et al. 2012, Djousse, Benkeser et al. 2013). During cardiac disease progression, FA oxidation and metabolism are impaired (Pellieux, Montessuit et al. 2009), concomitant changes in mitochondrial metabolism link to contractile dysfunction of the myocardium and to the decrease in cardiac efficiency (Fillmore, Mori et al. 2014). In the current study, we suggest that NO bioavailability is important in FA-dependent

mitochondrial oxygen consumption in atrial and ventricular myocardium of both normal and hypertensive hearts. In hypertension, nNOS is the predominant source of NO in HTN-LA and HTN-LV. FA increases nNOS-derived NO production in LV, but decreases nNOS-derived NO in LA, result in contrasting FA-dependent mitochondrial activity. In particular, mitochondrial activity was regulated by protein *S*-palmitoylation in both sham and HTN-LA, further investigation is needed. The results may provide novel insights into therapeutic targets to improve atrial myocardial function under pathological stress.

Conclusion & future prospective

FA-dependent metabolism is an important regulator of cardiac function in the heart. The key messages through my work are that FA plays distinctive roles in sham and HTN hearts, and there is a change in the oxidative phosphorylation in HTN through altered regulation between mitochondrial complexes and nNOS. In atria, nNOS activity is reduced by PA, which underlie mitochondrial dysfunction in HTN. $nNOS^{Ser1417}$ reduction through *S*-palmitoylation is responsible for the effects. These findings provide novel insights into therapeutic targets to improve FA-dependent myocardial metabolism and contractile function in HTN.

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국 문 초 록

정상 심장에 필요한 에너지 대부분은 지방산 대사로 생산된다. 하지만 질병 상태에서 지방산대사 약화되며 에너지는 포도당대사 위주로 변화 된다. 최근 연구에 따르면 고혈압 (HTN) 심장에서 지방산 대사가 미약해 진다고 알려져 있다. 신경성 산화 질소 합성 효소 (nNOS)는 미토콘드리아 복합체와 상호 작용을 하여 미토콘드리아 활성을 조절한다고 알려졌다. 현재까지 nNOS 가 지방산대사 조절과 기전에 대한 연구는 없다. 따라서, 저의 연구목적은 정상과 안지오텐신 II (Ang II)로 유도 된 HTN 쥐 심장에서 지방산에 의한 미토콘드리아 활성과 nNOS 에 의한 조절을 탐구하는 내용이다.

정상 쥐 심장 미토콘드리야에서 팔미 토일 카르니틴 (PC) 또는 팔미트 산 (PA)에 의해 산소 소모량(OCR)과 세포 내 ATP 는 증가되며, C-I 과 C-II 의 활성도 증가하였다. HTH 에서 PC 에 의해 C-I 활성은 증가 되였지만 C-II 의 활성은 감소되였다. 따라서 미토콘드리아 OCR 도 PC 에 의해 감소되였다. C-II 억제제 (malonate, 30mM) 또는 로테논 (5μM)으로 C-I 를 억제한 경우, 정상 쥐 심근 세포와 미토콘드리아에서 지방산에 의한 OCR 은 모두 감소되였다. 하지만 HTN 에서 malonate 를 처리하였을 경우 미토콘드리아 OCR 은 변화가 없었지만 로테논을 처리한 조건에서는 OCR 증가가 관찰 되였다. 따라서 정상 쥐 심장에서 지방산에 의한 미토콘드리아는 C-I 의 활성에 의해 증가되였고 HTN 에서 C-I 는 PC 에 의해 활성되였으나 C-II 의 활성은 감소되였다. nNOS 단백질의 발현은 정상과 HTN 심근 세포 또는 미토콘드리아에서 변화하지 않았고 nNOS 의 억제제 S-메틸-1-티오시트룰린 (SMTC)는 지방산에 의한 정상 쥐 심장세포 또는 미토콘드리아 OCR 과 ATP 증가에 영향을 미치지 않았다. HTN 에서는 SMTC 에 의해 ATP 가 변화 없었지만 OCR 는 증가하였다. 정상 쥐 심장에서 SMTC 는 C-I 의 활성을 증가 시켰지만 C-II 의 활성은 증가 시키지 않았다. HTN 에서 SMTC 는 C-I 및 C-II 활성을 모두 증가시켰다. 또한, PA 와 malonate 전처리 경우 OCR 는 SMTC 에 의해

증가되지만 PA 와 로테논 전처리 경우 SMTC 는 OCR 에 영향을 미치지 않은 것을 보아 nNOS 는 C-I 의 활성을 약화하여 OCR 를 조절하는 기전을 제시한다. HTN 에서 SMTC 는 로테논에 의해 증가한 OCR 을 더욱 증가 시켰지만 malonate 의 전처리에는 변화 주지 않은 것을 보아 HTN 에서 nNOS 에 의한 C-II 의 조절로 OCR 조절 되는 것을 알수 있다. 흥미롭게도 정상 쥐 심장 근세포에서 PA 에 의하여 유래되는 nNOS-NO 는 로테논에 의하여 증가되고, HTN 에서는 malonate 에 의해 nNOS-NO 는 부분적으로 감소된다.

지방산에 의한 미토콘드리아의 활성과 nNOS 의 조절을 심방근(LA)에서도 관찰하였다. OCR 는 HTN-LA 에서 정상보다 컸다. PA 는 정상 LA 에서 OCR 을 더 증가 시켰지만 HTN-LA 에서는 감소시켰다. SMTC 과 eNOS/nNOS 억제제 Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME)는 정상 LA 에서 PA 에 의해 증가한 OCR 를 감소 시켰지만 HTN-LA 에는 영향을 미치지 않았다. HTN-LA 에서 PA 는 $eNOS^{Ser1177}$ 과 $nNOS^{Ser1417}$ 및 NO 를 감소 시켰다. 하지만 sham-LA 에서는 변화없어 이는 HTN-LA 에서 NO 가 PA 에 의하여 감소하고 미토콘드리아 활성을 억제한 것으로 판단된다. *S*-팔미토이레이션은 PA 의 중요한 전사 후 변형이며, 따라서 *S*-팔미토이레이션 억제제인 2-브로모팔미트산 (2BP)를 사용하여 HTN-LA 에서 PA 에 의해 감소한 $nNOS^{Ser1417}$ 및 OCR 를 다시 증가시키는 것을 확인하였다.

저의 연구는 정상과 고혈압 심장에서 지방산에 의한 미토콘드리아 활성과 미토콘드리아 복합체 활성에 대한 nNOS 조절의 새로운 기전을 밝혀 냈고, 이로 인하여 nNOS 에 의한 대사 조절은 고혈압의 심근 기능 장애의 기초가 될 수 있음을 보여준다.

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주요어: 심근세포, 미토콘드리아, 미토콘드리아 활성, 복합체-I, nNOS, 고혈압.
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