Neuronal nitric oxide synthase modulation of intracellular Ca2+ handling overrides fatty acid-potentiation of cardiac inotropy in hypertensive rats

DOI:
10.1007/s00424-017-1991-1

Document Version
Accepted author manuscript

Published in:
Pflügers Archiv European Journal of Physiology

Neuronal nitric oxide synthase modulation of intracellular Ca^{2+} handling overrides fatty acid-potentiation of cardiac inotropy in hypertensive rats

Chun Li Jin[1,2], Ming Zhe Yin[1], Jin Chul Paeng[4], Seunggyun Ha[5], Jeong Hoon Lee[6], Peng Jin[7], Chun Zi Jin[2], Zai Hao Zhao[1], Yue Wang[1], Keon Wook Kang[4], Chae Hun Leem[6], Jong-Wan Park[7], Sung Joon Kim[1], Yin Hua Zhang[1,2,3]*

[1]Department of Physiology & Biomedical Sciences, Ischemic/hypoxic disease institute, Seoul National University College of Medicine, Seoul, Korea; [2]Yan Bian University Hospital, Yanji, Ji Lin, China; [3]Institute of Cardiovascular Sciences, University of Manchester, Manchester, Uk; [4]Department of Nuclear Medicine, Seoul National University Hospital, Seoul, Korea; [5]Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, and College of Medicine or College of Pharmacy, Seoul National University, Seoul, Korea.[6]Department of Physiology, Ulsan College of Medicine, Seoul, Korea; [7]Department of Pharmacology & Biomedical Sciences, Seoul National University, College of Medicine, Seoul, Korea

Running title: nNOS regulation of cardiac E-C coupling with fatty acid in hypertension

*Corresponding authors:Yin Hua Zhang MD PhD

Department of Physiology & Biomedical Sciences, Seoul National University, College of Medicine, Jongno Gu, 103 Dae Hak Ro, Seoul, Korea, 110-799;

E-mail: yinzhang87@gmail.com

Phone: +82 2 740 8223

Fax: +82 2 763 9667

Abstract

Cardiac neuronal nitric oxide synthase (nNOS) is an important molecule that regulates intracellular Ca^{2+} homeostasis and contractility of healthy and diseased hearts. Here, we examined the effects of nNOS on fatty acid (FA)-regulation of left ventricular (LV) myocyte contraction in sham and angiotensin II (Ang II)-induced hypertensive rats (HTN). Our results showed that palmitic acid (PA, 100 μM) increased the amplitudes of sarcomere shortening and intracellular ATP in sham but not in HTN despite that oxygen consumption rate (OCR) was increased by PA in both groups. Carnitine palmitoyl transferase I inhibitor, etomoxir (ETO), reduced OCR and ATP with PA in sham and HTN but prevented PA-potentiation of sarcomere shortening only in sham. PA increased nNOS-derived NO only in HTN. Inhibition of nNOS with S-methyl-l-thiocitrulline (SMTC) prevented PA-induced OCR and restored PA-potentiation of myocyte contraction in HTN. Mechanistically, PA increased intracellular Ca^{2+} transient ($[Ca^{2+}]_i$) without changing Ca^{2+} influx *via* L-type Ca^{2+} channel ($I\text{-}_{LTCC}$) and reduced myofilament Ca^{2+} sensitivity in sham. nNOS inhibition increased $[Ca^{2+}]_i$, $I\text{-}_{LTCC}$ and reduced myofilament Ca^{2+} sensitivity prior to PA supplementation; as such, normalized PA-increment of $[Ca^{2+}]_i$. In HTN, PA reduced $I\text{-}_{LTCC}$ without affecting $[Ca^{2+}]_i$ or myofilament Ca^{2+} sensitivity. However, PA increased $I\text{-}_{LTCC}$, $[Ca^{2+}]_i$ and reduced myofilament Ca^{2+} sensitivity following nNOS inhibition. Myocardial FA oxidation (^{18}F-fluoro-6-thia-heptadecanoidc acid, ^{18}F-FTHA) was comparable between groups but nNOS inhibition increased it only in HTN. Collectively, PA increases myocyte contraction through stimulating $[Ca^{2+}]_i$ and mitochondrial activity in healthy hearts. PA-dependent cardiac inotropy was limited by nNOS in HTN, predominantly due to its modulatory effect on $[Ca^{2+}]_i$ handling.

Keyword: cardiac myocyte, contraction, Ca^{2+} transients, fatty acid, myofilament Ca^{2+} sensitivity, nNOS, mitochondrial oxygen consumption

Introduction

Nitric oxide (NO) is an important regulator of myocardial contractility in healthy and diseased hearts [20,27]. Research from our own group and that of others has shown that NO modulates myocardial inotropy, facilitates lusitropy and is important in the maintenance of cardiac function under pathological stimuli [21,31,32]. Recent consensus is that neuronal nitric oxide synthase (nNOS) is the predominant isoform of NOS that regulates intracellular Ca^{2+} handling, myofilament Ca^{2+} sensitivity and contractility in cardiac myocytes from healthy and hypertensive rats [21,32,33]. In the myocytes from healthy heart, nNOS-derived NO attenuates basal contractility of cardiac myocytes by modulating the activities of L-type Ca^{2+} channels (LTCC) in the plasma membrane to reduce the amplitude of $[Ca^{2+}]_i$ transient or promoting Ca^{2+} decline by increasing phospholamban phosphorylation [26,34]. Recently, we have shown that nNOS protein expression and activity are up-regulated in left ventricular (LV) myocytes from hypertensive rat hearts whereas endothelial NOS (eNOS) protein expression is reduced [13]. nNOS facilitated myocardial lusitropy by reducing myofilament Ca^{2+} sensitivity; myofilament Ca^{2+} desensitization, *in turn*, increased Ca^{2+} handling [13,33].

Fatty acid (FA)-dependent beta-oxidation is an important source of myocardial ATP in healthy heart. As such, FAs are the predominant metabolic substrates that play crucial roles in cardiac metabolism and contractile function [6,18]. During disease progression, beta-oxidation is down-regulated and glycolysis becomes the predominant source of energy (metabolic shift) for the preservation of the pumping function of the heart [18]. In the failing myocardium, both glucose- and FA-dependent metabolism are limited, energy deficiency becomes an important precursor for impaired myocardial contraction. Until recently, the effects of FA on cardiac contractility in healthy and hypertensive rat hearts remain undetermined. Furthermore, the roles of nNOS on cardiac contraction in the presence of FA and the underlying mechanisms in healthy and hypertensive rat hearts needs to be explored.

Palmitic acid (PA) is one of the most common long chain saturated FAs for myocardial energetics [19]. FA oxidation can be affected by nNOS *in vivo* and *in vitro* in healthy and hypertensive rat hearts. Accordingly, we analyzed nNOS-regulation of myocyte contraction and Ca^{2+} handling with and without PA in LV myocytes from sham and angiotensin II (Ang II)-induced hypertensive rats.

Materials & Methods

Animals

Hypertension (HTN) was induced by infusing Ang II subcutaneously in Sprague-Dawley rats using osmotic minipumps and the rats were paired with a sham-operated group. Briefly, rats (of 8 weeks, male) were anesthetized with isoflurane (2.5 %) and osmotic minipumps (rats, Alzet model 2004) containing Ang II (200 μl, 6 mM, infusion rate 125 ng/min/kg) were implanted in the mid-scapular region for 4 weeks. 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 4 weeks using Non-Invasive 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 1996), and also conforms to the Institutional Animal Care and Use Committee (IACUC) in Seoul National University (IACUC approval No.: SNU-101213-1; SNU-160119-4-1).

Blood Pressure and LV myocyte dimensions in sham and hypertensive rats.

Both systolic and diastolic blood pressures (SBP and DBP) were increased from 1 week following Ang II infusion (125ng/min/kg) and continuously increased and heart rate was progressively slower up to the period studied. At 4 weeks, SBP, DBP and heart rate were significantly different between sham and HTN (**Table 1**). The diastolic sarcomere length was

decreased but the width of LV myocytes was not affected in HTN (**Table 1**). Membrane capacitance (C_m) of LV myocytes (in whole-cell patch clamp technique) was increased in HTN.

Table 1. Heart rate, Blood pressure, LV myocyte dimensions, Cell Capacitance in sham and hypertensive rats.

Characteristics	Sham			HTN			
	Sham value	(heart, n)	(cell, n)	HTN value	(heart, n)	(cell, n)	*p value*
Heart rate (bpm)	465.971±4.224	39		419.124±3.754**	40		< 0.0001
SBP (mmHg)	125.332±1.701	39		165.650±4.660**	40		< 0.0001
DBP (mmHg)	93.702±1.488	39		124.787±3.754**	40		< 0.0001
Cell width (μm)	27.658±0.494	24	124	28.066±0.838	22	51	0.3322
Cell sarcomere length (μm)	1.783±0.008	21	27	1.751±0.008*	20	24	0.0031
Cell Capacitance (pF)	182.060±6.417	25	70	196.765±5.259*	22	63	0.0413

SBP indicates systolic blood pressure and DBP, diastolic blood pressure.
* $p < 0.05$ compared with sham ;
** $p < 0.0001$ compared with sham ;
Values are expressed as means ± SEM

Isolation of left ventricular myocytes

LV myocytes were isolated enzymatically by Langendorff perfusion system as recently described [35]. Briefly, the rats were anesthetized with pentobarbital sodium (30 mg/kg, i.p.) and the hearts were extracted and rapidly mounted onto the Langendorff perfusion system. The isolated heart was perfused with nominal Ca^{2+}-free Tyrode solution for 10 minutes (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 minutes with the same solution with enzymes added (collagenase type 2, 1 mg/ml, Worthington Biochemical Co.; protease, 0.1 mg/ml, bovine serum albumin (BSA) 1.67 mg/ml; Ca^{2+} 0.05 mM). LV free wall was isolated and placed in a separate flask containing fresh collagenase type 2-containing solution for 8 minutes (oxygenated and maintained at 37◦C). Myocytes were harvested following a further 10-minute 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-mannitol 29, pH titrated to 7.40 using NaOH). The myocyte suspension was stored at room temperature and cells were used within 8 hours of isolation. A portion of LV myocytes were kept in high K^+ and low Cl^- Kraft-Brühe solution for patch clamp experiments (in mM: HEPES 10, KOH 70, L-gutamate 50, KCl 55, Taurine 20, KH_2PO_4 20, EGTA 0.5, $MgCl_2$ 3,Glucose 20, pH titrated to 7.30 using KOH) at 4 ◦C and myocytes were used within 8 hrs.

Measurement of LV myocyte contraction, Ca^{2+} transients & myofilament Ca^{2+} sensitivity

Isolated LV myocytes were superfused with Tyrode solution containing (in mM: NaCl 141.4, KCl 4, NaH_2PO_4 0.33, $MgCl_2$ 1, HEPES 10, Glucose 5.5, $CaCl_2$ 1.8, mannitol 14.5; pH titrated to 7.40 using NaOH) in the recording bath mounted on the stage of a high-resolution inverted microscope (Diaphot 200, Nikon, JP). Changes in sarcomere length and $[Ca^{2+}]_i$ transients were measured in LV myocytes by using a video-sarcomere detection system (IonOptix Corp). For $[Ca^{2+}]_i$ measurement, LV myocytes were pre-incubated with a $[Ca^{2+}]_i$ sensitive fluorescent dye, acetoxymethyl ester of Fura 2-AM (2 μM) in Tyrode solution containing 250 μM Ca^{2+} for 15 minutes in the dark (room temperature). After sedimentation, the supernatant was removed and LV myocytes were washed in Tyrode solution containing 500 μM Ca^{2+} for 10 minutes. The myocytes were kept in fresh Tyrode solution containing 500 μM Ca^{2+} before being used. Fura 2 AM-loaded myocytes were excited at wavelengths of 360nm and 380nm. The emission fluorescence was reflected through a barrier filter (510 ± 15nm) to a photomultiplier tube. The Fura-2 ratios of the F_{360}/F_{380} was analyzed as the relative changes of $[Ca^{2+}]_{i.}$

In some cells, sarcomere shortening and Fura-2 ratio were recorded simultaneously and phase-plane diagram of Fura-2 ratio *vs.* sarcomere length was used to assess the

myofilament Ca^{2+} sensitivity between sham and HTN. The rightward shift of the Fura-2 - sarcomere length trajectory during late relaxation of the twitch contraction indicates reduced myofilament response to Ca^{2+}. The $[Ca^{2+}]_i$ required for half relaxation (EC_{50}) was analyzed to quantify the relative changes in the myofilament Ca^{2+} sensitivity. Measurements from at least 10 steady state contractions were averaged for each intervention. All experiments were carried out at 36 ± 1 ◦C and field-stimulated at 2Hz [35].

Measurement of oxygen consumption rate (OCR) from LV myocytes

OCR was measured using a fluorescence-based oxygen sensor (NeoFox, Ocean Optics) connected to a phase measurement system (Instech). The sensor was calibrated every week according to the manufacturer's instructions. Briefly, isolated LV myocytes (density: 2 x $\mathbf{10^4}$/ml) were suspended in normal Tyrode's solution and was placed in a sealed chamber (300 μL). Record oxygen level in the chamber over 30 min period (37^oC) followed by 5 min recordings with carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, 20μM) to evaluate maximum OC of the myocytes in the chamber. Changes in the OCR relative to the maximal OC with PA with and without nNOS inhibitor were calculated over 30 min-period in sham and in HTN.

NO production

NO production was detected indirectly by measuring nitrite content in LV myocytes using NO assay kit (Sigma, Griess Reagent System). Briefly, LV myocytes were homogenized in HEPES Buffer solution (in mM: NaCl 141.4, KCl 4, NaH_2PO_4 0.33, $MgCl_2$ 1, HEPES 10, Glucose 5.5, $CaCl_2$ 1.8, mannitol 14.5, mixtures of phosphatase inhibitors and protease inhibitors, pH titrated to 7.40 using NaOH). After assaying the protein content with Bradford protocol, 50 μl of the supernatant of lysates were added to 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, protecting from light. Absorbance of the mixture was measured with a microplate plate reader (at 540 nm, PowerWave™ XS Microplate Spectrophotometer, BioTek Instruments, USA). Sodium nitrite (Sigma) was used as a standard.

L-type Ca^{2+} current recording

LV myocytes were transferred to the mounting chamber on the stage of an inverted microscope (Nikon) and perfused continuously with Tyrode solution contains (in mM: NaCl 141.4, KCl 4, NaH_2PO_4 0.33, $MgCl_2$ 1, HEPES 10, Glucose 5.5, $CaCl_2$ 1.8, D-mannitol 14.5, pH titrated to 7.40 using NaOH) at 35–37◦C. Patch pipettes with a resistance of 1.5–2.8 MΩ were used and L-type Ca^{2+} current (I_{Ca}) was recorded in the whole-cell configuration using an Axopatch 200A amplifier (Axon Instruments). I_{Ca} was elicited by 200 ms step depolarization from a holding potential of −40 mV to test potentials ranging from −60 to +40 mV. Steady-state I_{Ca} was assessed during a step to 0 mV at a stimulation frequency of 0.1 Hz. Membrane capacitance (C_m) was measured by applying a −10 mV hyperpolarizing ramp pulse immediately followed by a 10 mV depolarizing ramp of the same duration (100 ms) using the method by Golowasch J *et al.*, [10]. The range of membrane capacitance was also monitored directly from the window of Clampex 10.4. Recorded I_{Ca} were normalized to C_m and expressed as I_{Ca} density (in pA/pF). Intracellular pipette solution contains (in mM: CsOH 110, TEA-Cl 20, NaCl 10, HEPES 10, $MgCl_2$ 1, MgATP 5, EGTA 10, Aspartate 110, pH titrated to 7.20 using CsOH, Osmolarity 308 mOsm). Ca^{2+} influx *via* LTCC was compared by calculating the integral of I_{Ca}. The average of I_{Ca} integrals at 0 mV with PA was compared between sham and HTN. Data were analyzed in Clampfit 10.

PET/CT image acquisition and analysis of FA oxidation *in vivo*

^{18}F-fluoro-6-thia-heptadecanoidc acid (^{18}F-FTHA) was synthesized from benzyl-14-(R,S)-tosyloxy-6-thiaheptadecanoate.Three sham and Ang II-induced HTN rats (12 weeks) were involved in the PET imaging study using radiotracer ^{18}F-FTHA in a dedicated small animal PET/CT scanner (eXplore Vista CT, GE Healthcare). Following water and food fasting for 6 hours before PET/CT scanning, rats were anesthetized with 2% isoflurane and 2 L/min oxygen and were placed on the scanning bed with prone position and centered the heart in the field of view (FOV). ^{18}F-FTHA (5.0 ± 1.8 MBq) was administered *via* tail vein as a 0.5 ml bolus. In the small-animal PET/CT system, static PET image acquisition began 40 minutes after the tracer injection for 5 minutes. A CT scan was acquired for image co-registration after the PET acquisition. On separate days, PET/CT acquisitions with the same protocol were performed 10 minutes after admission of NOS inhibitors *i.v.* SMTC (1mg/kg) or L-NAME (5mg/kg). Two-dimensional ordered-subset expectation maximum (OSEM) algorithm was applied to PET image reconstruction. The reconstructed PET images were analyzed by a software package, AMIDE (ver.1.0.4, Stanford University, Palo Alto, CA). After drawing a spherical volume-of-interest (VOI) covering the entire heart, LV myocardium (0.55 cm^3-sized) of highest uptake was segmented in the VOI, in which mean uptake value of ^{18}F-FTHA was measured. The liver was chosen as a reference organ to calculate myocardium-to-liver ratio (MLR), for which mean uptake value of the liver was measured from a spherical VOI (0.57 cm^3-sized). [11,22]

Measurement of intracellular pH (pH_i)

Temporal intracellular H^+ was measured using a membrane permeant acetoxymethyl ester form of the fluorescent H^+-sensitive indicator, SNARF-1 AM (10 μM, 5–10 min, Molecular Probes, Eugene, OR). All experiments were performed at 37 °C. In some experiments, pH buffer capacity was increased with $NaHCO_3$ (25 mM) + CO_2 (5 %) instead of HEPES in the perfusion solution to examine the effect of pH on myocyte responses to PA.

ATP production

Intracellular ATP level was determined using luminescence measurement of LV myocytes (ATP Bioluminescent Assay Kit, Sigma-Aldrich, Saint Louis, MO, USA). Briefly, LV myocytes were incubated in HEPES buffer solution (in mM: NaCl 141.4, KCl 4, NaH_2PO_4 0.33, $MgCl_2$ 1, HEPES 10, Glucose 5.5, $CaCl_2$ 1.8, mannitol 14.5, pH 7.4 NaOH). Cells ($1x10^5$ /ml) were added to 200 μL of mammalian cell lysis solution (Tris-acetate buffer and 10 % T richloroacetic acid was lysis buffer) and vortex the cell suspension tube for 10 minutes. Supernatant (100 μL) was added to 96 well white microplate and ATP assay mix solution was added to the wells in dark adapt stand at room temperature for 3 minutes. Take an aliquot of the ATP standard solution and prepare a dilution series in Tris-acetate buffer from a concentration of 2×10^{-4}M down to blank.

Chemicals

Angiotensin II (Ang II, 6 mM) was used to induce hypertension. Tiron (1 mM, Sigma) scavenges superoxide. S-methyl-L-thiocitrulline acetate salt (SMTC, 100 nM, Sigma) and Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 1 mM, Sigma) were used to target NOS activity. Sodium nitroferricyanide (III) dihydrate (SNP, 10 μM, Sigma) is an exogenous NO donor. Palmitic acid (PA, 100 μM, Sigma) was used as the substrate for the effect of FA. Etomoxir sodium salt hydrate (ETO, 100 μM, sigma or 10 μM, Cayman) used to inhibit the activity of CPT-1(carnitine palmitoyltransferase 1). Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP, 20 μM, Sigma) was used to evaluate maximum oxygen consumption. ^{18}F-FTHA ([^{18}F] fluorothia-6-heptadecanoic acid) was used to monitor fatty acid metabolism *in vivo*.

Statistics

Data are expressed as means ± SEM or as relative to control (100%) and n indicates the number of cells used. For all comparisons, cells were obtained from a minimum of three hearts per treatment group per protocol. Data were analyzed using student's *t* tests or **ANOVA with multiple comparisons with Bonferroni correction.** A value of $p < 0.05$ was considered to be statistically significant.

Results

PA increased LV myocyte contraction in sham but not in hypertension

The effects of FA on contraction in isolated LV myocytes were examined in sham and in HTN. As shown in Fig.1 a-c, PA (100 μM) significantly increased sarcomere shortening in LV myocytes from sham ($p < 0.0001$, control *vs.* PA, n = 13). In contrast, the positive inotropic effect of PA was absent in HTN ($p = 0.1$, control *vs.* PA, n = 12). Pre-treatment of LV myocytes with an inhibitor of carnitine palmitoyltransferase I (CPT-1), ETO (10 μM, 30 min-1hr), abolished PA-enhancement of myocyte contraction in sham ($p = 0.37$, control *vs.* PA with ETO, n = 5, Fig.1d,f) without an effect at basal level. Furthermore, ETO did not affect myocyte contraction in HTN with or without PA ($p = 0.1$, control *vs.* PA with ETO, n = 5, Fig.1e,f).

Sustained pressure-overload in hypertension is able to increase reactive oxygen species (ROS) and induce oxidative stress in the myocardium [3]. In addition, FAs have been implicated in the myocardial oxidative stress and modulate myocyte contractility in murine heart [8]. To investigate whether increased ROS in HTN may have prevented PA-regulation of myocyte contraction, the effect of PA on LV myocyte was re-evaluated after incubation with a potent ROS scavenger, tiron (1 mM). Tiron pre-incubation did not affect myocyte contraction with PA in sham ($p < 0.0001$, Tiron *vs.* Tiron+PA, n = 8, Supplementary Fig.1) or

in HTN (p = 0.24, Tiron *vs.* Tiron+PA, n = 7, Supplementary Fig. 1), excluding the role of ROS in preventing PA-induced myocyte contraction in HTN.

We performed experiments to observe whether PA affects mitochondrial activity (oxygen consumption rate, OCR) in LV myocytes from sham and HTN rats. As shown in Fig. 2a,b, PA increased basal OCR in both groups (p=0.004 and p=0.004, n=10 and n=9, respectively). Pre-treatment of LV myocytes with CPT-1 inhibitor etomoxir (ETO, 10 μM, 30 min-1hr) prevented PA induced increases of OCR in sham (p = 0.045, ETO vs. ETO+PA, n = 6 and n = 9) and in HTN (p = 0.01, ETO vs. ETO+PA, n = 5 and n = 11). Similarly, *in vitro* analysis of ATP level in LV myocytes showed that PA increased intracellular ATP and ETO prevented the effect in sham (p=0.024, control vs. PA, n=4; p=0.0162, PA vs. ETO+PA, n=4, Fig.2c). In HTN, PA did not increase intracellular ATP, however, ETO significantly reduced intracellular ATP level in the presence of PA (p=0.209, control vs. PA, n=4; p=0.048, PA vs. ETO+PA, n=4, Fig.2d).

PA increased nNOS-derived NO in HTN, which in turn, prevents PA-stimulation of myocyte contraction

We aimed to identify PA-regulation of nNOS activity in LV myocytes from sham and HTN rats. As shown in Fig.3a, NO production (relative to control) was slightly but significantly reduced by PA in sham (p = 0.03, control *vs.* PA, n = 6). nNOS inhibition with SMTC did not affect PA-modulation of NO production (p = 0.09, PA *vs.* PA+SMTC, n = 6, Fig.3a). In contrast, NO production was increased by PA in HTN (p = 0.01, control *vs.* PA, n = 5, Fig. 3b). SMTC (100 nM, 30 min-1hr) and L-NAME (1 mM, 30 min-1hr) blocked NO increment by PA (p = 0.02, PA *vs.* PA with SMTC in HTN; p = 0.02, PA *vs.* PA+ L-NAME in HTN, Fig.3b), suggesting the role of nNOS.

Fig. 3c showed that inhibition of nNOS with SMTC did not affect PA-induced OCR increase in sham (p>0.05, SMTC and SMTC+PA, n=8 and n=10). In HTN, SMTC abolished PA-induced increase in OCR (p<0.05, SMTC and SMTC+PA, n=7 and n=6). These results suggest that PA-dependent mitochondrial activity is increased in both sham and HTN but this effect is attenuated with nNOS inhibition only in HTN.

Intriguingly, sarcomere shortening of LV myocyte was increased by PA following nNOS inhibition in HTN (SMTC, 100 nM, 30min-1hr; p = 0.0005, SMTC *vs.* PA + SMTC, n = 10, Fig.3f,g). In contrast, nNOS inhibition exerted no effect on PA-dependent myocyte contraction in sham (p < 0.0001, SMTC *vs.* PA + SMTC, n = 15, Fig. 3e,g). There is no difference in sarcomere shortening with PA between two groups following nNOS inhibition (p = 0.1, in sham and HTN with SMTC+PA). These results suggest that nNOS is important in restricting PA-enhancement of myocyte contraction in HTN.

Next, we examined FA-metabolism and the response to nNOS and NOS inhibition by quantifying ^{18}F-FTHA uptake *in vivo* with PET/CT scanning in LV myocardium from Sham and HTN rats. **As shown in supplementary Fig.2, myocardial ^{18}F-FTHA uptake was not different between two groups at basal (p = 0.2382, sham control *vs.* HTN control, n = 3 each group). Inhibition of nNOS with SMTC did not affect ^{18}F-FTHA uptake in sham (p = 0.187, control *vs.* SMTC) but increased it in HTN (p = 0.0284, control *vs.* SMTC). Subsequent infusion of L-NAME increased ^{18}F-FTHA uptake in sham (p = 0.0095, control *vs.* L-NAME, n = 3) and in HTN (p = 0.0397, control *vs.* L-NAME, n = 3). These results indicate that basal FA uptake is not different between sham and HTN; nNOS modulates myocardial FA uptake in HTN.**

nNOS modulation of Ca^{2+} handling prevents the inotropic effect of PA in HTN

Next, we evaluated PA-regulation of the key parameters of excitation-contraction coupling and the responses of these parameters to nNOS inhibition in sham and HTN.

Peak $I_{Ca\text{-}L}$ at 0 mV was reduced by PA in sham, however, inactivation of LTCC was slower (Supplementary Fig.3); as a result, total Ca^{2+} influx through LTCC ($I_{Ca\text{-}L}$ integral, $I\text{-}_{LTCC}$) was not changed by PA in sham (p = 0.4, n = 11, at 0 mV, Supplementary Fig.4). Inhibition of nNOS with SMTC increased peak $I_{Ca\text{-}L}$ with and without PA (p = 0.06, control *vs.* SMTC and p = 0.01, control *vs.* SMTC + PA, n = 11 & 7) and slowed inactivation kinetics further (Supplementary Fig.3). Accordingly, $I\text{-}_{LTCC}$ was increased after nNOS inhibition with and without PA in sham (p = 0.001 and p = 0.003, n = 11 and n = 7, respectively, Supplementary Fig.4).

In HTN, PA significantly reduced peak $I_{Ca\text{-}L}$ and slowed inactivation kinetics of LTCC (Supplementary Fig. 3) and $I\text{-}_{LTCC}$ at 0 mV was reduced (p = 0.0007, n = 8, Supplementary Fig.4). Inhibition of nNOS with SMTC significantly increased peak $I_{Ca\text{-}L}$ at 0 mV and slowed tau further (Supplementary Fig. 3). Consequently, $I\text{-}_{LTCC}$ was significantly increased following nNOS inhibition with PA in HTN (p = 0.001, PA *vs.* SMTC+PA, n = 8, at 0 mV, Supplementary Fig.4).

Next, we detected the changes in $[Ca^{2+}]_i$ following PA supplementation with and without nNOS inhibition in sham and HTN. As shown in Fig. 4, PA significantly increased systolic and diastolic $[Ca^{2+}]_i$ in sham (systolic $[Ca^{2+}]_i$: p = 0.002, n = 11; diastolic $[Ca^{2+}]_i$: p = 0.03, n = 11), but the effects were absent in HTN (systolic $[Ca^{2+}]_i$: p = 0.13, n = 13; diastolic $[Ca^{2+}]_i$: p = 0.31, n = 13). Inhibition of nNOS with SMTC significantly increased the amplitude of basal $[Ca^{2+}]_i$ in sham (p = 0.04, Fig. 4a,c,d) and PA no longer increased **the peak amplitude of $[Ca^{2+}]_i$** in the presence of SMTC in sham (p = 0.3, n = 11, Fig.4a,c,d).

In contrast, SMTC pre-treatment significantly reduced the amplitude of basal $[Ca^{2+}]_i$ in HTN (p = 0.043, n = 13 & 9, Fig.4b,e). In addition, PA significantly increased $[Ca^{2+}]_i$ in the presence of SMTC (p = 0.009, SMTC *vs.* SMTC+PA, n = 9, Fig.4 b,e). These results indicate that PA increased $[Ca^{2+}]_i$ in sham. nNOS inhibition restores PA-regulation of intracellular $[Ca^{2+}]_i$ in HTN.

PA regulation of myofilament Ca^{2+} sensitivity before and after nNOS inhibition in sham and HTN

Myofilament Ca^{2+} sensitivity is a vital regulator of myocyte contraction and relaxation. Importantly, alterations in the myofilament Ca^{2+} sensitivity are involved in Ca^{2+} binding to TnC and Ca^{2+} buffering capacity in the myofilament, consequently affects intracellular Ca^{2+} homeostasis [4,12,25]. Accordingly, we investigated the changes in the myofilament Ca^{2+} sensitivity by analyzing the relationship between sarcomere shortening and $[Ca^{2+}]_i$ with PA before and after nNOS inhibition in sham and HTN.

As shown in Fig. 5a & c, PA shifted the relaxation phase of the sarcomere shortening and $[Ca^{2+}]_i$ relationship to the right and increased EC_{50} in sham (p = 0.01, n = 11, Fig.5e). Inhibition of nNOS with SMTC reduced myofilament Ca^{2+} sensitivity under basal conditions (EC_{50}, p = 0.04, n = 11 & 11, Fig.5b, d, e) and abolished the relationship between sarcomere shortening and $[Ca^{2+}]_i$ with PA (EC_{50}, p = 0.2, n = 11, Fig.5e).

In contrast, PA did not affect sarcomere shortening and $[Ca^{2+}]_i$ relationship in HTN (EC_{50}, p = 0.7, n = 13, Fig.6a, c, e). Inhibition of nNOS sensitized the myofilament response to Ca^{2+} in HTN without PA (EC_{50}, p = 0.04, n = 13 & 9, Fig.6b, d, e) but PA significantly reduced myofilament Ca^{2+} sensitivity in the presence of SMTC (100 nM, 30 min-1hr) (EC_{50}, p = 0.01, n = 9, Fig.6e). These results indicate that PA modulates myofilament Ca^{2+} sensitivity in sham

and in HTN. nNOS maintains myofilament Ca^{2+} sensitivity in sham but reduces it in HTN, which *in turn*, masks the effects of PA on $[Ca^{2+}]_i$ and myocyte contractility in HTN.

Furthermore, intracellular pH ($[pH_i]$) was shown to be gradually decreased with PA (6–8 min) in both groups. Averaged results showed that PA significantly reduced pH_i ($p<0.05$, $p<0.05$ n=7, respectively, Supplementary Fig.5a, 5b). Increasing pH buffer capacity in PA using $NaHCO_3$ + CO_2 (5 %) weaken the changes in pH_i by PA in sham and in HTN ($p>0.05$, $p>0.05$, n=7, respectively, Supplementary Fig.5a, 5b).

Discussion

Our present study demonstrates that PA increases mitochondrial activity (via beta-oxidation) in LV myocytes from sham and hypertensive rat hearts but PA failed to increase myocyte contraction in hypertensive group, contrary to that in sham. Further analysis showed that PA increased nNOS-derived NO in LV myocytes from hypertensive rats and mediates PA-dependent increase in mitochondrial activity. Nevertheless, nNOS prevented PA-potentiation of myocyte contraction by modulating $[Ca^{2+}]_i$ handling. Particularly, PA reduced myofilament Ca^{2+} sensitivity and increased $[Ca^{2+}]_i$ in LV myocytes from sham. However, nNOS reduced myofilament Ca^{2+} sensitivity and Ca^{2+} influx *via* LTCC prior to PA application and limited myocyte responses to PA. Our results demonstrate that PA is an active regulator of myocyte contraction through altering intracellular Ca^{2+} handling in LV myocytes from both healthy and hypertensive rat hearts. nNOS in HTN restricts PA-regulation of myocyte contractility by controlling $[Ca^{2+}]_i$ handling alongside its regulation of mitochondrial activity and myofilament property (Fig. 7).

FAs are the predominant metabolic substrates to cardiac metabolism and activation of β-oxidation in the mitochondria with FA supplementation produces more energy in the myocardium for its contractile function under normal conditions. Our results clearly showed

that PA increased myocyte contraction, mitochondrial activity (OCR) and ATP production from healthy hearts and the responses were blocked by CTP-1 inhibitor (ETO), supporting the role of β-oxidation and FA metabolism. In addition, convincing evidence showed that PA increased myocyte contraction by affecting $[Ca^{2+}]_i$ handling in sham. I.e. PA increased the amplitude of $[Ca^{2+}]_i$ without changing Ca^{2+} influx through LTCC. On the other hand, myofilament Ca^{2+} sensitivity was reduced, which is seemingly contrary to PA potentiation of myocyte contraction. In fact, myofilament Ca^{2+} desensitization is associated with reduced Ca^{2+} binding affinity to TnC and/or $[Ca^{2+}]_i$ buffering capacity [12,25]. Therefore, it is possible that reduced myofilament Ca^{2+} sensitivity by PA increased the amplitude of $[Ca^{2+}]_i$, which *in turn*, contributed to PA-enhancement of myocyte contraction. Recently, our results have shown that FA-containing metabolic substrates increase myocyte contraction, reduce myofilament Ca^{2+} sensitivity in healthy rat hearts [37]; reduced intracellular pH_i, which is a potent modulator of myofilament Ca^{2+} sensitivity in cardiac and skeletal muscle [1,7,17,28], may mediate PA-dependent myofilament Ca^{2+} desensitization and the subsequent regulation of $[Ca^{2+}]_i$ by PA. Additional results with pH_i measurement confirmed the reduction of pH_i with PA in both sham and HTN (supplementary Fig. 5). In HTN, myofilament Ca^{2+} sensitivity was reduced before the application of PA; consequently, PA exerted little effect on myofilament Ca^{2+} sensitivity, $[Ca^{2+}]_i$ and myocyte contraction, suggesting the importance of myofilament Ca^{2+} desensitization in the regulation of intracellular Ca^{2+} homeostasis and contractile phenotype of PA. Increased cellular ATP level through cardiac metabolism may also modulate myofilament sensitivity to Ca^{2+} by affecting the phosphorylation of myofilament proteins those are involved in myocardial contractile function [2,9]. Furthermore, high concentrations of FAs (at mM range) have been implicated in the myocardial oxidative stress [8]. Intracellular ROS*, in turn,* either increase LV myocyte contraction by stimulating intracellular protein kinases (e.g. PKA) or by direct oxidation of proteins those are involved in myocyte contraction [36] or decrease myocyte contraction secondary to increased ROS in

the myocardium from hypertensive models [31]. Pre-incubation of LV myocytes with tiron exerted no effect on myocyte responses to PA in sham or in HTN (supplementary Fig. 1), excluding the role of ROS in affecting PA-induced myocyte contraction in either group.

Previously, we have shown that nNOS protein expression and activity were increased in hypertensive rat LV myocytes which is responsible for myofilament Ca^{2+} desensitization but enhanced $[Ca^{2+}]_i$ transient [13]. Here, PA increased nNOS-derived NO further in HTN and nNOS is responsible for greater reduction in Ca^{2+} influx through LTCC and myofilament Ca^{2+} desensitization and restricts PA-potentiation of myocyte contraction, confirming that nNOS-dependent modulation of $[Ca^{2+}]_i$ handling and myofilament Ca^{2+} sensitivity overrides the greater contraction induced by PA.

Although cardiac metabolism and β-oxidation may have downregulated in hypertrophy and in heart failure myocardium, it may not be the case in the model of the current study. Our previous reports clearly presented in echocardiography assessment that myocardial contraction was not altered despite pressure-overload in these rats [13]. It is possible that the metabolic status or substrate preference has not been modified in this *early stage* of hypertensive model yet. Indeed, *in vivo* examination of FA metabolism using PET/CT scanner in both sham and HTN showed that basal ^{18}F-FTHA uptake in the LV was not different between two groups. However, inhibition of nNOS with SMTC slightly but significantly increased FA metabolism in HTN without affecting it in sham. It is possible that greater contraction following myocardial nNOS inhibition may have increased energy demand via enhancing cardiac metabolism. On the other hand, systematic inhibition of nNOS may have activated “sympathetic drive” and greater norepinephrine release from neural innervation [16] may enhance myocyte contraction and therefore FA metabolism in HTN. In addition, subsequent treatment with L-NAME to block NOS significantly increased myocardial ^{18}F-FTHA uptake in both groups. NO has been implicated in modulating FA

uptake and utilization in the mammalian myocardium from normal heart [4,14,15,23,24] Furthermore, nNOS may affect myocardial function *via* regulating mitochondrial proteins since nNOS-derived NO has been shown to inhibit mitochondrial respiration chain, including complex I, III and IV, [5,29,30] and reduces mitochondrial oxygen consumption and affects cardiac metabolism. However, inhibition of NOS *in vivo* may affect hemodynamics of the cardiovascular system (e.g. hypertension), resulting in NOS-independent effect on myocardial metabolism (e.g. increase energy demand). Notably, the FA metabolism was increased significantly greater with L-NAME in sham compared to that in HTN (Supplementary Fig 2b), possibly due to prerequisite hypertension and smaller margin for the myocardial responses. The effects of NO and the NOSs on myocardial FA metabolism and its roles in myocardial contractile function remain to be determined.

In conclusion, our results demonstrate novel evidence that although FA-dependent metabolism is an important regulator of myocardial contraction in healthy and hypertensive rats, nNOS-derived NO is up-regulated by FA in hypertension, which *in turn*, modulates key elements of Ca^{2+} handling process and controls myocardial response to FA. The functional relevance and the mechanistic insights into FA-regulation of myocardial contractile function and its regulation by nNOS is important in better understanding the cardiac physiology under pressure-overload.

Conflicts of interests: none declared.

Sources of Funding

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2013068067); by the Brain Korea 21 Graduate Programme of the Korean Ministry of Education, Science and Technology, Seoul National University Hospital, the Korean Society

of Hypertension (2013), SK Telecom Research Fund (no. 3420130290) and from the National Natural Science Foundation of China (NSFC, 31460265).

References

1. Ashley CC, Moisescu DG (1977). Effect of changing the composition of the bathing solutions upon the isometric tension-pCa relationship in bundles of crustacean myofibrils. J Physiol 270 (3):627-652.
2. Best PM, Donaldson SK, Kerrick WG (1977). Tension in mechanically disrupted mammalian cardiac cells: effects of magnesium adenosine triphosphate. J Physiol; 265 (1):1-17.
3. Briones AM, Touyz RM (2010). Oxidative stress and hypertension: current concepts. Curr Hypertens Rep 12 (2):135-142.
4. Briston SJ, Dibb KM, Solaro RJ, Eisner DA, Trafford AW (2014). Balanced changes in Ca buffering by SERCA and troponin contribute to Ca handling during beta-adrenergic stimulation in cardiac myocytes. Cardiovasc Res; 104 (2):347-354.
5. Chouchani ET, Methner C, Nadtochiy SM, Logan A, Pell VR, Ding S, James AM, Cochemé HM, Reinhold J, Lilley KS, Partridge L, Fearnley IM, Robinson AJ, Hartley RC, Smith RA, Krieg T, Brookes PS, Murphy MP (2013). Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. Nat Med; 19 (6):753-759.
6. Doenst T, Nguyen TD, Abel ED (2013). Cardiac metabolism in heart failure: implications beyond ATP production. Circ Res; 113 (6):709-724.
7. Fabiato A, Fabiato F (1978). Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiace and skeletal muscles. J Physiol; 276:233-255.
8. Fauconnier J, Andersson DC, Zhang SJ, Lanner JT, Wibom R, Katz A, Bruton JD, Westerblad H. (2007). Effects of palmitate on Ca(2+) handling in adult control and ob/ob cardiomyocytes: impact of mitochondrial reactive oxygen species. Diabetes; 56 (4):1136-1142.
9. Godt RE (1974). Calcium-activated tension of skinned muscle fibers of the frog. Dependence on magnesium adenosine triphosphate concentration. J Gen Physiol; 63 (6):722-739.
10. Golowasch J, Thomas G, Taylor AL, Patel A, Pineda A, Khalil C, Nadim F. (2009). Membrane capacitance measurements revisited: dependence of capacitance value on measurement method in nonisopotential neurons. J Neurophysiol; 102 (4):2161-2175.
11. Hernandez AM, Huber JS, Murphy ST, Janabi M, Zeng GL, Brennan KM, O'Neil JP, Seo Y, Gullberg GT. (2013). Longitudinal evaluation of left ventricular substrate metabolism, perfusion, and dysfunction in the spontaneously hypertensive rat model of hypertrophy using small-animal PET/CT imaging. J Nucl Med; 54 (11):1938-1945.

12. Huke S, Knollmann BC (2010). Increased myofilament Ca2+-sensitivity and arrhythmia susceptibility. J Mol Cell Cardiol; 48 (5):824-833.
13. Jin CZ, Jang JH, Kim HJ, Wang Y, Hwang IC, Sadayappan S, Park BM, Kim SH, Jin ZH, Seo EY, Kim KH, Kim YJ, Kim SJ, Zhang YH. (2013). Myofilament Ca2+ desensitization mediates positive lusitropic effect of neuronal nitric oxide synthase in left ventricular myocytes from murine hypertensive heart. J Mol Cell Cardiol; 60:107-115.
14. Khan SA, Skaf MW, Harrison RW, Lee K, Minhas KM, Kumar A, Fradley M, Shoukas AA, Berkowitz DE, Hare JM. (2003). Nitric oxide regulation of myocardial contractility and calcium cycling: independent impact of neuronal and endothelial nitric oxide synthases. Circ Res; 92 (12):1322-1329.
15. Kolwicz SC, Jr., Purohit S, Tian R (2013). Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes. Circ Res; 113 (5):603-616.
16. Li D, Nikiforova N, Lu CJ, Wannop K, McMenamin M, Lee CW, Buckler KJ, Paterson DJ (2013). Targeted neuronal nitric oxide synthase transgene delivery into stellate neurons reverses impaired intracellular calcium transients in prehypertensive rats. Hypertension. 61(1):202-7.
17. Liou YM, Kuo SC, Hsieh SR (2008). Differential effects of a green tea-derived polyphenol (-)-epigallocatechin-3-gallate on the acidosis-induced decrease in the Ca(2+) sensitivity of cardiac and skeletal muscle. Pflugers Arch; 456 (5):787-800.
18. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC (2010). Myocardial fatty acid metabolism in health and disease. Physiol Rev; 90 (1):207-258.
19. Mancini A, Imperlini E, Nigro E, Montagnese C, Daniele A, Orrù S, Buono P. (2015). Biological and Nutritional Properties of Palm Oil and Palmitic Acid: Effects on Health. Molecules; 20 (9):17339-17361.
20. Massion PB, Feron O, Dessy C, Balligand JL (2003). Nitric oxide and cardiac function: ten years after, and continuing. Circ Res; 93 (5):388-398.
21. Niu X, Watts VL, Cingolani OH, Sivakumaran V, Leyton-Mange JS, Ellis CL, Miller KL, Vandegaer K, Bedja D, Gabrielson KL, Paolocci N, Kass DA, Barouch LA. (2012). Cardioprotective effect of beta-3 adrenergic receptor agonism: role of neuronal nitric oxide synthase. J Am Coll Cardiol; 59 (22):1979-1987.
22. Ohira H, deKemp R, Pena E, Davies RA, Stewart DJ, Chandy G, Contreras-Dominguez V, Dennie C, Mc Ardle B, Mc Klein R, Renaud JM, DaSilva JN, Pugliese C, Dunne R, Beanlands R, Mielniczuk LM. (2015). Shifts in myocardial fatty acid and glucose metabolism in pulmonary arterial hypertension: a potential mechanism for a maladaptive right ventricular response. Eur Heart J Cardiovasc Imaging 17(12):1424-1431.
23. Pellieux C, Montessuit C, Papageorgiou I, Lerch R (2009). Angiotensin II downregulates the fatty acid oxidation pathway in adult rat cardiomyocytes via release of tumour necrosis factor-alpha. Cardiovasc Res; 82 (2):341-350.

24. Recchia FA, McConnell PI, Loke KE, Xu X, Ochoa M, Hintze TH (1999). Nitric oxide controls cardiac substrate utilization in the conscious dog. Cardiovasc Res; 44 (2):325-332.
25. Robinson P, Griffiths PJ, Watkins H, Redwood CS (2007). Dilated and hypertrophic cardiomyopathy mutations in troponin and alpha-tropomyosin have opposing effects on the calcium affinity of cardiac thin filaments. Circ Res; 101 (12):1266-1273.
26. Sears CE, Bryant SM, Ashley EA, Lygate CA, Rakovic S, Wallis HL, Neubauer S, Terrar DA, Casadei B. (2003). Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. Circ Res; 92 (5):e52-59.
27. Shah AM, MacCarthy PA (2000). Paracrine and autocrine effects of nitric oxide on myocardial function. Pharmacol Ther, 86 (1):49-86.
28. Takahashi R, Shimazaki Y, Endoh M (2001). Decrease in Ca(2+)-sensitizing effect of UD-CG 212 Cl, a metabolite of pimobendan, under acidotic condition in canine ventricular myocardium. J Pharmacol Exp Ther; 298 (3):1060-1066.
29. Torres J, Darley-Usmar V, Wilson MT (1995). Inhibition of cytochrome c oxidase in turnover by nitric oxide: mechanism and implications for control of respiration. Biochem J; 312 (Pt 1):169-173.
30. Welter R, Yu L, Yu CA (1996). The effects of nitric oxide on elecontrolon transport complexes. Arch Biochem Biophys; 331 (1):9-14.
31. Zhang YH, Jin CZ, Jang JH, Wang Y (2014). Molecular mechanisms of neuronal nitric oxide synthase in cardiac function and pathophysiology. J Physiol; 592 (15):3189-3200.
32. Zhang YH, Casadei B (2012). Sub-cellular targeting of constitutive NOS in health and disease. J Mol Cell Cardiol; 52 (2):341-350.
33. Zhang YH (2016). Neuronal nitric oxide synthase in hypertension - an update. Clin Hypertens; 22:20.
34. Zhang YH, Zhang MH, Sears CE, Emanuel K, Redwood C, El-Armouche A, Kranias EG, Casadei B. (2008) Reduced phospholamban phosphorylation is associated with impaired relaxation in left ventricular myocytes from neuronal NO synthase-deficient mice. Circ Res; 102 (2):242-249.
35. Zhao ZH, Jin CL, Jang JH, Wu YN, Kim SJ, Jin HH, Cui L, Zhang YH. (2016). Assessment of Myofilament Ca2+ Sensitivity Underlying Cardiac Excitation-contraction Coupling. J Vis Exp (114).
36. Zhang YH, Dingle L, Hall R, Casadei B (2009). The role of nitric oxide and reactive oxygen species in the positive inotropic response to mechanical stretch in the mammalian myocardium. Biochim Biophys Acta; 1787 (7):811-817.
37. Zhao ZH, Youm JB, Wang Y, Lee JH, Sung JH, Kim JC, Woo SH, Leem CH, Kim SJ, Cui L, Zhang YH. Cardiac inotropy, lusitropy, and Ca2+ handling with major metabolic substrates in rat heart (2016). Pflugers Archiv; 468(11-12):1995-2006.

Figure legend

FIGURE 1 Effects of PA and inhibition of carnitine palmitoyltransferase 1 on myocyte contraction in sham and HTN. **A-C** Raw representative traces and the averages of sarcomere shortening in both groups. PA (100 μM) increased sarcomere shortening in sham but not in HTN. **D-F** Raw representative traces and the averages of sarcomere shortening following pre-treatment of LV myocytes with ETO (10 μM, 30 min-1hr). ETO abolished PA-enhancement of myocyte contraction in sham with little effect in HTN.

FIGURE 2 PA regulation of mitochondrial activity. **A-B**, PA increased mitochondrial activity (oxygen consumption rate, OCR) in LV myocytes from both sham and HTN rats. Pre-treatment of LV myocytes with CTP-1 inhibitor etomoxir (ETO, 10 μM, 30 min-1hr) prevented PA induced OCR in both groups. **C-D,** PA significantly increased intracellular ATP level in sham, which is abolished by ETO (10 μM, 30 min-1hr). In HTN, PA did not affect intracellular ATP but ETO significantly reduced it in the presence of PA.

FIGURE 3 Effects of PA on nNOS production of NO and nNOS regulation of oxygen consumption rate (OCR) and myocyte contraction in sham and HTN. **A & B.** NO production (nitrite level relative to control) was significantly increased by PA in HTN, which was prevented by SMTC (100 nM, 30 min-1hr) and L-NAME (1 mM, 30 min-1hr). PA failed to increase NO production in sham. Neither SMTC nor L-NAME affected NO production in the presence of PA. **C & D.** PA increased OCR in LV myocytes from sham and HTN rats. Pre-incubation with SMTC did not affect PA-induced OCR increase in sham. However, SMTC abolished PA-induced increase in OCR in HTN. **E-G.** Raw representative traces and the averages of sarcomere shortening with PA following pre-treatment of LV myocytes with nNOS inhibitor, SMTC (100 nM, 30-1hr), Myocyte contraction was remained to be increased by PA with SMTC in sham. However, PA increased sarcomere shortening following SMTC pre-treatment in HTN.

FIGURE 4 PA regulation of $[Ca^{2+}]_i$ with and without nNOS inhibition (SMTC, 100 nM, 30 min-1hr) in sham and HTN. **A, C&D** Representative raw traces and averaged values of $[Ca^{2+}]_i$ transient with PA before and after SMTC treatment in sham. PA significantly increased the peak $[Ca^{2+}]_i$ amplitude but failed to affect $[Ca^{2+}]_i$ after SMTC pre-treatment in sham. **B, E&F** Representative raw traces and averaged values for $[Ca^{2+}]_i$ with PA before and after SMTC treatment in HTN. PA failed to affect basal Ca^{2+} transient and SMTC reduced peak $[Ca^{2+}]_i$ in HTN. In the presence of SMTC, PA significantly increased $[Ca^{2+}]_i$ amplitude.

FIGURE 5 PA regulation of myofilament Ca^{2+} sensitivity before and after nNOS inhibition in sham. **A&B** representative raw traces of sarcomere shortening and $[Ca^{2+}]_i$ transient with PA before (A) and after nNOS inhibition (B) in sham. **C&D** Phase-plane diagram of Fura-2 ratio *vs.* sarcomere length with PA in the presence and absence of SMTC in sham. PA shifted the relaxation phase of the relationship to the right. **E** EC_{50} was increased by PA in sham, indicating reduced myofilament Ca^{2+} sensitivity. EC50 was increased in the presence of SMTC, PA no longer affected EC_{50} under these conditions.

FIGURE 6 PA regulation of myofilament Ca^{2+} sensitivity before and after nNOS inhibition in HTN. **A&B** representative raw traces of sarcomere shortening and $[Ca^{2+}]_i$ transient with PA before (**A**) and after nNOS inhibition (**B**) in HTN. **C&D** Phase-plane diagram of Fura-2 ratio *vs.* sarcomere length with PA in the presence and absence of SMTC in HTN. PA did not affect the relaxation phase of the relationship, indicating no effect on myofilament Ca^{2+} sensitivity. **E** EC_{50} was reduced in HTN compared to that in sham. SMTC reduced EC_{50} and restored EC_{50} increment by PA in HTN.

FIGURE 7 Schematic diagram illustrating FA-regulation of myocyte contraction and the regulation by nNOS in healthy and hypertensive rat hearts. PA increases myocyte contraction by enhancing $[Ca^{2+}]_i$ transient secondary to myofilament Ca^{2+} desensitization in

sham. PA increases pH_i and ATP *via* β-oxidation in the mitochondria, which may induce myofilament Ca^{2+} desensitization, subsequently increase $[Ca^{2+}]_i$. In HTN, PA stimulates nNOS activity, nNOS-derived NO reduces Ca^{2+} influx through LTCC, intracellular Ca^{2+} and myofilament Ca^{2+} sensitivity, as a result, overrides PA-induced increase of myocyte contraction and metabolism in HTN.

Supplementary Fig 1. Effects of ROS scavenger, tiron, on PA-regulation of myocyte contraction in sham and in HTN. **A-C** Representative raw traces and mean values of sarcomere shortening by PA with Tiron (1 mM). Sarcomere shortening was remained increased by PA in sham and was unaffected by PA in HTN, despite Tiron pre-treatment.

Supplementary Fig 2. Examination of FA uptake with PET/CT in sham and HTN rats. **A.** PET/CT images were obtained by using ^{18}F-FTHA in the LV at basal, in the presence of nNOS inhibitor, SMTC (1mg/kg) and L-NAME (5mg/kg). **B.** Averaged mean values of ^{18}F-FTHA uptake in the myocardium/liver between sham and HTN with and without NOS inhibition. nNOS inhibition induced small but significant increase in ^{18}F-FTHA uptake in HTN only. L-NAME significantly increased ^{18}F-FTHA uptake in both groups, with an effect significantly higher in sham compared to that in HTN.

Supplementary Fig 3. PA regulation of the peak $I_{Ca\text{-}L}$ density and inactivation kinetics with and without nNOS inhibition in sham and HTN. LTCC was stimulated by a step depolarization protocol from -60 mV to +40 mV (10 mV interval) for 200 ms (holding potential, -40 mV). **A-D** representative $I_{Ca\text{-}L}$ density with and without PA before and after nNOS inhibition (SMTC, 100 nM, 30 min-1hr). PA slightly but significantly reduced peak $I_{Ca\text{-}L}$ density at 0 mV in both groups. nNOS inhibition increased $I_{Ca\text{-}L}$ density at 0 mV and abolished PA-regulation in both groups. **E-H** Inactivation of $I_{Ca\text{-}L}$ was slowed by PA in both groups; nNOS

inhibition (SMTC, 100 nM, 30min-1hr) significantly reduce inactivation and abolish PA-dependent responses in sham and in HTN.

Supplementary Fig 4. PA regulation of the Ca^{2+} influx through LTCC. LTCC was stimulated by a step depolarization protocol from -60 mV to +40 mV (10 mV interval) for 200 ms (holding potential, -40 mV). **A & C.** Representative $I_{Ca\text{-}L}$ integral at 0 mV in sham and HTN before and after nNOS inhibition with and without PA. **B & D.** Averaged mean values showed that PA did not change $I_{Ca\text{-}L}$ integral in sham but significantly reduced it in HTN. nNOS inhibition increased $I_{Ca\text{-}L}$ integral with and without PA in both groups and abolished the response of PA in two groups.

Supplementary Fig 5a Regulation of intracellular pH_i by PA in sham. pHi (SNARF-1/AM) was significantly reduced by PA in HEPS buffer. Increase pH_i buffer capacity using $NaHCO_3$ + CO_2 (5 %) weaken the changes in pH_i by PA in sham.

Supplementary Fig 5b Regulation of intracellular pH_i by PA in HTN. pH_i (SNARF-1/AM) was significantly reduced by PA in HEPS buffer. Increase pH_i buffer capacity using $NaHCO_3$ + CO_2 (5 %) weaken the changes in pH_i by PA in HTN.