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Negligible effect of eNOS palmitoylation on fatty acid regulation of contraction

in ventricular myocytes from healthy and hypertensive rats

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Abstract

S-palmitoylation is an important post-translational modification that affects the translocation and the activity of target proteins in a variety of cell types including cardiomyocytes. Since endothelial nitric oxide synthase (eNOS) is known to be palmitoylated and the activity of eNOS is essential in fatty acid-dependent β -oxidation in muscle, we aimed to test whether palmitoylation of eNOS is involved in palmitic acid (PA)-regulation of left ventricular (LV) myocyte contraction from healthy (sham) and hypertensive (HTN) rats. Our results showed that PA, a predominant metabolic substrate for cardiac β -oxidation, significantly increased contraction and oxygen consumption rate (OCR) in LV myocytes from sham. L-NAME (N ω -nitro-L-arginine methyl ester hydrochloride) or eNOS gene deletion prevented PA-regulation of the myocyte contraction or OCR, indicating the pivotal role of eNOS in mediating the effects of PA in cardiac myocytes. PA increased the palmitoylation of eNOS in LV myocytes and depalmitoylation with 2 bromopalmitate (2BP, 100 μ M) abolished the increment. Furthermore, although PA did not increase eNOS-Ser¹¹⁷⁷, 2BP reduced eNOS-Ser¹¹⁷⁷ with and without PA. Intriguingly, PA-induced increases in contraction and OCR were unaffected by 2BP treatment. In HTN, PA did not affect eNOS palmitoylation, eNOS-Ser¹¹⁷⁷ or myocyte contraction. However, 2BP diminished eNOS palmitoylation and eNOS-Ser¹¹⁷⁷ in the presence and absence of PA but did not change myocyte contraction. Collectively, our results confirm eNOS palmitoylation in LV myocytes from sham and HTN rats and its up-regulation by PA in sham. However, such post-transcriptional modification plays negligible role in PA-regulation of myocyte contraction and mitochondrial activity in sham and HTN.

Keyword: cardiac myocyte, contraction, eNOS, oxygen consumption, palmitic acid, *S*-palmitoylation

Introduction

Fatty acids are the predominant metabolic substrates that play important roles in cardiac metabolism and myocardial function [3,11]. Palmitic acid (PA, a 16-carbon saturated fatty acid) which is one of the most common saturated fatty acids for myocardial ATP, has been implicated in the regulation of cardiac contraction. **However, the mechanism** of this regulation is not fully understood. Recent evidence indicates that nitric oxide (NO) that is derived from endothelial NOS (eNOS) may play a critical role in the regulation of energy production and fatty acid utilization by muscle. For example, Le Gouill *et al.* demonstrated that oxygen consumption in muscle from eNOS gene knockout (eNOS^{-/-}) mice is impaired compared to that in normal mice [8]. Furthermore, mitochondrial β -oxidation was reduced in eNOS^{-/-} mice [8]. Since NO is well established to affect myocardial function, including the modulation of contraction, energetics (metabolism) and signaling pathways [5,12], eNOS may regulate PA-dependent myocyte contraction in the heart.

S-palmitoylation is an important post-translational modification that affects the translocation and the activity of target proteins in the cells and the reversible nature of palmitoylation provides a potential mechanism for protein shuttling between intracellular compartments [9]. eNOS is known to be palmitoylated [10] and it is possible that the activity of eNOS is altered to play a part in fatty acid β -oxidation in cardiac myocyte and regulate myocardial contractility. Recently, we have shown that eNOS protein expression is reduced in **angiotensin II (Ang II)** - induced hypertensive myocardium [7]. Reduced NO bioavailability is able to impair cardiac metabolism and reduce the capacity of fatty acid oxidation [5,11]. We hypothesize that PA-regulation of myocyte contraction is impaired due to the lack of eNOS palmitoylation in hypertensive heart.

Here, we aimed to identify whether eNOS is palmitoylated by PA in **left ventricular (LV)** myocytes and how this post-translational modification affects eNOS activity (eNOS phosphorylation) in healthy heart and in hypertension. Furthermore, we aimed to identify whether eNOS palmitoylation contributes to PA-regulation of myocyte contraction and mitochondrial activity in healthy heart and whether this effect is diminished in hypertension.

Materials & Methods

Animals

Sprague-Dawley rats (10-12 weeks old, male) were used in the study. Hypertension was induced by infusing Ang II subcutaneously in rats using osmotic minipumps and they were paired with sham-operated group. Briefly, rats (of 8 wk old, male) were anesthetized with isoflurane (2.5 %). An osmotic minipump (Alzet model 2004, DURECT Corporation, San Francisco, California, USA.) containing Ang II (200 μ l, 6 mM, infusion rate 125 ng/min/kg) was 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 for 4 weeks by Non-Invasive Blood Pressure System, tail-cuff method (CODA, Torrington, CT, USA).

eNOS knockout mice (21- to 25-wk-old males) were provided by Professor Goo Taeg Oh's Laboratory (Ewha Womans University, Seoul, Korea).

Isolation of left ventricular myocytes

LV myocytes were isolated enzymatically by Langendorff perfusion system as recently described [14]. 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 a nominally Ca^{2+} -free 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 Corporation, Lakewood, New Jersey, USA; protease, 0.1 mg/ml, bovine serum albumin (BSA) 1.67 mg/ml; Ca^{2+} 0.05 mM). The LV free wall was isolated and placed in a separate flask containing fresh collagenase-only 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.

S-palmitoylation of eNOS

For blocking the free thiol, LV myocytes were diluted with blocking buffer (100 mM HEPES, 1 mM EDTA, 2.5 % SDS, 1 % MMTS, pH 7.5) and incubated at 40°C for 4 hrs. Following acetone precipitation, the pellet was rinsed five times with 70 % cold acetone and added in binding buffer (100 mM HEPES, 1.0 mM EDTA, 1% SDS, pH 7.5). When employed, an equal volume of freshly prepared 2M NH₂OH (pH adjusted to 7.0 with 2M NaCl) was added followed by prewashed thiopropyl-Sepharose (**Sigma-Aldrich, Seoul, Korea**). Binding reactions were carried out on a rotator at room temperature for 2.5 hrs. Each of supernatant was saved as the “total input.” Resins were washed at least 7 times with binding buffer. For immunoblot analysis, elution was performed using 2X Laemmli sample buffer supplemented with 100 mM DTT and heated to 95°C for 5 min. The obtained sample was separated *via* SDS-PAGE and immunoblotting with eNOS. S-palmitoylation of eNOS was compared in LV myocytes before and after palmitic acid treatment [4].

Measurement of LV myocyte contraction

Isolated LV myocytes were superfused with normal Tyrode solution containing (in mM: NaCl 141.4, KCl 4, NaH₂PO₄ 0.33, MgCl₂ 1, HEPES 10, Glucose 5.5, CaCl₂ 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 were measured in LV myocytes by using a video-sarcomere detection system (**IonOptix Corporation, Milton, Massachusetts, USA**). 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 [14].

Measurement of oxygen consumption rate (OCR) from LV myocytes

OCR was measured using a fluorescence-based oxygen sensor (**NeoFox, Ocean Optics, Dunedin, Florida, 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: 2 x 10⁴/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°C) followed by about 5 min recordings with carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, 20 µM) to evaluate maximum OC of the myocytes in the chamber. Changes in the OCR with PA with and without nNOS inhibitor were calculated over 30 min-period in sham and HTN.

Immunoblotting

LV myocytes were lysed in lysis buffer contained 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% Triton X-100, 5 mM EDTA with protease/phosphatase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cell lysates were then centrifuged at 14,000g for 30 min at 4 °C, and the supernatant 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 poly-vinylidene difluoride membranes in 25 mM Tris, 192 mM glycine, and 20 % methanol. Membranes were blocked in 1X TBS containing 1 % Tween-20 and 5 % bovine serum albumin (blocking solution) for 1 hr at room temperature with gentle rocking, and incubated overnight at 4 °C with anti-eNOS (BD Transduction Laboratories), eNOS-Ser¹¹⁷⁷ (BD Transduction Laboratories, San Francisco, California, USA), GAPDH (Santa Cruz, Dallas, Texas, 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.

NO production

NO production was detected indirectly by measuring nitrite content in LV myocytes using NO assay kit (Griess Reagent System, Sigma-Aldrich, Seoul, Korea). Briefly, LV myocytes were homogenized in HEPES Buffer solution (in mM: NaCl 141.4, KCl 4, NaH₂PO₄ 0.33, MgCl₂ 1, HEPES 10, Glucose 5.5, CaCl₂ 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-Aldrich) solution added to the wells and incubated for another 5-10 min at room temperature, protected 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-Aldrich) was used as a standard.

Chemicals

Angiotensin II (Ang II, 6 mM) was injected to the osmotic mini-pumps. S-methyl-L-thiocitrulline acetate salt (SMTTC, 100 nM, Sigma) and Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 1 mM, Sigma) were used to target nNOS and NOS activity. Sodium nitroferricyanide (III) dehydrate (SNP, 10 µM, Sigma) was an exogenous NO donor. Palmitic acid (PA, 100 µM, Sigma) was used to stimulate fatty acid oxidation. 2-bromopalmitic acid (2BP, 100 µM, Sigma) was an inhibitor of S-palmitoylation. Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, 20 µM, Sigma) was an uncoupling agent induced maximal oxygen consumption rate. Sodium hydrosulfite or hyposulfite (Na₂S₂O₄, Sigma-Aldrich) was used in calibration for OCR by minimizing O₂ level in the solution.

Statistics

Data are reported as mean ± SEM. 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 paired and unpaired *t* test. A value of *p*<0.05 was considered to be statistically significant. Data are reported as mean ± SEM.

Results

eNOS mediates PA-regulation of myocyte contraction

Fig.1A & D showed that PA (100 μ M) significantly increased sarcomere shortening in LV myocytes from sham rats ($p<0.0001$, control vs. PA in shams, $n=13$). However, the positive inotropic effect of PA was absent in the presence of a non-specific inhibitor of NOS, N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME, 1 mM, 30 min-1hr), ($p = 0.26$, control vs. PA in pre-treatment of L-NAME in shams, $n=8$; $p<0.001$, PA vs. L-NAME+PA in sham, $n=13$ and $n=8$, Fig.1B & D) and in LV myocytes from eNOS^{-/-} mice ($p=0.2927$, control vs. PA in eNOS^{-/-}, Fig.1C & E).

We performed additional experiments to observe whether eNOS affects mitochondrial activity (oxygen consumption rate, OCR) in LV myocytes from sham rats with and without eNOS inhibition and from eNOS^{-/-} mice. As shown in Fig.2A, PA significantly increased OCR in LV myocytes from sham at control but not with L-NAME ($p=0.004$, control vs. PA in shams, $n=10$ and $n=9$; $p=0.4935$, L-NAME vs. L-NAME+PA, $n=8$ each; $p=0.1951$, control vs. L-NAME in sham, $n=10$ and $n=8$; $p=0.008$, PA vs. L-NAME+PA, $n=9$ and $n=8$). This is in contrast to the inhibition of neuronal NOS (nNOS) with SMTC (100nM, 30min~1hr), where PA failed to affect the OCR ($p=0.0239$, SMTC vs. SMTC+PA in shams, $n=8$ and $n=10$, Fig.2A). In line with this result, PA failed to increase myocyte shortening following SMTC-pretreatment (data not shown). In addition, PA did not induce an increase in OCR in LV myocytes from eNOS^{-/-} mice, on the contrary, PA reduced OCR in eNOS^{-/-} ($p=0.0329$, control vs. PA in eNOS^{-/-} mice, $n=6$ and $n=7$, Fig. 2B). These data indicate that eNOS is essential in mediating PA-regulation of myocyte contraction through stimulating mitochondrial activity.

S-palmitoylation of eNOS and its regulation of eNOS-phosphorylation and OCR

Fig. 3A showed that PA (100 μ M) increased eNOS palmitoylation in LV myocytes from sham rats ($p=0.023$, control vs. PA in sham, $n=6$, Fig. 3A). Pre-treatment of LV myocytes with a palmitate analog that is the most commonly used to inhibit palmitoylation in cells, 2-bromopalmitic acid (2BP, 100 μ M, 10-30min), abolished PA-induced eNOS palmitoylation ($p=0.005$, PA vs. PA+2BP in sham, $n=6$).

Furthermore, we aimed to observe whether the palmitoylation of eNOS affects its activity by

regulating eNOS-Ser¹¹⁷⁷. As shown in Fig. 3B, eNOS-Ser¹¹⁷⁷ was not affected by PA in LV myocytes from sham. However, 2BP significantly reduced eNOS-Ser¹¹⁷⁷ with and without PA ($p=0.235$, control vs. PA; $p=0.02677$, control vs. 2BP; $p=0.00949$, PA vs. PA+2BP; $p=0.01294$, 2BP vs. PA+2BP in sham, $n=6$, Fig. 3B). **Paradoxically, the NO production (nitrite level relative to control), which was reduced by PA (84% of control, $p=0.045$, $n=6$), significantly increased by 2BP in sham (112%, $p=0.152$, Ctr vs. 2BP, $n=6$; 139%, $p=0.028$, PA vs. PA+2BP, $n=6$). Furthermore, neither SMTc nor L-NAME prevented the effect of 2BP (147%, $p=0.03$, SMTc vs. SMTc+PA+2BP, $n=8$; 149%, $p=0.02$, L-NAME vs. L-NAME+PA+2BP, $n=8$). Therefore, the effect of 2BP on eNOS-Ser¹¹⁷⁷ does not associate with myocyte NO level in sham.**

To examine whether eNOS palmitoylation affects the function of PA-dependent responses in LV myocytes, both OCR and myocyte contraction were detected with 2BP pre-treatment. As shown in Fig. 3C, pre-treatment LV myocytes with 2BP failed to affect PA-potential of OCR in sham ($p=0.004$, control vs. PA; $p<0.0001$, 2BP vs. PA+2BP; $p=0.0752$, PA vs. PA+2BP, Fig.3C). NOS inhibition with L-NAME prevented PA-dependent OCR increase in the presence of 2BP ($p<0.0001$, PA+2BP vs. L-NAME+PA+2BP, Fig. 3C).

Furthermore, treatment of LV myocytes with 2BP did not affect myocyte contraction with PA in sham ($p<0.0001$, control vs. PA, $p=0.0015$, PA vs. PA+2BP, $n=13$, Fig.4A & D). Pre-treatment of 2BP did not prevent PA-dependent myocyte contraction ($p=0.005$, 2BP vs. 2BP+PA, $n=8$, Fig.4B & E). Similarly, 2BP did not affect myocyte contraction in the presence of L-NAME, *i.e.* PA did not increase myocyte contraction in the presence of L-NAME and 2BP ($p=0.406$, L-NAME+PA vs. L-NAME+PA+2BP, $n=8$, Fig. 4C & F). These results exclude the effect of eNOS palmitoylation on eNOS-mediated and PA-induced myocyte contraction in sham.

Palmitoylation of eNOS in PA-regulation of LV myocytes contraction and OCR from HTN rats

In HTN, PA did not increase eNOS palmitoylation ($p=0.58$, control vs. PA, $n=4$, Fig.5A). However, 2BP significantly reduced eNOS palmitoylation in the presence and absence of PA ($p=0.04$, PA vs.

PA+2BP, Fig.5A), suggesting that basal eNOS is palmitoylated and PA does not affect it further. Similar to that in sham, 2BP significantly reduced eNOS-Ser¹¹⁷⁷ in HTN with and without PA (with PA, $p=0.038$, PA vs. PA+2BP; without PA, $p=0.05$, control vs. 2BP, $n=3$, Fig.5B). In addition, **the NO production was significantly increased by 2BP in the presence of SMTC and L-NAME (140%, $p=0.02$ SMTC vs. SMTC+PA+2BP, $n=8$; 180%, $p=0.01$, L-NAME vs. L-NAME+PA+2BP, $n=8$)**. Unexpectedly, 2BP reduced PA-induced OCR in HTN ($p=0.0177$, control and PA, $n=8$ and $n=9$; $p=0.0198$, PA vs. PA+2BP, $n=9$ and $n=10$; $p=0.2419$, 2BP vs. PA+2BP, $n=8$ and $n=10$, Fig.5C).

Finally, the effect of PA on myocyte contraction and the involvement of eNOS palmitoylation are examined. As shown in Fig.6, PA-dependent positive inotropic effect was absent in HTN ($p=0.3896$, **control vs. PA, $n=12$, Fig.6A & D**). This effect was unaffected by 2BP ($p=0.069$, 2BP vs. PA+2BP in HTN, $n=5$, **Fig.6B & E**). NOS inhibition with L-NAME significantly increased myocyte contraction with PA and 2BP did not affect such an effect (**Fig. 6C & F**), indicating the lack of an effect of eNOS and its palmitoylation on myocyte contraction in HTN. These results show clear evidence that eNOS is palmitoylated in LV myocytes from HTN and does not change myocyte contraction with or without PA.

Discussion

In the present study we showed convincing evidence that a long chain fatty acid, PA, significantly increased LV myocyte contraction from healthy rat heart. This effect was prevented by pharmacological inhibition of eNOS with L-NAME or with eNOS gene deletion (eNOS^{-/-}). Furthermore, PA increased mitochondrial activity (OCR) which was prevented by L-NAME or in eNOS^{-/-}. In contrast, inhibition of nNOS with SMTC did not affect PA-induced myocyte contraction or mitochondrial activity. These results suggest that the effects of PA on myocyte contraction and mitochondrial activity are mainly mediated **by eNOS but not by nNOS**. Immunoblotting experiments showed direct evidence that PA increased the palmitoylation of eNOS which was abolished by a potent blocker of palmitoylation, 2BP. Interestingly, depalmitoylation of eNOS with 2BP was

1 associated with a reduction of eNOS phosphorylation at Ser¹¹⁷⁷. However, 2BP, either pre-treatment
2 or post-treatment, did not affect the inotropic effect of PA or PA regulation of mitochondrial activity,
3 excluding the role of eNOS-palmitoylation as the mediating mechanism. In addition, eNOS was
4 shown to be palmitoylated in LV myocytes from HTN rats. Depalmitoylation of eNOS with 2BP did
5 not affect LV myocyte contraction in the presence and absence of PA, despite its inhibitory effect on
6 eNOS Ser¹¹⁷⁷ in HTN. To our knowledge, this is the first study to exam the influence of eNOS and its
7 palmitoylation in the regulation of LV myocyte contraction following supplementation of a favorable
8 metabolic substrate of the myocardium in healthy and hypertensive hearts.
9

10 Fatty acids are the predominant metabolic substrates for myocardial ATP and therefore play
11 essential roles in the contractility of the heart. During disease progression, glucose oxidation or
12 glycolysis becomes the predominant sources of energy whereas both glucose and fatty acid
13 metabolism are downregulated in the failing myocardium [13]. Understanding the mechanisms
14 regulating cardiac contractility through fatty acid-dependent metabolism in healthy and diseased
15 hearts are important in better defining the molecular targets underlying cardiac contraction in these
16 conditions. Here, our results showed that PA increased myocyte contraction and mitochondrial
17 activity (OCR). It is possible that PA exerts these effects through promoting β -oxidation in the
18 mitochondria because our additional studies showed that PA increased ATP production from LV
19 myocytes and both myocyte contraction and ATP level were normalized following the inhibition of
20 carnitine palmitoyltransferase, a key limiting factor of β -oxidation [6]. The effect of PA on myocyte
21 contraction was absent in HTN, indicating functional remodeling of cardiac metabolism in regulating
22 contraction under pressure-overload.
23

24 On the other hand, PA is also the essential substrate for a post-translational modification, *S*-
25 palmitoylation. As such, PA may affect myocyte contraction through changing the activities of the
26 mediator proteins. Intriguingly, our results showed that eNOS plays a critical role in mediating PA-
27 regulation of myocyte contraction. Furthermore, eNOS palmitoylation is significantly increased by
28 PA, which was reversed by pre-treatment of the inhibitor, 2BP. It is well established that the
29

1 palmitoylation of eNOS is associated with its translocation to plasma membrane (caveolae) and
2 increased its activity in endothelial cells [10]. There are evidences showed the interplays between
3 eNOS palmitoylation and phosphorylation, that is, inhibition of eNOS palmitoylation with another
4 potent inhibitor of palmitoylation, triacsin C, significantly reduced eNOS phosphorylation in cultured
5 endothelial cells [1]. Paradoxically, triacsin C increased NO level in these experiments [1]. Similar to
6 the responses in the report, our results showed that eNOS-Ser¹¹⁷⁷ was significantly reduced by 2BP in
7 the presence and absence of PA. Moreover, in line with the results shown in endothelial cells [1], 2BP
8 increased NO production in LV myocytes before and after PA supplementation. However, neither L-
9 NAME nor SMTC affected the increment of NO, excluding the roles of eNOS or nNOS. Although
10 2BP is a potent and reliable inhibitor of palmitoylation, it has been shown to exert off-target effects
11 [2]. Therefore, 2BP may regulate NO level in myocyte samples through alternative mechanisms – it is
12 speculative to suggest that 2BP increases cellular NO by preventing cysteine residues of NO-binding
13 proteins. Nevertheless, 2BP did not affect OCR with or without PA supplementation, *i.e.* PA
14 increased OCR in 2BP pre-treated LV myocytes, suggesting that 2BP does not affect the functional
15 regulation of PA on the mitochondria in LV myocytes (despite increased NO level). Accordingly,
16 2BP was used to exam the effect of de-palmitoylation on PA-regulation of myocyte contractility.
17 Similar to its effect on mitochondrial activity, 2BP did not affect myocyte contraction, either pre-
18 treated or post-treated, demonstrating that de-palmitoylation process, eNOS depalmitoylation in
19 particular, is not the major mechanism mediating PA-regulation of myocyte contraction.

20
21 In HTN, cardiac NOSs are remodeled, exhibiting reduced eNOS protein level and greater nNOS
22 protein expression and activity [7]. Furthermore, the residual eNOS was palmitoylated in HTN,
23 judging from the significant reduction of eNOS palmitoylation by 2BP with and without PA.
24 Palmitoylation of eNOS may have maintained the activity of eNOS since eNOS-Ser¹¹⁷⁷ was
25 minimized by 2BP and 2BP reduced PA-dependent OCR increment in HTN. However, the
26 significance of this modification remains uncertain. It is possible that reduced eNOS activity underlie
27 the lack of PA-dependent inotropic response on LV myocytes in HTN, regardless of its palmitoylation

1 since 2BP did not affect myocyte contraction in HTN. **Inhibition of cardiac NOSs with L-NAME**
2
3 **significantly increased myocyte contraction in HTN, similar results were shown with SMTc,**
4 **suggesting nNOS modulation of myocyte contraction by PA in HTN.** Notably, 2BP did not affect
5 myocyte contraction even in the presence of L-NAME, excluding the involvement of eNOS
6 palmitoylation in myocyte contraction by PA in HTN.
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12 Taken together, our observations suggest a role of eNOS in mediating PA-regulation of myocyte
13 contraction in healthy rat heart through stimulating mitochondrial activity. Although PA increases
14 eNOS palmitoylation and maintains the phosphorylation of eNOS at Ser¹¹⁷⁷, we present clear evidence
15 to show that eNOS palmitoylation is not the main mechanism to mediate PA-dependent inotropic
16 response in healthy heart or the lack of this increment in HTN. The significance of eNOS and its
17 post-transcriptional modification in regulating cardiac metabolism and contraction in healthy and
18 diseased heart needs further investigation, which may provide novel insights into therapeutic targets
19 to improve myocardial contractile function.
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33

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Figure legend

Fig.1 eNOS is essential in mediating PA-regulation of myocyte contraction. Changes in sarcomere length were measured in LV myocytes by using a video-sarcomere detection system (IonOptix Corp). **A&B, D** PA (Palmitic acid, 100 μM) significantly increased sarcomere shortening in LV myocytes from sham rats (sarcomere shortening, $p < 0.0001$, $n=13$). The positive inotropic effect of PA was prevented by L-NAME pre-treatment (1 mM, 30 min-1hr) (sarcomere shortening, $p < 0.001$, PA vs. L-NAME+PA in sham, $n=8$). **C&E** PA failed to affect myocyte contraction from eNOS^{-/-} mice. (sarcomere shortening, $p = 0.2927$, $n=7$)

Fig.2 eNOS regulation of mitochondrial activity (oxygen consumption rate, OCR) in LV myocytes from rats and eNOS^{-/-} mice. OCR was measured using a fluorescence-based oxygen sensor (NeoFox,

Ocean Optics) connected to a phase measurement system (Instech). Bars represent average values $\pm$ SEM. **A** PA significantly increased OCR in LV myocytes from sham at control. L-NAME (1mM, 30min~1hr) prevented PA-induced OCR increase, but nNOS with SMTC (100nM, 30min~1hr) failed to affect the OCR by PA (OCR, μ M.30min, $p<0.0001$, control vs. PA; $p=0.008$, PA vs. L-NAME+PA; $p = 0.0239$, SMTC vs. SMTC+PA in shams). **B** PA did not induce an increase in OCR in eNOS^{-/-} mice (OCR, μ M.30min, $p=0.0329$, control vs. PA). **C** Flow chart shows: eNOS mediates PA-regulation of myocyte contraction through stimulating mitochondrial activity (oxygen consumption rate) in cardiac myocytes.

Fig.3 The palmitoylation and phosphorylation of eNOS by PA and its effects on mitochondrial activity in sham LV myocyte. **A** PA (Palmitic acid, 100 μ M) increased eNOS-palmitoylation in LV myocytes from sham rats ($p=0.023$, control vs. PA) and 2BP (100 μ M) prevented eNOS-palmitoylation ($p=0.005$, PA vs. PA+2BP in sham, $n=6$). **B** eNOS-Ser¹¹⁷⁷ was not increased by PA in LV myocytes from sham ($p=0.235$, control vs. PA). 2BP significantly reduced both basal eNOS-Ser¹¹⁷⁷ and eNOS-Ser¹¹⁷⁷ with PA in sham ($p=0.02677$, control vs. 2BP; $p = 0.00949$, PA vs. PA+2BP; $p=0.01294$, 2BP vs. PA+2BP). **C** 2BP showed negligible effect on PA-regulation of OCR; NOS inhibition with L-NAME prevented PA-dependent OCR increase.

Fig.4 Effect of eNOS-palmitoylation on PA-induced myocyte contraction in sham. **A, B, D&E** Post or pre-treatment of LV myocytes with 2BP did not reduce PA-induced myocyte contraction (sarcomere shortening, $p=0.0015$, PA vs. PA+2BP, post-treatment 2BP, $n=13$; $p=0.005$, 2BP vs. 2BP+PA, pre-treatment, $n=8$). **C&F** NOS inhibition (L-NAME, 1mM, 30min -1hr) abolished PA-dependent increase of myocyte contraction even in the presence of 2BP in sham (Sarcomere shortening, $p=0.406$, L-NAME+PA vs. L-NAME+PA+2BP, $n=8$). Bars represent average values $\pm$ SEM.

Fig.5 Palmitoylation of eNOS in LV myocytes from hypertensive rats and the effects on OCR

A In hypertension, PA did not increase eNOS palmitoylation ($p=0.58$, control *vs.* PA in HTN, $n=4$). However, 2BP significantly reduced basal eNOS palmitoylation in the presence and absence of PA ($p=0.04$, PA *vs.* PA+2BP in HTN). **B** Similar to that in sham, 2BP significantly reduced eNOS-Ser¹¹⁷⁷ in HTN with and without PA ($p=0.038$, PA *vs.* PA+2BP; $p=0.05$, control *vs.* 2BP, $n=3$). **C** 2BP reduced PA-induced OCR in HTN ($p=0.0177$, control and PA, $n=8$ and $n=9$; $p=0.0198$, PA *vs.* PA+2BP, $n=9$ and $n=10$; $p=0.2419$, 2BP *vs.* PA+2BP in HTN, $n=8$ and $n=10$).

Fig.6 Effect of 2BP on LV myocyte contraction from hypertensive rats. **A&D** PA-dependent positive inotropic effect was absent in HTN ($p=0.3896$, control *vs.* PA in HTN, $n=12$). **B&E** PA-dependent positive inotropic effect was unaffected by 2BP pre-treatment ($p=0.069$, 2BP *vs.* PA+2BP in HTN, $n=5$). **C&F** NOS inhibition with L-NAME significantly increased myocyte contraction with PA ($p=0.001$, L-NAME *vs.* L-NAME+PA in HTN, $n=7$), 2BP did not affect such an effect ($p=0.2668$, L-NAME+PA *vs.* L-NAME+PA+2BP in HTN, $n=7$).