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Metabolic substrate regulation of cardiac myocyte contraction and insulin response in normal and hypertensive rats

정상 및 고혈압 백서에서 대사물질에 의한 심근수축 변화와 기전

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Metabolic substrate regulation of cardiac myocyte contraction and insulin response in normal and hypertensive rats

by

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ABSTRACT

Fatty acid-dependent metabolism is predominant in cardiac ATP production that maintains normal contractile function of the heart. However, excessive fat or fatty acids (metabolic syndrome) are the precursors of cardiovascular complications, such as ventricular arrhythmias (sudden cardiac death), heart failure and stroke. Insulin resistance in muscle is one of the key underlying mechanisms for the adverse effects of increased metabolites. Until recently, investigation of metabolic substrates' regulation of cardiac contractile function and insulin responsiveness *in vitro* is lacking. Therefore, we design to study whether supplementation of metabolic substrates (oleic acid 200μM, palmitic acid 100μM, linolic acid 100μM, lactate 1 mM, pyruvate 100μM and carnitine 50μM) to normal tyrode (NT) perfusate (termed nutrition full, NF) affects basal and beta-adrenergic left ventricular (LV) myocyte contractility and changes insulin response in normal and angiotensin II (Ang II)-induced hypertensive rat hearts.

Our results demonstrated that basal and isoprenaline (ISO, 100 nM)-stimulated myocyte shortening (field stimulation, 2Hz, 36 ± 1 ^{o}C) were significantly increased with NF in LV myocytes from normal and hypertensive rat hearts. In NT, insulin (10 nM) abolished ISO-increase in LV myocyte contraction from normal rats. This effect was prevented by nitric oxide synthase (NOS) inhibitor, L-NG-nitroarginine methyl ester (L-NAME, 1mM) but not by neuronal NOS (nNOS) inhibition with S-methyl-L-thiocitrulline (SMTC, 100 nM), suggesting eNOS-mediated anti-adrenergic response of insulin. In NF, insulin did not change basal or ISO-stimulated myocyte contraction in either group, suggesting reduced insulin response. Furthermore, L-NAME did not affect myocyte contraction in the presence or absence of

insulin in NF.

On the other hand, ISO induced spontaneous contractions (arrhythmias) in NF and the percentage of arrhythmic incidence was significantly greater in hypertension. Ranolazine (10 μM), inhibitor of carnitine palmitoyl transferase 1 (CPT-1), that is known to inhibit late Na^+ current, did not affect NF-enhancement of myocyte contraction in normal or hypertensive hearts but significantly reduced arrhythmias in both groups. Interestingly, SMTC significantly increased arrhythmias in normal but reduced it in hypertension, suggesting contrasting roles nNOS play in the rhythmic contraction in the presence of metabolic substrates between normal and hypertension.

Taken together, our results demonstrate that metabolic substrate supplementation improves myocyte contraction but impairs insulin response and induces arrhythmias with beta-adrenergic stimulation. nNOS plays an important role in cardiac arrhythmogenesis in the presence of metabolic stress.

Keywords: fatty acids, metabolic substrates, beta-adrenergic stimulation, insulin response, hypertension, nitric oxide synthase, left ventricular myocyte

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CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

NF= nutrition full solution

nNOS =neuronal nitric oxide synthase

eNOS= endothelial nitric oxide synthase

FA= fatty acid

HF= heart failure

HP= hypertension

LV= left ventricular

NO= nitric oxide

ROS= reactive oxygen species

INS= insulin

ISO= Isoprenaline

NAC= N-Acety-I-cysteine

SMTC= S-methy-1thiocitrulline

L-NAME= nitro-L-arginine methyl ester hydrochloride

RANO= ranolazine

Ph= peak height

Rv=return velocity

Ang II= angiotensin II

INTRODUCTION

The heart is a pump that works rhythmically against pressure and volume loads for approximately 100,000 beats and pumps 7600 liters of blood per day throughout lifetime. Therefore, the contractile function of the heart is crucial for supplying oxygen/nutrients and for removing the waste products through circulation system. In broad terms, cardiac contraction is governed by excitation-contraction coupling (E-C coupling) and myocardial metabolism. The former mechanism centralizes Ca^{2+} signals as a mediator between the membrane electrical activity and contractile myofibrils. The latter mechanism emphasizes the metabolic pathways and ATP production. Respectively, these two mechanisms are fairly well established in myocardial physiology and pathology. Until recently, almost all E-C coupling studies are conducted with glucose as the sole metabolic substrate (~10 mM instead of 5.6 mM) despite the fact that fatty acids are the predominant fuel for ATP in healthy cardiac myocytes. Therefore, our current understandings and the interpretations of the E-C coupling and cardiac contraction are based almost entirely on the results performed with glucose containing Tyrode solution (10 mM). Importantly, emerging evidence show that the E-C coupling and myocardial metabolism processes actually interact with each other, *i.e.* Ca^{2+} release from the SR is the source of Ca^{2+} influx into mitochondria through uniporter, which play critical roles in regulating intracellular signaling, energetic homeostasis and myocardial function [Kohlhaas et al., 2010]. Therefore, Ca^{2+} handling may interplay with cardiac metabolism dynamically during metabolic transition. However, how incorporation of cardiac metabolism regulates myocardial contraction in healthy and diseased heart remains unknown.

Myocardial metabolism in healthy and diseased heart

Normal myocardium consumes at least 6 Kg of ATP per day to maintain the pumping function of the heart. This amount has to be supplied constantly through metabolic pathways since myocardium has limited store for ATP (ATP vanishes within 6-10 beats) [Braunwald's Heart Disease, Text book].

In healthy adult heart, fatty acid oxidation (FAO) is the predominant source of ATP (accounts for ~70-90% of cellular ATP). FAs those are supplied from chylomicron triacylglycerol or albumin bound free FAs enter the cardiac myocytes by various FA transporters on the plasma membrane (e.g. FAT/CD36, FA binding protein and FA transport protein). FAs in the cytosol immediately esterified to long chain fatty acyl CoA and shuttled into mitochondrial matrix after the formation of long chain acylcarnitine by carnitine palmitoyltransferase I (CPT-I), a key enzyme that determines the fate of FA for beta-oxidation. Acetyle CoA carboxylase (ACC) and malonyl CoA decarboxylase (MCD) are important enzymes that produce and degrade malonyl CoA, the endogenous inhibitor of CPT-1. The acylcarnitine is converted back to fatty acyl CoA by CPT 2 in the inner membrane of mitochondria and enters the fatty acid beta-oxidation cycle, producing acetyl CoA, NADH, and FADH2 and subsequently ATP. In addition, FAO is dynamically controlled by transcriptional regulation of proteins involved in FAO by factors such as peroxisome proliferator activated receptor (PPAR) and peroxisome proliferator activated receptor gamma co-activator (PGC-1).

The other metabolic pathway, glucose-dependent glycolysis and oxidation account for 10-30% of ATP production in the myocardium under normal conditions. Glucose enters cytosol *via* transporters (GLUT-1 and GLUT-4) and converts to glucose-6-phosphate (G6P) which undergoes irreversible glycolytic pathway to produce two molecules of ATP, NADH and two pyruvate. Pyruvate can then transport into mitochondria and is decarboxylated to acetyl CoA by pyruvate

dehydrogenase (PDH). Acetyl CoA from both glucose and fatty acids enter tricarboxylic acid cycle (TCA) and electron transport chain (ETC) to produce ATP. During the progression of heart diseases, the availability and the uptake of metabolic substrates are altered and favor glycolysis to FAO (metabolic paradigm shift). However, at advanced failing stage, almost all the steps of glycolysis and FAO are dysregulated, account for ATP depletion in the failing myocardium [JM. Berthiaume et al., 2012]. Furthermore, metabolic shift is also associated with increased serum fatty acid level in heart disease patients [McDaniel HG et al., 1985]. Currently, fatty acids are emerging to be instrumental in affecting adverse myocardial function and remodeling through lipotoxicity, oxidative stress or inducing inflammation [*C*. Vigouroux et al., 2011].

On the other hand, over-nutrition and metabolic syndrome are becoming global threatens to the public health worldwide. Accumulating evidences demonstrate that metabolic syndrome is associated with type 2 diabetes, atheroscrosis, coronary artery diseases, arrhythmias (atrial fibrillation & sudden cardiac death), hypertension and heart failure [Whaley-Connell A et al., 2011]. Integration of E-C coupling and cardiac metabolism reveals better understanding of impaired myocardial contraction in human failing myocardium.

Insulin signaling in normal and diseased heart function

Insulin is a hormone that plays an important role in coupling metabolism with cardiovascular physiology and pathology. Insulin binds to insulin receptors in the plasma membrane of cardiac myocytes, leading the activation of mainly two signaling pathways: Ras-MAPK, which results in cell proliferation; and PI3K-Akt-eNOS, which results in metabolic modulation and cardiovascular protection. Among the insulin-activated signaling cascades, PI3K-Akt-eNOS-NO represents a special link between insulin and the cardiovascular system with regard to health to pathology [Q.Yu et al., 2011]. Upon stimulation, activation of the insulin receptor

tyrosine kinase that phosphorylates insulin receptor substrate-1 (IRS-1) leads to the activation of PI3K and subsequent activation of phosphoinositide-dependent protein kinase-1 (PDK-1). Downstream, serine-threonine kinase Akt is then phosphorylated and activated, which directly causes eNOS Ser^{1177} phosphorylation, and subsequently increases NO production within minutes [Montagnani M et al., 2002]. Consequently, insulin regulates the translocation of glucose and fatty acid transporters to plasma membrane and increases the uptake and metabolism of these metabolic substrates in cardiac myocytes [Luiken JJ et al., Diabetes, 2002]. Parallel and balanced insulin signaling between PI3K-Akt-eNOS and Ras-MARK maintains normal cardiovascular growth, metabolism and function in healthy conditions.

Impairment of insulin-PI3K-Akt-eNOS-NO signaling defines a key characteristic of insulin resistance and cardiovascular dysfunction. In diseased heart, where Akt activation and eNOS phosphorylation are decreased, together with hyperglycaemia-induced eNOS glycosylation by O-GlcNAc modification, leads to reduced eNOS-derived NO generation and mitochondrial dysfunction with resultant enhanced .O2- production, eNOS uncoupling, and oxidative stress [Q Yu et al., 2011].

It is becoming clear that excess nutrients in the form of fat and carbohydrates promote insulin resistance in cardiovascular tissue as well as conventionally insulin-responsive tissue such as muscle, fat and liver [Sowers JR et al., 2011; Whaley-Connell A et al., 2011; Pulakat L et al., 2011]. In addition to over-nutrition, several other mechanisms, such as enhanced activation of the renin-angiotensin-aldosterone sytem (RAAS), hypertension, and inflammation further antagonize insulin metabolic signaling [Lastra G et al., 2009]. Although increased oxidative stress has mostly been implicated in insulin resistance, emerging evidence indicate that over-nutrition, Ang II and aldosterone promote insulin resistance through the activation of the mammalian target of rapamycin (mTOR)/S6 kinase (S6K1) signaling pathway [Sowers JR et al., 2011]. This action leads to decreased protein kinase B

(Akt) activation/downstream insulin signaling and to reduced myocardial and skeletal muscle glucose uptake, impaired nitric oxide (NO) production in endothelial cells, and reduced myocardial glycogen synthase activity and generation of ATP [Varma S et al., 2008; Malhotra JD et al., 2011]. Insulin resistance and dysregulated mitochondrial fatty acid oxidation cause increased myocardial accumulation of fatty acids and reduced myocardial glucose and fatty acid utilization [Sowers JR et al., 2011].

Constitutive nitric oxide synthase & cardiac metabolism

Constitutive NOS is well known to be involved in the regulation of myocardial function. Under physiological conditions, NOS-derived nitric oxide regulates almost all aspects of cellular function, including increasing myocardial distensibility, reducing stiffness, lowering diasolic pressure, facilitating relaxation, fatty acid uptake and utilization [Moncada S et al., 1991; Paulus WJ et al., 1994; shah AM et al., 2000]. NO is generated by NOS, which catalyzes the conversion of L-arginine to L-citrulline in a reaction that requires O_2 and cofactors [Palmer RM et al., 1988]. Currently, three distinct isoforms of NOS have been identified and cloned: two are constitutively expressed in cardiac myocytes (neuronal NOS or nNOS or NOS1, endothelial NOS or eNOS or NOS3).

eNOS is described and purified from the bovine vascular endothelium [Pollock JS et al., 1991] and studied extensively in many other tissues afterwards. eNOS traffics from the cytoplasmic face of cavolae to intracellular domains, such as the Golgi complex or the nucleus, and can bind to the RyR following left ventricular injury [Feng Y et al., 1999; Martinez-Moreno M et al., 2005; Grasselli A et al., 2008]. eNOS activity was thought to be principally regulated by intracellular Ca^{2+}; however, more recent evidence has suggested phosphorylation at Ser^{1177} residue and activation of the enzyme by the serine-threonine kinases, *e.g.* Akt murine thymoma viral oncogene homolog Akt, also known as Protein Kinase B [Dimmeler S et al.,

1999; Fulton D etal., 1999] or protein kinase A (PKA) in response to fluid shear stress [Dixit M et al., 2005]. More recently, eNOS activity has been shown to be increased in response to beta3-adrenergic receptor stimulation [Brixius K et al., 2004] which may be critical to maintain a sustained activation of this enzyme by reducing dissociation of calmodulin from activated eNOS in the presence of intracellular Ca^{2+} [McCabe TJ et al., 2000] and moderate beta-adrenergic responses.

nNOS is firstly purified from the rat cerebellum [Bredt DS et al., 1990] and is the major source of NO in the nervous system. In 1999, Xu et al. identified that nNOS is expressed in mammalian myocardium and functionally regulates SERCA reuptake of Ca^{2+} into sarcoplasmic reticulum. Over the last decade, accumulating evidence have shown that nNOS modulates cardiac inotropy and facilitates lusitropy by targeting protein phosphatase activity and PKA-dependent decrease of L-type Ca^{2+} channel activity [Sears CE, et al., Circ Res, 2003] and increase of phospholamban phosphorylation [Zhang YH et al., Circ Res, 2008]. In addition, nNOS is shown to modulate the activities of NADPH oxidase and xanthine oxidoreductase [Kinugawa S et al., 2005; Zhang YH et al., 2009], thereby controlling the redox homeostasis in the myocardium. So far the role of nNOS in insulin regulation of myocardial contractile function was unknown.

Aim of the study:

In the present study, we aim to investigate

1) Whether metabolic substrates supplementation affects basal and beta-adrenergic stimulated contraction of LV myocytes isolated from normal and hypertensive rats;

2) Whether metabolic substrates supplementation affects insulin response in LV myocytes from normal and hypertensive rats.

3) Examine the role of nNOS in regulating LV myocyte contraction following metabolic substrate supplementation in normal and hypertensive rats.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (12wk old, male) were used in this study. In some experiments, rats were subjected to angiotensin II (Ang II) infusion subcutaneously using osmotic minipump for 4wks. Those animals were paired with sham-operated groups. Briefly, rats (of 8wk old, male) were anesthetized with isoflurane (2.5%). Osmotic minipumps for rats (Alzet model 2004) containing Ang II (200μl, 6mM, infusion rate 125 ng/min/Kg) were implanted in the mid of scapulars region under sterile condition. Sham-operated animals underwent the same surgical procedure, except for the pump insertion (Figure 2). Blood pressures were measured every 3 days from 3 days *before* the surgery by using Non-Invasive Blood Pressure System, tail-cuff method (CODA, Torrington, CT, USA). Rats were warmed at 37℃ for 25min before the measurement; each value used in the analysis was the means of ten readings (Figure 3).

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-120822-7, SNU-101213-1-1).

Isolated left ventricular myocytes

Rats were anesthetized with pentobarbital sodium (30 mg/kg, i.p.), pinned out and the thorax was rapidly opened up. The heart was exposed and the thymus gland removed to expose the aorta. A suture was looped around the aorta and then an incision was made in the aorta into with a cannula (made from a blunted 19G needle,

filled with Ca^{2+} free isolation solution 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) was inserted. The suture was tightened in order to secure the cannula and the heart was removed from the chest cavity. The cannulated heart was then secured to the Langendorff perfusion system taking care not to introduce any bubbles into the coronary circulation, as this is known to affect the efficiency of the isolation procedure.

The heart was perfused with Ca^{2+}-free isolation solution, which was aerated with 100% oxygen and maintained at 37℃, for 10 min and then with a primary enzyme solution, containing 1mg/ml of collagenase type II (Worthington biochemical Co., Lakewood, NJ, USA), 0.1mg/ml of protease, 1.67mg/ml bovine serum albumin (BSA) and 50uM Ca^{2+} in isolation solution, which was also aerated with 100% oxygen and maintained at 37℃, for 8min. At the end of this period, the heart was removed from the perfusion system and the needle, suture, aorta and any extraneous tissue removed so that the heart could be accurately weighed. Once weighed, the atria and the right ventricles were carefully dissected away and the LV was dissected into smaller pieces and put in a 50 ml Erlenmeyer flask with 10 ml of secondary enzyme solution (containing 1mg/ml collagenase, 1.67 mg/ml BSA and 50 μM Ca^{2+} in isolation solution).

The flask was shaken at 80 rev/min of 10 min in a 37 ℃ water bath, after which it was taken out and the LV tissue allowed to settle, and then the supernatant solution filtered into a centrifuge tube. A further 10 ml of the secondary enzyme solution was added to the remaining LV tissue which was again shaken at 80 rev/min for a further 10 min of 1% BSA in isolation solution was added to the supernatant in the centrifuge tube which was then centrifuge at 600 rpm for 2 min. After this, the supernatant was removed and the pellet containing the LV myocytes was re-suspended in storage solution (in mM: NaCl 20, KCl 5.4, $MgSO_4$ 5, Taurine 20, HEPES 10, Na-pyruvate 5, Glucose 5.5, $CaCl_2$ 0.2, mannitol 29; pH titrated to 7.40 using NaOH). The yield of rod shaped LV myocytes ranged from 50 to 80% of

the total number of cells isolated. The myocyte suspension was stored at room temperature and cells were used within 8 hours of isolation.

Measurement of LV myocyte contraction

The recording bath was made up of a Perspex-sided chamber with a microscope coverslip floor, mounted on the stage of a high-resolution inverted microscope (Diaphot 200, Nikon, JP) supported on a pneumatic anti-vibration table. HEPES-buffered perfusion solution (in mM; NaCl 141.4, KCl 4, NaH_2PO_4 0.33, HEPES 10, Glucose 5, mannitol 14.5, $CaCl_2$ 1.8, $MgCl_2$ 1; pH titrated to 7.40 using NaOH) perfused by a speed entered from the left hand side of the bath after being heated to 36±1 ℃ by passage through a thermostatically controlled heater situated at the entrance to the perfusion bath. The solution was subsequently removed by suction from the right hand side of the bath ensuring a constant and adjustable flow of the perfusion solution across the recording bath chamber.

A suspension of LV myocytes in storage solution was placed in the recording bath and the cells allowed to settle to the bottom of the bath. Once settled, perfusion solution was perfused at a constant rate (2 ml/min) and the temperate maintained at 36±1 ℃.

LV myocytes were stimulated with electrodes placed in the bath and connected to a digital stimulator (Medical Systems Corp, Greenvale, NY, USA). Stimulation voltage was maintained at 10V and frequency was maintained at 2 Hz.

Changes in myocyte sarcomere length were displayed on the computer by the acquisition program (IonOptix Corp, Milton, MA, USA, Figure 1). And recordings were stored on-line for subsequent off-line analysis. Measurements from at least 10 steady state contractions were averaged for each cell.

Chemicals

Angiotensin II (Ang II, Sigma) was used to induce hypertension. L-NAME (1mM, Sigma) and SMTC (100 nM, Sigma) was used to target eNOS and nNOS activity, respectively. Isoproterenol (ISO, 10-50 nM) was used to stimulate amplitude of LV myocyte contraction. Membrane permeable amid (14-22, PKI, 100nM, Merck Biosciences, Darmstadt, Germany) was used to inhibit the activity of PKA. Insulin (10 nM, sigma) was used to stimulate intracellular metabolism, NAC (1mM, sigma) was used to reduce intracellular oxidation. Ranolazine (10μM, sigma) was used to inhibit the activity of CPT-1. Metabolic substrates were supplemented to the normal tyrode (NT) solution (Table 1).

Statistics

Data are expressed as means ± s.e. 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 one way ANOVA or student's unpaired test. A value of $P < 0.05$ was considered to be statistically significant.

Table 1. Chemical compositions in "nutrition full-NF" solution.

Normal Tyrode	**Metabolic substrate**
NaCl (141.4mmol)	**Palmitic acid (100 μM)**
KCl (4mmol)	**Oleic acid (200 μM)**
NaH_2PO_4 (0.33mmol)	**Linoleic acid (100 μM)**
$MgCl_2$ (1mmol)	**Pyruvate (100 μM)**
HEPES (10mmol)	**Lactate (1 mM)**
Glucose (5.5mmol)	**Carnitine (50 μM)**
$CaCl_2$ (1.8mmol)	
Mannitol (14.5mmol)	
Nutrition Full Solution(NF)=Normal Tyrode+ Metabolic substrates	

The composition and the concentrations of metabolic substrates are modified from rodent's blood sample results (Bhagavan et al., 2009). It should be noted that lower end concentrations are chosen for the metabolic substrates with a fairly wide concentration range.

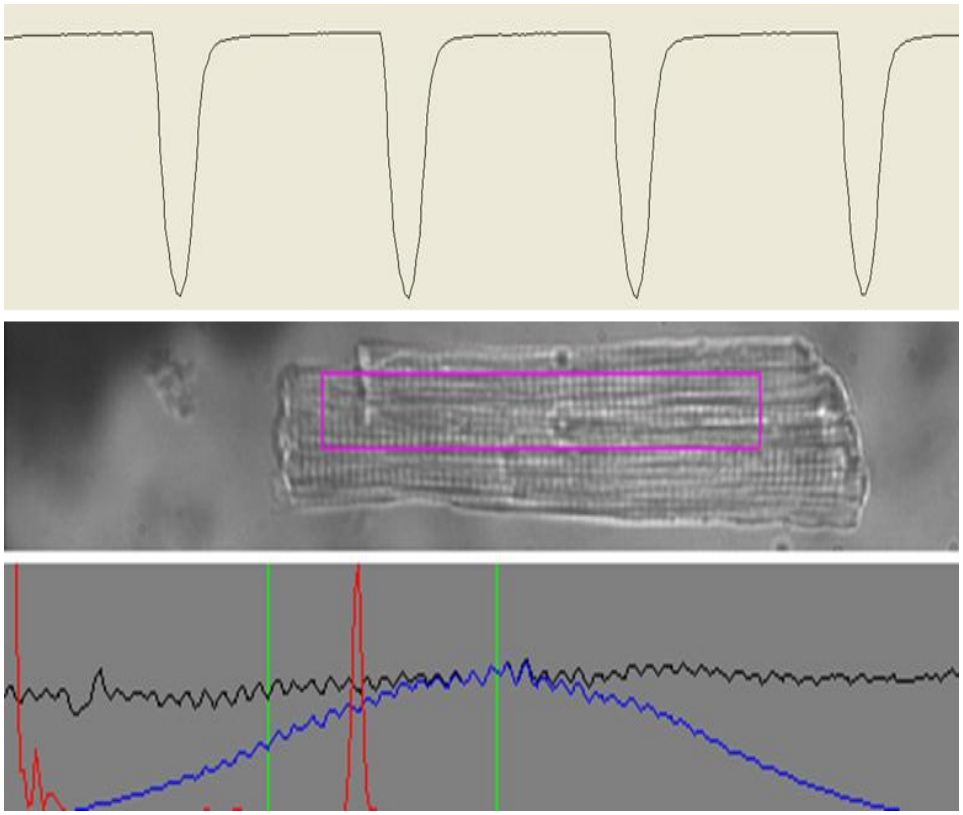

Figure 1. Recordings of sarcomere length in isolated myocyte using IonOptix software

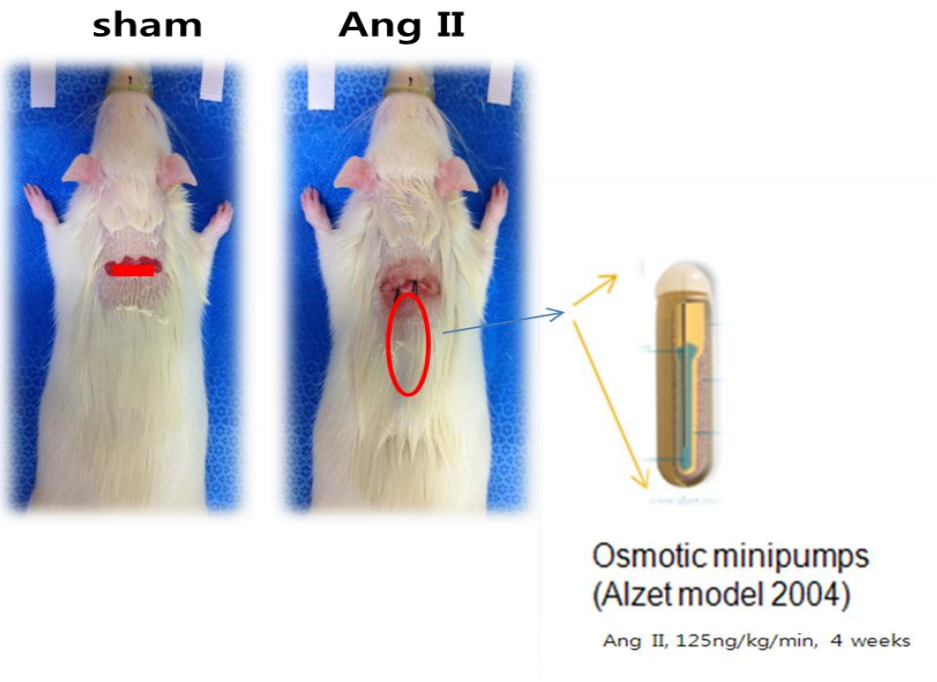

Figure 2. Sham and hypertension model (induced by angiotension II infusion *in vivo*).

Ang II-induced hypertensive rats (*via* osmotic minipump, 125 ng/min/kg, 4 weeks).

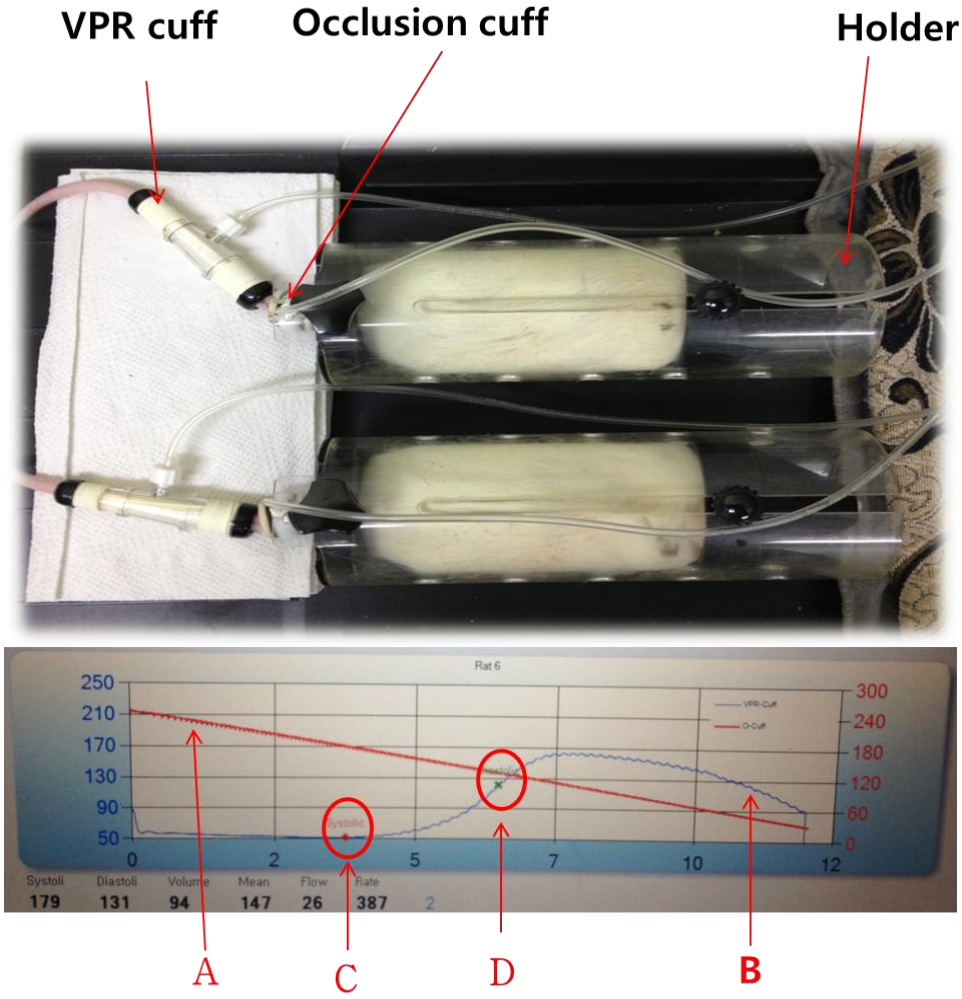

Figure 3. Non-invasive tail-cuff blood pressure measurement.

A. Occlusion cuff (O-cuff) waveform, which is displayed as a thin red line. B. Volume Pressure Recoding sensor cuff (VPR-cuff) waveform, which is displayed as a thick blue line. C. The red maker on the VPR-cuff waveform shows where the event of systolic blood pressure occurred. D. The green marker on the VPR-cuff waveform shows where the event of diastolic blood pressure occurred.

RESULTS

Part I:

Effect of metabolic substrates supplementation on LV myocyte contractile function in normal rat hearts

Metabolic substrates supplementation (NF) increases LV myocyte contraction and facilitates the speed of relaxation

In NT, the amplitude of sarcomere length of LV myocytes from normal heart in response to field stimulation is 1.773 μm (±0.005, n=100). Beta-adrenergic stimulation with isoprenaline (ISO, 10-50 nM) significantly increased the amplitude of sarcomere length and increased the return velocity of relaxation (P<0.001 for contraction; P<0.001 for relaxation, n=29, Figure 4C, D).

Next, the effect of NF on LV myocyte contraction was determined. As shown in Figure 4B, after myocyte contraction reached steady-state, perfusion of NF solution induced time-dependent biphasic changes: reduced contraction after 1-2 min of NF perfusion (changes in sarcomere length, 0.006 ± 0.078, P=0.02, n=29, n=22), followed by gradual increase in the sarcomere length which reaches the steady-state at about 5 min (P<0.001, n=29, n=22, Figure 4C). Application of ISO increased myocyte shortening further (0.014 ± 0.219, P<0.001, n=22). Return velocity was also enhanced by NF and by NF+ISO (P<0.001, n=22 with NF; P<0.001, n=22 with NF+ISO Figure 4D). Notably, ISO in the presence of NF induced spontaneous contractions (arrhythmias) in almost all the myocytes tested (P<0.001, n=22, Figure 4E).

In order to determine whether free fatty acids can still affect LV myocyte contraction, LV myocyte contraction was assessed with 3 types of FAs. As shown in Figure 5A, the amplitude of LV myocyte shortening was significantly increased

with 3FA, and application of ISO increased myocyte shortening further. (0.011±0.147, P=0.002, n=9 with 3FA; 0.017±0.235 P<0.001 with 3FA+ISO, Figure 5B).

These result demonstrated that NF supplementation in NT solution increased both basal and beta-adrenergic receptor-stimulated LV myocyte contraction in normal rats. Importantly, beta-adrenergic stimulation induced arrhythmias in the presence of the metabolic substrates supplementation.

Effect of antioxidant, N-acetylcysteine (NAC) on NF-increased LV myocyte contraction and NF-induced arrhythmias

Metabolic substrates supplementation may stimulate cardiac metabolism (oxidative phosphorylation) and increase the superoxide production from mitochondria. Since superoxide has been implicated to activate protein kinase A [Eaton P et al., 2012] which may potentiate myocyte contraction, we tested whether NF-increased LV myocyte contraction is mediated by intracellular superoxide. As shown in Figure 6B, incubation of LV myocytes with NAC (1 mM, 20 min) did not affect NF-induced increase in LV myocyte contraction (P=0.10, n=22, n=16). Similarly, ISO was able to further increase myocyte contraction under these conditions (P<0.001, n=16), although quantitatively smaller compared to that without NAC (Figure 6C). It should be noted that NAC pre-treatment reduced the number of contracting myocytes before and after NF application. In addition, NF+ISO significantly increased the frequency of arrhythmias in the presence of NAC (P<0.001, n=20, n=13, Figure 6E). The return velocity parallels the changes of myocyte shortening, therefore, this parameter will not be mentioned in the following sections.

These results suggest that increased LV myocyte contraction by NF is independent of intracellular superoxide. Furthermore, basal oxidative status is required for normal rhythmic contraction of cardiac myocytes.

Effect of carnitine palmitoyltransferase I (CPT-1) on NF-regulation of LV myocyte contraction

Under normal conditions, fatty acid-dependent metabolism accounts for ~60-90% of ATP production in cardiac myocytes, as such, CPT-1 transport of the derivative of fatty acid, carnitine, a substrate for beta-oxidation in mitochondria plays a pivotal role in the energy supply and cardiac function [N. Fillmore, G.D. Lopaschuk et al., 2013]. Therefore, we aim to examine whether inhibition of CPT-1 may attenuate the positive inotropic effect of NF. As shown in Figure 7B & C, ranolazine (10 μM) did not affect myocyte inotropy in NT, NF or in NF+ISO (P=0.16, P=0.74; P=0.33, n=22, n=6). Interestingly, ranolazine significantly decreased the frequency of arrhythmias (NF+ISO and NF+ISO+RANO: P=0.05, n=22, n=6 Figure 7E).

Effect of insulin on NF-induced LV myocyte contraction

Insulin is an important peptide that regulates glucose and fatty acid-dependent metabolism in cardiac myocytes and therefore plays an important role in the maintenance of myocardial function [Randle et al., 1963]. It has been shown that insulin exerts its function by attenuating beta-adrenergic receptor signaling [Feng Gao et al., 2008]. Accordingly, we examined whether insulin affects LV myocyte contraction in the presence and absence of NF. As shown in Figure 8A & C, in NT, insulin did not affect the amplitude of sarcomere length under basal conditions (between NT and NT+INS: P=0.18, n=29). However, it abolished beta-adrenergic stimulation of myocyte contraction (sarcomere length between NT+ISO and NT+INS+ISO: P<0.0001, n=29, n=29). In contrast, insulin did not affect the amplitude of sarcomere length in NF or that in NF+ISO (between NF and NF+INS: P=0.995, n=20; between NF+ISO and NF+ISO+INS: P=0.30, n=20 Figure 8B & C).

Insulin exerts its function by binding to insulin receptor on the plasma membrane of cardiac myocytes. Activation of insulin receptors stimulates Ras-mediated MAPK signaling to initiate proliferation or PI3K-dependent and eNOS mediated

signaling pathways to affect metabolism [Q. Yu et al., 2010]. Therefore, it is possible that the anti-adrenergic effect of insulin in NT is mediated by eNOS. To test this hypothesis, insulin response were observed in LV myocytes pre-incubated with L-NAME (1mM, 30 min), a non-specific inhibitor of cardiac NOSs. As shown in Figure 9A & C, L-NAME per-treatment restored ISO-stimulation of LV myocyte contraction in NT (P=0.03, n=7). In contrast, L-NAME did not change the amplitude of sarcomere length under basal conditions or after ISO treatment in NF (between NF and NF+ISO P=0.19, n=8; between NF+INS+ISO P<0.001, n=8 Figure 9B & D). Interestingly, inhibition of cardiac NOS with L-NAME significantly increased the frequency of arrhythmias in NF+ISO (between NF+ISO and NF+ISO+L-NAME, P=0.03, n=20, n=8, Figure 9E).

Since nNOS has been shown to be the endogenous NOS that regulates basal and beta-adrenergic stimulated myocardial function [Sears CE et al., 2003; Zhang YH et al., 2008], the involvement of nNOS in insulin-regulation of myocyte contraction was also investigated. As shown in Figure 10, nNOS inhibition with SMTC (100 nM, 30min) did not affect basal LV myocyte contraction in NT with and without insulin (between NT and SMTC in NT, P=0.45, n=29, n=22; NT+INS and NT+INS+SMTC, P=0.36, n=29, n=22). In addition, it did not restore insulin-abolishment of the positive inotropic effect of ISO in NT (between NT+INS+SMTC and NT+INS+ISO+SMTC, P=0.30, n=22). Furthermore, SMTC did not affect LV myocyte contraction in NF or in NF+ISO (between NF+INS and NF+INS+SMTC, P=0.29, n=10, n=10; between NF+INS+ISO and NF+INS+ISO+SMTC P=0.26, n=20, n=10 Figure 10B & C). However, SMTC significantly increased the frequency of arrhythmias in NF+ISO (between NF+ISO and NF+ISO+SMTC, P=0.03, n=20, n=11 Figure 10E).

Taken together, these results demonstrate that in LV myocytes from normal rat heart, metabolic substrates supplementation abolished insulin-induced and eNOS-mediated anti-adrenergic response. Moreover, nNOS controls the myocyte from irregular beating under beta-adrenergic and metabolic stress conditions.

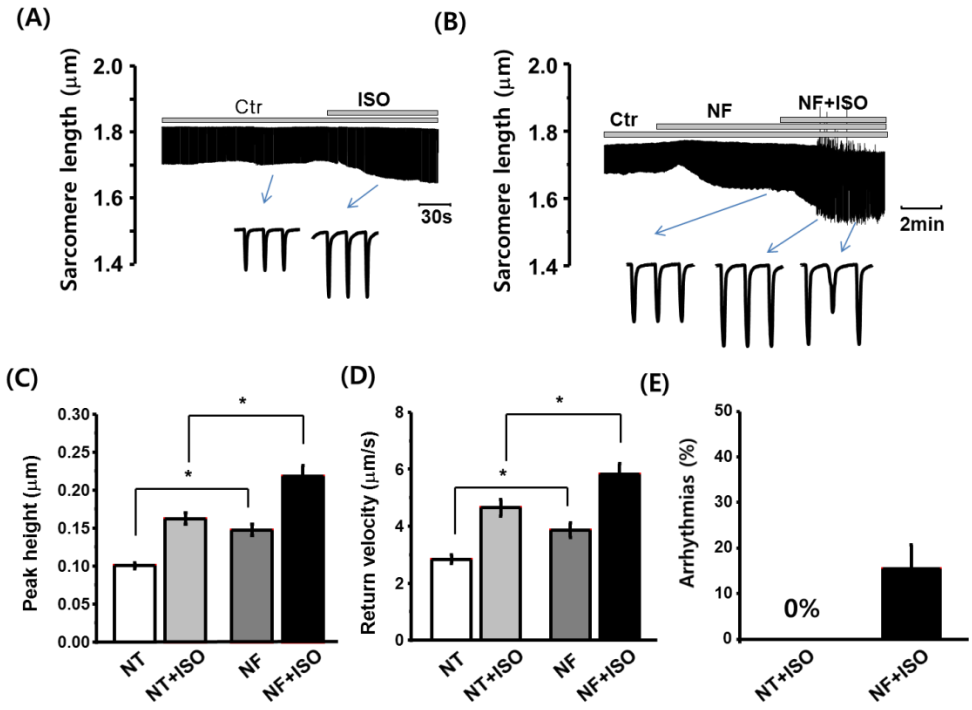

Figure 4. NF significantly increased LV myocyte contraction in normal rat heart and induced arrhythmias in the presence of beta-adrenergic stimulation with isoprenaline.

A,B. Representative raw traces of sarcomere shortening and re-lengthening in the absence (left) and presence (right) of NF. C,D. Average values of the amplitude of sarcomere shortening was greater with NF under basal conditions (between NT and NF, $P<0.0001$, n=29, n=22) and after ISO pre-treatment (between NT+ISO and NF+ISO , $P=0.0003$, n=29, n=22) D. Average values of the amplitude of return velocity was similar with sarcomere shortening.(between NT and NF, $P=0.0007$, n=29, n=22; between NT+ISO and NF+ISO, $P=0.01$, n=29, n=22 .) E. Mean values of the percentage of arrhythmias induced between NT+ISO and NF+ISO.

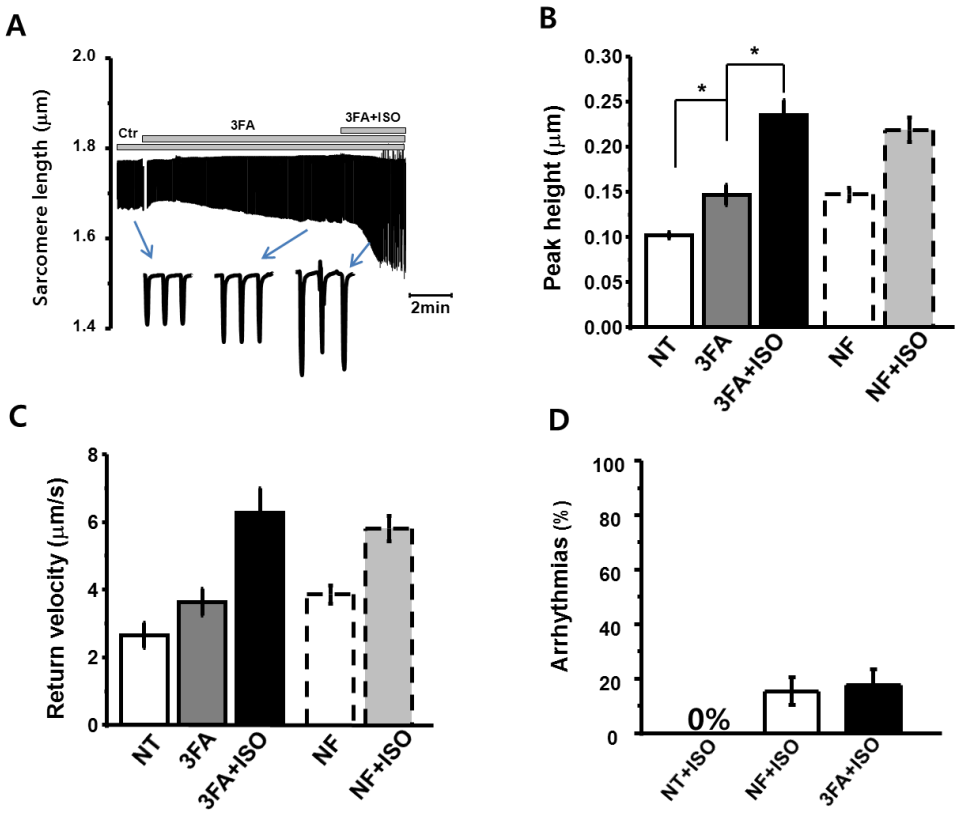

Figure 5. Supplementation of 3 types of fatty acids significantly increased LV myocyte contraction in normal rat heart.

A. Representative raw traces of sarcomere shortening and re-lengthening in the presence of 3FA. B. Average values of the amplitude of sarcomere shortening was greater with 3FA under basal conditions (between NT and 3FA, P=0.005, n=9) and after ISO pre-treatment (between 3FA and 3FA+ISO, P<0.001, n=9). C. Average values of the amplitude of return velocity were similar with sarcomere shortening. (Between NT and 3FA, P=0.008, n=9; between 3FA and 3FA+ISO, P=0.001, n=9). D. Average percentage of arrhythmias was increased in the presence of 3FA.

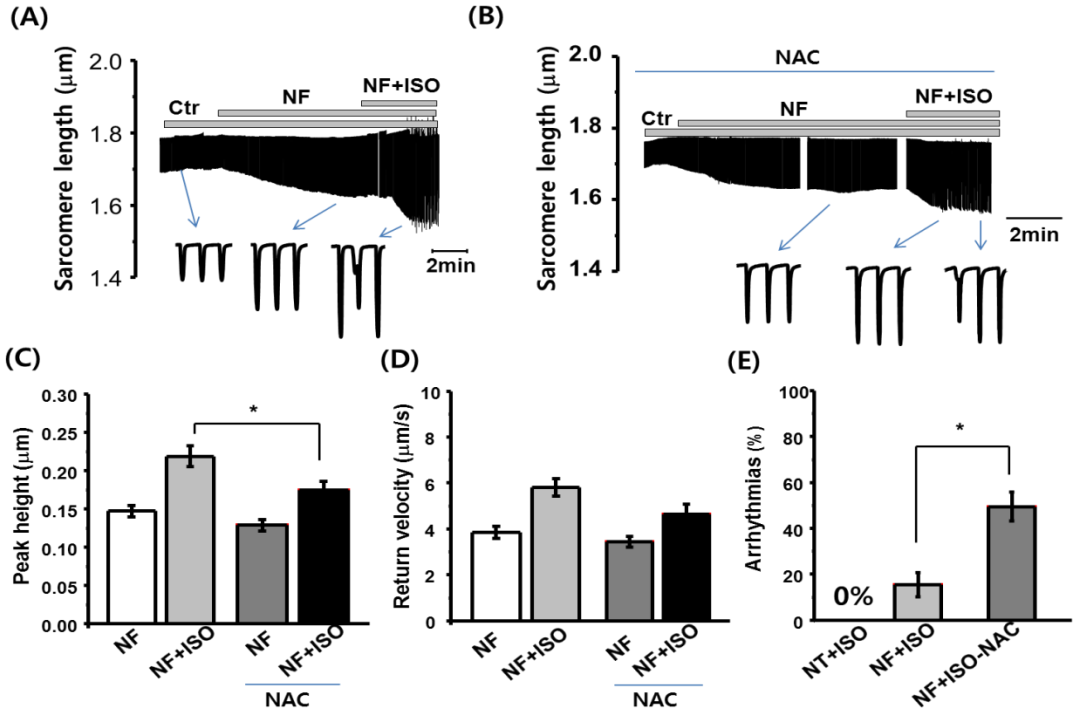

Figure 6. Potent antioxidant, NAC, did not block NF enhancement of LV myocyte contraction in the presence or absence of isoprenaline in normal rat. Paradoxically, arrhythmias were significantly increased with NF in NAC pre-treated myocytes in normal rat heart.

A,B. Representative raw traces of sarcomere shortening and re-lengthening in the absence (left) and presence (right) of NAC (1mM, 20min). C. Average values of the amplitude of sarcomere shortening were significantly reduced by NAC in NF+ISO (P=0.02, n=22, n=16), but not affect in NF. D. RV was not affect by NAC. E. NAC increased the percentage of arrhythmias appearing (between NF+ISO and NF+ISO+NAC P=0.0003, n=20, n=13).

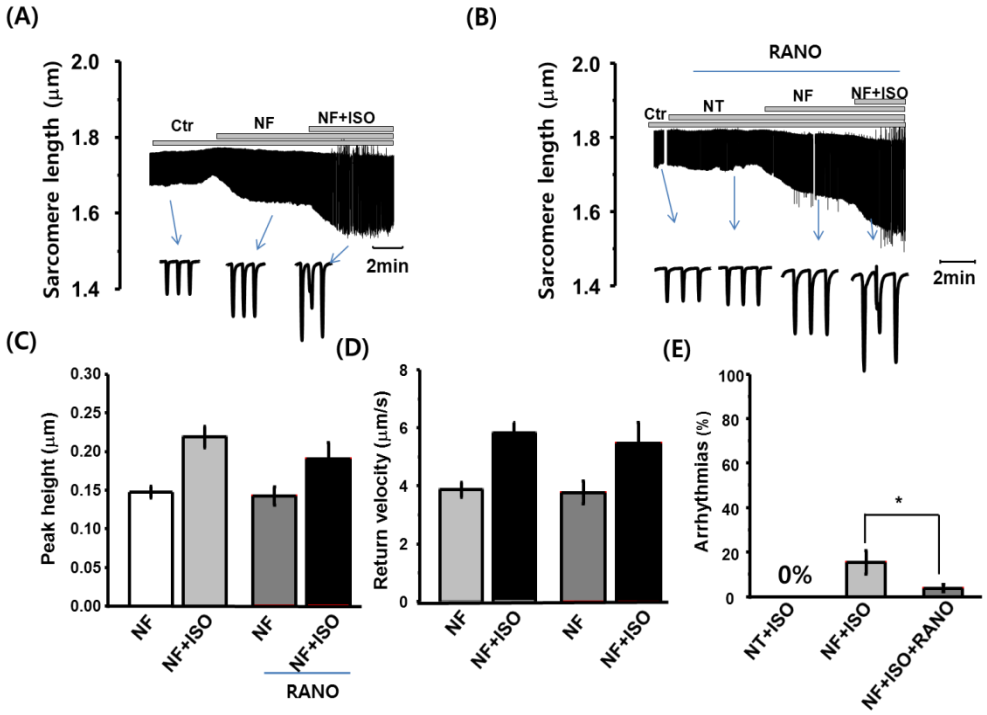

Figure 7. Ranolazine did not affect NF enhancement of LV myocyte contraction in the presence or absence of isoprenaline in normal rat heart. However, arrhythmias were significantly reduced by ranolazine.

A,B. Representative raw trace of sarcomere shortening and re-lengthening in the absence (left) and presence (right) of Ranolazine (10uM). C,D. Average values of the amplitude of sarcomere shortening and return velocity were not significantly change. E. Arrhythmias were significantly reduced by ranolazine.

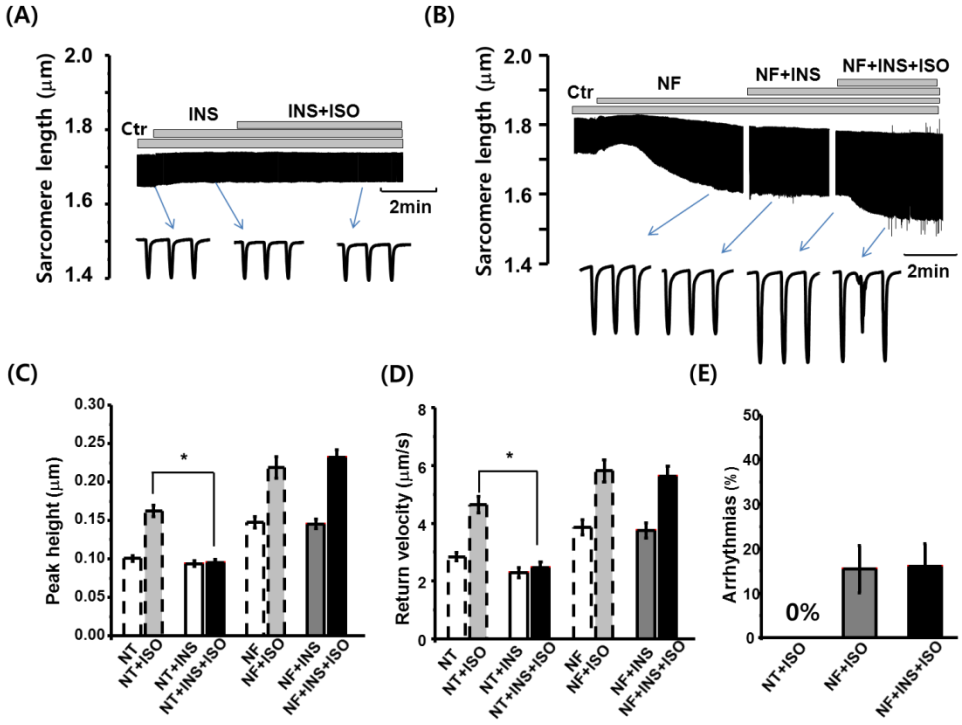

Figure 8. Insulin exerted anti-adrenergic effect on LV myocyte contraction in NT in normal rat hearts. However, insulin did not affect basal and beta-adrenergic stimulation of myocyte contraction in NF.

A,B. Representative raw traces of sarcomere shortening and re-lengthening in the absence of insulin (10nM) in NT and NF. C,D. Insulin significantly blunted the isoprenaline stimulation of LV myocyte contraction (P<0.0001, n=29, n=29) and return velocity from NT (P<0.0001, n=29, n=29), but not in NF. E. The average percentage of arrhythmias was not significantly affected by insulin in NF+ISO (P=0.9257, n=20, n=21).

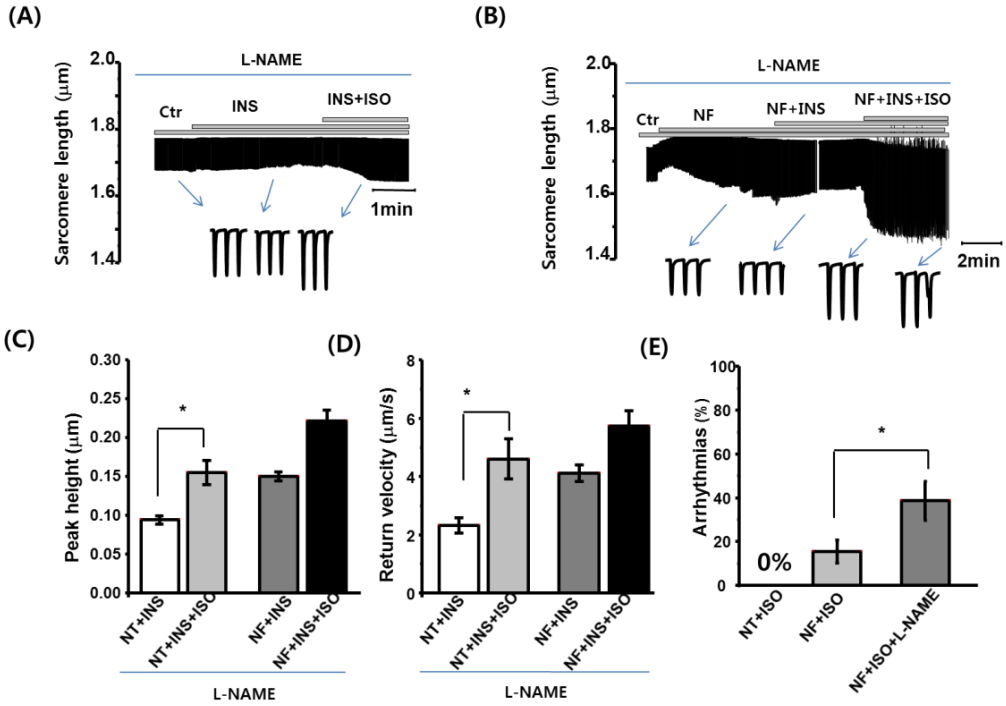

Figure 9. eNOS inhibition with L-NAME restored ISO-stimulation of myocyte contraction in NT in normal rat hearts. L-NAME did not affect basal and ISO-induced myocyte inotropy in the presence of NF.

Representative raw traces (A) and mean values of the amplitudes of the sarcomere shortening with restring doses of L-NAME (1mM, 30min)(B) (*P*=0.03, n=7). Relaxation (RV) was affected also (C) (*P*=0.00879, n=7). B,C. L-NAME did not affect the sarcomere shortening and RV in presence insulin in NF. The average percentage of arrhythmias was increasing in presence L-NAME in NF+ISO (E)

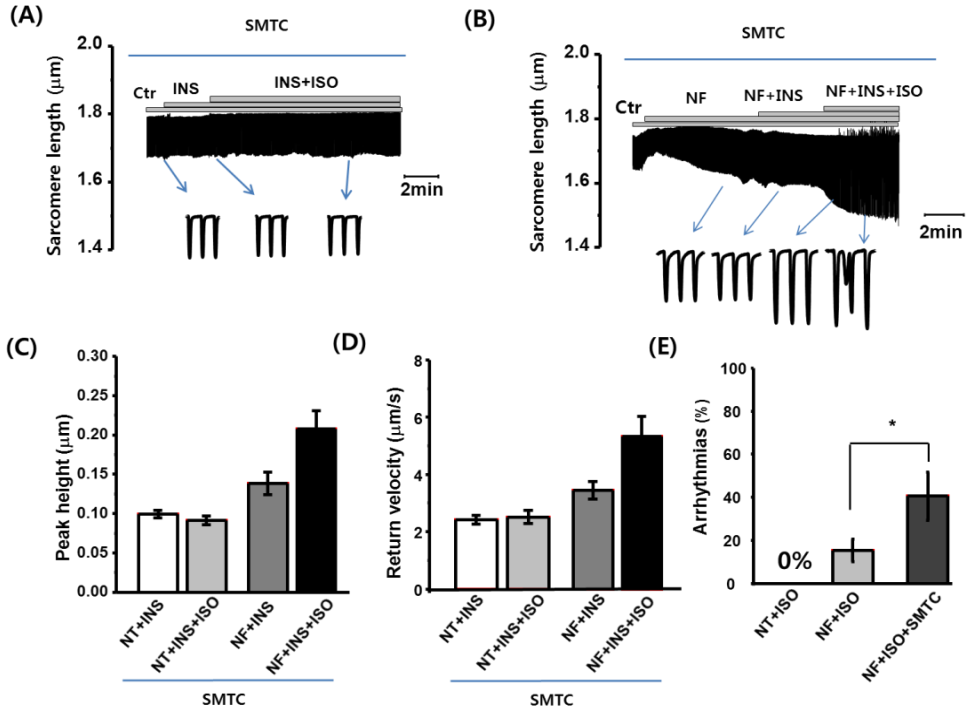

Figure 10. nNOS inhibition with SMTC did not affect insulin-dependent inotropic response in NT or in NF in normal rat hearts.

A, B, C. Representative raw traces and mean values of the amplitudes of the sarcomere shortening were not restore in presence insulin in NT and NF(Between NT and NT+INS+ISO *P*=0.3, n=22, n=22.) D. Relaxation (RV) was unaffected. E. Average percentage of arrhythmias was increasingly in presence SMTC in NF+ISO (Between NF+ISO and NF+ISO+SMTC *P*=0.2927, n=20, n=11).

Metabolic substrate supplementation and excitation-contraction coupling in cardiac myocyte in normal rat.

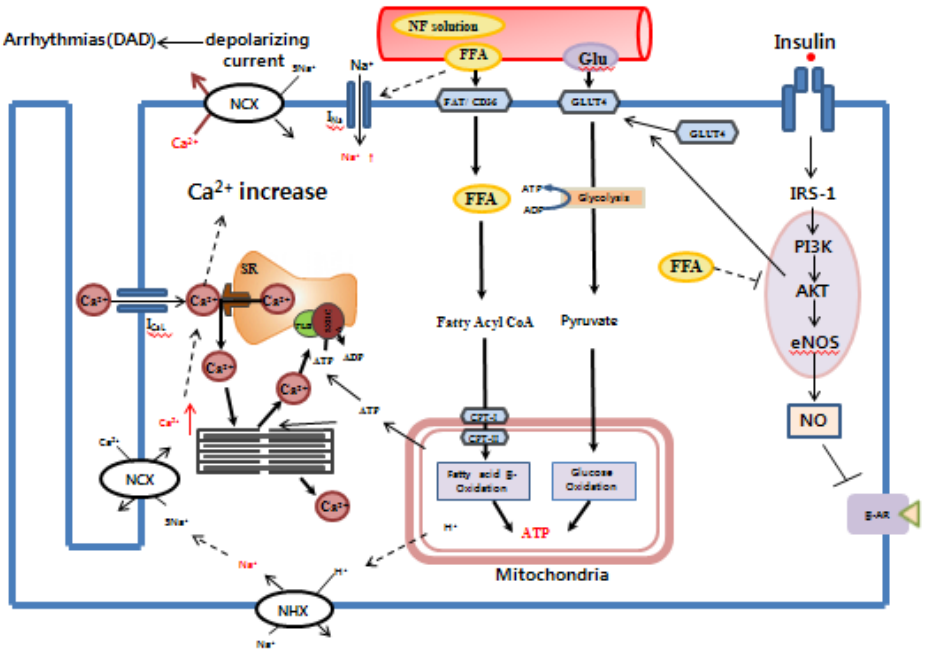

Figure 11. Schematic diagram of metabolic substrate supplementation and excitation-contraction coupling in a normal LV myocyte.

Metabolic substrates potentiated LV myocyte contractility in normal hearts possibly via increasing cardiac metabolism in mitochondria. However, it abolished insulin-induced and eNOS-mediated anti-adrenergic response. Furthermore, ISO in the presence of NF induced spontaneous diastolic contractions (arrhythmias) in almost all the myocytes.

Part II:

Effect of metabolic substrates supplementation on LV myocyte contractile function in Ang II-induced hypertensive rat hearts

Systemic blood pressure was increased and heart rate was significantly decreased

Systolic and diastolic blood pressures were continuously monitored from 3 days before the operation and every 3 days after the operation. As shown in Figure 12A, both systolic and diastolic blood pressures were increased 3 days after Ang II infusion (between sham and Ang II: systolic $P<0.001$, n=49, n=52; diastolic $P<0.001$, n=50, n=53) and continuously increased up to the period studied (Figure 12A). At 4 wks, systolic blood pressure was 131.4±1.9 mmHg in shams *vs*. 156.9±2.8 mmHg with Ang II ($P<0.001$, n=52, n=51); diastolic blood pressure was 97.2±1.4 mmHg in shams *vs*. 118.5±2.3 mmHg with Ang II ($P<0.001$ n=51, n=51). Heart rate was gradually but significantly lower in Ang II rats (heart rate, beat/min: 460.7±3.8 in shams and 448.3±5.0 with Ang II 4w, $P=0.004$, n=51, n=51, Figure 12B).

Effect of metabolic substrates supplementation (NF) on LV myocyte contraction and relaxation from hypertensive rats

Before NF application, basal cell shortening in NT was not different between

sham and hypertension (amplitude of sarcomere length: 0.005 ± 0.096 μm in sham and 0.007 ± 0.085 μm in hypertension; *P*=0.18, n=22, n=8). However, beta-adrenergic stimulation with ISO failed to increase myocyte contraction in hypertension (amplitude of sarcomere length: *P*<0.001, n=29 in normal, n=35 in Ang II, Figure 13).

Figure 13B & C showed that NF application significantly increased myocyte contraction and ISO (50 nM) increased it further (amplitude of sarcomere shortening: *P*=0.007, n=8 with NF; *P*<0.001, n=8 with ISO). In addition, NF+ISO induced arrhythmias in LV myocytes, the frequency of arrhythmias was significantly higher than that in normal rats (*P*=0.009, n=20, n=9 Figure 13E).

In order to determine whether free fatty acids can still affect LV myocyte contraction, LV myocyte contraction was assessed with 3 types of FAs. As shown in Figure 14A, the amplitude of LV myocyte shortening was significantly increased with 3FA, and application of ISO increased myocyte shortening further. (0.006±0.141, *P*=0.004, n=5 with 3FA; 0.017±0.183 *P*=0.04 with 3FA+ISO, Figure 14B).

These results suggest that metabolic substrates supplementation increased contraction in LV myocyte from hypertensive rats. As expected, significantly higher arrhythmias was induced in LV myocytes from hypertensive rats in the presence of metabolic stress.

Effect of antioxidant, N-acetylcysteine (NAC) on NF-increased LV myocyte contraction and arrhythmias in hypertension

Pressure overload is associated with myocardial oxidative stress and adverse metabolic and functional remodeling of LV myocardium [Hayakawa T et al., 2001]. Therefore, we aim to determine whether antioxidant can change NF-induced LV myocyte phenotypes in hypertension. Pre-incubation of LV myocytes with

antioxidant, NAC (1mM, 20min) significantly reduced LV myocyte contraction under basal conditions (NT: *P*=0.008, n=35, n=5) and in the presence of ISO (NT+ISO: *P*=0.002, n=35, n=5) (Figure 15A &C).

Interestingly, NAC did not affect NF-increase of basal and ISO-stimulated LV myocyte contraction in hypertension (NF: *P*=0.7, n=8, n=5; NF+ISO: *P*=0.13, n=8, n=5 Figure 15B & C). More surprisingly, the frequency of arrhythmias become significantly higher with NAC (*P*=0.05, n=9, n=5 Figure 15E).

The results suggest that the positive inotropic effect of NF was not mediated by increased oxidative status in LV myocytes from hypertensive rats. However, oxidation of certain proteins is necessary for maintaining rhythmic LV myocyte contraction after beta-adrenergic stimulation in metabolic stress.

Effect of carnitine palmitoyltransferase I (CPT-1) on NF-regulation of LV myocyte contraction in hypertension

Metabolism is shifted from fatty acid predominant pathway to glucose-dependent metabolism or glycolysis in pressure overload or in diseased heart. Although NF induced increase in LV myocyte contraction was insensitive to CPT-1 in normal conditions, we aim to determine whether CPT-1 inhibition have any effect on the inotropic effect of NF in hypertension. As shown in Figure 16B & C, ranolazine (10 μM) didn't affect the LV myocyte contraction in NF or in NF+ISO (the amplitude of sarcomere shortening: with NF *P*=0.96, n=8, n=5; with NF+ISO *P*=0.67, n=8, n=5). Importantly, ranolazine significantly decreased the frequency of arrhythmias in NF+ISO (*P*=0.003, n=9, n=5, Figure 16E).

Effect of insulin on NF-induced LV myocyte contraction in hypertensive rats

Next, we examined whether insulin response is affected in the presence of NF in hypertension. As shown in Figure 17A, insulin did not affect LV myocyte

contraction in NT; in addition, ISO did not increase myocyte contraction in the presence of insulin (between NT+INS and NT+INS+ISO: *P*=0.90, n=15). ISO still increased the contraction with L-NAME in the presence of insulin (insulin response in L-NAME pretreated LV myocytes: *P*= 0.01, n=5 Figure 17B). In fact, ISO *only* did not increase LV myocyte contraction in NT (Figure 13A).

We have shown previously that nNOS protein expression and activity was significantly increased in LV myocytes from hypertensive rats, but eNOS protein level was significantly reduced [Jin CZ et al., 2013]. It is possible that increased nNOS blunts ISO-stimulation of LV myocyte contraction. To test this possibility, LV myocytes were pre-treated with SMTC (100 nM, 30 min) and ISO response was re-examined. As shown in Figure 17C, SMTC pre-treatment restored ISO-induced increase in LV myocyte contraction (the amplitude of sarcomere shortening: *P*<0.001, n=11), suggesting tonic inhibition of beta-adrenergic signaling by nNOS in LV myocytes from hypertensive rats. Furthermore, ISO increased LV myocyte contraction in the presence of insulin in SMTC-pre-treated LV myocytes (data not shown).

Insulin response was tested after NF treatment. As shown in Figure 18A, the amplitude of sarcomere length was not affected by insulin treatment in NF before and after ISO treatment (between NF and NF+INS P=0.20, n=13; between NF+ISO and NF+INS+ISO *P*=0.74, n=8, n=13). Neither SMTC nor L-NAME affected beta-adrenergic stimulated LV myocyte contraction with insulin (NF+INS+ISO & NF+INS+ISO+SMTC: *P*=0.004, n=7 Figure 18A&B; NF+INS+ISO & NF+INS+ISO+L-NAME *P*=0.01 n=5 Figure 18A&C). Interestingly, SMTC pre-treatment significantly reduced the frequency of arrhythmias that was induced by NF+ISO (Figure 18E).

These results suggest that nNOS up-regulation blunts beta-adrenergic response in LV myocytes from hypertensive rats. Metabolic substrate supplementation impaired insulin response in LV myocytes from hypertensive rats. nNOS is important in controlling rhythmic contraction in hypertensive rat LV myocardium.

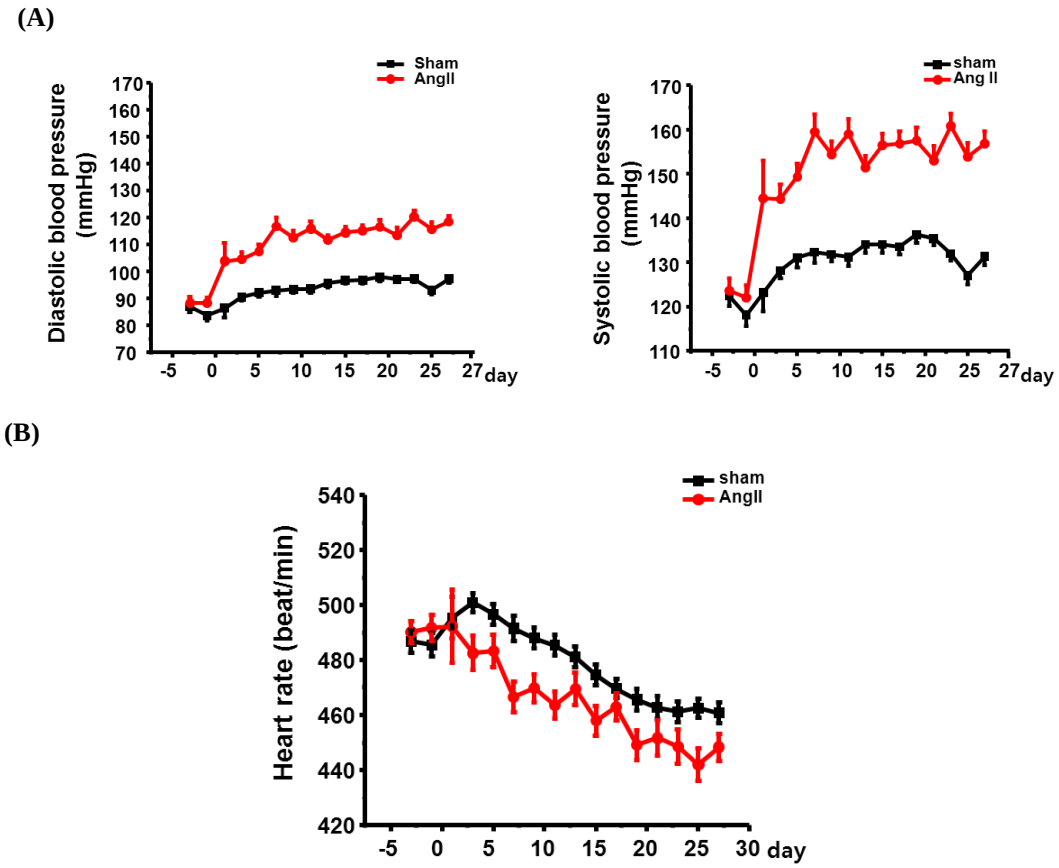

Figure 12. Systolic and diastolic blood pressure measurements in normal and in hypertensive rat.

A. Time course of the mean systolic (left) and diastolic (right) blood pressures in Ang II-rats vs. sham. High blood pressure develops from 1 wk infusion with Ang II and continuously increases until 4 wks (systolic blood pressure, $P<0.001$, n=51, n=51; diastolic blood pressure, $P<0.001$. n=51, n=51). B. Heart rate was significantly lower in Ang II 4w rats (sham: $P<0.001$, n=51; Ang II: $P<0.001$, n=50).

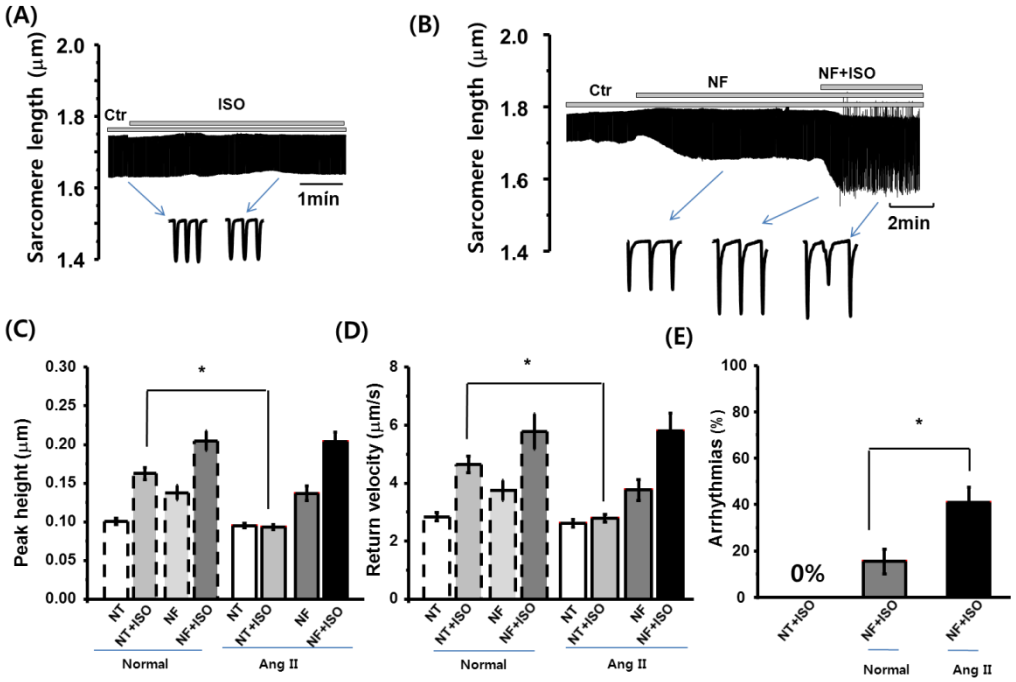

Figure 13. Beta-adrenergic response was abolished by nNOS in NT; NF significantly increased LV myocyte contraction in hypertensive rat and increased arrhythmias in the presence of isoprenaline.

A,B. Representative raw traces showed that the stimulation of isoprenaline was disappeared, and NF significantly increased LV myocyte contraction. C,D. The mean values of the amplitude of peak height and RV was abolished significantly (Peak height in normal and Ang II, $P<0.0001$, n=29, n=35; RV $P<0.0001$, n=29, n=35). However, it didn't affect in the presences of NF condition. E. The arrhythmias was significantly increasing in hypertension rat heart than normal ($P=0.00872$, n=20, n=9).

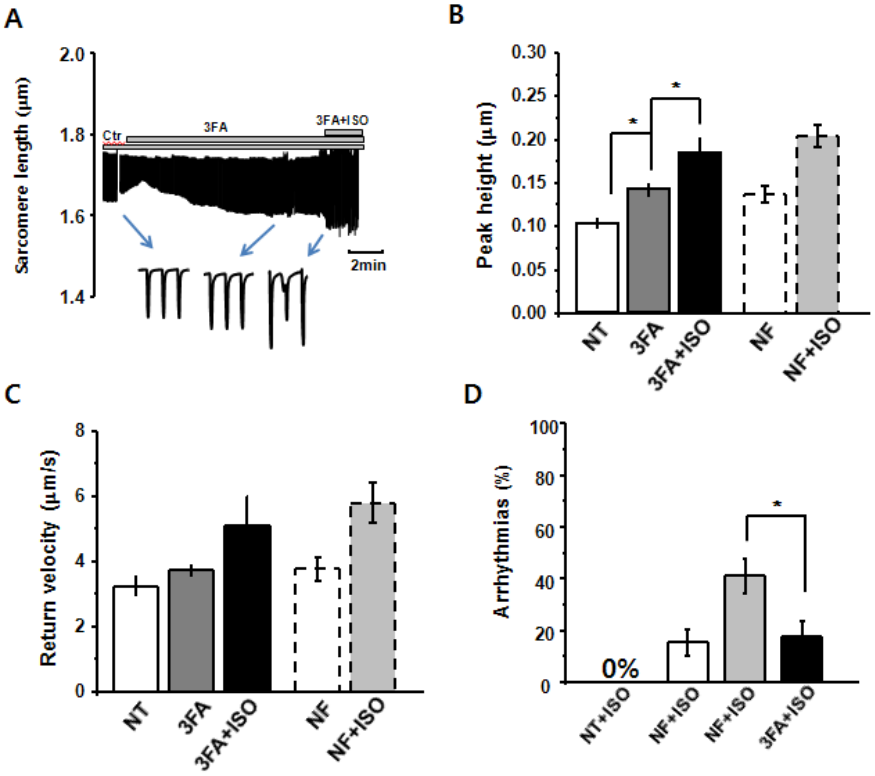

Figure 14. Supplementation of three types of fatty acids significantly increased LV myocyte contraction in hypertensive rat heart.

A. Representative raw traces of sarcomere shortening and re-lengthening in the presence of 3FA. B. Average values of the amplitude of sarcomere shortening was greater with 3FA under basal conditions (between NT and 3FA, *P*=0.004, n=5) and after ISO pre-treatment (between 3FA and 3FA+ISO, *P*=0.04, n=5). C. Average values of the amplitude of return velocity was similar with sarcomere shortening. D. Average percentage of arrhythmias was increasingly in presence in 3FA+ISO than NT+ISO, however less than in the presence of NF+ISO.

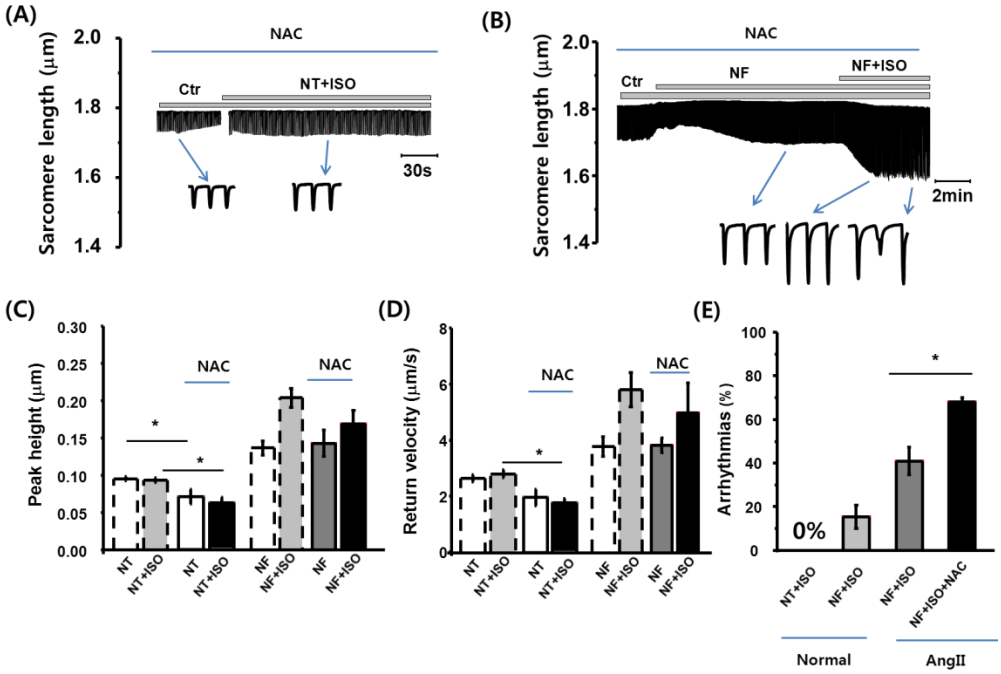

Figure 15. NAC did not restore isoprenaline-stimulation of myocyte contraction in sham or in hypertensive rat in NF, However, the mean values of arrhythmias percentage was increased in the existence of NAC.

A,B,C,D. Representative raw traces and mean values of the amplitudes of the sarcomere shortening and RV were not restored. Instead the peak height and RV were decreased in the presences of NAC under the basal condition (NT: *P*=0.00791 n=35, n=5 NT+ISO: *P*=0.003, n=35, n=5; RV NT+ISO: P=0.007, n=35, n=5). E. The mean values of arrhythmias percentage was increasing by NAC (Between NF+ISO and NF+ISO+NAC in Ang II *P*=0.005, n=9, n=5).

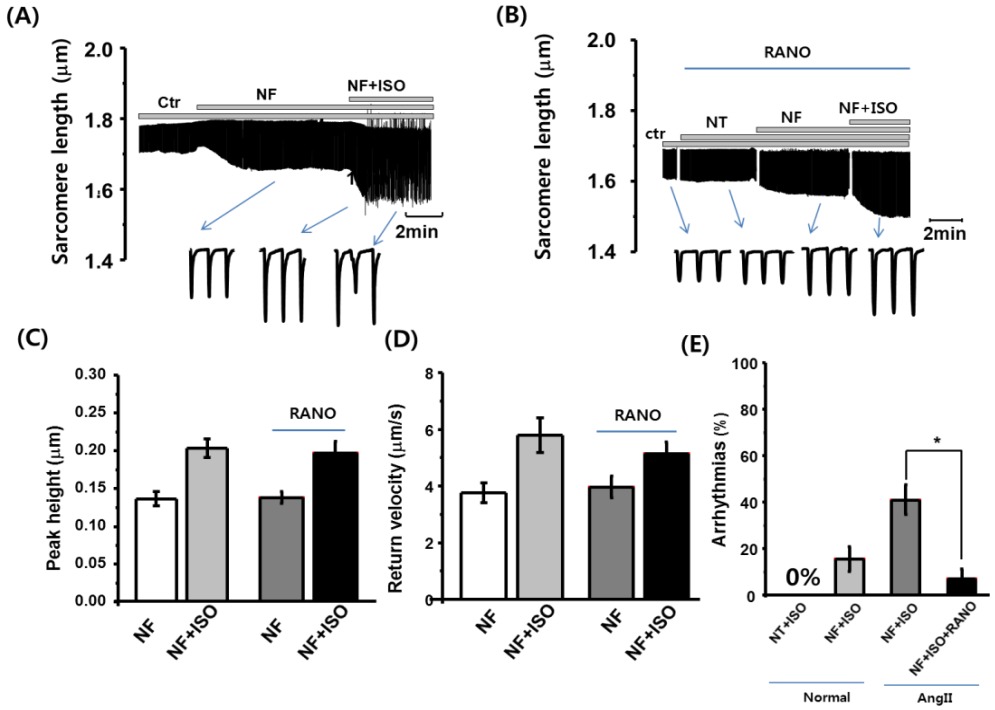

Figure 16. Ranolazine did not affect LV myocyte sarcomere shortening in hypertensive rat. However, it significantly reduced the incidence of arrhythmias.

A,B. Representative raw traces of sarcomere shortening and re-lengthening in the absence (left) and presence (right) of Ranolazine (10uM). C. Average values of the amplitude of sarcomere shortening was not different between basal and pre-treatment Ranolazine. E. The mean values of percentage of arrhythmias were significantly decreased by Ranolazine verge on 0% (*P*=0.003, n=9, n=5).

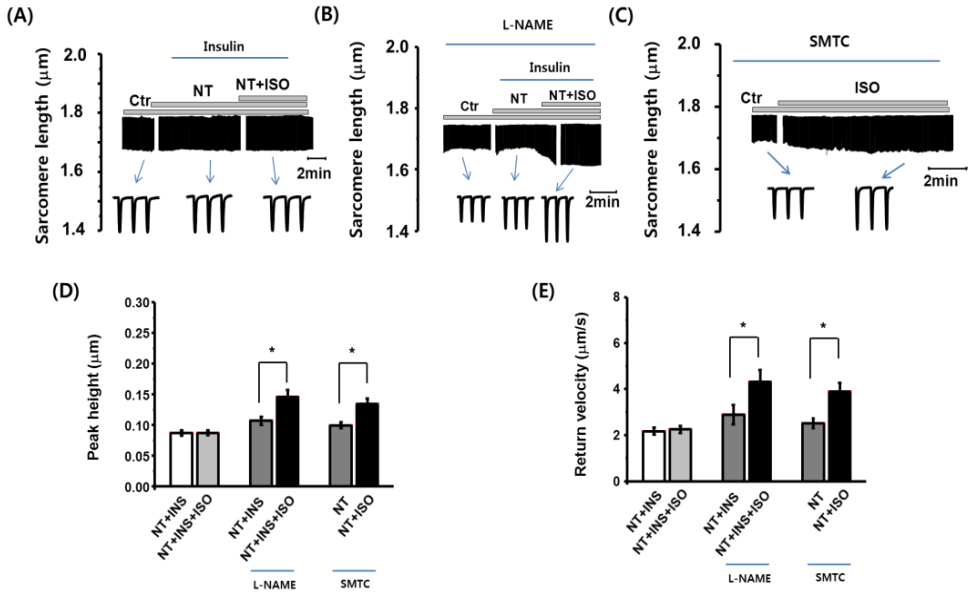

Figure 17. nNOS-regulation of sarcomere shortening in the presence of insulin in NT and in hypertensive rat.

A. Representative raw traces and mean values of the amplitudes of the sarcomere shortening in the presence of insulin. (NT+INS & NT+INS+ISO *P*=0.9, n=15). B,D. Representative raw traces and mean values of the amplitudes of the sarcomere shortening pre-treated L-NAME in the presence of the insulin. (*P*=0.01, n=5). C,D. Beta-adrenergic stimulation of myocyte contraction pre-treated SMTC (*P*<0.001, n=11).

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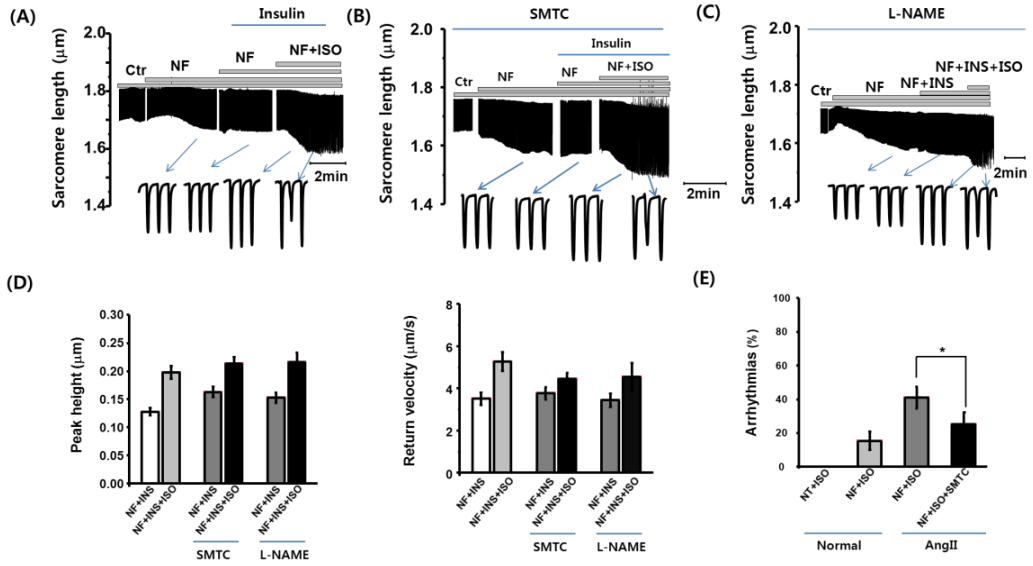

Figure 18. nNOS- or eNOS-regulation of sarcomere shortening in the presence of insulin in NF in hypertensive rat.

A,D. Raw trace and mean values of sarcomere shortening in the presence of insulin with NF (P<0.001, n=13). B,C,D. Amplitude of sarcomere shortening and mean values of contraction in the presence of SMTC or L-NAME with NF. (P<0.001, n=7 in SMTC; P<0.001, n=5). E. SMTC significantly reduced the mean values of arrhythmias percentage than basal only with NF+ISO.

Metabolic substrate supplementation and excitation-contraction coupling in cardiac myocyte in hypertensive rat.

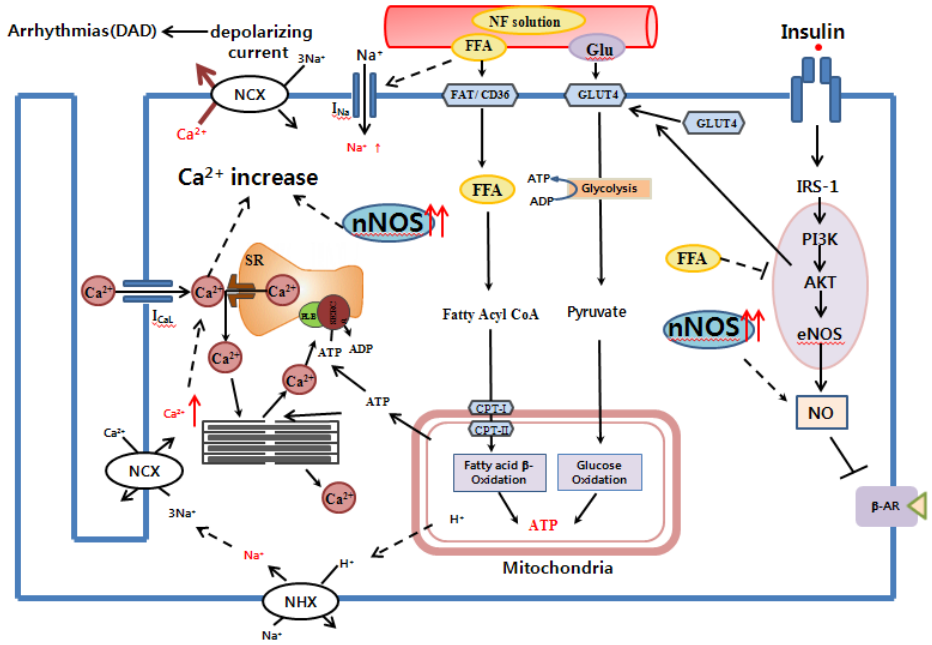

Figure 19. Schematic diagram of metabolic substrate supplementation and excitation-contraction coupling in LV myocytes in hypertensive rats.

Metabolic substrate potentiated LV myocyte contractility in hypertensive hearts. However, it abolished insulin-induced and eNOS-mediated anti-adrenergic response. Furthermore, ISO in the presence of NF induced spontaneous contractions (arrhythmias) in almost all the myocytes and the arrhythmias percentage is more than normal heart. nNOS is important in controlling rhythmic contraction in hypertensive rat LV myocardium.

DISCUSSION

Metabolic substrate supplementation and LV myocyte contractile function in normal and hypertensive rat heart

Part I results showed that the NF supplementation in NT solution increased both basal and beta-adrenergic receptor-stimulated LV myocyte contraction in normal or hypertensive rats. Importantly, beta-adrenergic stimulation induced arrhythmias in the presence of the metabolic substrate supplementation and this effect was significantly increased in hypertension. None of these effects were mediated by ROS, since NAC did not reduce NF increment of myocyte contraction or arrhythmias. In contrast, NAC increased arrhythmias in the presence of beta-adrenergic stimulation in both groups, suggesting pivotal role of ROS in maintaining normal rhythm of cardiac contraction. Surprisingly, CPT-1 inhibitor ranolazine did not affect the amplitude of myocyte contraction in NF in normal or in hypertensive rats. However, 3 FA only significantly increased LV myocyte contraction in both normal and hypertensive rats. It is feasible to consider that FA and other metabolic substrates (e.g. pyruvate, lactate and glucose) may be compromised ATP production and increase myocyte contraction. At least in this model, fatty acid-dependent myocyte beta-oxidation was not affected in hypertension.

Metabolic substrates' supplementation and insulin response

One of our key findings is that insulin response is impaired in the presence of NF. This is because insulin abolished beta-adrenergic stimulation of myocyte contraction *via* eNOS (but not nNOS) in NT but did not affect the basal and beta-adrenergic stimulated sarcomere length in NF. It is not known whether this is due to the reduction of NO bioavailability (dysfunction of eNOS or nNOS) or impaired

insulin receptors and signaling in cardiac myocytes. Our preliminary experiments with palmitic acid have shown that intracellular Ca^{2+} level was increased which may increase the activities of eNOS and nNOS rather than decrease them. Nevertheless, inhibition of eNOS or nNOS did not affect NF-increment of myocyte contraction in the presence or absence of insulin. Therefore, it is necessary to examine NO production in the presence of metabolic substrate supplementation.

Our results also show that nNOS does not contribute to the anti-adrenergic effect of insulin in normal rats, suggesting that eNOS is the predominant downstream signaling that mediates the effect of insulin. Previously, we have shown that eNOS protein expression in cardiac myocytes was significantly reduced in hypertensive rats [Jin CZ et al., 2013]. In contrast, nNOS becomes the prime source of NO in cardiac myocytes from hypertensive rats. Interestingly, our results show that endogenous nNOS activity attenuates the positive inotropic effect of beta-adrenergic stimulation in LV myocytes from hypertension and inhibition of nNOS restored increased myocyte contraction by ISO. We did not test whether nNOS activity can be affected by insulin stimulation in hypertension. It is known that hypertensive subjects are often associated with impaired insulin response or insulin resistance; whether the remodeling of NOS system may play a part in insulin resistance remains investigated.

Metabolic substrate supplementation and arrhythmias

Increased metabolic status (metabolic syndrome), in particular, increased serum fatty acids is often associated with increased coronary artery diseases and sudden cardiac death due to ventricular arrhythmias. Although fatty acids and systematic inflammation (e.g. increased plasma cytokines) are suggested to be involved in the arrhythmogenesis, the mechanistic insights remain unclear. Interestingly, our results show that arrhythmias are induced by NF in the presence of beta-adrenergic

stimulation, and such arrhythmias are further intensified in hypertension, well in line with clinical statistics that hypertension is an important precursor of arrhythmias and sudden cardiac death. Chronic pressure overload or mechanical disturbance induces remodeling of cellular ion channels those shape cardiac action potential and intracellular Ca^{2+} homeostasis which causes abnormal contraction. Metabolic substrates' regulation of these arrhythmogenetic factors may potentiate the pathogenic effect. It is important to study the cellular mechanisms underlie the phenotype to understand molecular mechanisms mediating metabolites' induction of arrhythmias, particularly in hypertension. Nevertheless, we have found that ranolazine, a late Na^{+} current inhibitor, significantly attenuated arrhythmias in both shams and in hypertension. Interestingly, nNOS inhibition with SMTC significantly increased the incidence of arrhythmias in shams but SMTC significantly reduced arrhythmias in hypertension. We have shown recently that SMTC decreases the amplitude of Ca^{2+} transient in sham LV myocytes but increases it in hypertension. It is possible that different intracellular Ca^{2+} status may have contributed to the SR Ca^{2+} loading and spontaneous Ca^{2+} release.

NO is a ubiquitous cellular messenger that plays a key role in regulating cardiac Ca^{2+} homeostasis and function. Although it is not clear how NF affects NO production in our experimental conditions, inhibition of nNOS with SMTC or with L-NAME significantly increased arrhythmias in the presence of NF, suggesting that NO derived from nNOS may represent a cardioprotective signaling pathway that lower the threshold for arrhythmogenesis. We have not measured intracellular Ca^{2+} in the presence of NF in this study. However, it is possible to postulate that increases in cardiac metabolism with NF may lead to an elevation of intracellular H^{+} and Na^{+}/H^{+} exchanger, bring in more Na^{+} in the cytosol which in turn activate Na^{+}/Ca^{2+} exchanger to induce a depolarizing current. This depolarizing current, if of sufficient size and occurring at diastole, leads to a delayed afterdepolarization (DAD) that can trigger a new action potential and ectopic beat. Adrenergic receptor stimulation potentiate the processes therefore increase the incidence of arrhythmias.

nNOS has been well established to reduce intracellular Ca^{2+} level and SR Ca^{2+} loading therefore exerts anti-arrhythmic effect.

In conclusion, we have found that metabolic substrates supplementation increased basal and beta-adrenergic stimulated myocyte contraction, impaired insulin response and induced arrhythmias in normal and hypertension. Therefore, we reveal an *in vitro* cardiac model of "metabolic syndrome". Detailed mechanisms underlying the phenomenon will facilitate our understandings of cardiac dysfunction that is associated with metabolic syndrome.

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국문 초록

대사증후군과 고혈압은 심실 부정맥에 의한 돌연 심장사, 심부전, 뇌졸증과 같은 심혈관계 합병증의 원인으로서 잘 알려져 있다. 아울러 심근의 인슐린 저항성은 대사에 의한 심장질환의 주요한 기전임이 잘 밝혀져 있다.

최근까지도 대사물질에 의한 심근수축력 및 인슐린 반응성에 관한 연구와 실험적 증거들은 아직 부족하다. 그러므로 본 연구에서는 정상 쥐와 안지오텐신 II로 유도된 고혈압 쥐 심근세포에의 외부 관류액으로 대사물질(올레산 200 μM, 팔미트산 100 μM, 리놀레익산 100 μM, 젖산 1 mM, 피루브산 100 μM, 카니틴 50 μM)이 보충된 Normal Tyrode (Nutrition Full : NF라 명명) 용액을 흘려줌으로써, 베타 아드레날린 수용체가 조절하는 심근세포의 수축성 및 인슐린 반응 변화를 관찰하고 그 상관관계를 규명하고자 한다.

실험결과에서 NF 용액을 세포 밖에 흘려주었을 경우 정상 쥐와 고혈압 쥐 모두에서 NT 용액을 흘려주었을 때보다 정상 수축력이 증가하였다. 뿐만 아니라 NF 용액과 함께 베타-아드레날린 수용체의 효능제로서 알려진 Isoproterenol (ISO, 50 nM) 처리 시에는 심근 수축력이 더 커지는 변화를 관찰하였다.

정상 쥐의 심근세포에서 NT 용액을 관류시켰을 때 나타나는 ISO에 의한 심근 수축 반응은 인슐린(10 nM)을 함께 처리하였을 경우에는 관찰되지 않았다. 이러한 인슐린에 의하여 차단된 ISO에 의한 심근 수축반응은 질소산화물 합성 효소(nitric oxide synthase, NOS)의 비특이적 억제제인 L-NG-nitroarginine methyl ester (L-NAME, 1 mM)에 의하여 회복되었으나, nNOS 특이적 억제제인 S-methyl-L-thiocitrulline (SMTC, 100 nM)에 의해서는 여전히 차단되어 있음을 관찰하였다. 이는 NT 용액을 심근세포에 관류시켰을 경우, 인슐린에 의한 특이적인 eNOS 활성화가 아드레날린 반응을 억제함을 시사한다.

또한, NF 용액과 인슐린을 함께 심근세포에 흘려주었을 경우에서는 정상 수축력과 ISO 처리 시에 증가하는 수축성 변화가 모두 일어나지 않았다. 이는 심근세포에 대사물질이 보충될 경우, 인슐린의 반응성이 감소하였음을 의미한다. 하지만 NT 용액을 흘려주었을 때와는 다르게 NF 용액에서는 인슐린의 유무와 관계없이 심근세포의 수축력은 L-NAME에 의해 영향을 받지 않았다.

NF 용액에서 ISO에 의해 나타나는 심근세포의 부정맥의 발생과 빈도수는 정상 쥐에서보다 고혈압 쥐에서 상당히 증가하였다. Carnitine palmitoyl transferase 1 (CPT-1)의 억제제인 Ranolazine (10 μM)은 정상 쥐와 고혈압 쥐 모두에서 NF 용액을 통해 강화된 심근 수축력에는 영향을 미치지 못하였지만 부정맥은 상당히 감소시켰다. 흥미롭게도 정상 쥐의 심근세포에서는 SMTC를 처리할 경우 부정맥이 상당히 증가되었으나, 고혈압 쥐에서는 오히려 감소되었다. 이는 대사기질 존재 시, nNOS가 정상 쥐와 고혈압 쥐 심근세포의 정상적인 수축에 있어서 서로 상반되는 역할을 한다는 것을 나타낸다.

결론적으로, 백서심근세포에서 대사기질을 보충할 경우 베타-아드레날린 반응에 의한 부정맥이 발생하였고 인슐린 반응은 손상되었다. 또한 고혈압 쥐에서는 nNOS가 대사성 스트레스에서 심장 부정맥을 발생시키는데 중요한 역할을 하고 있음을 알 수 있다.

주요어 : 지방산, 대사 기질, 베타-아드레날린 자극, 인슐린, 고혈압, 산화 질소 합성 효소, 좌심실 심근세포

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