Electrical stimulation of cardiomyocytes activates mitochondrial matrix metalloproteinase causing electrical remodeling

Thomas P. Vacek, Naira Metreveli, Neetu Tyagi, Jonathan C. Vacek, Sebastian Pagni, Suresh C. Tyagi

A part of this study was supported by NIH grants; HL-71010, HL-74185 and HL-88012.

Corresponding author. Fax: +1 502 852 6239.
E-mail address: suresh.tyagi@louisville.edu (S.C. Tyagi).

1. Introduction

Heart failure (HF) involves abnormalities in systolic and/or diastolic function and increases the propensity for reentry arrhythmias [15]. Persistent increased in cardiac sympathetic drive contributes to myocardial toxicity, leading to the decline in cardiac contractility.

Homocysteine (Hcy), a sulfur-containing amino acid, whose higher concentrations in blood known as hyperhomocysteinemia (HHcy) and have been linked independently to heart disease [5,17]. Homocysteine (HCY) induces interstitial cardiac fibrosis leading to heart failure [3]. During heart failure, increase in load cause increase in oxidative stress leading to activation of matrix metalloproteinases (MMPs) [21].

MMPs are membrane-bound; zinc dependent endopeptidases involved in extracellular matrix proteins and are required for ECM remodeling. Considering the large family of MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are the key ones involved in the ECM remodeling of heart with both changing demands and response to injury [23]. Both are known to break down non-fibrillar-forming type IV collagen and denatured interstitial collagens [1].

MMP-9 activation in HHcy decreases the collagen/elastin ratio and increases the fibrosis between endothelium and myocytes, and is arrhythmogenic [18,20,23]. MMP-9 is playing an important role in a wide variety of physiological conditions and disease states, some of which include normal development, inflammation, wound healing and repair and vascular diseases [4]. We and others have shown the presence of MMP-9 in the cardiac mitochondria (mtMMP) [9,15]. Hcy was shown to activate mtMMP-9 in mitochondria with an enlargement of mitochondria in addition to disruption of cristae that resulted in contractile dysfunction [14,16]. MMP-9 has also been shown to destroy SERCA pump, resulting in decreased calcium transient.

Calcium is a primary regulator of contraction in heart muscle in both initial depolarization and subsequent contraction [10]. Calcium-dependent MMP-9 activation has been demonstrated in cells [13]. Hoit et al. showed atrial remodeling by electronic pacing that resulted in systolic and diastolic dysfunction included chamber stiffness; there was an increase in MMP-9 and decrease in its tissue inhibitor (TIMP-4) with no change in calcium pumps [6]. Russo et al. showed that MMP-2 expression was significantly increased in electrically-stimulated denervated skeletal muscle compared to control [19]. MMP-2 is known to be involved in fibrotic proliferation and connective tissue remodeling [11].

A decrease in contractility is undoubtedly a major contributing factor in the pathophysiology of a significant portion of the patients with heart failure. A reasonable therapeutic goal in these patients would then be to increase cardiac contractility either acutely...
or long term [2]. Recently, we showed that novel electrical stimulation activates mtMMP-9. Based on these results, we hypothesis that increased contraction frequency induced the activation of mitochondrial MMP (mtMMP) that has already been linked to myocyte mechanical dysfunction.

2. Methods

2.1. Mice and experimental protocol

C57BL/6j mice procured from The Jackson Laboratory (Bar Harbor, ME) were housed in a controlled environment on 12:12 h light–dark cycle. Mice were fed with normal mouse chow. Animal experimentation was performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Louisville. All animal care and use procedures were carried out according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86–23, revised 1985) and the regulations of the Animal Welfare Act.

2.2. Adult ventricular myocyte isolation

Single Ventricular myocytes from the adult mice heart were isolated according to the protocol as described elsewhere [15]. Perfusion buffer was prepared from basic buffer, glucose and butanedione monoxime (BDM) (120.0 mM NaCl, 1.0 mM KCl, 0.6 mM KH2PO4, 0.6 mM Na2HPO4, 1.2 mM MgSO4·H2O, 10 mM Na-HEPES, 4.6 mM NaHCO3, 30 mM Tauroen, 10 mM BDM, 5.5 mM Glucose and pH 7.0). The perfusion system was prepared as follows: The temperature of the circulation water bath was set so that the temperature of the outflow liquid at the tip of the cannula (20 g needle with the hub filed flat and smooth) is 37 °C. Flow rate was set at 4.0 ml/min. Prior to the cardiomyocyte isolation, about 100 ml of distilled water was run through the system. Then the system was perfused with perfusion buffer for at least 5 min.

A mouse of at least 20 g was injected with heparin (1000 U/kg; i.p.). Then the mouse was anesthetized with 2,2,2-trimethoxyethanol (240 mg/kg; i.p.). The chest was wiped with 70% ethanol. A skin incision was made revealing the xiphoid process. The rib cage was completely cut starting at the xiphoid process running up the chest cavity. To avoid heart damage, the diaphragm was cut as well. The heart was secured with forceps and all vessels were cut. The aorta was cut to leave the maximal length, which is important for rapid cannulation. The dissected heart was immediately placed in a Petri dish containing ice-cold calcium free perfusion buffer (pH 7.4). To expose the aorta, all the remnant excess tissue was removed and discarded. While holding the aorta with two fine forceps, a vertically-mounted cannula was inserted until the tip of the needle reached the aortic valve. The heart was secured on the needle with a small brass clip and was immediately perfused with perfusion buffer at a flow rate of 4.0 ml/min. The aorta was tied to the needle with silk thread. The time from the heart dissection until the start of perfusion should not exceed 1 min.

The heart was perfused with perfusion buffer for 2 min, or until the outflow from apex was cleared from blood. Then the perfusion with digestion buffer consisting of 29 ml perfusion buffer and 1.0 ml of 10 mg/ml Liberase Blendzyme 4 (Roche Diagnostics Corp. Indianapolis, IN) was continued for 7–12 min. At the end of the perfusion, the tissue became soft, swollen and light pink.

After the perfusion, the heart was cut from the needle just below the atria using sterile fine scissors and is placed in a Petri dish with 10 ml room temperature incubation buffer (135 mM NaCl, 4 mM KCl, 1 mM MgCl2, 10 mM HEPES, 0.33 mM Na2HPO4, 10 mM glucose, 10 mM BDM, pH 7.4). The heart was cut in half and the tissue was gently teased into small pieces with fine forceps. The obtained suspension of cardiomyocytes was gently pipetted up and down with a plastic pipette (2 mm tip) several times. Then the cells were transferred to a 15 ml sterile polypropylene conical tube and 10, 20, 30, and 30 μl of a 100 mM CaCl2 solution is added at 5 min intervals. The final content of calcium was 1.2 mM. Isolated myocytes were maintained at room temperature in this buffer.

2.3. Cell shortening /re-lengthening

Mechanical properties of the ventricular myocytes were determined using a video-based edge-detection system (IonOptix, Milton, MA), as described elsewhere [25]. The myocytes were field stimulated at a rate of 1 and 4 Hz using a pair of platinum wires placed on the opposite sides of the dish chamber and connected to a MyoPacer Field Stimulator (IonOptix). Cardiomyocyte contractility was controlled by electrical stimulation. Mechanical properties of isolated ventricular myocytes were assessed by video-based edge detection. An inverted microscope, a low light-level video camera and a computer-based motion analyzer are used to track the movement of cell edges.

The isolated myocytes were diluted approximately tenfold with contractility buffer (135 mM NaCl, 4 mM KCl, 1 mM MgCl2, 10 mM HEPES, 0.33 mM Na2HPO4, 10 mM glucose, 10 mM BDM, 1.2 mM CaCl2) and placed on a Teflon glass coverslip dish mounted on the stage of an inverted microscope (Olympus, IX-70). The cells were field stimulated to contract by the MyoPacer field stimulator through a pair of platinum electrodes at a frequency of 1.0 Hz, pulse duration of 4 ms and amplitude of 10 v. The image of the myocyte is obtained with an Ion Optix Myo Cam camera side-mounted onto the microscope and displayed on a computer monitor using the Soft-Edge software. Typically 10–15 individual myocytes are recorded before replacing the myocytes with a fresh dilution of un-stimulated myocytes. This prevents exhaustion of the myocytes due to prolonged stimulation. Fifteen to twenty myocytes were analyzed for each heart. The cells being studied were scanned every 8.3 ms so that the amplitude and velocity of shortening and lengthening can be recorded with good fidelity. The displacements of cell edges at both ends of the myocyte were detected and converted to an analog voltage signal, which is then digitized and stored for off-line analysis. Steady-state twitches [8–10] were analyzed for cell length changes using the Soft-Edge software and averaged for each myocyte. Cell shortening and re-lengthening were assessed by percentage of peak shortening (%PS), time to 90% peak shortening (TPS90), time to 90% re-lengthening (TR90) and maximal velocities of shortening and re-lengthening (±dL/dt).

2.4. Intracellular fluorescence measurement of calcium

Intracellular calcium was determined using a dual-excitationfluorescence photomultiplier system (IonOptix), as described elsewhere [25]. A separate cohort of myocytes was loaded with calcium-specific radio-metric fura 2-AM dye (1 μmol/l) for 30 min in the dark, and the fluorescence was recorded. Myocytes were placed in a dish chamber on the stage on an Olympus IX-70 inverted microscope and imaged through a Fluor ×100 objective. Cells were exposed to light emitted by a 75 W lamp and were passed through either 360 or 380 nm filters (bandwidths will be placed at ±15 nm) while being stimulated to contract at 1.0 and 4.0 Hz. Fluorescence emission was detected between 480 and 520 nm by a photomultiplier tube after exciting the cells at 360 nm for 0.5 s and then at 380 nm for the duration of their coding protocol. A 360 nm scan was repeated at end of the experiments, and the qualitative changes in intracellular calcium concentration were interpreted from the ratio. The calcium transients were measured as changes in fura fluorescence intensity (FFI). FFI was determined as the difference
between the levels of calcium in systolic and diastolic settings (FFI = peak FFI – baseline FFI). The time course of calcium fluorescence signal decay (the duration at which calcium transient decays 67% from the peak level) was calculated to determine intracellular calcium clearing rate. The myocytes included in the cell shortening/re-lengthening and calcium measurement studies met the following criteria: (1) rod-shaped with clear striation pattern; (2) quiescent in the absence of electrical stimulation; (3) stable mechanical behavior at 0.25 or 2 Hz and 37 °C for 15 min and (4) absence of sarcolemmal blebs.

2.5. In-gel-gelatin zymography

mtMMP-9 activity of mitochondrial fraction was measured using gel-gelatin zymography. Briefly, isolated myocyte, washed two times in PBS, and incubated in the protein extraction buffer (in mMol/L: 50 mM Tris pH 7.4, 5 mM EDTA, 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride,0.1% Triton X-100), for 1 h on ice. The homogenate was centrifuged for 600 g for 5 min. Pellet one that consists of nuclei and whole cells was discarded. The supernatant was centrifuged again at 14,000 g for 45 min to acquire mitochondrial fraction. Protein concentration was assayed using the Bradford method. Samples were electrophoretically resolved with 10% SDS-PAGE containing 2 mg/ml gelatin as a substrate. At the end, gel was incubated in renaturation buffer (2.5% TritonX-100) for 30 min to remove SDS, rinsed in distilled water, and then incubated overnight at 37 °C in water bath in activation buffer (50 mM Tris-HCl, pH 7.4 and 5 mM CaCl_2). Gel was stained using 0.5% Coomassie blue R-250 for 1 h. MMP activity was detected as a white band on a dark blue background and quantity densitometrically using Un-Scan software (Silk Scientific, Orem, UT).

2.6. Statistics

The contractility measurements were performed on 12–15 myocytes from 6 to 8 hearts in each group. Values were mean ± SEM. Significance was carried out by paired Student’s t-test. p < 0.05 was considered significant.

3. Results

The results suggested that mitochondrial MMP-9 was activated in the myocyte mitochondria with increased contraction frequency (Fig. 1). It was demonstrated that mtMMP-9 was significantly elevated 25% at 1 Hz stimulation relative to 0 Hz (p < 0.05, n = 3) and elevated 40% at 4 Hz stimulation relative to 0 Hz (p < 0.01, n = 3).

There was a decrease in cell shortening at higher contraction frequency that resulted in the following length shortening relative to control, i.e. 0.80 decrease in length shortening relative to 1 Hz (p < 0.05, n = 3) (Fig. 2A–F).

The levels of calcium transient were reduced with increase in calcium transient relative to 1 Hz (p < 0.05) (Fig. 3A–E).

Although number of frequency was higher, the magnitude of myocyte lengthening was shorter in 4 Hz stimulation than 1 Hz (Fig. 4).

4. Discussion

Calcium is a primary regulator of contraction within the heart; transients are important for both sinoatrial depolarization and myocardial contraction [11]. An inward calcium transient is responsible for the depolarization (Phase 0) of sinoatrial node as well as the plateau phase (Phase 2) of myocardial depolarization and contraction [24]. Moreover, the contraction of cardiomyocytes

![Fig. 1. Zymographic analysis of myocyte mitochondrial (mt) MMP-9 activity. Myocytes were stimulated at 0, 1 and 4 Hz; mitochondria were isolated using differential centrifugation and analyzed on 1% gelatin-gel zymography (gel panel). The bar graphs represent the accumulative densitometric scan data from n = 3 in each group. *p < 0.05 compared to 0 Hz.](image)

![Fig. 2. Single myocyte lengthening stimulated at 1 versus 4 Hz. (A) lengthening at 1 Hz; (B) at 4 Hz; (C) maximum rate of relaxation; (D) maximum rate of contraction; (E) cell shortening; (F) time to release calcium. Each bar represents mean ± SEM from n = 3. *p < 0.05 compared to 1 Hz.](image)
Our lab has worked largely with homocysteine, a sulfur-containing amino acid that has been shown to be an independent risk factor for heart disease [17,21]. Hcy-induced calcium-dependent PKC can phosphorylate ERK½ to activate intracellular MMP-9 that proceeds with vascular remodeling in vitro [13]. Hence, calcium is an important inducer of MMP activity. It is known that calpain-1 disrupts inner mitochondrial membrane electron gradient that results in an accumulation of reactive oxygen species within mitochondria [14]. These ROS can activate mitochondriar MMP as well as diffusing from mitochondria to cytosol to activate intracellular MMP [14]. Activation of MMP-9 via ROS can occur either directly or via ERK½ pathways that have been shown to activate MMP-9 [14].

Our lab investigated Hcy-induced activation of mtMMP via Hcy agonizing NMDA-R receptor and increasing calcium within the cell; we also investigated its effect on mitochondria via ultra structural analysis and mitochondrial swelling assay; results showed that there was an enlargement of mitochondria as well as fragmentation of cristae that resulted in contractile dysfunction [12,15]. This is logical considering how disruption of inner mitochondrial membrane would destroy the electric potential necessary to generate ATP. Contractile dysfunction was measured via the following: decrease in percent cell shortening, maximal rate of contraction and maximal rate of relaxation. Moreover, decrease of calcium transient amplitude was faster in wild type compared with HHCy. This was confirmed using pharmacological blocker of NMDA-R i.e. MK-801, in presence of Hcy; contractile function returned [12,22]. In fact, the contractile dysfunction in previous experiments resembles the data that we have collected upon stimulation of myocytes. This is strong evidence with the data that we have collected that calcium mishandling within cell can lead to mtMMP activation in the mitochondria. This is associated with an increase in contraction frequency and decrease in myocyte contractility.

This can have serious implications in arrhythmogenesis. Abnormal electric pacing via ectopic electrical stimulation within the heart could possibly lead to fibrotic remodeling and long-term contractile dysfunction. Moreover, electrical stimulation or pacing as a therapeutic measure should be evaluated closely in higher pacing ranges.

In rat choroidallantois membranes, calcium was shown to be necessary for the development of a complex of MMPs that proceed with connective tissue degradation [24]. Remodeling induced by electronic pacing has been demonstrated in atria of dogs; rapid pacing produced the following results: systolic, diastolic dysfunc-

Under physiological electrical stimulation, more calcium enters the cell from a greater number of action potentials [11]. Moreover, the interval for calcium uptake is decreased; therefore, more calcium remains in the cell with subsequent contractions. A shorter interval may prevent NCX from extruding calcium quick enough; hence, intracellular calcium may accumulate to greater levels and increase force of contraction as more calcium binds to more TnC and unveils greater number of actin binding sites. SERCA uptake and release of calcium proceed at a greater rate to accommodate the higher number of action potential. Electrical stimulation of astroglia and fibroblast cells also resulted in induction of heat shock protein (hsp70) gene whose role is to maintain protein integrity and aid in protein folding [19].

**Fig. 3.** Single myocyte calcium (340/380 nm) transient was analyzed at 1 Hz (A) versus 4 Hz (B). (C) Cell shortening (%); (D) rate of calcium release; (E) time to release calcium. Each bar represents mean ± SEM from n = 3. *p < 0.05 compared to 1 Hz.

**Fig. 4.** Representative tracing of myocyte lengthening at 1 and 4 Hz.
References