Hypothermia/rewarming disrupts excitation-contraction coupling in cardiomyocytes

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Submitted 3 November 2015; accepted in final form 16 March 2016

Schaible N, Han YS, Hoang T, Arteaga G, Tveita T, Sieck G. Hypothermia/rewarming disrupts excitation-contraction coupling in cardiomyocytes. Am J Physiol Heart Circ Physiol 310: H1533–H1540, 2016. First published March 18, 2016; doi:10.1152/ajpheart.00840.2015.—Hypothermia/rewarming (H/R) is poorly tolerated by the myocardium; however, the underlying intracellular basis of H/R-induced cardiac dysfunction remains elusive. We hypothesized that in cardiomyocytes, H/R disrupts excitation-contraction coupling by reducing myofilament Ca2+ sensitivity due to an increase in cardiac troponin I (cTnI) phosphorylation. To test this hypothesis, isolated rat cardiomyocytes (13–15 cells from 6 rats per group) were electrically stimulated to evoke both cytosolic Ca2+ ([Ca2+]cyto) and contractile (sarcomere shortening) responses that were simultaneously measured using an IonOptix system. Cardiomyocytes were divided into two groups: 1) those exposed to hypothermia (15°C for 2 h) followed by rewarming (35°C; H/R); or 2) time-matched normothermic (35°C) controls (CTL). Contractile dysfunction after H/R was indicated by reduced velocity and extent of sarcomere length (SL) shortening compared with time-matched controls. Throughout hypothermia, basal [Ca2+]cyto increased and the duration of evoked [Ca2+]cyto transients was prolonged. Phase-loop plots of [Ca2+]cyto vs. contraction were shifted rightward in cardiomyocytes during hypothermia compared with CTL, indicating a decrease in Ca2+ sensitivity. Using Western blot, we found that H/R increases cTnI phosphorylation. These results support our overall hypothesis and suggest that H/R disrupts excitation-contraction coupling of cardiomyocytes due to increased cTnI phosphorylation and reduced Ca2+ sensitivity.

Animal models have demonstrated that systolic function or cardiac muscle force generation is significantly reduced after hypothermia/rewarming (H/R) (14, 16, 38, 40). The intracellular mechanisms underlying excitation-contraction coupling and muscle force generation appear to be disturbed following H/R, which could be explained either through a reduction in evolved transient cytoplasmic Ca2+ ([Ca2+]cyto) responses or decreased Ca2+ sensitivity of force generation. Normally, [Ca2+]cyto is maintained at a low basal level (~100 nM) and increases transiently in response to electrical stimulation. The intensity and duration of evoked [Ca2+]cyto transients are proportionally transduced into contractile responses via tropolin-based thin filament regulation, specifically [Ca2+]cyto binding to cardiac troponin C (cTnC) (1). However, phosphorylation of cardiac troponin I (cTnI) can alter the contractile response independent of [Ca2+]cyto levels, thereby influencing the Ca2+ sensitivity of the contractile response (31). Previously, in isolated rat papillary muscle strips, we found that cardiac contractile dysfunction induced by H/R was associated with a reduction in Ca2+ sensitivity (rather than reduced transient [Ca2+]cyto responses) and increased cTnI phosphorylation (14).

To better understand the physiological basis of H/R induced contractile dysfunction at a cellular level, we developed an in vitro model of rewarming stress that allows for simultaneous tracking of [Ca2+]cyto and contractile response in isolated cardiomyocytes during H/R. We hypothesize that in cardiomyocytes, H/R disrupts excitation-contraction coupling in cardiomyocytes by reducing myofilament Ca2+ sensitivity due to an increase in cTnI phosphorylation.

NEW & NOTEWORTHY

We found that profound hypothermia followed by rewarming increases cardiac troponin I (cTnI) phosphorylation and reduces Ca2+ sensitivity and contractile function in isolated cardiomyocytes. We conclude that this mechanism underlies rewarming shock observed in accidental hypothermia patients, suggesting a novel therapeutic target.

ACCIDENTAL HYPOTHERMIA victims are at risk of fatal cardiovascular system failure upon rewarming referred to as rewarming shock. Although accidental hypothermia is associated with low incidence, claiming ~1,500 death certificates in the U.S. per year (4), it is accompanied with high mortality (estimates range from 50–80%) (21). The underlying cause of rewarming shock is not clearly understood, which is essential to develop optimal rewarming strategies for accidental hypothermia victims.

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Hypothermia/rewarming protocol. Cardiomyocytes incubated in M199 were placed in a cell chamber and perfused with Tyrode solution for 30 min to 1 h to allow stabilization before the experimental protocol was started. During this time the cardiomyocytes were electrically stimulated at a rate of 0.5 Hz using a pair of platinum electrodes controlled by a MyoPacer stimulator (IonOptix). Thereafter, the cardiomyocytes were subjected to a H/R protocol (illustrated in Fig. 1) in which the temperature of the cell chamber was cooled from 35°C to 15°C in 30 min. Temperature was then maintained at 15°C for 2 h, followed by rewarming to 35°C over a 30-min period. After rewarming, temperature was maintained at 35°C for 1 h. For time-matched control, cardiomyocytes were maintained at 35°C in the cell chamber in for 4 h.

Simultaneous [Ca2+]_{cyto} and contractile response measurements. Cardiomyocytes were loaded with 0.5 μM fura-2 AM for 10 min at 35°C. After loading, the cells were washed twice and continuously perfused with Tyrode solution maintained at 35°C and aerated with 95% O2-5% CO2. The methods for simultaneous measurements of [Ca2+]_{cyto} and contractile responses using an IonOptix system have been previously described (14). Briefly, in isolated cardiomyocytes, [Ca2+]_{cyto} (fura-2 fluorescence at 340 and 380 nm) and contractile responses were induced by continuous electrical stimulation (see above) at 0.5 Hz (every 2 s). Figure 1 depicts the timing of measurements where each measurement represents the ensemble average of 30-s periods representing 15 evoked responses. An initial measurement was obtained before the H/R protocol was initiated and then repeated every hour across the 4-h H/R protocol.

The level of [Ca2+]_{cyto} was determined based on calibration of the ratio of Fura-2 fluorescence (R = 340/380 nm) using the equation described by Grynkiewicz et al. (12). Given the Ca2+ affinity of Fura-2 is temperature dependent, the dissociation constant (Kd) was determined at 35°C and 15°C based on an in vitro (cell-free) calibration performed using Fura-2 pentapotassium (Fura-2 PP) salt. The Kd is determined from the x-intercept of the double-log plot of free Ca2+ vs. Fura-2 fluorescence.

**Western blot.** Cardiomyocytes were homogenized in RIPA buffer containing 2% SDS and PMSF and kept on ice or at 4°C during all subsequent steps. Homogenized samples were solubilized by sonication for three 10-s intervals in a cold room. Samples were then spun in a refrigerated centrifuge at 10,000 g for 15 min. Protein content was assayed by the Lowry assay (Bio-Rad DC protein assay). The samples were denatured in 1× sample buffer at 100°C for 3 min, fractionated by SDS-PAGE, and transferred to the polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Transferred proteins on separate PVDF membranes were detected with specific antibody for either total cTnI, p-TnI at Ser23/24, catalytic subunit of protein kinase A (PKA), or actin and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The detected proteins were visualized and quantified by Kodak Imaging System.

**Chemicals and solutions.** Tyrode solution (in mM) contained 137 NaCl, 5.4 KCl, 0.5 MgCl2, 1.8 CaCl2, 0.33 NaH2PO4, 10.0 HEPES, and 10.0 glucose and was adjusted to pH 7.4 with NaOH. Enzyme solution was composed of Tyrode solution supplemented with 0.2 mM CaCl2, 0.6 mg/ml Type II Collagenase (Worthington) and 0.1 mg/ml protease with 1% bovine serum albumin. KB solution (in mM) contained 70 KOH, 50 l-glutamic acid, 40 KCL, 0.5 MgCl2, 1 K2HPO4, 0.5 EGTA, 10 HEPES, 5 pyruvic acid, 5 Na2ATP, 5.5 glucose, 20 taurine, and 5 creatine and was pH adjusted to 7.38 with KOH. M199 (M3769, Sigma; in mM) was supplemented with 5 creatine, 2 carnitine, 5 taurine, 10 NaHCO3, and 10 HEPES and adjusted to pH 7.4 with NaOH. Antibodies used for Western blot were specific for cTnI (Fitzgerald), p-TnI at Ser23/24 (Cell Signaling), catalytic subunit of PKA (Abcam), and actin (Cytoskeleton). Unless otherwise stated, all chemicals were purchased from Sigma.

**Statistical methods.** The experiments were designed according to a power analysis (power = 80%, α = 0.05) to include 6 animals per group. All results are presented as means ± SE. Differences between CTL and H/R over time were compared using a two-way ANOVA. If significant differences were found, Student’s t-test was used post hoc to compare values within equivalent time points. Comparison of CTL and H/R for cTnI phosphorylation levels was made using two-tailed, unpaired Student’s t-test. Differences were considered to be significant at P < 0.05.

**RESULTS**

Contractile dysfunction occurs following H/R. By comparison to time-matched controls (CTL), H/R exposed cells showed evidence of diminished contractile behavior during hypothermia and during initial rewarming (Fig. 2). Specifically, H/R slowed sarcomere shortening velocity during rewarming. Also H/R slowed the rate of sarcomere relaxation during hypothermia exposure (1-, 2-h time point, Fig. 2) and rewarming (3-h time point, Fig. 2). Although resting sarcomere length remained constant near 1.7 μm throughout the protocol for CTL cells, H/R exposed cells showed decreased resting length during hypothermia. The extent of sarcomere shortening significantly increased through hypothermia. However, immediately after rewarming, the extent of sarcomere shortening fell to a level less than CTL, indicating contractile dysfunction as a result of H/R exposure. However, during the subsequent 1-h period of rewarming (4-h time point, Fig. 2) the contractile response of H/R-exposed myocytes recovered.

[Ca2+]_{cyto} increases and slows during hypothermia. The evoked [Ca2+]_{cyto} response in H/R cells, despite undergoing alterations during hypothermia, recovered to a similar state as CTL cells after rewarming (3 h time point, Fig. 3) and were maintained 1 h after rewarming (4-h time point, Fig. 3). Basal [Ca2+]_{cyto} levels progressively rose throughout hypothermia compared with CTL, and returned to the same level as CTL cells upon rewarming. The peak or maximum [Ca2+]_{cyto} level
in response to stimulation progressively increased and plateaued around the 2-h time point in CTL and H/R. Two opposing aspects of the kinetics of the \(\text{[Ca}^{2+}\text{]}_{\text{cyto}}\) transient were measured: the time-to-peak (TTP) reflecting the action of Ca\(^{2+}\) release channels, and the time-to-50% relaxation (TT50%) reflecting the action of Ca\(^{2+}\) reuptake channels. In CTL cells, TTP and TT50% were maintained throughout the protocol. However, in H/R cells, both TTP and TT50% were dramatically slowed during hypothermia and returned to normal during rewarming.

**Myofilament Ca\(^{2+}\) sensitivity is reduced after H/R.** An advantage of simultaneous measurement of contraction and \(\text{[Ca}^{2+}\text{]}_{\text{cyto}}\) allows for a dynamic assessment of their relationship by plotting as a phase-loop (Fig. 4) where normalized sarcomere length changes span the \(y\)-direction while corresponding \(\text{[Ca}^{2+}\text{]}_{\text{cyto}}\) levels span the \(x\)-direction. A single evoked contractile response starts at the upper left corner of the phase-loop diagram and travels clockwise. Compared with CTL, H/R phase-loops are shifted rightward during hypothermia and rewarming but reverse to normal at 1 h after rewarming (4-h time point, Fig. 4). The rightward shift of the phase-loop indicates that Ca\(^{2+}\) sensitivity of the myofilament was reduced during and immediately after exposure to H/R but recovers within 1 h after rewarming.

To confirm that a decrease in myofilament Ca\(^{2+}\) sensitivity (in light of the rightward shift in phase-loop) was consistent across multiple cells exposed to H/R, phase-loop plots for 6 cells from each group were constructed and analyzed by determining the \(\text{[Ca}^{2+}\text{]}_{\text{cyto}}\) required for 50% shortening/relaxation (Fig. 4). In H/R cells compared with CTL cells, the \(\text{[Ca}^{2+}\text{]}_{\text{cyto}}\) required for 50% shortening/relaxation increased during hypothermia and immediately after rewarming, which indicates a decrease in myofilament Ca\(^{2+}\) sensitivity after H/R. Myofilament Ca\(^{2+}\) sensitivity returned to normal during 1 h after rewarming (4-h time point, Fig. 4).

**Cardiac TnI phosphorylation and the catalytic subunit of PKA is increased after H/R.** Phosphorylation of cTnI was determined by calculating the ratio of p-TnI to total cTnI protein expression (Fig. 5). The ratio of p-TnI/TnI was significantly increased in cells exposed...
to H/R compared with CTL. The increase in cTnI phosphorylation began during hypothermia and progressively increased during continued hypothermia exposure. However, cTnI phosphorylation gradually reverses during rewarming (Fig. 5).

Expression of the catalytic subunit of PKA was determined relative to actin expression. Similar to the increase in p-TnI, expression of the catalytic subunit of PKA increased progressively during hypothermia and gradually declined during rewarming (Fig. 5).

**DISCUSSION**

The results of the present study support the hypothesis that H/R disrupts excitation-contraction coupling in cardiomyocytes by reducing myofilament Ca$^{2+}$ sensitivity due to an increase in cTnI phosphorylation. These results in isolated cardiomyocytes are consistent with the contractile dysfunction induced by H/R in rat papillary muscle (14), and the left ventricular dysfunction induced by H/R in rat heart (10, 16, 38, 40). These results are also consistent with the clinical observation of ventricular dysfunction during rewarming shock following accidental hypothermia (21). The results of the present study provide new information regarding the time course of H/R disruption of excitation-contraction coupling without the complication of loading effects. These results also indicate that recovery occurs post rewarming.

The H/R-induced decrease in myofilament Ca$^{2+}$ sensitivity is well illustrated by the rightward shift in the phase-loop plots of sarcomere length change (contraction) vs. [Ca$^{2+}$]$_{cyt}$ during evoked contractile responses. The phase-loop diagram represents the dynamic behavior of the contractile response to the transient increase in [Ca$^{2+}$]$_{cyt}$. Spurgeon et al. (33) first described the use of phase-loop plots of simultaneously measured [Ca$^{2+}$]$_{cyt}$ and contractile responses in isolated cardiomyocytes to assess changes in myofilament Ca$^{2+}$ sensitivity. These investigators clearly validated that a leftward shift of the phase-loop plot occurred during Ca$^{2+}$ sensitization, whereas a rightward shift occurred during desensitization. More recently, we (14) and others (22, 25) have also demonstrated phase-loop relationships of simultaneously measured [Ca$^{2+}$]$_{cyt}$ and contractile responses to explore changes in Ca$^{2+}$ sensitivity of rat myocardium. In the present study, we show a rightward shift in the phase-loop plot of [Ca$^{2+}$]$_{cyt}$ and contractile responses...
after H/R reflecting a decrease in myofilament Ca\(^{2+}\) sensitivity (i.e., there was an increase in \([\text{Ca}^{2+}]_{\text{cyto}}\) at 50% relaxation). Furthermore, we also found that H/R induced a rightward shift in the contraction portion of the phase-loop plot (i.e., there was an increase in \([\text{Ca}^{2+}]_{\text{cyto}}\) at 50% shortening). This rightward shift in the contraction portion of the phase-loop plot reflects an increased delay between an elevation in \([\text{Ca}^{2+}]_{\text{cyto}}\) and the contractile response and may reflect a decrease in cross-bridge cooperativity.

In the present study, we found that the H/R-induced decrease in myofilament Ca\(^{2+}\) sensitivity (i.e., there was an increase in \([\text{Ca}^{2+}]_{\text{cyto}}\) at 50% relaxation). Furthermore, we also found that H/R induced a rightward shift in the contraction portion of the phase-loop plot (i.e., there was an increase in \([\text{Ca}^{2+}]_{\text{cyto}}\) at 50% shortening). This rightward shift in the contraction portion of the phase-loop plot reflects an increased delay between an elevation in \([\text{Ca}^{2+}]_{\text{cyto}}\) and the contractile response and may reflect a decrease in cross-bridge cooperativity.

In the present study, we found that the H/R-induced decrease in myofilament Ca\(^{2+}\) sensitivity of cardiomyocytes was associated with an increase in cTnI phosphorylation and PKA catalytic subunit expression. The observed increase in cTnI expression is consistent with our previous study of rat papillary muscle (14) and other cardiac pathophysiological states such as decompensated heart failure (29) and sepsis (36), where decreased Ca\(^{2+}\) sensitivity contributes to contractile dysfunction in association with increased in cTnI phosphorylation at Ser 23/24. The functional significance of phosphorylation at Ser 23/24 by PKA activation is well recognized as a key mechanism underlying reduced Ca\(^{2+}\) sensitivity in cardiomyocytes (35, 41). Accordingly, we found that the catalytic subunit of PKA parallels the trend of p-TnI increase during H/R and supports the role of PKA underlying cTnI phosphorylation. However, phosphorylation of cTnI at Ser 23/24 can be targeted by other kinases in addition to PKA, namely protein kinase C (PKC) and cyclic GMP-dependent protein kinase (PKG) (18).

It is important to note that H/R had no lingering effect on evoked \([\text{Ca}^{2+}]_{\text{cyto}}\) responses, even though the evoked \([\text{Ca}^{2+}]_{\text{cyto}}\) responses during hypothermia were markedly affected. The effect of hypothermia on \([\text{Ca}^{2+}]_{\text{cyto}}\) regulation in cardiomyocytes was characterized by a progressive increase in basal \([\text{Ca}^{2+}]_{\text{cyto}}\), and a prolongation of the evoked transient \([\text{Ca}^{2+}]_{\text{cyto}}\) response reflected by a slowing of both the
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Fig. 5. Western blot analysis of cTnI phosphorylation at Ser 23/24 site (p-TnI) and the catalytic subunit of PKA (PKAcat). cTnI phosphorylation was quantified as a ratio of total cTnI (p-TnI/TnI) and catalytic PKA was quantified relative to actin. Each graph shows the trend of H/R-exposed cardiomyocytes (as % of CTL) over the experimental time course, where CTL is always at 35°C, while H/R starts at 35°C at 0 h, is at 15°C during 1 h and 2 h (hypothermia), and returns to 35°C at 3 h (rewarming) and during an additional 1 h. *Significant difference (P < 0.05).

The increased contractile response during hypothermia that we observed is consistent with previous reports of a robust inotropic response of the myocardium to hypothermia (19, 28). Hypothermia-induced changes in the contractile response include 1) progressive decrease in resting sarcomere length, 2) progressive decrease in contractile shortening velocity, 3) prolongation of contractile response, and 4) increased extent of sarcomere length shortening. During initial rewarming, resting sarcomere length partially but not completely reverses and the duration of the contractile response returns to pre-hypothermia values. Yet, sarcomere length shortening velocity is slower and thus the extent of shortening is reduced compared with CTL. Changes in resting length during hypothermia is likely due to prolongation of both the evoked [Ca^{2+}]_{cyto} and contractile response, with insufficient time between stimuli to fully return to baseline. The slowing of sarcomere length shortening velocity induced by hypothermia persists after rewarming. With a slowing of sarcomere length shortening velocity, the extent of sarcomere length shortening will entirely depend on the duration of the contractile response. During hypothermia, there is a prolonged contractile response with an increased extent of shortening, but after rewarming the duration of the contractile response is normalized resulting in a decreased extent of shortening. Given an additional hour after rewarming, these altered contractile states of H/R cells return to normal as defined by CTL cells.

A number of previous studies have reported that hypothermia is associated with a Ca^{2+} overload in the heart (2, 11, 16, 19, 34, 40) and the results of the present study, which focused on [Ca^{2+}]_{cyto}, are generally consistent with these observations. However, we did not examine changes in [Ca^{2+}] in the sarcoplasmic reticulum (SR), mitochondria, nucleus, and other organelles that store Ca^{2+} within cells. It is possible that changes in [Ca^{2+}] in any of these intracellular structures might also induce molecular mechanisms that could diminish contractile function following H/R. For example, changes in mitochondrial [Ca^{2+}] may increase reactive oxygen species (ROS) generation and lead to mitochondrial disruption (1, 3) and altered oxyradical modification of proteins. Indeed, we previously examined the impact of hydrogen peroxide-induced thiol modification (oxidation) in skeletal muscle fibers (24), which decreased Ca^{2+} sensitivity. Although a full assessment of the involvement of hypothermia/rewarming-induced oxyradical...
modification is beyond the scope of the present study, its involvement cannot be excluded.

The clinical importance of our results brings focus to enhancing Ca\(^{2+}\) sensitivity via reducing cTnI phosphorylation as a therapeutic target in accidental hypothermia victims. Since a drug that directly targets cTnI phosphorylation is not yet available, the use of catecholamines or Ca\(^{2+}\) sensitizers may seem to be plausible alternatives for the treatment of H/R; however, neither option is unequivocally indicated based on the current study. In the case of catecholamines, their inotropic effect is mediated through β-receptor activation to increase cAMP and enhance the evoked [Ca\(^{2+}\)]\(_{cyto}\) response. However, as shown in this study, there was no significant reduction in the evoked [Ca\(^{2+}\)]\(_{cyto}\) transient following H/R. In fact, it has been shown that catecholamines and elevation of cAMP can induce cTnI phosphorylation (17, 32), which would be counterindicated in resolving the H/R-induced increase in cTnI phosphorylation and reduction in Ca\(^{2+}\) sensitivity. In agreement, we previously found that catecholamine (epinephrine) treatment does not resolve H/R-induced cardiac dysfunction in an in vivo rat model but actually has a negative inotropic effect (7, 13, 15, 37). With respect to Ca\(^{2+}\) sensitizers, we are unaware that any have been shown to unequivocally target TnI phosphorylation. In previous studies, we (6) and others (27) found that the Ca\(^{2+}\) sensitizer levosimendan improves the pressure/volume relationship of the heart after H/R in an in vivo rat model. Most studies indicate that levosimendan exerts its positive inotropic effects by targeting Ca\(^{2+}\) binding to cTnC or by inhibiting phosphodiesterase 3 (PDE3) breakdown of cAMP (9, 23). Rungatscher and colleagues (27) reported that levosimendan mitigated the H/R-induced increase in cTnI phosphorylation and suggested that this was the underlying mechanism of enhanced contractility in their in vivo rat model. However, based on this result, we conducted experiments exploring the effects of levosimendan on cTnI phosphorylation in isolated cardiomyocytes, but could not confirm that levosimendan had any mitigating effect on the H/R-induced increase in cTnI phosphorylation. Thus, in isolated cardiomyocytes, levosimendan does not appear to directly target cTnI phosphorylation. We also considered other Ca\(^{2+}\) sensitizers such as thiadiazone compounds (e.g., EMD 57033), but these appear to influence actomyosin interactions directly without targeting thin filament regulation (30). In agreement, we previously showed that the inotropic effect of EMD 57033 persisted in cTnI deficient cardiomyocytes (5). Further investigation is required to identify pharmacological agents capable of targeting H/R-induced cTnI phosphorylation and cardiac dysfunction.

**Conclusion.** In cardiomyocytes, H/R decreases myofilament Ca\(^{2+}\) sensitivity due to an increase in cTnI phosphorylation. The intracellular mechanism responsible for changes in cTnI phosphorylation is associated with PKA activation as well as elevated [Ca\(^{2+}\)]\(_{cyto}\) levels during hypothermia.

**GRANTS**

We acknowledge the following sources of funding: National Heart, Lung, and Blood Institute Grant ST-32-HL-105355 (N. Schaible) and the Norwegian Air Ambulance Foundation (Y. S. Han).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

N.S., Y.-S.H., G.M.A., T.T., and G.C.S. conception and design of research; N.S., Y.-S.H., and T.H. performed experiments; N.S., Y.-S.H., and T.H. analyzed data; N.S., Y.-S.H., and T.T. interpreted results of experiments; N.S. prepared figures; N.S. and Y.-S.H. drafted manuscript; N.S., Y.-S.H., and G.C.S. edited and revised manuscript; N.S., Y.-S.H., T.H., G.M.A., T.T., and G.C.S. approved final version of manuscript.

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AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00840.2015 • www.ajpheart.org


