β-adrenergic stimulation after rewarming does not mitigate hypothermia-induced contractile dysfunction in rat cardiomyocytes

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Abstract

Victims of severe accidental hypothermia are frequently treated with catecholamines to counteract the hemodynamic instability associated with hypothermia-induced cardiac contractile dysfunction. However, we previously reported that the inotropic effects of epinephrine are diminished after hypothermia and rewarming (H/R) in an intact animal model. Thus, the goal of this study was to investigate the effects of Epi treatment on excitation-contraction coupling in isolated rat cardiomyocytes after H/R. In adult male rats, cardiomyocytes isolated from the left ventricle were electrically stimulated at 0.5 Hz and evoked cytosolic [Ca^{2+}] and contractile responses (sarcomere length shortening) were measured. In initial experiments, the effects of varying concentrations of epinephrine on evoked cytosolic [Ca^{2+}] and contractile responses at 37° were measured. In a second series of experiments, cardiomyocytes were cooled from 37° to 15°C, maintained at 15°C for 2 h, then rewarmed to 37°C (H/R protocol). Immediately after rewarming, the effects of epinephrine treatment on evoked cytosolic [Ca^{2+}] and contractile responses of cardiomyocytes were determined. At 37°C, epinephrine treatment increased both cytosolic [Ca^{2+}] and contractile responses of cardiomyocytes in a concentration-dependent manner peaking at 25-50 nM. The evoked contractile response of cardiomyocytes after H/R was reduced while the cytosolic [Ca^{2+}] response was slightly elevated. The diminished contractile response of cardiomyocytes after H/R was not mitigated by epinephrine (25 nM) and epinephrine treatment reduced the exponential time decay constant (Tau), but did not increase the cytosolic [Ca^{2+}] response. We conclude that epinephrine treatment does not mitigate H/R-induced contractile dysfunction in cardiomyocytes.

Key words: Accidental hypothermia, rewarming, epinephrine, inotropic effects, cardiac contractile function, catecholamines, cardiomyocyte

Abbreviations:

H/R: Hypothermia and rewarming

Epi: Epinephrine

PKA: Protein kinase A

cTnI: Cardiac troponin I

$[Ca^{2+}]_{cyt}$: Cytosolic $[Ca^{2+}]$

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Introduction

The effect of medications administered during hypothermia may be significantly altered due to temperature-dependent changes in pharmacokinetics and pharmacodynamics [2]. Still, many accidental hypothermia victims receive inotropic drugs due to circulatory dysfunction, which clinically manifests as reduced arterial blood pressure and cardiac output [38,42]. This condition, sometimes referred to as rewarming shock, is associated with reduced cardiac contractile function caused by hypothermia and rewarming (H/R) [38,41].

Generally, β-adrenergic receptor agonists such as epinephrine (Epi) are widely used to improve hemodynamic function during acute cardiovascular failure [26]. However, the inotropic response to Epi seems to be reduced at core temperatures < 30°C and may remain depressed after rewarming [13,40]. While most clinical guidelines discourage the use of Epi at core temperatures < 30°C [8,20], the evidence to support this recommendation is limited [15].

At normothermia (37°C), Epi causes positive inotropic effects by stimulating the β_1-receptor in cardiomyocytes, which lead to an elevation of cyclic AMP (cAMP), subsequently activating PKA to phosphorylate numerous molecular targets [1,25,31]. In turn, this enhances Ca^{2+} influx to the cytosol via phosphorylation of L-type Ca^{2+} channels and increased sarcoplasmic reticulum Ca^{2+} reuptake and release [1]. Previously, we found that the increase in cardiac cAMP content after *in vivo* administration of Epi was significantly higher at 15°C compared to 37°C [7]. Thus, β-receptor function appears intact at low temperatures and may even show increased sensitivity during hypothermia [44,45].

Experimental studies using both *in vivo* and *in vitro* models show that H/R-induced cardiac contractile dysfunction is associated with increased protein kinase A (PKA) mediated phosphorylation of cardiac troponin I (cTnI) and reduced myofilament Ca^{2+} sensitivity

[5,10,14,27,33,39,41]. Furthermore, hypothermia can lead to a significant accumulation of myocardial intracellular [Ca^{2+}**]** causing a Ca^{2+} overload that may persist after rewarming [16,46]. The goal of the present study was to investigate the mechanisms underlying the apparent lack of inotropic effects of Epi after H/R. For this purpose, we examined effects of Epi treatment after H/R on excitation-contraction coupling in isolated rat cardiomyocytes. We hypothesize that the cytosolic [Ca^{2+}] ($[Ca^{2+}]_{cyt}$) response will be increased after H/R and aggravated by subsequent Epi treatment, and that Epi will not provide a positive inotropic effect in cardiomyocytes exposed to H/R.

Methods

2.1. Animals

Twelve male Sprague Dawley rats weighing 250 – 350 g (Envigo, Madison, WI, USA) were used in this study. The Mayo Clinic Institutional Animal Care and Use Committee (IACUC) approved the experimental protocol. Anesthesia was induced with intraperitoneal injections of ketamine (90 mg/kg; Zoetis Inc., Kalamazoo, MI, USA) and xylazine (10 mg/kg; Biomed-MTC, Animal Health Inc., Cambridge, ON, Canada). After confirming the anesthetic status by verifying the lack of a toe pinch reflex withdrawal, a thoracotomy was performed, the heart was excised, and the animal was euthanized by exsanguination.

2.2. Cardiomyocyte isolation

Isolation of left ventricular cardiomyocytes was performed as previously described in detail [33]. After excision, the heart was connected to a modified Langendorff system via cannulation of the aorta, and retrogradely perfused with oxygenated zero Ca^{2+} Tyrode solution for 5 min, after which the zero Ca^{2+} Tyrode solution was replaced by an enzyme solution containing type II collagenase, and perfused for another 10 min, causing digestion of the myocardium. The heart was then minced and filtered through a stainless-steel strainer (~200 μm). The isolated cardiomyocytes were centrifuged at 1000 rpm for 1 min and incubated with Kraft-Brühe solution at 37°C for 30 min, and subsequently suspended into Cardiac Myocyte Medium (ScienCell Research Laboratories) at 37°C for another 30 min. More than 60% of isolated cardiomyocytes from each heart were viable, as identified by trypan blue exclusion. Only viable cardiomyocytes maintaining a rod-shaped morphology with clear sarcomeric striation patterns and a stable contractile response to electrical field stimulation were included in the study. Throughout the

experiment, cardiomyocytes were electrically stimulated at 0.5 Hz using a MyoPacer stimulator (IonOptix, Westwood, MA, USA). The isolated cardiomyocytes were divided into two groups: 1) concentration-dependent effects of Epi were tested at 37°C and 2) effects of Epi were tested after H/R (Figure 1A)

2.4. Experimental protocol

Concentration-dependent effects of Epi in normothermic cardiomyocytes

In an initial series of experiments, the concentration-dependent effects of Epi on $[Ca^{2+}]_{cyt}$ and contractile responses (sarcomere length shortening) were tested in cardiomyocytes at 37°C. For each experiment, a single cardiomyocyte from the same rat heart were used and exposed to increasing concentrations of Epi (in nM; 0, 5, 25 and 50). The cardiomyocytes were perifused with each concentration of Epi for 5 min, followed by a washout with Ca^{2+} Tyrode solution for 10 min before another 5 min perifusion with the subsequent Epi concentration (Figure 1B).

Hypothermia/rewarming protocol

In a separate set of experiments, cardiomyocytes were cooled from 37°C to 15°C over 30 min, then kept at 15°C for 2 h, followed by a 30 min rewarming to 37°C (Figure 1C). Cardiomyocytes from the same rat heart were used in each H/R experiment. Simultaneous measurements of $[Ca^{2+}]_{cyt}$ and contractile responses were carried out in the same cardiomyocyte before cooling (baseline) and immediately after rewarming in the H/R protocol with and without Epi (25 nM) treatment. The time between post-H/R measurements with and without Epi treatment was not more than 10 minutes. The concentration of Epi was determined from the results of the initial series of studies.

2.3. Measurements of $[Ca^{2+}]_{cyt}$ and contractile responses to electrical field stimulation

Simultaneous measurements of $[Ca^{2+}]_{cyt}$ and contractile responses to electrical field stimulation were carried out as previously reported [12,33]. Briefly, the isolated cardiomyocytes were loaded with 1 μM fura-2 AM in 0.1% DMSO for 10 min at 37°C, after which the cells were washed twice and subsequently perfused with Ca^{2+} Tyrode solution aerated with 95% O_2 and 5% CO_2. The cardiomyocytes were electrically stimulated at a frequency of 0.5 Hz and $[Ca^{2+}]_{cyt}$ and contractile responses were measured simultaneously using an IonOptix system [23,33]. With this technique, simultaneous measurement of $Ca^{2+}]_{cyt}$ and contractile responses are limited to a single cardiomyocyte at a time. Thus, only one cardiomyocyte per animal can be studied. Calibration of the ratio (R=340/380 nm) of fura-2 fluorescence was used to determine the absolute value of $[Ca^{2+}]_{cyt}$ expressed in nM [11].

2.5. Chemicals and solutions

Epi was purchased from Millipore Sigma (St. Louis, MO). Ca^{2+} Tyrode solution (in mM) contained 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 10.0 HEPES, and 10.0 glucose and was adjusted to pH 7.4 with NaOH. The enzyme solution was composed of zero-Ca^{2+} Tyrode solution supplemented with 0.2 mM $CaCl_2$, 0.6 mg/ml type II collagenase (Worthington), and 0.1 mg/ml protease with 1% bovine serum albumin. Kraft-Brühe solution (in mM) consisted of 70 KOH, 50 $_L$-glutamic acid, 40 KCL, 0.5 $MgCl_2$, 1 KH_2PO_4, 0.5 EGTA, 10 HEPES, 5 pyruvic acid, 5 Na_2ATP, 5.5 glucose, 20 taurine, and 5 creatinine, and was adjusted to pH 7.38 with KOH. Cell medium (#6201; ScienCell) contained 500 ml of basal medium, 25 ml

of fetal bovine serum (FBS, Cat. No. 0025), 5 ml of cardiac myocyte growth supplement (CMGS, Cat. No. 6252), and 5 ml of penicillin/streptomycin solution (P/S, Cat. No. 0503).

2.6. Statistical analyses

The number of cardiomyocytes included in each protocol was determined based on a power analysis to detect a 20% difference for each outcome measure ($\beta = 80\%$, $\alpha = 0.05$). The distribution of each outcome measure was tested for normality using the Shapiro-Wilk test. In the experiment examining the Epi concentration-response relationship in normothermic cardiomyocytes, data were statistically analyzed using a one-way ANOVA for repeated measures. For the H/R experiment, the effects of H/R and Epi treatment on the outcome measures were analyzed using a two-way ANOVA for repeated measures. Inter-animal variability was excluded by the ANOVA. If overall significant differences were found, a *post-hoc* Student's t-test with Bonferroni correction was used for further analysis in both the Epi concentration-response experiment and the H/R experiment. Differences were considered significant at $P < 0.05$.

Results

The data from the experiment examining Epi concentration-response relationships at 37°C are included as supplemental figures, as these results were not used to test the hypothesis but were used to select the concentration of Epi for the H/R experiment. The results from the H/R experiment are presented as % change from baseline pre-hypothermia. The absolute values are presented in supplemental table 1.

Concentration-dependent effects of Epi on $[Ca^{2+}]_{cyt}$ *and contractile responses at 37°C*

Representative tracings of $[Ca^{2+}]_{cyt}$ and sarcomere length shortening responses of cardiomyocytes to electrical field stimulation at 37°C and at different Epi concentrations are shown in supplemental figure 1. A significant increase in the peak evoked $[Ca^{2+}]_{cyt}$ response was observed only at Epi concentrations of 25 and 50 nM of Epi (Supplemental figure 2). All three concentrations of Epi (5, 25 and 50 nM) significantly increased the extent of sarcomere length shortening, the velocity of shortening and the rate of relaxation compared to no treatment (Supplemental figure 3). The enhanced contractile responses with Epi were concentration dependent. At Epi concentrations of 25 and 50 nM, both $[Ca^{2+}]_{cyt}$ and contractile responses reached a plateau, and there were no significant differences between 25 and 50 nM Epi. A phase-loop plot comparing $[Ca^{2+}]_{cyt}$ and contractile responses (Supplemental figure 4) revealed that 25 and 50 nM Epi treatment had no effect on the $[Ca^{2+}]_{cyt}$ at which 50% relaxation occurred (i.e., Ca^{2+} sensitivity). However, the $[Ca^{2+}]_{cyt}$ at which 50% contraction occurred was significantly increased after treatment with 25 and 50 nM of Epi. Thus, we found that Epi increased both $[Ca^{2+}]_{cyt}$ and contractile responses in a concentration-dependent fashion reaching a plateau at 25 nM. Based on these findings, an Epi concentration of 25 nM was selected for the H/R experiment.

Epi does not mitigate H/R-induced contractile dysfunction

Figure 2 shows representative tracings of evoked $[Ca^{2+}]_{cyt}$ and contractile responses at 37°C before (baseline pre-hypothermia) and after H/R with and without Epi (25 nM) treatment. Compared to baseline pre-hypothermia, the cardiomyocytes exposed to H/R demonstrated a significant reduction of both the extent of sarcomere length shortening, the velocity of shortening and rate of relaxation, which was not mitigated after Epi treatment (Figure 3).

H/R-induced changes of evoked $[Ca^{2+}]_{cyt}$ responses are not affected by Epi treatment

Cardiomyocytes exposed to H/R showed a small but significant increase of the peak evoked $[Ca^{2+}]_{cyt}$ response compared to baseline pre-hypothermia $[Ca^{2+}]_{cyt}$ responses. However, both basal (resting) $[Ca^{2+}]_{cyt}$ and the amplitude of evoked $[Ca^{2+}]_{cyt}$ transients were unchanged (Figure 4). The kinetics of evoked $[Ca^{2+}]_{cyt}$ transients were affected by H/R; the exponential time decay constant (Tau) of the $[Ca^{2+}]_{cyt}$ transient was significantly increased, reflecting a delay in the sequestration of $[Ca^{2+}]_{cyt}$. In contrast, the time-to-peak $[Ca^{2+}]_{cyt}$ response was reduced after H/R, reflecting a more rapid influx of Ca^{2+} to the cytosol. These H/R-induced alterations of evoked $[Ca^{2+}]_{cyt}$ responses were unaffected by Epi treatment, except for Tau, which returned to baseline pre-hypothermia levels. Dynamic assessment of the relationship between $[Ca^{2+}]_{cyt}$ and sarcomere shortening (phase-loop plots) (Figure 5) demonstrated that the $[Ca^{2+}]_{cyt}$ required for 50% relaxation and contraction were unchanged after H/R and unaffected by Epi treatment.

Discussion

This study confirmed previous observations that contractility of cardiomyocytes is reduced immediately after H/R [8–10, 23]. Importantly, we found that the H/R-induced contractile dysfunction was not mitigated by 25 nM Epi, a concentration that elicited a positive inotropic effect 37°C (normothermia). We found that the positive inotropic effect of Epi at normothermia involves an increase in the evoked $[Ca^{2+}]_{cyt}$ response, whereas Epi treatment immediately after H/R did not significantly change the amplitude of the evoked $[Ca^{2+}]_{cyt}$ response or the kinetics of $[Ca^{2+}]_{cyt}$ transients. Our findings are consistent with previous studies demonstrating reduced inotropic effects of Epi when administered *in vivo* during cooling or rewarming [7,17,40]. However, in the intact animal model, the reduced inotropic effects of Epi administered during hypothermia were suggested to be the result of the marked increase in systemic vascular resistance, causing an abrupt elevation of cardiac afterload [7]. Thus, this study provides new information showing that Epi fails to improve contractile function in cardiomyocytes when administered after rewarming.

In general, the lack of inotropic effects of Epi appear to depend on the severity of hypothermia. American and European resuscitation guidelines recommend lowering the core temperature to between 32-36°C (Targeted Temperature Management, TTM) for 24 h in patients who remain unresponsive following a return of spontaneous circulation after cardiac arrest [28,29]. At this temperature range (i.e., mild hypothermia), experimental data show positive inotropic effects of Epi [9,24]. In contrast, preclinical studies in different species report reduced or even negative inotropic effects of Epi at temperatures below 30°C [7,17,32,40].

There is a discrepancy between available guidelines addressing use of Epi during resuscitation of hypothermic patients. Due to the potential of adverse effects and insufficient evidence showing benefits of Epi treatment, both the European Resuscitation Council and other expert panel guidelines recommend withholding Epi until a temperature of 30°C is reached [8,20]. The same recommendation was found in previous American Heart Association guidelines, but was rescinded in their latest version given the limited amount of studies not showing benefits of treatment [15]. This lack of evidence to guide treatment makes circulatory dysfunction in hypothermic patients a difficult condition to manage in the clinical setting [6,30]. The existent need for therapeutic guidelines can be underlined by clinical reports showing that 38 – 66 % of patients who suffered severe accidental hypothermia received catecholamine support to manage hemodynamic instability during rewarming [18,22,42]. This hemodynamic instability may occur during or after rewarming and is characterized by an uncompensated reduction of arterial blood pressure and cardiac output [38]. If left untreated, it may result in compromised blood flow to vital organs and failure to maintain aerobic metabolism. The presence of hemodynamic instability during rewarming is found to be a stronger predictor for poor outcomes than the severity of hypothermia itself [22].

The present experimental model used to investigate cardiac contractile dysfunction in response to H/R is relevant to severe accidental hypothermia. Previous studies using this isolated cardiomyocyte model have reported that the H/R-induced contractile dysfunction can be associated with increased PKA-mediated phosphorylation of serine residues at position 23 and 24 (Ser23/24) of cTnI, and consequently reduced myofilament Ca^{2+} sensitivity [33,34]. In another study using transgenic mice lacking the cTnI Ser23/24 phosphorylation site, the

transgenic mice returned to normal cardiac contractile function after rewarming, in contrast to wildtype mice that displayed H/R-induced contractile dysfunction [39]. These findings indicate an underlying role of cTnI Ser23/24 phosphorylation and reduced myofilament Ca^{2+} sensitivity in developing H/R-induced contractile dysfunction. These fundamental physiological changes induced by H/R may be aggravated by using Epi, which is found to induce cTnI Ser23/24 phosphorylation through β-receptor stimulation and increased cAMP levels [19,36]. In agreement, a previous study from our research group showed significantly higher levels of cAMP after Epi treatment during hypothermia compared to normothermia [7]. High concentrations of cAMP after catecholamine treatment are reportedly toxic to cardiac cells, inducing an unphysiological increase of $[Ca^{2+}]_{cyt}$ via phosphorylation of L-type calcium channels, which can cause mitochondrial Ca^{2+} overload and impair energy production [6,21,43]. In fact, accumulation of intracellular Ca^{2+} is reported to occur during prolonged hypothermia [16,47], owing to increased duration of evoked Ca^{2+} transients causing elevation of basal $[Ca^{2+}]_{cyt}$ [33]. Consequently, an added Ca^{2+} load during β-stimulation can explain the reduced inotropic effects of Epi during hypothermia [6]. In support of this argument, a study using Langendorff-perfused isolated rat hearts found that adding Ca^{2+} to the perfusate enhanced the inotropic effects of Epi during normothermia, whereas it led to reduced stroke volume and cardiac output during Epi treatment at hypothermia [35].

In addition, we have reported that H/R-induced contractile dysfunction is associated with the increased formation of reactive oxygen species, which may serve to reduce myofilament Ca^{2+} sensitivity via oxidative modification of contractile proteins [34]. However, we found no changes in the phosphorylation status of cardiac myosin binding protein C in cardiomyocytes displaying H/R-induced contractile dysfunction compared to normothermic controls [12].

In the present study, basal $[Ca^{2+}]_{cyt}$ was unchanged after rewarming compared to pre-hypothermia. This finding resonates with a previous study in cardiomyocytes demonstrating hypothermia-induced accumulation of $[Ca^{2+}]_{cyt}$ that returned to pre-hypothermic levels after rewarming [33]. We found a significantly reduced sarcomere length shortening in combination with increased peak $[Ca^{2+}]_{cyt}$ after rewarming, consistent with impaired contractile function due to reduced myofilament Ca^{2+} sensitivity and in line with previous studies [12,14,33,34]. Based on the phase-loop plot, in which changes in sarcomere length plotted against the corresponding $[Ca^{2+}]_{cyt}$ can be dynamically assessed, we were unable to confirm the change in myofilament Ca^{2+} sensitivity after H/R (figure 5). Previous studies using this method have shown that a leftward and rightward shift of the phase-loop plot is associated with Ca^{2+} sensitization and desensitization, respectively [33,37]. Nonetheless, myofilament Ca^{2+} sensitivity ultimately depends on the relationship between $[Ca^{2+}]_{cyt}$ and force generated by the myocardium, and the actual loaded force development in this model of externally unloaded cardiomyocytes remains essentially unknown [3,4].

The present data does not support our hypothesis that the increased $[Ca^{2+}]_{cyt}$ response after H/R was aggravated by Epi treatment. Other than a decrease of the exponential time decay constant (Tau) of the $[Ca^{2+}]_{cyt}$ transient, reflecting an accelerated sequestration of $[Ca^{2+}]_{cyt}$, Epi treatment after H/R had no effect on the $[Ca^{2+}]_{cyt}$ response. However, we did not measure $[Ca^{2+}]$ in other intracellular pools such as the sarcoplasmic reticulum or mitochondria, which was beyond the scope of the this study but should be subject to future research.

In summary, we found that Epi, given in a concentration that generates a positive inotropic effect at normothermia, does not mitigate H/R-induced contractile dysfunction when administered after rewarming from a 2 h period of hypothermia at 15°C. Our data are acquired from rat

cardiomyocytes and do not allow for direct translation to human clinical medicine. We included only a single cardiomyocyte per animal, which is a limitation with using this methodological approach and may contribute to the observed degree of variability between cardiomyocytes in this study. Taken together, we interpret our findings to support the European Resuscitation Council recommendations to withhold Epi treatment during acute management of severe accidental hypothermia. Future studies are warranted to determine the specific intracellular mechanisms underlying the blunted inotropic response to Epi treatment after H/R, and to investigate other therapeutic targets than the β -adrenergic receptor pathway to mitigate H/R-induced cardiac contractile dysfunction.

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Figure legends:

Figure 1: Experimental protocol for examining the concentration-dependent effects of Epi on normothermic (37^{o}C) $[Ca^{2+}]_{cyt}$ and sarcomere length (SL) shortening responses of cardiomyocytes to electrical field stimulation (A); for each experiment, a single cardiomyocyte isolated from the same rat heart was used and exposed to increasing concentrations of Epi (in nM; 0, 5, 25 and 50) Experimental protocol for examining the effect of H/R without/with Epi (25 nM) on the evoked $[Ca^{2+}]_{cyt}$ and sarcomere length (SL) shortening responses of cardiomyocytes (B); a single cardiomyocyte isolated from the same rat heart underwent the H/R protocol. Measurements were carried out at baseline and after rewarming without/with Epi (25 nM).

Figure 2: Representative tracings of the $[Ca^{2+}]_{cyt}$ and sarcomere length (SL) shortening responses to electrical field stimulation before H/R (A, C). After H/R, the $[Ca^{2+}]_{cyt}$ response to electrical field stimulation returned to pre-H/R levels (B), while the extent of sarcomere length (SL) shortening was reduced (D). Treatment with Epi (25 nM) had no significant effect on the $[Ca^{2+}]_{cyt}$ response to electrical field stimulation post-H/R (B) nor did it mitigate the reduction in SL shortening post H/R (D).

Figure 3: Summary of the effects of H/R with and without Epi (25 nM) treatment on the extent of sarcomere length (SL) shortening (A), the velocity of SL shortening (B), the SL relaxation rate (C), the time to peak SL shortening (D), and the time to 50% relaxation of SL shortening (E). H/R reduced the extent of SL shortening (A) and slowed shortening velocity (B) as well as relaxation rate (C), compared to pre-H/R. However, there was no effect of H/R on the time to peak SL shortening (D) or the time to 50% SL relaxation (E). Treatment with Epi (25 nM) did

not alter the effects of H/R on the evoked contractile responses. For each experiment, cardiomyocytes from the same rat heart were used, and exposed to 25 nM Epi. A two-way repeated measures ANOVA was used to analyze results. * $p < 0.05$ compared to pre-H/R and to post-H/R with Epi, n =6 (number of animals).

Figure 4: Summary of the effects of H/R with and without Epi (25 nM) treatment on basal $[Ca^{2+}]_{cyt}$ (A), peak $[Ca^{2+}]_{cyt}$ response evoked by electrical field stimulation (B), the amplitude of the evoked $[Ca^{2+}]_{cyt}$ response (peak $[Ca^{2+}]_{cyt}$ – basal $[Ca^{2+}]_{cyt}$) (C), time to peak $[Ca^{2+}]_{cyt}$ response (D) and Tau of the $[Ca^{2+}]_{cyt}$ response (E). H/R had no effect on basal $[Ca^{2+}]_{cyt}$ but increased the peak $[Ca^{2+}]_{cyt}$ response, the time to peak $[Ca^{2+}]_{cyt}$ and the Tau of the $[Ca^{2+}]_{cyt}$ response compared to pre-H/R. Treatment with Epi (25 nM) reduced the Tau of the $[Ca^{2+}]_{cyt}$ response, but did otherwise not alter the effects of H/R on the evoked $[Ca^{2+}]_{cyt}$ response. For each experiment, cardiomyocytes from the same rat heart were used, and exposed to 25 nM Epi. A two-way repeated measures ANOVA was used to analyze results. * $p < 0.05$ compared to pre-H/R and to post-H/R with Epi, n =6 (number of animals).

Figure 5: Phase-loop plots (A) in which $[Ca^{2+}]_{cyt}$ was plotted vs. SL shortening in response to electrical stimulation had no significant shifts between pre-hypothermia and post-hypothermia with/without Epi. For each experiment, cardiomyocytes from the same rat heart were used, and exposed to 25 nM Epi. A two-way repeated measures ANOVA was used to analyze results. n =6 (number of animals).

Supplemental figure legends:

Supplemental Figure 1: Raw tracings of $[Ca^{2+}]_{cyt}$ (A) and sarcomere length (SL) shortening (B) responses of cardiomyocytes to electrical field stimulation at different Epi concentrations. A total of 25 evoked $[Ca^{2+}]_{cyt}$ (C) and SL shortening (D) responses were averaged for different Epi concentrations.

Supplemental Figure 2. Treatment with Epi increased peak $[Ca^{2+}]_{cyt}$ (C) and absolute $[Ca^{2+}]_{cyt}$ (D) response to electrical filed stimulation in a concentration-dependent manner, but had no effect on basal $[Ca^{2+}]_{cyt}$ (B), time to peak (E), and Tau (F). For each experiment, cardiomyocytes from the same rat heart were used, and exposed to increasing concentrations of Epi (in nM; 0, 5, 25 and 50). A one-way repeated measures ANOVA was used to analyze results. * $p < 0.05$ compared to 0 nM Epi, n =6 (number of animals).

Supplemental Figure 3: Treatment with Epi increased extent of sarcomere length (SL) shortening (B), SL shortening velocity (C), and SL relaxation velocity (D) in response to electrical field stimulation, but did not affect time to peak (E) and time to 50% relaxation (E). For each experiment, cardiomyocytes from the same rat heart were used, and exposed to increasing concentrations of Epi (in nM; 0, 5, 25 and 50). A one-way repeated measures ANOVA was used to analyze results. * $p < 0.05$ compared to 0 nM Epi, n =6 (number of animals).

Supplemental Figure 4: Treatment with Epi changed the phase-loop plots of $[Ca^{2+}]_{cyt}$ and sarcomere length (SL) shortening in a concentration-dependent manner (A and B). Epi treatment

increased the extent of SL shortening (A). In B, relative SL changes are plotted against $[Ca^{2+}]_{cyt}$. The concentration of $[Ca^{2+}]_{cyt}$ at which 50% relaxation of SL occurred (EC50 relaxation) was not affected by Epi treatment, supporting no change in Ca^{2+} sensitivity in response to Epi (C). The concentration of $[Ca^{2+}]_{cyt}$ at which 50% sarcomere length (SL) shortening occurred (EC50 contraction) was shifted rightward following Epi treatment (A, B and D), which indicates that the positive ionotropic effect of Epi was not due to increased Ca^{2+} sensitivity. Arrows in (A) represent the direction of phase-loop plots. * $p < 0.05$ compared to 0 nM Epi, n =6 (number of animals).