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Myocardial mechanical dysfunction and calcium overload following rewarming from experimental hypothermia *in vivo* ☆

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Abstract

Rewarming patients from accidental hypothermia are regularly complicated with cardiovascular instability ranging from minor depression of cardiac output to fatal circulatory collapse also termed ''rewarming shock''. Since altered Ca^{2+} handling may play a role in hypothermia-induced heart failure, we studied changes in Ca^{2+} homeostasis in *in situ* hearts following hypothermia and rewarming. A rat model designed for studies of the intact heart in a non-arrested state during hypothermia and rewarming was used. Rats were core cooled to 15 °C, maintained at 15 °C for 4 h and thereafter rewarmed. As time-matched controls, one group of animals was kept at 37 °C for 5 h. Total intracellular myocardial Ca^{2+} content ($[Ca^{2+}]_i$) was measured using $^{45}Ca^{2+}$. Following rewarming we found a significant reduction of stroke volume and cardiac output compared to prehypothermic control values as well as to time-matched controls. Likewise, we found that hypothermia and rewarming resulted in a more than six-fold increase in $[Ca^{2+}]_i$ to 3.01 ± 0.43 μmol/g dry weight compared to 0.44 ± 0.05 μmol/g dry weight in normothemia control. These findings indicate that hypothermia-induced alterations in the Ca^{2+}-handling result in Ca^{2+} overload during hypothermia, which may contribute to myocardial failure during and after rewarming.

Keywords: Calcium overload; Cardiovascular function; Rewarming shock; Myocardial intracellular calcium; Rat

The success rate when rewarming patients from accidental profound hypothermia has not been improved the last five decades and mortality is reported to be 52–80% depending on methods of rewarming [19]. One important factor that may explain the low success rate in treating hypothermic patients is that rewarming following accidental hypothermia is often associated with cardiovascular collapse, clinically termed ''rewarming shock''. This condition is recognized as a progressive reduction of cardiac output and a sudden fall in arterial blood pressure. The underlying pathophysiology explaining this condition, however, is largely unknown. Deterioration of cardiovascular function is known to occur when rewarming the intact animal heart in a non-arrested state following exposure to experimental hypothermia [7,22,23,25,29,32,34,35]. In these experiments hypothermia-induced heart failure has been proposed with a contractile dysfunction not related to foregoing ischemia as will be the case following rewarming from hypothermic cardio-pulmonary bypass used for cardiac surgery. [12,21,32].

Impaired Ca^{2+} handling is described as a key factor in the pathophysiology of normothermic heart failure [36], and may also play a central role in hypothermia-induced heart failure. Low temperature is known to both decrease myofilamental Ca^{2+}-sensitivity and increase myocardial contractility [10]. These two, seemingly contradictory

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functional changes, are induced already when lowering temperature from 36 to 30 °C [14]. The underlying mechanism for temperature-induced increase of force of contraction in cardiac muscle preparations has been shown to be associated with elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) [24]. Increase of $[Ca^{2+}]_i$ in response to hypothermia has previously been reported in isolated perfused hearts subjected to a hypothermic cardiac arrest [1,3,27,30]. To our best knowledge no data concerning changes in myocardial $[Ca^{2+}]_i$ in *in situ* rewarmed hearts subjected to a non-arrested hypothermic state, however, has so far been reported. Thus, the aim of the present study was to examine changes in total myocardial $[Ca^{2+}]_i$ in an intact animal model following rewarming from experimental hypothermia. To do this we used a rat model, in which we previously have shown significant deterioration of myocardial mechanical function following rewarming from 4 to 5 h hypothermia at 15–13 °C [12,13,34]. Our hypothesis is that hypothermia-induced myocardial failure was associated with increase in myocardial $[Ca^{2+}]_i$ in this intact animal model.

Materials and methods

Wistar rats (males, 377 ± 21 g) were used in the experiments. The rats had a microbiological status according to the recommendation of FELASA (Federation of European Laboratory Animal Science Associations) and were provided from Harlan UK Limited, England. On arrival animals were quarantined for 1 week. Housing during experiments was performed in accordance with guidelines for accommodation and care of animals (article 5 of European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, Strasbourg, 18.III.1986). Free food and water access were permitted. Experimental protocol was approved by Norwegian Animal Research Authority (NARA) and conducted accordingly.

Anaesthesia

Anaesthesia was induced by sodium pentobarbital 50 mg/kg body weight i.p., followed by a continuous infusion of 7.5 mg/kg/h through an i.v. line in right jugular vein extended to the right auricle. The normothermic group was given anaesthesia during experiments. In the hypothermic group the infusion was terminated when cooling was started due to hypothermia created anaesthesia and reduced drug metabolism. During rewarming pentobarbital infusion was started after 30 °C.

Respiratory support

The rat was placed on the operating table in a supine position. The trachea was opened and a tracheal tube inserted. All animals had spontaneous and sufficient ventilation at core temperatures above 20 °C. At core temperatures below 20 °C normoventilation was achieved by a volume-controlled small animal respirator (New England rodent ventilator, model 141, New England Instruments, Medway, MA) using room air.

Core cooling and rewarming

The animals in the hypothermia group were cooled and rewarmed by circulating cold (10 °C) or warm (40 °C) water (Thermo stated water bath type RTE-110, Neslab Instruments, Newington, NH) through U-shaped polyethylene tubes placed in esophagus and the lower bowels, and through the double-layered operating table made of hollow aluminum. Normothermia in control animals was maintained by circulating warm (37–38 °C) water through the operating table and tube system. Core temperature was continuously monitored using a thermocouple wire positioned in the aortic arch via right femoral artery, connected to a thermocouple controller (Thermoalert TH-5, Columbus Instruments, Columbus, OH). Cooling lasted 60 min, while rewarming lasted 120 min.

Hemodynamic measurements

A fluid-filled catheter (22G) was implanted in left femoral artery for continuous recording of arterial pressure. Left ventricular (LV) pressure was measured through a fluid-filled catheter (20G) placed in the left ventricle via the right common carotid artery. LV pressure was differentiated to obtain the maximum rate of rise of LV pressure (LV dP/dt_{max}). Catheters were connected to Transpac III transducers (Abbot, North Chicago, IL). The signals from the pressure transducers were amplified to 0–10 V and passed to a 12-bit analogue to digital converter (BNC 2090, National Instruments, Austin, TX). Signal processing and analysis were performed with the help of a special computer program developed at our department using a commercially available software package (LabVIEW v.6.0, National Instruments, Austin, TX). Cardiac output was measured by thermodilution technique [20], by injecting saline (0.1–0.15 ml) precooled in ice water through the i.v. line positioned in the right auricle. The change in temperature was recorded from the thermocouple positioned in the aortic arch through the right femoral artery. Thermodilution curves were recorded on a Linearcorder (Type Mark II, WR3101, Watanabe Instruments Corp., Japan). These curves were digitalized with Calcomp digitizing table (model: 23180, Calcomp Digitzer Products Division, Anaheim, CA) and cardiac output was calculated with a program designed with the LabView package. Cardiac output was calculated as the mean of three measurements.

Blood gases measurement

Blood gases, oxygen saturation, pH and base excess were measured in 0.15 ml arterial blood samples taken from femoral artery after surgery, at 15 °C and after

rewarming to 37 °C in hypothermic group and three times in normothermic control group (at baseline and after 3 and 5 h at 37 °C). Samples were analyzed by RapidLab 800 blood gas analyzer (Chiron Diagnostics Corp., USA).

Measurement of intracellular Ca^{2+} content

The method using radiolabeled calcium ($^{45}Ca^{2+}$) for measuring total intracellular calcium content $[Ca^{2+}]_i$, as described by Tani and Neely [31], was modified to an *in vivo* experiment, 20 μCi of $^{45}Ca^{2+}$ (ARX-102 Calcium-45, American Radiolabeled Chemicals Inc., St. Louis, MO), diluted in 0.5 ml saline, was injected through the left ventricle catheter as soon as instrumentation was completed. In order to measure time to equal distribution of $^{45}Ca^{2+}$ throughout the body, pilot experiments were performed in which the activity of $^{45}Ca^{2+}$ in blood was measured in blood samples collected at time intervals following the $^{45}Ca^{2+}$ injection. We found a rapid reduction in $^{45}Ca^{2+}$ activity in the plasma, reaching a steady state level 120 min after injection. Furthermore, in order to washout the extracellular $^{45}Ca^{2+}$ in the myocardium, hearts were excised and perfused in a Langendorff system with Krebs–Henseleit bicarbonate buffer containing glucose 11.1 mmol/l and Ca^{2+} 2.4 mmol/l at room temperature. Pilot studies were conducted in order to determine washout time. In these experiments the coronary effluent samples were collected and the $^{45}Ca^{2+}$ activity determined. During the first minute of washout $^{45}Ca^{2+}$ activity in the coronary perfusate decreased to a constant level that was found not to change further over the next 14 min. From these experiments a 3-min washout period was chosen. Total intracellular calcium content in the myocardium was measured in hearts freeze-clamped following this 3-min perfusion period. The hearts were vacuum dry-frosted for 24 h (Christ Alpha 1-4, Medizinischer Apparatebau, Osterode, Harz, Germany) and subsequently pulverized for 3 min at 1600 rpm by a micro-dismembrator (Braun Messungen AG, Germany). Thereafter 80–90 mg of homogenate was extracted in 1 ml ice-cold 0.42 M perchloric acid for 10 min and then centrifuged for 10 min (Kubota 1700 centrifuge, Cubota Corp., Tokyo, Japan) at 3000 rpm. The $^{45}Ca^{2+}$ activity in the supernatant was determined. In order to determine the specific activity of the isotope, an arterial blood sample, drawn immediately before terminating the experiment, was centrifuged (5 min) and the $^{45}Ca^{2+}$ activity as well as calcium concentration in plasma was determined using a liquid scintillation spectrometer (Model 1900 TR, Packard Instrument Company, USA) and RapidLab 800 blood gas analyzer (Chiron Diagnostics Corp., USA), respectively. Intracellular Ca^{2+} content was calculated from the tissue radioactivity, the specific activity of the plasma and weight of the dried homogenized heart tissue. It should be emphasized that this method cannot distinguish between intracellular pools of Ca^{2+}, namely cytosolic, sarcoplasmic reticulum and mitochondrial. Therefore, only total myocardial intracellular calcium content was measured.

Experimental protocol

Following instrumentation and $^{45}Ca^{2+}$ infusion the animals were stabilized for 2 h before starting measuring basal function. The animals were divided into two groups—one hypothermic group and one normothermic control group. In the hypothermic group rats were cooled to 15 °C and maintained at 15 °C for 4 h before rewarming to normothermia. In the control group animals were kept at 37 °C for 5 h. At the end of experiments myocardial Ca^{2+} content was determined as described. Two animals that did not survive hypothermia and rewarming were not included.

Statistics

Results are presented as means and SEM. Differences in hemodynamic values and myocardial calcium content between groups were compared using two-tailed independent Student's *t*-test. To assess hemodynamic changes of corresponding values in hypothermic group during cooling and rewarming two-tailed paired Student's *t*-test was used. Differences were considered significant at $p < 0.05$.

Results

Control animals

Hemodynamic function

All control animals survived the experiments, and hemodynamic function remained stable during experiments (5 h at 37 °C). Core temperature remained stable through the whole experiment (Figs. 1 and 2).

Myocardial intracellular Ca^{2+} content

Intracellular myocardial calcium content ($[Ca^{2+}]_i$) measured at the end of experiment was 0.44 ± 0.05 μmol/g dry weight. This is comparable to $[Ca^{2+}]_i$ measured in normothermic, isolated rat hearts [2] (Fig. 3).

Hypothermia/rewarmed animals

Hemodynamic function (Fig. 1 and 2)

Cooling. During cooling there was a dynamic reduction of all hemodynamic variables, except for stroke volume (SV). At 15 °C both mean arterial pressure (MAP) and left ventricular (LV) systolic pressure (LVSP) were reduced by 65–70%, whereas heart rate (HR) and LV dP/dt_{max} were reduced to 13 and 4% of their prehypothermic baseline values, respectively. Due to technical limitations we were not able to measure cardiac output (CO) below 20 °C. However, already at 20 °C CO was reduced by 38% and total peripheral resistance (TPR) by 35%, while SV was

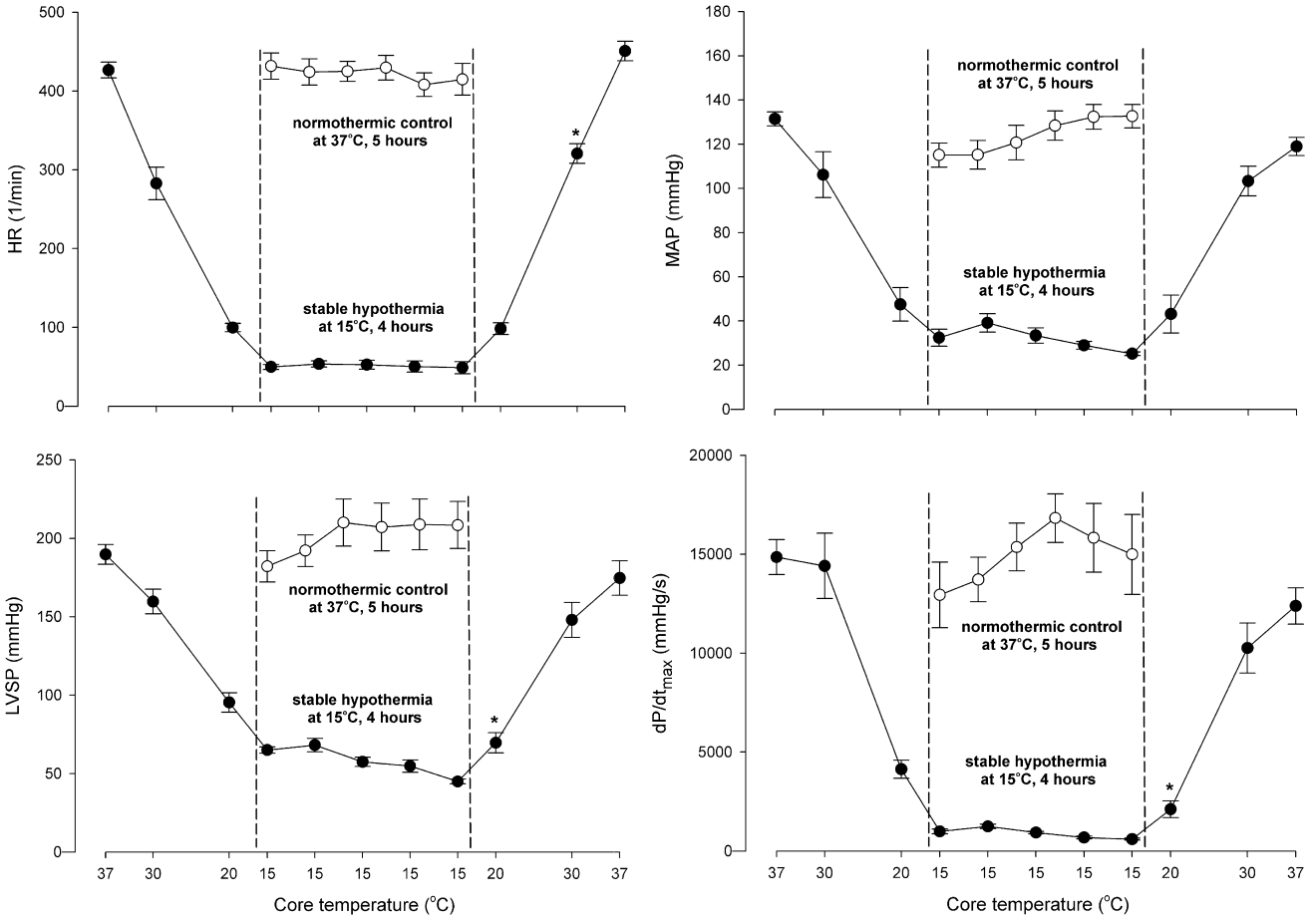

Fig. 1. HR, MAP, LVSP and dP/dt_{max} in hypothermic group and normothermic control. Heart rate (HR), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP) and maximal speed of increase of left ventricle pressure (dP/dt_{max}) in hypothermic and time-matched normothermic control animals. (●)—hypothermic animals; (○)—normothermic control animals. Values are mean and SEM. $^{*}p < 0.05$ vs. corresponding value during cooling.

increased almost three-fold. During the 4 h at 15 °C there were no further changes in any of the hemodynamic variables measured.

Rewarming. Recovery of hemodynamic function following rewarming was calculated as relative values in comparison to their prehypothermic baseline values. Following rewarming CO and SV were both significantly reduced to 77% of baseline values, while TPR was increased by 28%. Although LVSP and LV dP/dt_{max} were reduced to 91% and 83%, respectively, these changes did not reach statistical significance ($p = 0.06$ and 0.054, respectively). HR and MAP, returned to baseline levels, and LV end-diastolic pressure (LVEDP) remained unchanged during experiments (the last one is not presented in figures).

Myocardial intracellular Ca^{2+} content (Fig. 3)

In the present study myocardial $[Ca^{2+}]_i$ in rats exposed to 4 h hypothermia (15 °C) followed by rewarming was almost seven times higher than in normothermic, time-matched control rats (3.01 ± 0.43 vs 0.44 ± 0.05 μmol/g dry weight, respectively, $p < 0.0001$). Two rats died between 34 °C and 37 °C during rewarming and results from these were not included. Unlike hearts from surviving animals we found visible signs of infarct in both these hearts, but $[Ca^{2+}]_i$ (0.97 and 2.55 μmol/g dry weight) was not higher in these hearts compared to hearts from surviving animals.

Discussion

The present experiments show that rewarming following 4 h of profound hypothermia induces significant reduction in cardiac output and stroke volume, and an almost seven-fold increase in intracellular myocardial tissue calcium content. Taken together, this indicates that hypothermia/rewarming-induced calcium overload in the myocardium may contribute to the posthypothermic myocardial failure.

In general, an increase in intracellular Ca^{2+} content ($[Ca^{2+}]_i$) appears to be involved in both short- and long-term compensatory mechanisms tending to maintain cardiac output both in physiologic and pathophysiologic conditions. However, an inappropriate elevation of $[Ca^{2+}]_i$ may cause calcium overload and eventually contribute to induction of heart failure. Thus, calcium overload may be

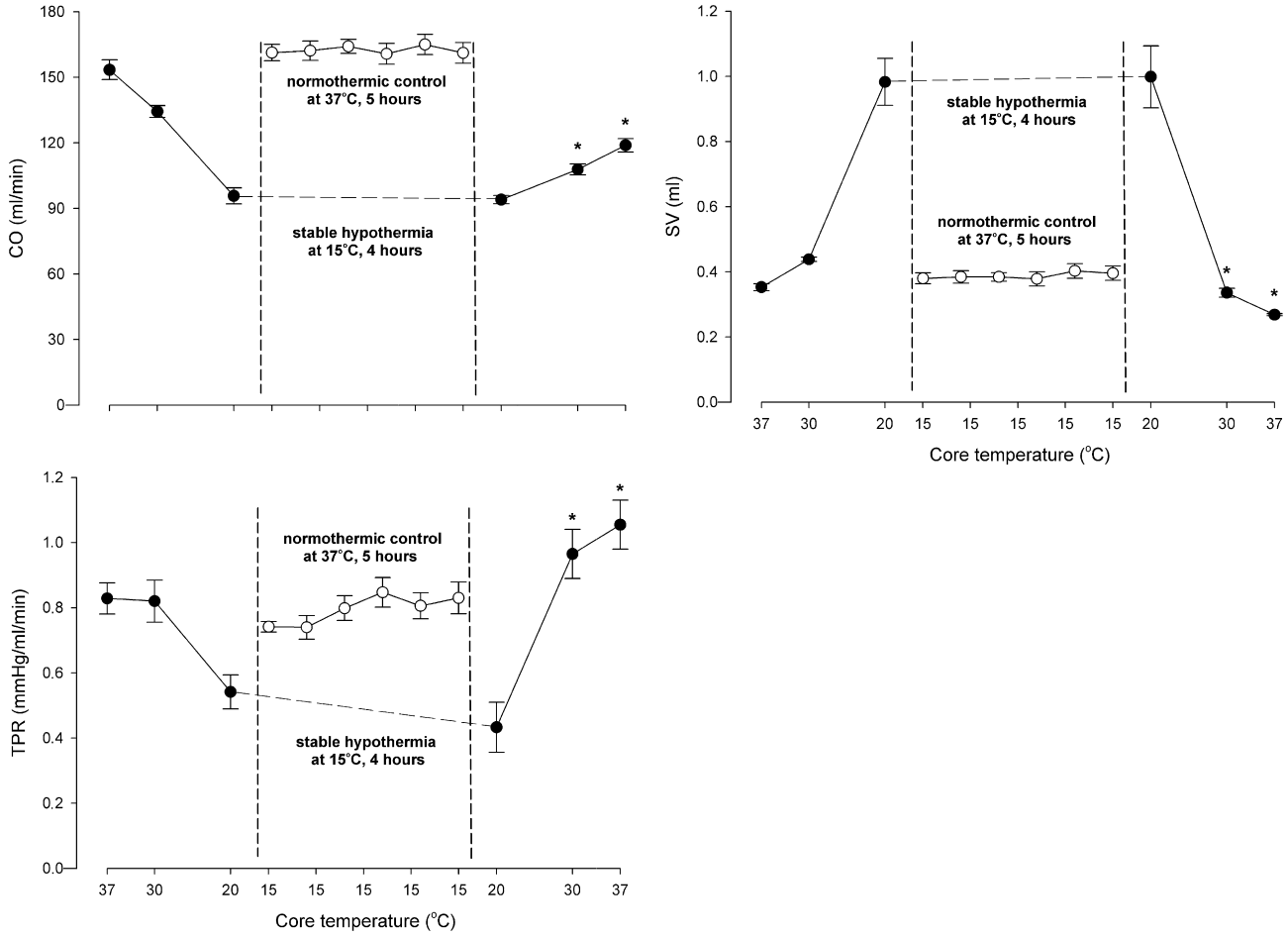

Fig. 2. CO, SV and TPR in hypothermic group and normothermic control. Cardiac output (CO), stroke volume (SV) and total peripheral resistance (TPR) in hypothermic and time-matched normothermic control animals. (●)—hypothermic animals; (○)—normothermic control animals. Values are mean and SEM. $^{*}p < 0.05$ vs. corresponding value during cooling.

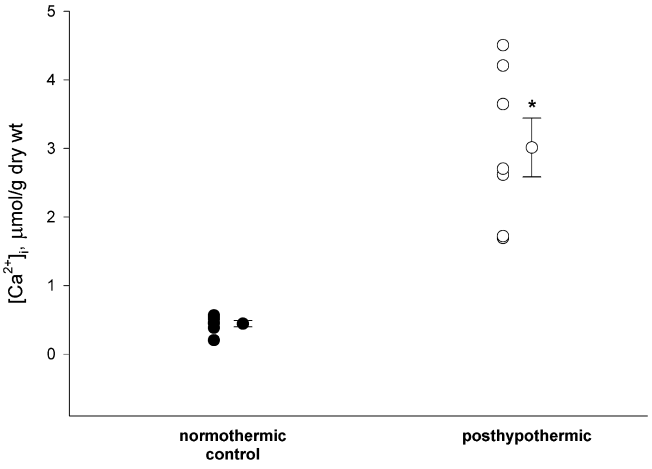

Fig. 3. Intracellular myocardial calcium content in hypothermic group and normothermic control. Intracellular calcium content in hearts from time-matched normothermic control rats, and hearts from rats following 4 h hypothermia (15 °C) and rewarming. Error bars represent SEM. $^{*}p < 0.05$ vs. normothermic control values.

defined as an excessive increase in intracellular Ca^{2+} that cannot be handled in a normal way and therefore resulting in abnormal cell function [36]. Calcium overload is extensively studied following myocardial ischemia/reperfusion where Ca^{2+} overload is regarded an important substrate for reduced contractile function [8,16].

Previous reports have shown increased intracellular Ca^{2+} during hypothermia in isolated cardiomyocytes [5,9,17,37] as well as in isolated rodent hearts exposed to 10 °C and hypothermic cardiac arrest [1,3,27,30]. In isolated perfused hearts the hypothermia-induced Ca^{2+} accumulation was only partially normalized following rewarming [1,3,30]. Despite obvious differences between *in vitro* and *in vivo* experimental conditions, the present experiments demonstrate that also the intact heart show Ca^{2+} overload after rewarming from profound hypothermia. Also, to the best of our knowledge, this study demonstrate for the first time that mechanical dysfunction following rewarming of the intact heart where spontaneous circulation was maintained during hypothermia, was associated with an increase in myocardial calcium content. Research aimed at exploring the underlying mechanisms of this hypothermia-induced calcium overload is essential in the effort to improve treatment of cardiac failure during rewarming from accidental hypothermia.

In a recent study using the same experimental model we studied global oxygen supply during hypothermia (5 h at 15 °C) and rewarming, and found that reduced myocardial mechanical function after rewarming was not related to myocardial hypoxia or ischemia during hypothermia and rewarming [12]. Thus, in the present study other mechanisms than global myocardial ischemia must be responsible for calcium overload following rewarming from 4 h at 15 °C. Despite previous reports of Ca^{2+} accumulation during hypothermia [5,9,17,37] the mechanism behind this hypothermia-induced Ca^{2+} overload is less elucidated. Several mechanisms are known to control $[Ca^{2+}]_i$ [6]; extracellular Ca^{2+} enter the cytoplasm via sarcolemmal L-type Ca^{2+} channels and the Na^+/Ca^{2+}-exchanger (NCX), whereas mitochondrial Ca^{2+} enters cytoplasm through the mitochondrial Na^+/Ca^{2+}-exchanger. Sarcolemmal Ca^{2+} entry induces Ca^{2+} release from SR via SR Ca^{2+} release channel/RyR (Ca^{2+}-induced Ca^{2+}-release, CICR). During a normal action potential the L-type Ca^{2+} channel is the dominant pathway inducing this release. Removal of Ca^{2+} from the cytoplasm is taken care of by four possible mechanisms: (1) back to SR by SERCA2; (2) through NCX, (3) through SL Ca^{2+}-ATPase; and (4) mitochondrial uptake by Ca^{2+}-uniporter and/or mitochondrial Na^+/Ca^{2+}-exchanger. Although all ion transport is affected by temperature, it is known that the Na^+/K^+-ATPase is markedly inhibited during temperature reduction [11]. Thus, inhibition of Na^+/K^+-ATPase will eventually give rise to elevation of $[Na^+]_i$ which may create a reversed mode of the NCX resulting in Ca^{2+} uptake during hypothermia [4]. The fact that the NCX is less affected by temperature than the Na^+/K^+ ATPase makes this possibility very likely (Q_{10} coefficients of 1.35 and 3.0, respectively) [28,30]. As the activity of SERCA2 [15,18], SL Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter are known to be markedly slower at low temperature (2–3 times slower at 25 °C) as compared to at 37 °C [4], give rise to the hypothesis that a depressed Ca^{2+} extrusion and buffering may contribute to further increase of $[Ca^{2+}]_i$.

In addition, the hypothermia-induced cardiac contractile dysfunction may be due to reduced content of high energy phosphates available for myofilamental contraction potentially due to a mitochondrial Ca^{2+} accumulation resulting in reduced oxidative phosphorylation [26]. In support of this hypothesis we have in the same model shown a significant reduction of ATP in hearts following rewarming [34]. Likewise, using electron microscopy we have reported morphologic changes in the mitochondria during hypothermia as well as after rewarming, including mitochondrial swelling and electron-dense inclusions, both indicating compromised mitochondrial function [33]. Finally, it has been suggested that rewarming may induce a stunning-like state [35]. Although the mechanism leading to contractile failure in stunned myocardium is not clear, it has been proposed to be associated with changes in Ca^{2+} handling leading to Ca^{2+} overload [38]. Thus, although the role of a stunning phenomenon in posthypothermic myocardial dysfunction remains to be elucidated, we propose that Ca^{2+} overload, as observed in the present study, may play an important deleterious role causing myocardial failure during and after rewarming.

In conclusion, our findings indicate that hypothermia-induced alterations in the Ca^{2+}-handling result in Ca^{2+} overload during hypothermia/rewarming, which may contribute to myocardial failure during and after rewarming.

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