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Myocardial calcium overload during graded hypothermia and after rewarming in an *in vivo* rat model

R. M. Wold,[1] T. Kondratiev[1] and T. Tveita[1,2]

1 *Anaesthesia and Critical Care Research group, Institute of Clinical Medicine, University of Tromsø, Tromsø, Norway*

2 *Division of Surgical Medicine and Intensive Care, University Hospital of North Norway, Tromsø, Norway*

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Correspondence: R. M. Wold, MD, Anaesthesia and Critical Care Research group, Institute of Clinical Medicine, University of Tromsø, 9037 Tromsø, Norway.E-mail: ragnhild.m.wold@uit.no

Abstract

Aim: Mechanisms underlying cardiac contractile dysfunction during and after rewarming from hypothermia remain largely unknown. We have previously reported myocardial post-hypothermic calcium overload to be the culprit. The aim of the present study was to measure changes in myocardial $[Ca^{2+}]_i$ during graded hypothermia and after rewarming in an anesthetized, intact rat model, using the $^{45}Ca^{2+}$ technique.

Methods: Rats were randomized and cooled to 15 °C. Hearts were excised and perfusion-washed to remove extracellular calcium after 0.5 h of hypothermia (n = 9), 4 h of hypothermia (n = 8), and after 4 h of hypothermia and 2 h rewarming (n = 9). A normothermic group, kept at 37 °C for 5 h, served as control (n = 6). $[Ca^{2+}]_i$ was determined in perchloric acid extracts of heart tissue. Spontaneous cardiac electromechanic work was maintained during hypothermia without cardiac arrest or ischaemia.

Results: Between 0.5 and 4 h at 15 °C, a six-fold increase in cardiac $[Ca^{2+}]_i$ was observed (0.55 ± 0.10 vs. 2.93 ± 0.76 μmol (g dry wt)$^{-1}$). Rewarming resulted in a 33% decline in $[Ca^{2+}]_i$, but the actual value was significantly above the value measured in control hearts.

Conclusion: We show that calcium overload is a characteristic feature of the beating heart during deep hypothermia, which aggravates by increasing duration of exposure. The relatively low decline in $[Ca^{2+}]_i$ during the rewarming period indicates difficulties in recovering calcium homoeostasis, which in turn may explain cardiac contractile dysfunction observed after rewarming.

Keywords calcium, cardiac, contractile dysfunction, hypothermia, rat.

Despite continued improvements in medical therapy, the success rate when rewarming patients from accidental hypothermia has not been improved over the last five decades, and mortality rate is reported to be between 52 and 80% depending on methods of rewarming (MacLean & Emslie-Smith 1977). These patients often present with impaired cardiac function, cardiac arrhythmias or even circulatory arrest, which may explain such high mortality (MacLean & Emslie-Smith 1977). From animal studies, we know that cooling and rewarming the intact heart in a non-arrested state result in cardiac dysfunction, which in its fulminant form is recognized as a progressive reduction of cardiac output (CO) and a sudden fall in arterial blood pressure (Blair *et al.* 1956, Popovic & Kent 1965, Steen *et al.* 1980, Tveita 2000). In addition, severity of hypothermia-induced cardiac dysfunction seems dependent on duration and depth of cooling (Tveita *et al.* 1996a, Tveita 2000), which implies that one should strive for limiting exposure time to hypothermia.

The hypothermia-induced cardiac dysfunction suggests that heart failure plays a role in the pathophysiology of hypothermia, and hence, this may be a decisive factor to the outcome from hypothermia and subsequent rewarming. However, because of limited knowledge of the pathophysiology of accidental hypothermia, guidelines for

treatment are missing. To establish such guidelines, it is necessary to have detailed understanding of the pathophysiology including the hypothermia- and rewarming-induced cardiac dysfunction. This topic is sparsely elucidated and motivates to study myocardial function and Ca^{2+} homoeostasis during cooling and rewarming.

Cardiac intracellular Ca^{2+} content ($[Ca^{2+}]_i$) is central in excitation–contraction coupling, and in the contraction itself, and is necessary for adequate function of the heart. From *in vitro* experiments, it is well known that cooling cardiac myocytes and isolated hearts rapidly creates an increase in $[Ca^{2+}]_i$ (Shattock & Bers 1987, Bers *et al.* 1989, Stowe *et al.* 1993, 1995, 1999, Aasum *et al.* 1994, Gambassi *et al.* 1994, Steigen *et al.* 1994, Puglisi *et al.* 1996, Aasum & Larsen 1997a,b, Schiffmann *et al.* 2001, Groban *et al.* 2002, Shutt & Howlett 2008). The elevation of $[Ca^{2+}]_i$ is probably caused by temperature-induced changes in cellular components handling Ca^{2+} and other ions that are slowed by cooling. Examples are Na^+/K^+-ATPase (Isenberg & Trautwein 1975, Steigen *et al.* 1994), Na^+/Ca^{2+}-exchanger (Shattock & Bers 1987) and SERCA2 (Labow *et al.* 1993). Initially, an increase in cytoplasmic $[Ca^{2+}]$ enhances cardiac contractility by increasing the number of crossbridges recruited for force development, but above a certain level, the cardiac myocytes become Ca^{2+} overloaded, which results in mechanical or electrical dysfunction that may entail cardiac dysfunction (Vassalle & Lin 2004).

In the *in vivo* setting, Ca^{2+} regulation during hypothermia is not fully elucidated, and whether Ca^{2+} accumulates during hypothermia remains unknown. However, previous experiments from our group have shown a sixfold increase in $[Ca^{2+}]_i$ combined with deterioration of myocardial contractile function after rewarming from 4-h stable hypothermia (15 °C) (Kondratiev *et al.* 2008). This is recognized as Ca^{2+} overload, but whether this evolves during hypothermia or solely during rewarming remains unknown. Therefore, the aim of this study is to elucidate cardiac Ca^{2+} regulation in hypothermia by measuring $[Ca^{2+}]_i$ during hypothermia and after rewarming. Our hypothesis is that hypothermia *in vivo* elevates $[Ca^{2+}]_i$, and that the hypothesized elevation of $[Ca^{2+}]_i$ is maintained during rewarming. Moreover, because hypothermia-induced cardiac dysfunction seems to be dependent on duration and depth of hypothermia (Tveita 2000), we also want to investigate if exposure time to hypothermia has an impact on $[Ca^{2+}]_i$ homoeostasis.

Materials and methods

Animals

Wistar rats (males, 325 ± 93 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 five 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 introduced i.p. by 55 mg (kg body weight)$^{-1}$ pentobarbital sodium and fentanyl 50 μg (kg body weight)$^{-1}$, followed by a continuous infusion of 7.5 mg (kg h^{-1})$^{-1}$ pentobarbital sodium and 50 μg (kg h^{-1})$^{-1}$ fentanyl through an intravenous line in the right jugular vein extended to the right auricle. In the normothermic group, anaesthesia was continued during experiments, whereas in the hypothermic groups, anaesthesia infusion was terminated when cooling was started because of hypothermia created anaesthesia and reduced drug metabolism.

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, USA) using room air. To ensure sufficient ventilation, blood gasses were analysed.

Experimental setup

The experimental setup allowing core cooling and rewarming and hemodynamic measurements have been previously described in detail (Kondratiev *et al.* 2008). Briefly, the animals were cooled and rewarmed by circulating water (thermostated water bath type RTE-110; Neslab Instruments, Newington, NH) through *U*-shaped tubes placed in oesophagus and lower bowels and a double-layered operating table circulated water, while core temperature was monitored using thermocouple wires positioned in the aortic arch, connected to a thermocouple controller (Thermoalert TH-5; Columbus Instruments, Columbus, OH, USA). Cooling lasted about 1 h, while rewarming lasted about 2 h. Arterial pressure and left

ventricular (LV) pressure were continuously recorded through catheters connected to Transpac III transducers (Abbot, North Chicago, IL, USA), and these signals were amplified to 0–10 V and passed to a 12-bit analogue to digital converter (BNC 2090; National Instruments, Austin, TX, USA). From LV pressure, maximum rate of left ventricular pressure rise (LV dP/dt_{max}) and maximum rate of left ventricular pressure decline (LV dP/dt_{min}) were obtained. Signal processing and analysis were performed with the help of a special computer program developed using LabVIEW v.6.0, National Instruments, Austin, TX, USA. Cardiac output (CO) was measured by thermodilution technique (Merrick *et al.* 1980). The curves were digitalized with a Calcomp digitizing table (model: 23180; Calcomp Digitizer Products Division, Anaheim, CA, USA), and CO was calculated with a program designed with the LabView package. Stroke volume (SV) was calculated from CO and HR (heart rate), and total peripheral resistance (TPR) from systolic and diastolic pressure and CO. CPO was calculated from MAP, CO and the conversion factor 2.22×10^{-3}. Because of technical limitations in the thermodilution technique CO, CPO, SV and TPR were measured at 20 °C, not 15 °C.

Blood gases, oxygen saturation, pH, base excess and $[Ca^{2+}]$ 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 analysed by RapidLab 800 blood gas analyzer (Chiron Diagnostics, Emeryville, CA, USA). All blood samples were analysed at 37 °C (alpha-stat strategy).

Measurement of intracellular Ca^{2+} content, $[Ca^{2+}]_i$

The measurement of $[Ca^{2+}]_i$ by the use of radiolabeled calcium ($^{45}Ca^{2+}$) in an *in vivo* experiment has been previously described in detail (Kondratiev *et al.* 2008). Briefly, 20-μ Ci of $^{45}Ca^{2+}$ (ARX-102 Calcium-45; American Radiolabeled Chemicals, St. Louis, MO, USA) was injected. From pilot experiments, we found a rapid reduction in $^{45}Ca^{2+}$ activity in the plasma, reaching a steady state level 2 h after injection. Pilot experiments also showed that the time needed to wash out extracellular $^{45}Ca^{2+}$ in the hearts in a Langendorff system was 1 min, and a washout period of 3 min was decided used. The perfusion pressure was 100-cm H_2O (~75 mmHg), and coronary flow rate (mL/min) was measured by timed collection of the coronary effluent. The hearts were subsequently freeze-clamped and vacuum dry-frosted (Christ Alpha 1–4; Medizinischer Apparatebau, Osterode, Harz, Germany) and then pulverized by a micro-dismembrator (Braun Messungen AG, Germany). 80–90 mg of the homogenate was extracted in perchloric acid and centrifuged (Kubota 1700 centrifuge; Cubota, Tokyo, Japan). 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. 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, Downers Grove, IL, USA) and RapidLab 800 blood gas analyser (Chiron Diagnostics) respectively. Intracellular Ca^{2+} content was calculated from the tissue radioactivity, the specific activity of the plasma and the dry weight of the hearts.

It should be emphasized that this method cannot distinguish between intracellular pools of Ca^{2+}, namely cytosolic, sarcoplasmic reticulum and mitochondrial. Hence, only total myocardial $[Ca^{2+}]_i$ was measured.

Experimental protocol

Following instrumentation and $^{45}Ca^{2+}$ infusion, the animals were stabilised for 2 h before starting measuring basal function. They were divided into four groups – hypothermic group kept at 15 °C for 0.5 h (15 $°C_{0.5\ h}$), hypothermic group kept at 15 °C for 4 h (15 $°C_4$ h), hypothermic group rewarmed from a 4 h period of hypothermia (37 $°C_{15°C,4\ h}$) and a time-matched control group kept at 37 °C for 5 h (37 $°C_{5\ h}$) (Fig. 1). At the end of experiments, myocardial $[Ca^{2+}]_i$ was determined as described.

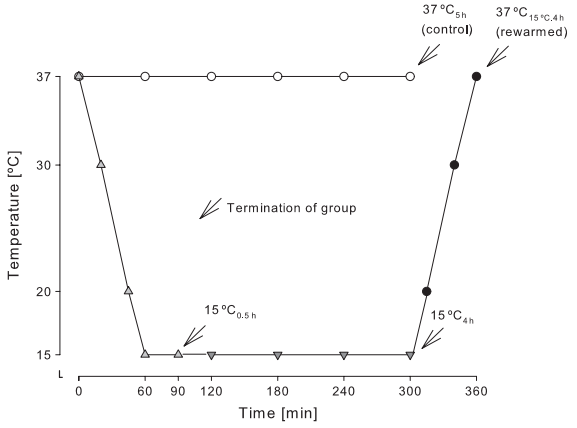

Figure 1 The experimental protocol; $[Ca^{2+}]_i$ was measured in hearts from rats following 0.5 h (15 $°C_{0.5\,h}$) or 4 h (15 $°C_{4\,h}$) of hypothermia, after rewarming from 4 h of hypothermia (37 $°C_{15°C4\,h}$), and in time-matched normothermic-control rats (37 $°C_{5\,h}$). Arrow indicates end of experiment in each group.

Animals that did not survive hypothermia and rewarming were not included.

Statistics

Results are presented as mean ± SE. Differences in hemodynamic values and myocardial $[Ca^{2+}]_i$ between groups were compared using two-tailed independent Student's *t*-test. Hemodynamic changes within one group were assessed in the cooled and rewarmed group by One-Way ANOVA. A *P*-value <0.05 was considered significant.

Results

Time-matched control group, 37 °C$_{5\ h}$

Myocardial intracellular Ca^{2+} ($[Ca^{2+}]_i$) was 0.35 ± 0.06 μmol (g dry weight)$^{-1}$ at the end of experiment (5 h at 37 °C) (Fig. 2). This is comparable with $[Ca^{2+}]_i$ determinations performed in this model previously by means of ^{45}Ca technique (Kondratiev *et al.* 2008), and to similar measurements in isolated normothermic rat hearts (Aasum & Larsen 1999).

Hemodynamic function remained stable during the experiment (Figs 3 and 4), and all control animals survived.

Myocardial $[Ca^{2+}]_i$ in hypothermic groups

$[Ca^{2+}]_i$ was 0.55 ± 0.10 μmol (g dry weight)$^{-1}$ in the group maintained at 15 °C for 0.5 h. This was not statistically different from control levels (Fig. 2).

$[Ca^{2+}]_i$ was 2.33 ± 0.53 μmol (g dry weight)$^{-1}$ in the group maintained at 15 °C$_{4\ h}$, which is more than a sixfold increase compared with the control group (Fig. 2).

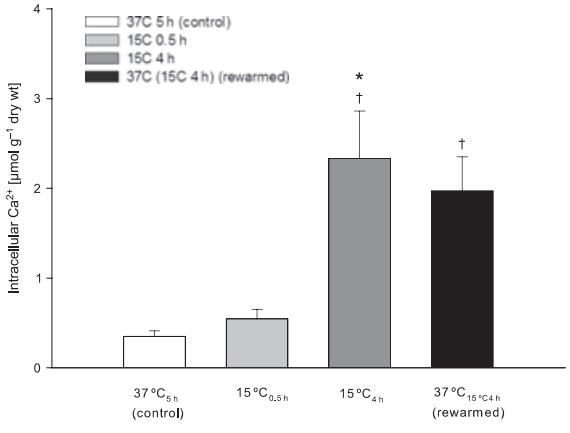

Figure 2 Intracellular myocardial calcium content ($[Ca^{2+}]_i$). †$P < 0.05$ vs. control (37 °C$_{5\,h}$), *$P < 0.05$ vs. 15 °C$_{0.5\,h}$.

Myocardial $[Ca^{2+}]_i$ in rewarmed animals following 15 °C$_{4\ h}$ (37 °C$_{15\ °C,4\ h}$)

$[Ca^{2+}]_i$ was 1.97 ± 0.38 μmol (g dry weight)$^{-1}$ in the post-hypothermic group (Fig. 2). This is almost a sixfold increase from control and comparable to data from other experiments (Aasum & Larsen 1999, Kondratiev *et al.* 2008). One rat in this group had abnormally high $[Ca^{2+}]_i$. In this particular experiment, we had problems with placing the ventricular catheter. The manipulation of the catheter could have induced contractile dysfunction. All data from this animal were therefore excluded.

Hemodynamic function in animals undergoing hypothermia and rewarming

During cooling, there were no significant differences in hemodynamic variables between the three hypothermic groups. Within group comparisons are done in the one undergoing hypothermia and rewarming (Table 1 and Figs 3 and 4).

At 15 °C$_{0\ h}$, cooling had decreased both mean arterial pressure (MAP) and left ventricular (LV) systolic pressure (LVSP) by 75–80%, and heart rate was only 14% of normothermic values (Fig. 3a,b,c). Maximum rate of left ventricular pressure rise (dP/dt_{max}) was 6% of baseline value (Fig. 3d), and maximum rate of left ventricular pressure decline (dP/dt_{min}) was 5% of baseline value (Table 1). Because of technical limitations, we were not able to measure cardiac output (CO) below 20 °C. At 20 °C, CO was reduced by 42%, total peripheral resistance (TPR) reduced by 34%, stroke volume (SV) was almost threefold increased (Fig. 4), whereas CPO was 30% of baseline value.

At 15 °C$_{0.5\ h}$, hemodynamic parameters were unchanged from corresponding values at 15 °C$_{0\ h}$. At 15 °C$_{4\ h}$, MAP, LVSP, LV dP/dt_{max}, LV dP/dt_{min} were all significantly lowered, 21–50% compared with 15°C$_{0\ h}$ (Fig. 3 and Table 1). No episodes of arrhythmias were observed except for single ectopic ventricular beats.

After rewarming to 20 °C, following 15 °C$_{4\ h}$, CO, SV and CPO were all significantly reduced compared with the same values during cooling at 20 °C (Fig. 4a, b and Table 1). After rewarming was completed (37 °C), CO, SV and CPO were all significantly reduced to 67%, 60% and 34% respectively from baseline values (start of experiment). On the contrary, TPR was increased by 60% from baseline values (Fig. 4c). HR, MAP, LVSP and dP/dt_{max} returned to pre-hypothermic levels (Fig. 3). LV dP/dt_{min} was significantly reduced compared with pre-hypothermic value (Table 1). LV end-diastolic pressure (LVEDP)

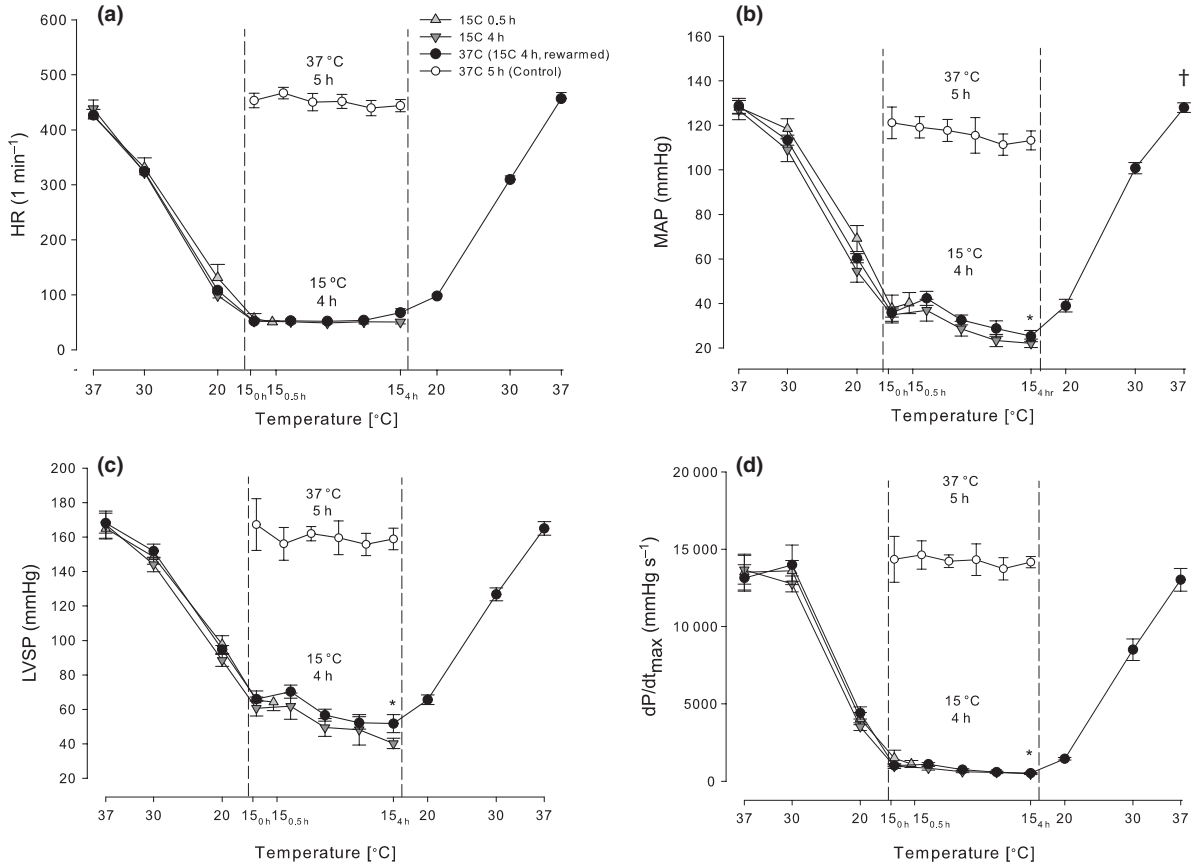

Figure 3 Heart rate (HR; a), mean arterial pressure (MAP; b), left ventricular systolic pressure (LVSP: c) and maximal speed of increase of left ventricular pressure (dP/dt_{max}; d) in hypothermic groups and normothermic controls. $^{\dagger}P < 0.05$ vs. control. $^{*}P < 0.05$ vs. corresponding value within group.

remained within normal limits during experiments (Table 1). No episodes of arrhythmias were observed except for single ectopic ventricular beats.

Blood gases

The alpha-stat strategy was followed. During 0.5-h stable hypothermia at 15 °C pH was about 7.4 in both hypothermic groups. After 4-h hypothermia (15 °C) and following rewarming from hypothermia (4 h 15 °C), metabolic acidosis was observed (Table 2).

Discussion

In the present study, we found that cardiac $[Ca^{2+}]_i$ after 4-h hypothermia was substantially elevated when compared both to 0.5-h hypothermia and normothermic controls. Moreover, the increase in $[Ca^{2+}]_i$ remained during rewarming in concert with a significant hypothermia-induced cardiac dysfunction. The present results indicate that the earlier observed post-hypothermic Ca^{2+} overload *in vivo* is established during hypothermia and that duration of hypothermia is crucial to elevation of $[Ca^{2+}]_i$. This finding adds to our understanding of the pathophysiology of hypothermia rewarming-induced cardiac dysfunction and substantiates the importance of reducing exposure time to hypothermia by starting rewarming as soon as possible.

Many researchers have investigated the effects of hypothermia on mammalian heart tissue *in vitro*, and it is well known that cooling elevates $[Ca^{2+}]_i$ within few minutes (Shattock & Bers 1987, Bers *et al.* 1989, Stowe *et al.* 1993, 1995, 1999, Aasum *et al.* 1994, Gambassi *et al.* 1994, Steigen *et al.* 1994, Puglisi *et al.* 1996, Aasum & Larsen 1997a,b, Schiffmann *et al.* 2001, Groban *et al.* 2002, Shutt & Howlett 2008). On the contrary, we show in the present study that short-time hypothermia *in vivo* does not induce an immediate rise in myocardial $[Ca^{2+}]_i$ (15 $°C_{0.5h}$). These divergent findings of $[Ca^{2+}]_i$ *in vitro* and *in vivo* after short-term hypothermia may be explained by the possibility that *in vivo* hearts tolerate low temperature better because *in vitro* hearts, or cells, are exposed to

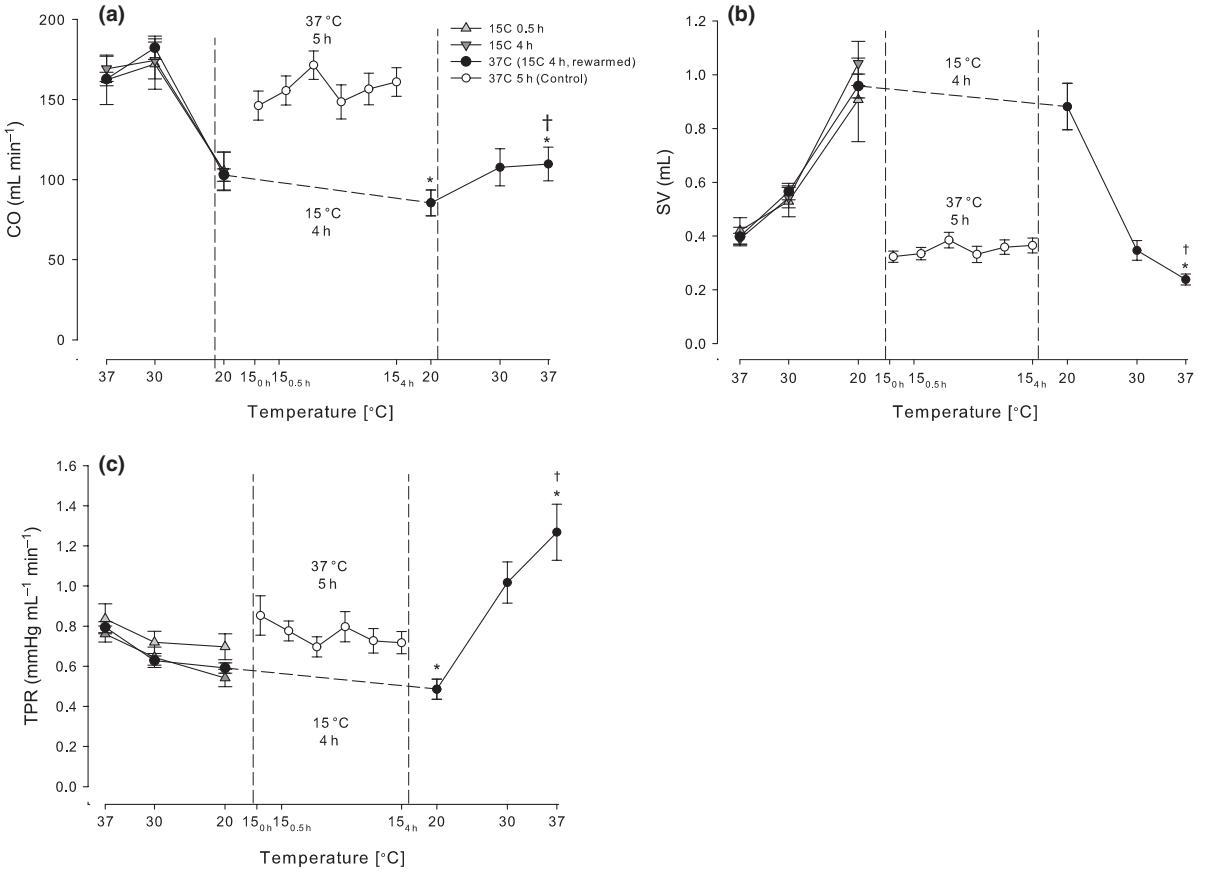

Figure 4 Cardiac output (CO; a), stroke volume (SV; b) and total peripheral resistance (TPR; c) in hypothermic groups and normothermic controls. $^{\dagger}P < 0.05$ vs. control. $*P < 0.05$ vs. corresponding value within group.

Table 1 Hemodynamic variables in animals which were cooled and rewarmed (37 $°C_{15\,°C,\ 4\,h}$) and in the group kept hypothermic for 0.5 h (15 $°C_{0.5\,h}$)

	Pre-hypothermic	Hypothermic 0 h	Hypothermic 0.5 h	Hypothermic 4 h	Post-hypothermic
HR (beats min^{-1})	427 ± 28	52 ± 1	51 ± 2	68 ± 7	457 ± 11
MAP (mmHg)	129 ± 3	36 ± 2	40 ± 5	25 ± 3*	128 ± 2†
CO (mL min^{-1})	163 ± 4	103 ± 4		85 ± 8*	110 ± 11*†
CPO (W)	47 ± 2	14 ± 1		7 ± 1*	31 ± 3*
SV (mL $beat^{-1}$)	0.40 ± 0.03	0.96 ± 0.04		0.88 ± 0.09	0.24 ± 0.02*†
TPR (mmHg min mL^{-1})	0.79 ± 0.03	0.59 ± 0.03		0.49 ± 0.05*	1.27 ± 0.14*†
LVSP (mmHg)	168 ± 6	66 ± 1	64 ± 5	52 ± 5*	165 ± 4
LVEDP (mmHg)	5 ± 1	12 ± 1	13 ± 2	8 ± 0	5 ± 1
dP/dt_{max} (mmHg s^{-1})	13143 ± 856	902 ± 84	1116 ± 229	525 ± 45*	13025 ± 734
dP/dt_{min} (mmHg s^{-1})	−11363 ± 712	−577 ± 38	−498 ± 51	−377 ± 35*	−9081 ± 478*

HR, heart rate; MAP, mean arterial pressure; CO, cardiac output; CPO, cardiac power output; SV, stroke volume; TPR, total peripheral resistance; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt_{max}, maximum rate of left ventricular pressure rise; dP/dt_{min}, maximum rate of left ventricular pressure decline. Because of technical limitations, CO, SV and TPR are measured at 20 °C, not at 15 °C.

$*P < 0.05$ vs. corresponding temperature within group.

$^{\dagger}P < 0.05$ vs. normothermic, time-matched controls (shown in Figs 3 and 4)

a dramatic change of their environment before they are exposed to hypothermia. Another explanation would be that in the present experimental setting the $^{45}Ca^{2+}$ technique was not sensitive enough to detect smaller changes of $[Ca^{2+}]_i$. Moreover, despite no significant changes in $[Ca^{2+}]_i$ after 0.5 h of hypothermia

Table 2 pH, O_2 and CO_2 values of arterial blood in response to hypothermia and rewarming

	Control	Hypothermic 0.5 h	Hypothermic 4 h	Post-hypothermic
pH	7.41 ± 0.01	7.37 ± 0.03	7.14 ± 0.03	7.16 ± 0.05
pO_2 (kPa)	11.7 ± 1.1	26.8 ± 2.4	23.1 ± 1.6	9.8 ± 1.0
pCO_2(kPa)	3.8 ± 0.1	3.7 ± 0.5	2.2 ± 0.2	3.1 ± 0.3

Values are mean ± SE. Hypothermic values are not temperature adjusted.

Ca^{2+} might be accumulated in some and reduced in other cellular compartments, which might have consequences for myocardial contractile function. In addition, the depressed myocardial contractile function observed during cooling and after 0.5-h hypothermia (compared to pre-hypothermic values) is shown to be reversed by rewarming and associated to a more favourable outcome (MacLean & Emslie-Smith 1977, Kondratiev *et al.* 2005). Thus, this suggests that an elevation of Ca^{2+} to critically high levels is not established at this point.

With respect to the outcome of hypothermia in a clinical setting, the effects of prolonged hypothermia and the ensuing rewarming are more important than short-time hypothermia; following 4 h at stable experimental hypothermia (15 °C), we observe elevated myocardial cell $[Ca^{2+}]_i$. To the best of our knowledge, the present study is the first conducted in an intact animal model where myocardial cell $[Ca^{2+}]_i$ is measured during long-term hypothermia, and hence, we are not able to compare with results from other studies. The post-hypothermic $[Ca^{2+}]$ values are comparable to our previous *in vivo* observations by Kondratiev *et al.* (2008) and to *in vitro* experiments in isolated rat and guinea pig hearts (40 min at 10 °C) where $[Ca^{2+}]$ is elevated in both species (Aasum & Larsen 1999). Compared with rat, the guinea pig heart is believed to more closely resemble the human heart with respect to calcium handling, and hence, this supports our conclusion in the present study where we draw lines into human medicine.

The underlying mechanisms of hypothermia-induced elevation of Ca^{2+} are yet not fully explored, but it is known that the components regulating Ca^{2+} homoeostasis, such as the Na^+/Ca^{2+} exchanger (NCX) and Na^+/K^+-ATPase are affected by temperature. This is discussed in detail in our previous paper (Kondratiev *et al.* 2008).

In the present study, elevated myocardial cell $[Ca^{2+}]_i$ after 4 h at 15 °C was observed simultaneously with indications of hypothermia-induced cardiac dysfunction (decreased LVSP, LV dP/dt_{max} and LV dP/dt_{min}). These alterations in $[Ca^{2+}]_i$ remained during rewarming accompanied by reduced MAP, CO and CPO. Impairment of hemodynamics during hypothermia and rewarming is demonstrated in numerous reports (Blair *et al.* 1956, Popovic & Kent 1965, Steen *et al.* 1980, Tveita 2000). During cooling, end systolic volume decreases (Tveita & Sieck 2012), but during the rewarming phase, isovolumetric pressure is depressed and ventricular wall shortening reduced, whereas diastolic function is reported to be intact (Tveita *et al.* 1994, 1996a, 1998). Hence, the observed hypothermia-induced cardiac dysfunction is mainly because of a compromised systolic function. Based on the present results, we suggest that the underlying mechanism of hypothermia rewarming-induced contractile dysfunction may be 'Ca^{2+} overload'. Calcium overload is defined as mechanical or electrical contractile failure induced by elevated $[Ca^{2+}]_i$ in the heart (Vassalle & Lin 2004). With respect to impaired electrical function as result of Ca^{2+} overload, no episodes of arrhythmias were observed during the present experiments. This is explained by the fact that rodents and smaller mammals are less susceptible to hypothermia-induced arrhythmias compared with humans (Popovic & Kent 1965) who at low temperatures commonly presents with cardiac dysrrhythmias.

Ca^{2+} overload may induce cardiac contractile dysfunction through different pathways. In hypothermia and rewarming, an increase in reactive oxygen species (ROS) (Camara *et al.* 2003, Riess *et al.* 2004), decreased ATP synthesis and damaged mitochondrial ultrastructure (Tveita *et al.* 1996a, 1998, Aupperle *et al.* 2007) have been reported. Such findings are signs of alterations in mitochondrial function, which again likely affects cardiac contractility. In normothermia, Ca^{2+} homoeostasis is more extensively investigated and linked to mitochondrial dysfunction and affection of myocardial contractile function: (i) elevated $[Ca^{2+}]_i$ speeds up energy consumption because of the Ca^{2+}- and ATP-dependent myofilament interaction, and the ATP-dependent Ca^{2+} pumps in SR and sarcolemma (Bers 2001), (ii) extra-mitochondrial Ca^{2+} (resting) >1 μM may open the mitochondria permeability transition pore (MPTP), which enables free passage into the mitochondria of molecules <1.5 kDa, which causes mitochondrial depolarization, leading to uncoupling of oxidative phosphorylation, limits ATP synthesis, induces formation of ROS, mitochondrial swelling and even cell death (Tani 1990, Hüser *et al.* 1998, Ghafourifar & Richter 1999, Bers 2001, Davidson & Duchen

2006, Grimm & Brdiczka 2007, Halestrap 2009, Lemasters *et al.* 2009) and (iii) elevated cytoplasmic [Ca^{2+}] in cardiomyocytes induces mitochondrial Ca^{2+} accumulation (Reimer & Jennings 1992, Dedkova & Blatter 2007). Mitochondrial Ca^{2+} uptake entails demolishing events by activating NO production (Dedkova & Blatter 2007), which promotes cytochrome C release, decreases ATP synthesis and opens up the MPTP. Such scenarios may create serious negative consequences for cardiac contractility and may all be because of elevated cytoplasmic [Ca^{2+}].

It has to be mentioned that elevation of Ca^{2+} in normothermia is known to initially improve myocardial performance and hemodynamics, but seemingly because of a dysfunctional elevation of this ion over time, Ca^{2+} overload occurs (Tani & Neely 1989, Vassalle & Lin 2004). Such biphasic course in cardiac function in response to increased [Ca^{2+}] is also observed in isolated hypothermic rat hearts (28 °C) (by elevating [Ca^{2+}] in the perfusate), but the positive effect was less pronounced (Schiffmann *et al.* 2001). In the present study, in addition to the hypothermia-rewarming-induced contractile dysfunction, we did observe the transient hypothermia-induced increase in SV during cooling, which may illustrate these paradoxical effects of [Ca^{2+}] on cardiac contractile function. Other mechanisms behind the increase of SV may be the negative staircase of the rat heart or increased Ca^{2+} responsiveness of the myofilaments (Post *et al.* 2010), and/or a substantial decrease in systolic volume (Tveita & Sieck 2012). However, these effects should remind us that lowering body temperature can also be taken advantage of as the effect of mild hypothermia shear similarities with positive inotropic agents as deliberately used during therapeutic hypothermia (Bernard *et al.* 2002) and that increase of $[Ca^{2+}]_i$ may not always be a sign of deteriorations in Ca^{2+} handling. Thus, the term Ca^{2+} overload should be used with caution because this term pre-judices a state of elevated calcium content. This illustrates that the pathophysiology of hypothermia- rewarming-induced contractile dysfunction is complex and that Ca^{2+} overload may be one of the many underlying mechanisms. Other suggested mechanisms behind hypothermia- rewarming-induced cardiac contractile dysfunction are increased phosphorylation of cardiac troponin I (protein kinase A dependent) (Han *et al.* 2010), increased interstitial fluid accumulation (Tveita *et al.* 1994, 1998) and a stunning-like state (Filseth *et al.* 2010).

In addition, one might emphasize that cardiac dysfunction alone may not explain the whole truth about high mortality rates after rewarming from hypothermia. Hypothermia is suggested to induce inflammatory responses (Yenari & Han 2006, Tveita *et al.* 2012), alter capillary function, increase leakage of plasma protein and to induce metabolic acidosis and possibly reduce circulating blood volume (Tveita *et al.* 1996b, Kondratiev *et al.* 2005). All these factors may contribute to post-hypothermic circulatory collapse.

Conclusion

In the present study, we found that in the intact, beating, hypothermic heart calcium overload evolves during long-lasting hypothermia and is maintained during rewarming. Thus, we conclude that duration of hypothermia is crucial to increase myocardial $[Ca^{2+}]_i$ *in vivo,* and that the myocardial calcium overload is not reversed by rewarming. This disturbance in calcium homoeostasis may initiate cardiac dysfunction observed after hypothermia and rewarming and suggests that limiting time of exposure to hypothermia is important for a successful outcome. Further studies in the field of hypothermia-induced cardiac dysfunction must aim at investigating the effects of measures that promote normalization of calcium homoeostasis during rewarming.

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Conflict of interests

The authors declare that they have no competing interests.

Authors' contributions

RMW participated in the design of the study, carried out the experiments, performed the statistical analysis, drafted the manuscript and approved the final manuscript. TK participated in the design of the study, carried out the experiments, participated in statistical analysis and drafting the manuscript. TT participated in the design and coordination of the study, helped to draft the manuscript and read and approved the final manuscript.

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