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Cardiovascular effects of levosimendan during rewarming from hypothermia in rat ☆

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ABSTRACT

Background: Previous research aimed at ameliorating hypothermia-induced cardiac dysfunction has shown that inotropic drugs, that stimulate the cAMP, – PKA pathway via the sarcolemmal β-receptor, have a decreased inotropic effect during hypothermia. We therefore wanted to test whether levosimendan, a calcium sensitizer and dose-dependent phosphodiesterase 3 (PDE3) inhibitor, is able to elevate stroke volume during rewarming from experimental hypothermia.
Methods: A rat model designed for circulatory studies during experimental hypothermia (4 h at 15 °C) and rewarming was used. The following three groups were included: (1) A normothermic group receiving levosimendan, (2) a hypothermic group receiving levosimendan the last hour of stable hypothermia and during rewarming, and (3) a hypothermic placebo control group. Hemodynamic variables were monitored using a Millar conductance catheter in the left ventricle (LV), and a pressure transducer connected to the left femoral artery. In order to investigate the level of PKA stimulation by PDE3 inhibition, myocardial Ser23/24-cTnI phosphorylation was measured using Western-blot.
Results: After rewarming, stroke volume (SV), cardiac output (CO) and preload recruitable stroke work (PRSW) were restored to within pre-hypothermic values in the levosimendan-treated animals. Compared to the placebo group after rewarming, SV, CO, PRSW, as well as levels of Ser23/24-cTnI phosphorylation, were significantly higher in the levosimendan-treated animals.
Conclusion: The present data shows that levosimendan ameliorates hypothermia-induced systolic dysfunction by elevating SV during rewarming from 15 °C. Inotropic treatment during rewarming from hypothermia in the present rat model is therefore better achieved through calcium sensitizing and PDE3 inhibition, than β-receptor stimulation.

Introduction

Case-reports show that the human body can survive core temperatures down to 13.7 °C and up to 7 h of hypothermic cardiac arrest [6,18]. Although survival is reported in such extreme situations, the mortality rate of accidental hypothermia is still described to be between 29% [30] and 80% [17]. A report from Melbourne showed that 13% of patients admitted to the emergency department had a core temperature below 35 °C. This patient group had a threefold independent risk of death [11]. The complications related to hypothermia is also acknowledged in surgical procedures, as use of therapeutic hypothermia during aortic surgery is related to increased need for inotropic support [2]. Thus, finding optimal strategies for treatment of patients subjected to therapeutic hypothermia and victims of accidental hypothermia is essential.

Abbreviations: PDE3, phosphodiesterase III; CO, cardiac output; LV, left ventricle; cTnC, cardiac troponin C; cTnI, cardiac troponin I; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PVDF, polyvinylidene difluoride; MAP, mean arterial pressure; SV, stroke volume; TPR, total peripheral resistance; SR, sarcoplasmic reticulum; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEDP, left ventricular end-diastolic pressure; CI, cardiac index; SW, Stroke work; $LVdp/dt_{max}$, maximum rate of left ventricular pressure change; PRSW, preload recruitable stroke work.

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Contributing to the high mortality of accidental hypothermia, rewarming is often complicated by cardiac dysfunction. Although this condition [29] was described as early as in 1826 by the French surgeon Moricheau-Beaupré [7], the pathophysiology behind it is not yet completely understood. Animal studies show that hypothermia-induced cardiac dysfunction is related to calcium overload [33], but not oxygen deficiency [13]. Further studies have tested inotropic drugs in order to counteract development of this condition. Among tested drugs are the β-agonists epinephrine and isoproterenol, which at normal core temperatures mediate positive inotropic effects by stimulating the cyclic AMP (cAMP), – protein kinase A (PKA) pathway. Remarkably, preclinical studies demonstrate diminished or adverse cardiovascular effects of these drugs when applied to treat hypothermia-induced cardiac dysfunction. Rather than giving positive inotropic effect, increased cardiac afterload, and lack of elevated SV dominated the hemodynamic response to β-agonists in hypothermic animals [14,12,8,28].

The calcium sensitizer levosimendan has potential in this setting. Acting through binding of cardiac troponin C (cTnC), levosimendan provides inotropic effect by stabilizing the calcium–cTnC–cTnI complex. In this way, levosimendan accelerates the cross-binding between actin and myosin [20]. In high concentrations levosimendan also function as a PDE3 inhibitor [25,3]. Inhibition of PDE3 will however increase cAMP and PKA, and thus induce Ser23/24-phosphorylation of cTnI. In a previous study carried out in our lab, we showed that contractile dysfunction after rewarming was related to increase of PKA-induced Ser23/24-cTnI phosphorylation [9], known to reduce myofilament calcium-sensitivity [19]. In contrast to epinephrine [28], which only had positive inotropic effect above 28 °C, administration of the PDE3 inhibitor milrinone demonstrated positive inotropic effect also during cooling below 28 °C [27]. Thus, in spite of the assumed increase in PKA-mediated Ser23/24-cTnI phosphorylation, PDE3 inhibition shows favorable effects on LV cardiac function at low core temperatures. According to clinical studies [16] we therefore wanted to test a high dose of levosimendan (bolus: 24 μg/kg, continuing infusion: 0.6 μg/kg/min) to make use of the combined effect of calcium sensitizing and PDE3 inhibition and explore whether this has potential to ameliorate hypothermia-induced cardiac dysfunction. To achieve this, we tested the effect of levosimendan on cardiac function during rewarming from 15 °C, using our rat model designed for hemodynamic measurements where spontaneous cardiac activity is maintained at all temperatures [33,13,14,12,8,28].

Materials and methods

Male Wistar rats (270–346 g) were used. The animals were provided by Charles River and quarantined for 1 week on arrival. Housing was provided in accordance with guidelines for accommodation and care of animals (article 5 of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, Strasbourg, 18.III.1986). The rats had a microbiological status according to the recommendation of the Federation of European Laboratory Animal Science Associations. Free access to food and water was permitted at all times. The experimental protocol was approved by the Norwegian Animal Research Authority and conducted accordingly.

Anesthesia

Anesthesia was introduced intraperitoneally by pentobarbital sodium (55 mg/kg) and fentanyl (50 μg/kg), followed by a continuous infusion of 7.5 mg/kg/h pentobarbital sodium and 50 μg/kg/h fentanyl through an intravenous line in the right jugular vein, extended to the right auricle. The anesthesia infusion was maintained at all hours in normothermic animals. In hypothermic animals, infusion was terminated at 30 °C during cooling and restarted at the same temperature during rewarming, due to hypothermia-induced anesthesia and reduced drug metabolism. The animals were monitored by toe-pinch for any sign of discomfort so that additional anesthesia could be provided if necessary. This is a well-established method for testing the effects of analgesic drugs in rodents and has been extensively tested in rats [4]. Toe pinch has been used for this purpose in all studies in the present model [33,13,14,12,8,28].

Respiratory support

Animals were 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 >20 °C. Below 20 °C, ventilation was achieved by a volume-controlled small-animal respirator (New England rodent ventilator, model 141, New England Instruments, Medway, MA) using room air. Normoventilation was achieved through adjusting ventilation in accordance with blood gas analyzes (ABL 800 blood gas analyzer, Bergmann diagnostika). During controlled ventilation, the alpha-stat strategy was followed.

Core cooling and rewarming

Animals were cooled and rewarmed by circulating cold or warm water (Thermo stated water bath type RTE-110, Neslab Instruments, Newington, NH) through an U-shaped polyethylene tube placed in the lower bowel. The tube was inserted gently to avoid harm of the intestine. In addition, the double-layered operating table made of hollow aluminum was circulated by temperature-adjusted water. Core temperature was continuously monitored using a thermocouple wire, positioned in the lowest part of the esophagus connected to a thermocouple controller (Thermalert Th-5, Bailey Instruments). Cooling and rewarming of the animals each lasted 1 1/2 h, while the hypothermic period (15 °C) lasted 4 h (Fig. 1).

Hemodynamic measurements

Hemodynamic variables were obtained using a pressure–volume conductance catheter (SPR-838, Millar Instruments Inc.,

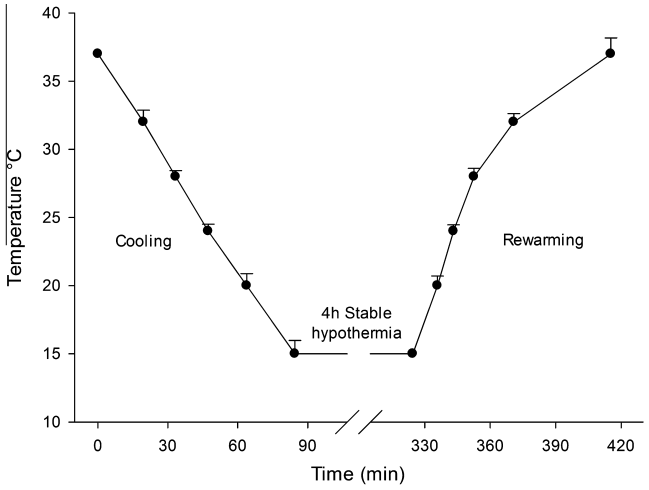

Fig. 1. Temperature profile of the experiments in rats assigned to either of the hypothermic groups, showing the cooling (37–15 °C) and rewarming (15–37 °C) rates and the stable hypothermic period (15 °C): Values are mean ± SEM.

Texas). The miniaturized 2.0 French pressure–volume conductance catheter allowed for assessment of *in vivo* left ventricular (LV) mechanical function in rats [1]. A constant sinusoidal alternating current (0.02 mA root means square at 20 kHz) was applied to drive the conductance catheter, through which changing conductance was used for the measurement of blood volume. Volume measurements in this study included parallel conductance (G_p). Further description of this method and temperature calibration of the catheter is described in detail in a previous report [8]. The load-independent LV-contractility index preload recruitable stroke work (PRSW) was measured by occluding the inferior vena cava transiently under the diaphragm. According to Filseth et al. this method was applied only prior to cooling and after rewarming, as caval occlusions at low temperatures does not induce changes in PV loops that can be applied to calculate PRSW [5]. To monitor peripheral vascular responses during cooling and rewarming, mean arterial pressure (MAP) was measured using a pressure transducer connected to a fluid-filled catheter (22G) inserted into the left femoral artery. During experiments, hemodynamic measurements were recorded at the following temperatures: 37, 32, 28, 24, 20 and 15 °C.

Experimental design

After surgery, the animals were given 1 ml saline and allowed to rest for 1 h before start of experiments. Levosimendan or placebo-levosimendan (placebo) was administered through an i.v. line in the left femoral vein, extended to the inferior caval vein. Infusion was started after 3 h of normothermia or hypothermia. The content of the placebo drug is identical to levosimendan except for absence of the active substance. Animals in hypothermic groups were core cooled to 15 °C and maintained at this temperature for 4 h, before rewarming to 37 °C. In the normothermic group, animals were held at 37 °C for 5 h.

Normothermic levosimendan group (n = 6)

After 3 h, animals received a bolus dose of 24 μg/kg of levosimendan infused over a period of 10 min, followed by a continuous 0.6 μg/kg/min infusion during the last 2 h of experiments.

Hypothermic levosimendan group (n = 7)

After 3 h at stable hypothermia (15 °C), animals were infused with a bolus dose of 24 μg/kg of levosimendan over 10 min, followed by a continuous infusion of 0.6 μg/kg/min during the last hour of hypothermia and till rewarming was completed.

Hypothermic placebo group (n = 7)

After 3 h of stable hypothermia (15 °C), animals were infused with a bolus dose of 24 μg/kg placebo for 10 min, followed by a continuous infusion of 0.6 μg/kg/min during the last hour of hypothermia and till rewarming was completed.

Measurement of cTnI phosphorylation

After successful rewarming, blood was removed by rapid flushing of sterile saline through the jugular catheter as described earlier [26]. The heart was quickly isolated, the LV dissected out and flash-frozen in liquid nitrogen. Measurements of the cTnI phosphorylation level on PKA associated sites Ser23/24 were performed using Western blot. The tissue was homogenized with a standard cell lysis buffer (Cell signaling) with 1 mM PMSF (Sigma). The protein level was measured with Lowry assay (Bio-Rad DC protein Assay). 45 μg of protein was loaded in each well separated with SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Transferred proteins on PVDF membranes were detected with specific antibodies for either total cTnI (Fitzgerald) or phosphorylated cTnI at Ser23/24 (Cell Signal) and visualized by a chemo luminescent for detecting horseradish peroxidase (Bio-Rad). The bands were quantitatively analyzed using molecular imaging software (v. 4.0.1 Kodak). The amount of phosphorylation is measured as phosphorylation rate (density of phosphorylated Ser23/24-cTnI divided by total cTnI).

Measurement of cTnI release

After experiments were completed, arterial blood was sampled from the left femoral artery. Blood was centrifuged and the plasma extracted from the tubes. Plasma-cTnI was then analyzed, using a high sensitivity rat cTnI ELISA kit, Life Diagnostics, Inc., West Chester, PA, USA.

Statistics

Changes from baseline in hemodynamic variables were compared by One-way repeated measures ANOVA. When significant differences were found, Dunnett's method was used to compare values within group vs. baseline. Differences in cTnI release between groups were analyzed using one-way ANOVA on ranks. When significant differences were found, Dunn's method was used to compare values between groups. PRSW data within each group were compared using a paired *t*-test. Differences between hypothermic groups at same temperatures and cTnI phosphorylation between the hypothermic groups after rewarming were measured using a two-tailed, unpaired Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Hemodynamics

Normothermia (Fig. 2)

Hemodynamic effects of levosimendan infusion in normothermic controls (37 °C). Compared to values measured at start of experiment (baseline), levosimendan infusion (120 min) caused significant elevation of stroke volume (SV), cardiac output (CO), heart rate (HR), maximum rate of LV pressure change ($LVdp/dt_{max}$) and stroke work (SW), whereas total peripheral resistance (TPR) decreased. Hemodynamic stability, up to 5 h at 37 °C, has been documented in numerous other studies using the present intact rat model [33,12].

Stable hypothermia

Levosimendan and placebo groups (15 °C). At the end of the 4 h stable hypothermia period, 1 h after start of placebo or levosimendan infusions in the two groups, most indexes of hemodynamic function were significantly reduced from their prehypothermic baseline values. Exceptions were left ventricular end-diastolic pressure (LVEDP), left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV) and SV, which remained unchanged. TPR was increased only in the placebo group (Figs. 3 and 4).

At the end of 4 h stable hypothermia and 1 h of levosimendan or placebo infusion, TPR was significantly lower and HR significantly higher in the levosimendan group compared to the placebo group.

Rewarming (Figs. 3, 4 and 7)

Placebo group (37 °C). After rewarming from 15 °C, SV, CO, SW and PRSW were all significantly reduced when compared to prehypothermic baseline values. TPR remained significantly elevated, when compared to prehypothermic baseline.

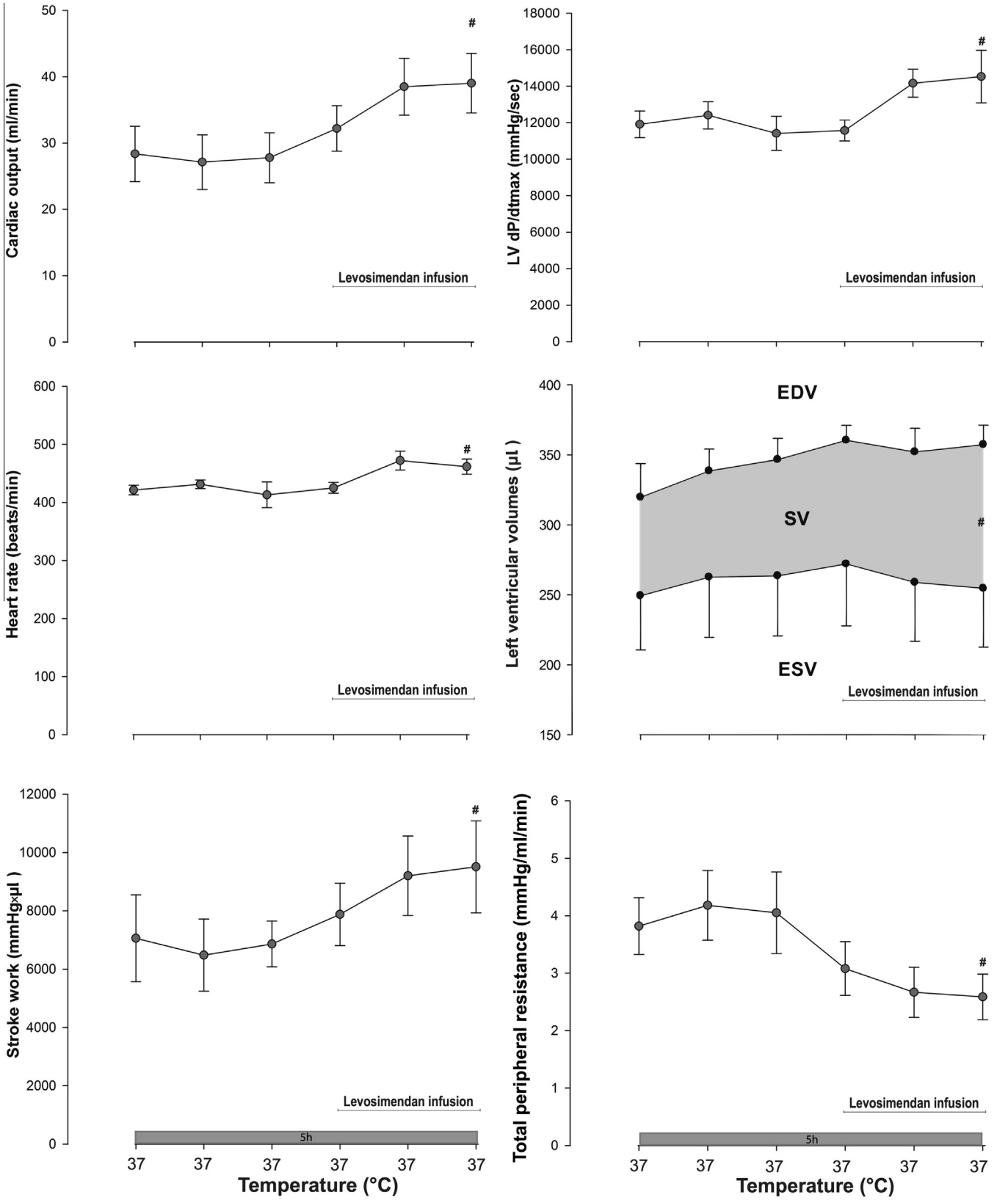

Fig. 2. Hemodynamic parameters in the normothermic control group, receiving levosimendan (bolus dose: 24 µg/kg, continuous infusion: 0.6 µg/kg/min) during the last 2 h of experiments. EDV: End-diastolic volume, SV: Stroke volume, ESV: End-systolic volume. Values are mean ± SEM. #Significantly different from within group baseline ($p < 0.05$). *Significant difference between groups ($p < 0.05$).

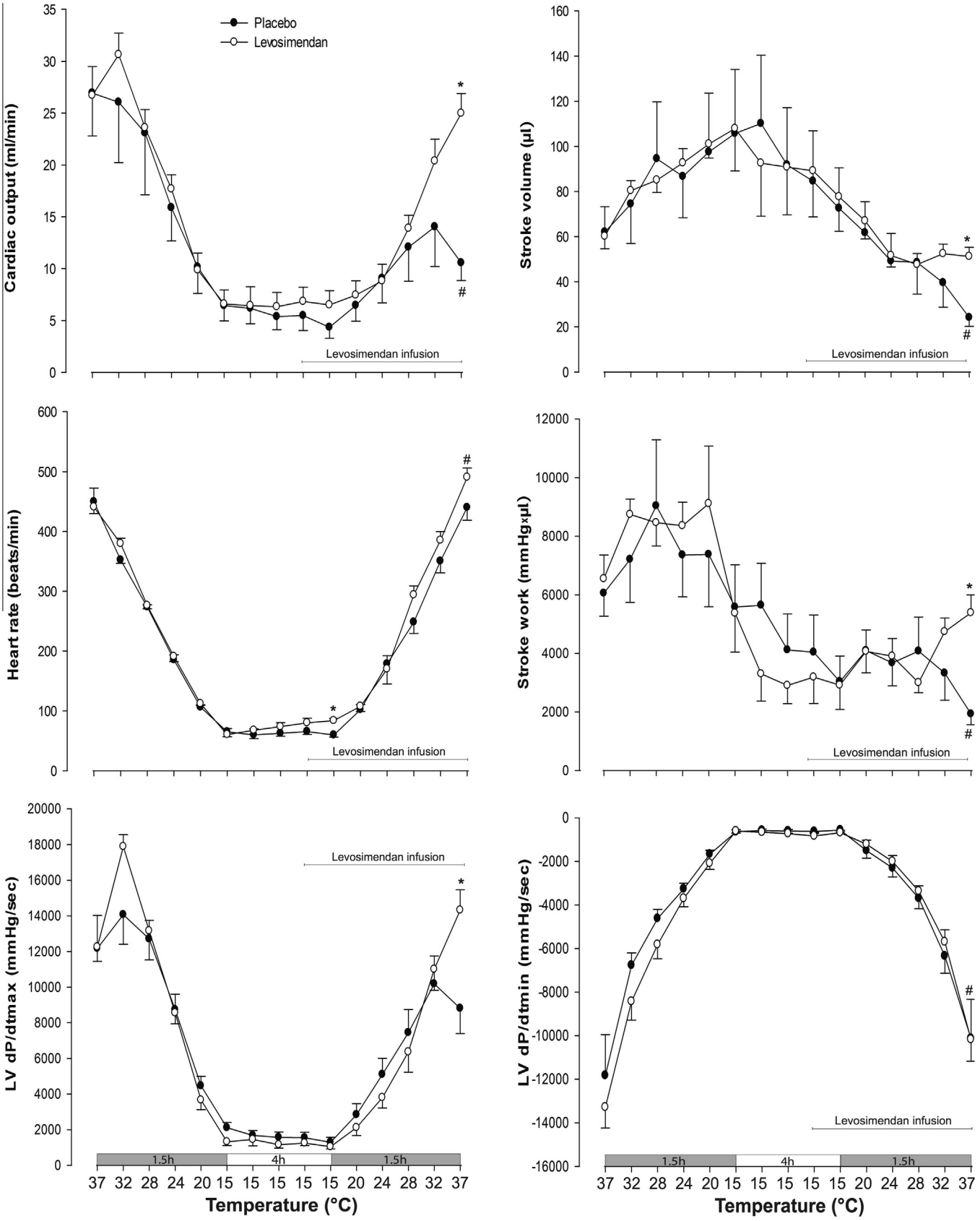

Fig. 3. Hemodynamic parameters in the two hypothermic groups, receiving either levosimendan or placebo (bolus dose: 24 µg/kg, continuous infusion: 0.6 µg/kg/min) during the last hour of hypothermia and during rewarming. Values are mean ± SEM. #Significantly different from within group baseline ($p < 0.05$). *Significant difference between groups ($p < 0.05$).

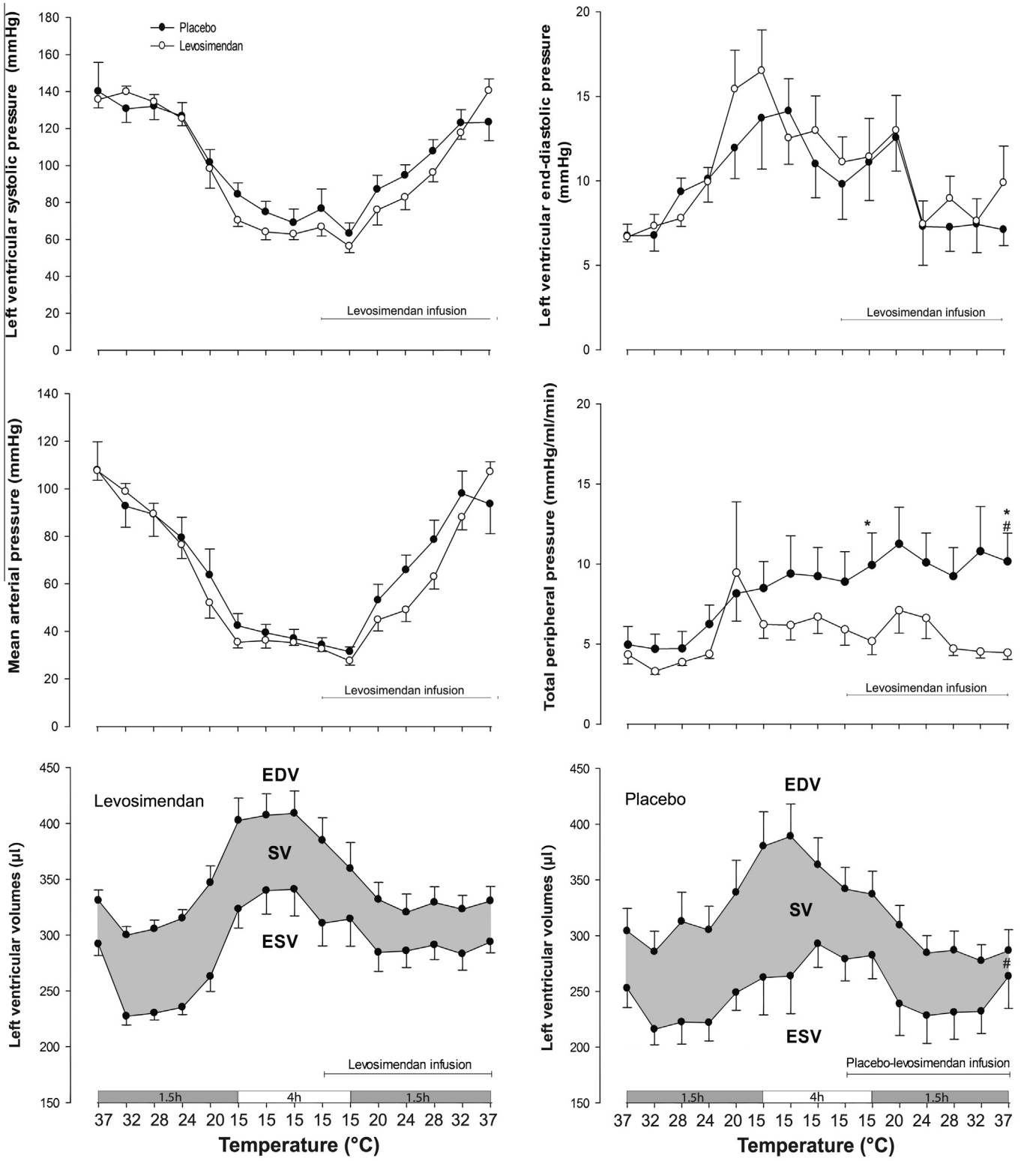

Fig. 4. Hemodynamic parameters in the two hypothermic groups, receiving either levosimendan or placebo (bolus dose: 24 µg/kg, continuous infusion: 0.6 µg/kg/min) during the last hour of hypothermia and during rewarming. Values are mean ± SEM. #Significantly different from within group baseline ($p < 0.05$). *Significant difference between groups ($p < 0.05$).

Levosimendan group (37 °C). In the levosimendan group rewarming caused a return to within pre-hypothermic baseline values of most cardiac variables. Minimum rate of LV pressure change ($LVdp/dt_{min}$) was decreased after rewarming, while HR was increased.

Differences between rewarmed levosimendan and placebo groups (37 °C). After rewarming SV, CO, $LVdp/dt_{max}$, SW and PRSW were significantly higher in the levosimendan group compared to the placebo group. TPR was significantly lower in the levosimendan group when compared to the placebo group.

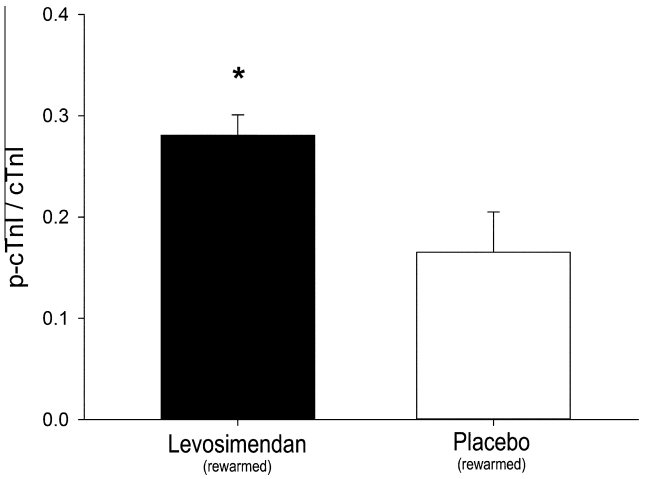

Fig. 5. cTnI phosphorylated at the Ser23/24 site in fraction of total cTnI, measured in left-ventricular tissue from excised hearts after rewarming in the two hypothermic groups, receiving either levosimendan or placebo (bolus dose: 24 μg/kg, continuous infusion: 0.6 μg/kg/min) during the last hour of hypothermia and during rewarming: Values are mean ± SEM. *Significant difference between groups ($p < 0.05$).

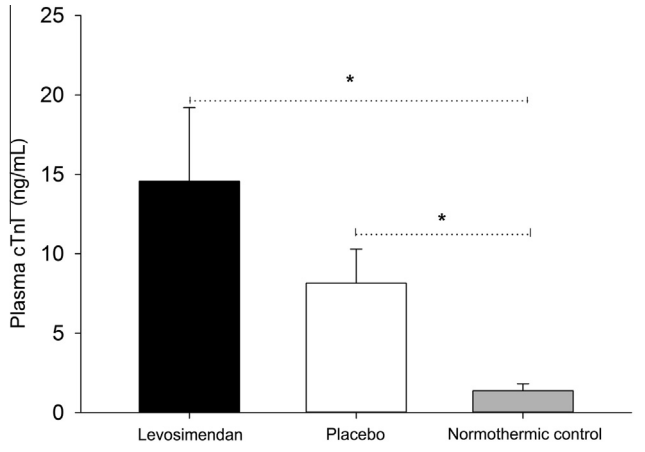

Fig. 6. Plasma-cTnI levels in the normothermic control group, receiving levosimendan (bolus dose: 24 μg/kg, continuous infusion: 0.6 μg/kg/min) during the last 2 h of experiments and in the two hypothermic groups, receiving either levosimendan or placebo (bolus dose: 24 μg/kg, continuous infusion: 0.6 μg/kg/min) during the last hour of hypothermia and during rewarming: Values are mean ± SEM. *Significant difference between groups ($p < 0.05$).

Phosphorylation of cTnI (Fig. 5)

After rewarming, Ser23/24-cTnI phosphorylation was significantly increased in the levosimendan group when compared to the placebo group. Phosphorylation results are displayed as a ratio between Ser23/24-cTnI phosphorylation and total cTnI protein.

cTnI release (Fig. 6)

Level of plasma-cTnI release at the end of experiments showed significant differences when comparing the normothermic group with both the rewarmed levosimendan group, and rewarmed placebo group. No significant differences were found between the rewarmed groups.

Discussion

Levosimendan administered during the last hour of hypothermia (4 h, 15 °C) and throughout rewarming to 37 °C caused a

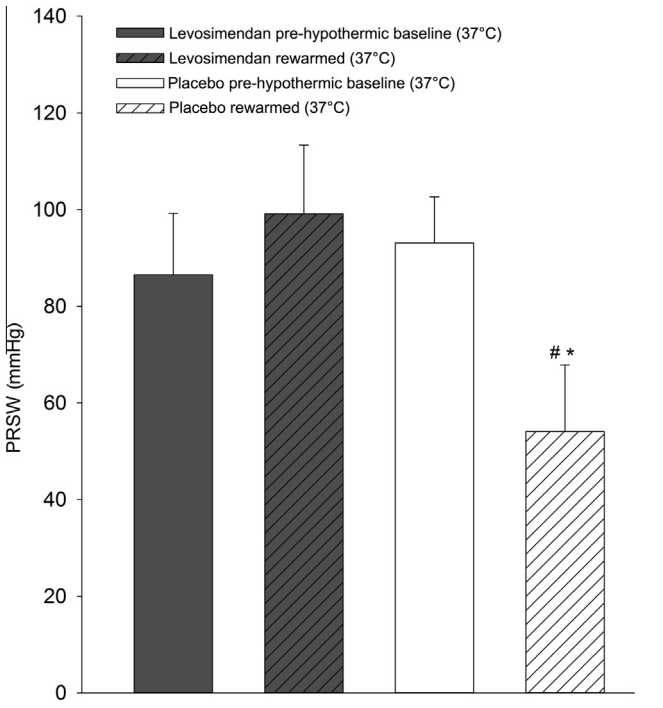

Fig. 7. Preload recruitable stroke work before cooling and after rewarming in the two hypothermic groups, receiving either levosimendan or placebo (bolus dose: 24 μg/kg, continuous infusion: 0.6 μg/kg/min) during the last hour of hypothermia and during rewarming: Values are mean ± SEM. #Significantly different from within group baseline ($p < 0.05$). *Significant difference between groups ($p < 0.05$).

return to within prehypothermic levels of SV, CO and PRSW. This is essentially different from the significant reduction of these variables after rewarming in the placebo group, demonstrating the positive inotropic effect of levosimendan during both hypothermia and rewarming.

Several inotropic drugs have been tested in the pursuit to pharmacologically elevate SV during rewarming from hypothermia. The present study demonstrates that levosimendan ameliorates hypothermia-induced cardiac dysfunction by elevating SV to prehypothermic levels after rewarming. This stand in stark contrast to our previous studies on β-receptor agonists, demonstrating that the inotropic properties of such drugs are altered during hypothermia and rewarming [14,12,8,28]. In more detail, only low-dose (0.125 μg/min) epinephrine showed beneficial hemodynamic effects when administered throughout rewarming from 15 °C. Elevation of SV was not achieved by higher epinephrine doses like in normothermia and vasoconstriction was induced [12], This shows that low core temperatures narrow the therapeutic window of inotropic treatment with β-agonists. The apparent challenges in providing cardiovascular pharmacological support during rewarming of hypothermic patients is reflected by recommendations in both the European [23] and American [31] guidelines, where administration of drugs is recommended only at core temperatures above 30 °C. Our findings indicate that use of inotropic support during hypothermia as well as rewarming from low core temperatures can provide favorable hemodynamic effects and ameliorate hypothermia-induced cardiac dysfunction. In the present study, we have demonstrated these favorable effects during rewarming after 4 h exposure to 15 °C, when applying a levosimendan-dose equivalent to what is defined as a high dose in clinical medicine. Others have also reported positive effects of lower doses of levosimendan during 15 min exposure to core-temperatures at 13–15 °C in preclinical studies of thoracic surgery [21,22]. These findings indicate

that the positive inotropic effect of levosimendan may appear at a wider therapeutic window than for β-receptor agonists during hypothermia and rewarming.

The present experiment shows that levosimendan facilitates Ser23/24-cTnI phosphorylation when administered during rewarming from 15 °C. Phosphorylation of cTnI is well documented and three main phosphorylation sites are explored: Ser23/24, Ser43/45 and Thr144, which are targeted by various kinases. PKA, a downstream kinase from cAMP in the pathway stimulated by β-receptor agonists and PDE3 inhibition, has shown great specificity for Ser23/24 [24]. Phosphorylation of this site is associated with reduced myofilament calcium-sensitivity [19]. Interestingly, Han et al. found that site-specific cTnI phosphorylation at Ser23/24 was increased after rewarming from 15 °C in rat papillary muscle and related this to contractile dysfunction [9]. The ability of levosimendan to support cardiac function and avoid stimulation of the cAMP–PKA pathway, thereby preventing further Ser23/24-cTnI phosphorylation, has earlier been used to describe the positive inotropic effect of this drug during rewarming after a short exposure to hypothermia [22]. However, positive inotropic effect of stimulating the cAMP, – PKA pathway through PDE3-inhibition during hypothermia, have already been observed in our rat model, using milrinone [27]. Levosimendan is also known to inhibit PDE3 in high doses as used in the present study [25,3], and we found increased Ser23/24-cTnI phosphorylation when compared to the placebo group after rewarming. This indicates that levosimendan facilitated cAMP, – PKA mediated Ser23/24-phosphorylation of cTnI via PDE3 inhibition and was observed in the presence of restored contractile function (PRSW) in the levosimendan group after rewarming. We therefore suggest that the combined PDE3 inhibition and calcium sensitizing mediated by levosimendan overcomes the potential negative inotropic effects of increased Ser23/24-cTnI phosphorylation [9]. Furthermore, at low core temperatures the importance of PDE3 inhibition might be enhanced due to hypothermia-induced cTnI phosphorylation [9], which is reported to decrease the calcium-sensitizing effect of levosimendan [10]. Despite offering positive inotropic effect, levosimendan-treatment did not protect against release of the myocardial injury marker plasma-cTnI. Increased release of this injury marker after rewarming also in the hypothermic placebo group, but not in the normothermic controls, shows that hypothermia caused myocardial tissue injury.

The positive inotropic effect of PDE3 inhibition reported in the present and previous studies [27], strongly indicates that stimulation of the cAMP, – PKA pathway through inhibition of cAMP degradation is favorable during rewarming. Efforts to stimulate this pathway through β-receptor agonists however, have demonstrated impaired inotropic properties of such drugs already during cooling to moderate hypothermia (34–33 °C) [8,28]. One mechanism facilitating this could be hypothermia-induced changes in β-receptor function. Interestingly, both increased and decreased β-receptor sensitivity is reported at low temperatures [15,32] and underlying mechanisms for diminished effect of β-agonists during hypothermia still remains unclear. Thus, more knowledge about the effects of hypothermia on β-receptor function is needed.

Conclusion

The present experiment shows positive inotropic effect of levosimendan during rewarming, when combining the calcium sensitizing and the PDE3 inhibitory effects offered by a high-dose of this drug. We therefore suggest that at low core temperatures, pharmacologic efforts to elevate cardiac function are better achieved by stabilization of the cTnC–cTnI complex and prevention of cAMP breakdown, rather than by attempting to increase cAMP formation via β-receptor stimulation.

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