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Pharmacological approaches to management of hypothermia-induced cardiac dysfunction

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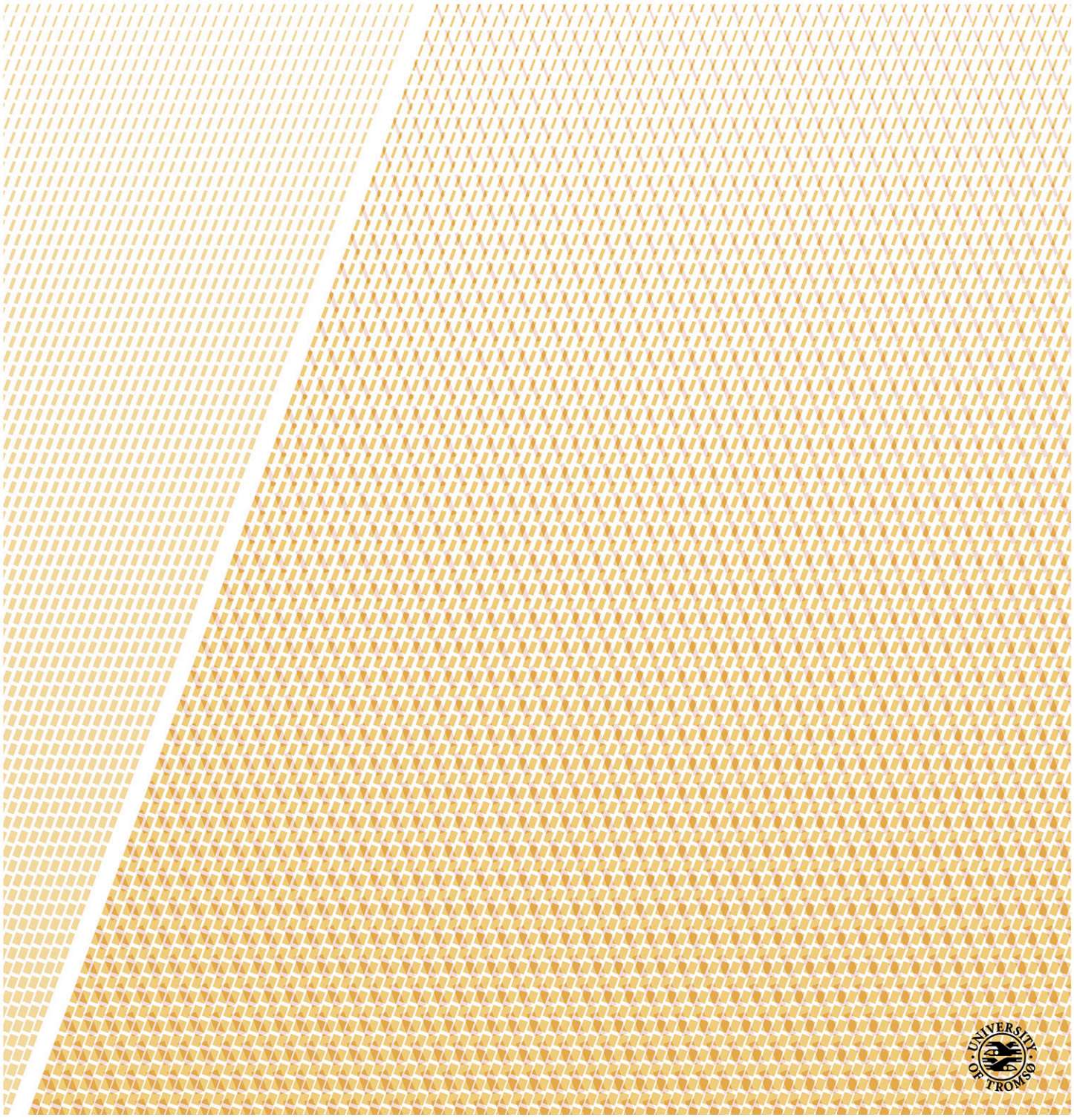

PHARMACOLOGICAL APPROACHES TO MANAGEMENT OF HYPOTHERMIA-INDUCED CARDIAC DYSFUNCTION

Content

1. Acknowledgements

My introduction to medical research came through admission to the research student program during my second year as a medical student in 2006/2007. This gave me a unique opportunity to find a research group within the medical faculty at the Arctic University of Norway (former University of Tromsø) that suited my academic interest. After hearing about the hypothermia research in the Anesthesia and Critical Care Research Group, I immediately visited Professor Torkjel Tveita, who warmly welcomed me to the group. I wish to express my gratitude to Torkjel for being an enthusiastic supervisor and door opener throughout my years as a medical research student and PhD student, always eager to listen to new ideas and encouraging of creative thoughts. His ability to see potential quality in such ideas, before looking for limitations has been of great inspiration and outmost importance for developing my interest for medical research while working on the present thesis.

I would like to thank all my co-workers in the Anesthesia and Critical Care Research Group, with special thanks to Timofei Kondratiev for teaching me how to work with animal models and for always being able to fix seemingly impossible challenges during surgery in the in vivo rat model. I want to thank Brage Håheim for being a great friend and skilled colleague. The interesting discussions I have with him and Torstein Schanche and their ability to find humor in experiments failing after 12 hours in the lab, is greatly appreciated. I am grateful for working with researchers like Ole Magnus Filseth and Jan Harald Nilsen with vast clinical experience and patience to answering my questions, which has increased my appreciation of the clinical relevance of our research.

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I am very thankful for the discussions I have with my parents and the support they as well as the rest of my family and friends have given me. Finally, I would like to give a special thanks to Turid, for always being supportive and for her immediate enthusiasm about the idea of living a year in Glasgow.

2. Abstract

We performed randomized, controlled experimental studies in an intact rat model and in isolated rat cardiomyocytes with the following aims:

Paper I

To investigate the effects of epinephrine during hypothermia and after rewarming and determine hypothermia-induced effects on in vivo and in vitro cardiac β-receptor sensitivity.

Paper II

To describe hemodynamic responses to the phosphodiesterase 3 (PDE3) inhibitor milrinone when compared to saline infusion during rewarming from deep, stable hypothermia (15°C).

Paper III

To describe hemodynamic response and phosphorylation of cardiac troponin I (cTnI) during rewarming from deep, stable hypothermia with use of the calcium sensitizer and PDE3 inhibitor levosimendan, compared to animals given placebo.

Main results and conclusions

β-receptor sensitivity is increased in hypothermia (15°C) compared to normothermia (37°C) (paper I), but administering epinephrine at 15°C had adverse effects, expressed with increased afterload and negative inotropy. Cardiac dysfunction during rewarming from stable hypothermia is however ameliorated by PDE3 inhibition alone (paper I) and combined with calcium sensitizing (paper III). PDE3 inhibition through levosimendan possesses the ability to

increase cTnI phosphorylation after rewarming from stable hypothermia (paper III). Treatment of hypothermia-induced cardiac dysfunction is therefore better achieved through intracellular strategies like PDE3 inhibition and calcium sensitizing than β-receptor stimulation.

3. List of papers

The studies in this thesis were carried out between 2007-2014 at the Anesthesia and Critical Care Research Group at the Department of Clinical Medicine at the Arctic University of Norway in Tromsø. The included papers in this thesis are listed below and will be referred to by their numerals.

Paper I

Negative inotropic effect of epinephrine in the presence of increased β-receptor sensitivity during hypothermia. Erik Sveberg Dietrichs, Torstein Schanche, Timofei Kondratiev, Svein Erik Gaustad, Georg Sager, Torkjel Tveita.

Paper II

Milrinone ameliorates cardiac mechanical dysfunction after hypothermia in an intact rat model.

Erik Sveberg Dietrichs, Timofei Kondratiev, Torkjel Tveita

Paper III

Cardiovascular effects of levosimendan during rewarming from hypothermia in rat.

Erik Sveberg Dietrichs, Brage Håheim, Timofei Kondratiev, Gary Sieck, Torkjel Tveita.

4. Abbreviations

AHA: American heart association

PDE3: Phosphodiesterase III

cTnI: Cardiac troponin I

CO: Cardiac output

LV: Left ventricle

MAP: Mean arterial pressure

HR: Heart rate

SV: Stroke volume

TPR: Total peripheral resistance

SR: Sarcoplasmic reticulum

cAMP: Cyclic adenosine monophosphate

NO: Nitric oxide

LVEDV: Left ventricle end-diastolic volume

LVESV: Left ventricle end-systolic volume

LVEDP: Left ventricle end-diastolic pressure

CI: Cardiac index

SW: Stroke work

$LVdP/dt_{max}$: Maximum rate of LV pressure change

$LVdP/dt_{min}$: Minimum rate of LV pressure change

P_{min}: Minimum LV pressure

Tau: The isovolumic relaxation constant

PKA: Protein kinase A

SERCA: Sarcoplasmic Ca^{2+} uptake pump

G_p: Parallel conductance

RVU: Relative volume unit

PVDF: polyvinylidene difluoride

CGP: [^{3}H]-CGP12177

IC50: The half maximal inhibitory concentration

5. Introduction

5.1 Definitions of severity

There is largely a consensus defining hypothermia as core temperatures below 35°C. Authors describing the severity of hypothermia have however used different definitions. Popovic (1974) suggested that temperatures above 32°C should be considered mild hypothermia, moderate between 32 - 22°C, deep 22 - 8°C and profound below 8°C [1]. In 1986, Moss suggested that 35 - 32°C should be defined as mild hypothermia, 32 - 28°C as moderate and severe hypothermia below 28°C [2]. This is also the definition used by the European Resuscitation Council [3]. The American Heart Association (AHA) use a definition described by Polderman and Herold [4], where mild hypothermia is defined as temperatures above 34°C, moderate between 34-30°C and severe below 30°C [5]. This is the definition which was found to be the most clinically relevant in the guidelines on accidental hypothermia for the health care services in Northern Norway [6], defining 30°C as a critical limit between "safe" and "unsafe" hypothermia. On this background, the AHA definition is also used in the present thesis.

5.2 Classification

Hypothermia can be classified in different categories according to how body temperature is lowered. *Acute hypothermia* occurs when a person is exposed to severe cold stress and the body is not able to prevent a drop in core temperature by shivering and centralizing blood distribution and thus is cooled down before energy reserves are exhausted. Immersion in cold water or intoxication from alcohol or other drugs combined with low ambient temperatures can lead to acute hypothermia. *Subacute hypothermia* describes a condition were the cold stress is less severe and cooling only occurs when energy reserves are exhausted. This

condition is most commonly found in climbers and people immersed in warmer water. *Chronic hypothermia* is a consequence of prolonged exposure to moderate cold stress, where core temperature decreases over time. This is most commonly seen in elderly people, who are subjected to poor housing during winter. *Submersion hypothermia* is hypothermia secondary to submersion in ice-cold water [7]. Rapid cooling of the brain due to inhalation of cold water with subsequent cooling of blood in the lungs is thought to differentiate this condition from hypoxic drowning [8]. *Therapeutic hypothermia* is deliberate use of hypothermia to avoid brain damage, mainly in comatose survivors following cardiac arrest. For this purpose, patients are most often cooled down to 32-34°C after resuscitation [9]. Therapeutic hypothermia is also used in surgical procedures like aortic arch surgery, where temperatures down to 15°C are used [10].

5.3 Background

Accidental hypothermia is a condition found in individuals that have lost the ability to maintain a body core temperature above 35°C [11]. If this lowering of core temperature happens in an otherwise healthy person without presence of any sedatives like anesthesia, sedative drugs or alcohol, the sympathetic nervous system will induce a fight and flight response and thus increase metabolism. The energy provided will enable muscle shivering and at the same time the body will centralize blood supply in order to conserve body temperature [8]. This depletion of energy stores and oxygen makes the victim of accidental hypothermic different from a patient subjected to therapeutic hypothermia. The activation of a sympathetic response in the latter group will mainly be prevented by the use of sedative drugs and opioids [12] and hypothermia is therefore used to reduce metabolism and protect against brain damage in cardiac arrest survivors [13]. When core temperatures in victims of

accidental hypothermia decline towards what is defined as severe hypothermia (<30°C), the fight and flight response will however dissipate. The resulting reduction of cerebral metabolic demand facilitates the possibility to survive cardiac arrest caused by accidental hypothermia for several hours [14]. When these patients are rewarmed, a hypothermia-induced cardiac dysfunction [15] often complicates treatment and is a severe threat to survival. In patients subjected to induced hypothermia, heart failure is also a common finding [16]. To ameliorate such heart failure, inotropic support is sensible but several studies have shown that such treatment is challenging in hypothermic animals [17-19]. This thesis is aimed at finding inotropic drugs suitable for treating this condition and to understand why traditional inotropic drugs working through the β-adrenergic receptor pathway have reduced inotropic effects [17-20] during hypothermia.

5.4 Who are affected?

5.4.1 History

Accidental hypothermia occurs in written sources dating back to Hippocrates (460-370 B.C.). One of the earliest known incidents with mass casualties caused by hypothermia happened when the Greek officer Xenophon lead 10000 men through the mountains of Armenia in 400 BC. Only 4000 of these soldiers survived [21]. Perhaps more famous is the casualties of Hannibal's campaign against the Romans in 218 BC. While working its way through the Alps, his army of men and elephants encountered bad weather and 20000 men perished in the mountains [22]. Military campaigns gone wrong due to cold weather have also happened in armies more adapted to harsh conditions. When retreating over the mountains from Norway in January 1719, 6000 Swedish soldiers were surprised by bad weather. It is estimated that 50 %

died of hypothermia [23]. The first accurate clinical descriptions of this condition came some years later during Napoleons Russian campaign in 1812 and were written by his surgeons. Their inventive work was of great importance for the evolvement of emergency medicine. One of these surgeons, Dominique Jean Larrey, described how hypothermic soldiers died during rewarming. His colleague Moricheau-Beaupré further described the victims' weakening pulse and fixed eyes [22]. In addition to describe the detrimental effects of accidental hypothermia, Larrey was also able to see the therapeutic potential of cooling. Being the first to describe that cold body parts may withstand asphyxia longer than their warm counterparts, he is by some ascribed to be the father of therapeutic hypothermia [24].

In more recent times with increasingly brutal methods of war, getting accustomed to environmental conditions by keeping sufficient supply lines of food, fuel and clothes proved harder in the 20th century. On the Eastern front in World War I, eight per cent of all casualties were due to cold. [21]. The 15th of October 1941, Hitler made a decision that might have affected the outcome of the Second World War. Due to lack of progression in the Soviet Union, it was decided that the attack was to continue through the winter, instead of halting it and wait for spring. To perform this, commanders were ordered to prioritize fuel and ammunition and to leave winter clothing behind [21]. Improper clothing led to massive amounts of hypothermic casualties throughout the next winters on the Eastern Front. The decisive Battle of Stalingrad left 1.1 million Russians and 800000 Germans dead. Although it is impossible to quantify, undoubtedly a large number of these soldiers died from hypothermia in the harsh winter [25]. Many perished further north as well. In fact, the biggest Arctic battle of all times took place at the border between Russia and Norway [26]. When the Russians pushed the German troops into Northern Norway in the autumn of 1944, the German command decided to evacuate Finnmark, the northernmost county of Norway. Included in this

decision was expelling all inhabitants from an area as big as Denmark and burning all buildings that could be used by the advancing Russians as protection from the harsh weather conditions. However, more than 20000 of Finnmark's inhabitants decided to defy German threats of execution and decided to stay behind [27]. In order to stay out of German sight and survive, they inhabited caves, mines and improvised huts in the mountains during the last Arctic winter of the Second World War [28]. Cold exposure was a big problem also in naval units. Of all the branches of any military service in the Second World War, the German submarine crews had the highest mortality. 70 % of 40000 men serving in the submarine service died, many of them from hypothermia. It is believed that the high mortality rate of the submarine and flight crews at sea was the background for one of the most inconceivable war-crimes in world history, the atrocious Dachau hypothermia experiments [21, 29]. Also regular sailors were at a high risk of dying from hypothermia or drowning. 3638 Norwegian merchant navy sailors died during the Second World War. In comparison, 2000 Norwegian soldiers died [30].Thus, it is evident that exposing people to un-physiological situations like war elicits unusual and unnecessary casualties, among them hypothermia.

In times of peace, mass casualties of accidental hypothermia are related to big accidents, where people are exposed to a cold environment, e.g. seawater. After the Titanic sunk on April 14th in 1912, 1500 victims were accounted as drowned. However, the first ship that came to rescue the passengers arrived one and a half hour after the sinking and only picked up the 710 persons in the lifeboats. Most of the 1500 victims, already reckoned as dead, were at that time floating in the calm sea, as they were wearing life-wests. On this background Shetty published a letter in The Lancet in 2003, changing the cause of death among the passengers of the Titanic from drowning to accidental hypothermia [31]. If provided todays knowledge and a theoretical access to proper rewarming facilities, a similar accident today could have had a

much higher survival rate. In a smaller scale, this was shown after the Danish Præstø Fjord accident, where 7 victims of accidental hypothermia were found with their heads submersed in water and were rewarmed with good outcome following hours of cardiac arrest [32].

Hypothermia is also a threat for those who voluntarily expose themselves to harsh conditions found in the Arctic and Antarctic. Some of these explorers have described their own experiences with hypothermia in Polar areas. Fridtjof Nansen reached farther north than any other known human being had ever been in 1895. On this expedition, Nansen and his companion Johansen used kayaks to reach Franz Josef's land on their return. After reaching the archipelago, they almost lost their kayaks when they drifted ashore while the two men were left on an ice float. Nansen jumped into the ice-cold water and swam after the kayaks. In his memoirs he described his hypothermic state and how Johansen saved him with skilled use of passive rewarming [33].

In 1911 a race for reaching the South Pole ended with Roald Amundsen and his Norwegian team reaching it the 14th of December. This team was however not the only competing for conquering the pole. Among the others, only the British expedition led by Robert Falcon Scott reached the South Pole, a month after Roald Amundsen. Scott's group perished during the return from the pole, in his diary found on his body 8 months after his death, Scott described the harsh conditions with ambient temperatures between -30° and -44°C the last month of the expedition. A continuous storm trapped Scott in his tent for 9 days, and he succumbed from starvation and hypothermia only 18 km from a big food and fuel depot [34].

5.4.2 Today

The elderly

In modern times, accidental hypothermia is mainly affecting people exposed to low ambient temperatures without capabilities to isolate themselves from the environment. It is therefore affecting poor and elderly and is associated with fuel poverty during winter [35]. Different from more acute accidental hypothermia, older people found with low core temperatures often have an impaired sense of low temperature and suffer from chronic hypothermia [36]. In Ireland, hypothermia is associated with being old and living alone in scarcely populated areas. Being old was also associated with not having properly insulated houses and central heating [37]. This finding is also consistent with findings from Glasgow, were the mean age of hypothermic patients found indoors was 76 years. The death rate in these victims of mainly chronic hypothermia was more than 4 times higher than in victims of presumably acute hypothermia, that were found outside [38]. Old people that were living alone and had associated problems as confusion or neglect were at greater risk for hypothermia. This shows that regular visits from health personnel or family is important for old people at risk for hypothermia and other conditions associated with living alone. Interestingly, this apparent increase in deaths of hypothermia due to poor insulation and fuel poverty is not associated with increasing latitudes. Both in England and Wales excess mortality was found in the middle-aged and old population during winter. This increase was markedly higher than in Norway and especially Iceland, where a similar increase in mortality during winter was not found [39]. The difference might be associated with traditions for insulating and heating of houses as well as general living conditions.

Socioeconomic challenges

Among poor people, the lack of properly insulated accommodation and sources of heat is probably one of the most important causes of hypothermia. In Tokyo, the leading cause of

accidental death among the homeless is accidental hypothermia [40]. In The United States, urban poverty contributes to a high percentage of hypothermia deaths and several of these occur in warm areas like Florida and Texas [41]. In New Mexico, Native Americans are 30 times more likely to die from hypothermia than other residents in the same state. 90 % of these deaths were associated with alcohol intoxication [42]. Other types of drug intoxication are also associated with hypothermia. In Victoria, Australia 21 % of GHB intoxicated patients were hypothermic over a 16 month period [43]. However, alcohol intoxication seems to be a more common finding in victims of accidental hypothermia, with ethanol detected in femoral blood from 43 % of fatalities included in an accidental hypothermia study from Northern Sweden [44]. Apart from alcohol, benzodiazepines (34 %) were the most common drugs detected in blood samples, while anti-depressives and opiates were found in 28 % and 14 % of the cases respectively. Hypothermia secondary to alcohol intoxication seems to be a common finding. In a study on Dutch adolescents (age 11-17) admitted for alcohol intoxication, accidental hypothermia was found in 43 % of subjects. Hypothermia was more common in boys and correlated to blood alcohol concentration [45].

Shipwrecks

Exposure to harsh climates with low ambient temperatures and bad weather is not only affecting people in times of war. Fishermen, hunters, reindeer herders and other inhabitants of the north have been exposed to cold conditions in arctic and subarctic areas for thousands of years. In recent years the activity in the arctic areas has been increasing, due to the melting ice cap [46] and an increase in search for oil and gas resources in these areas. The Alexander Kielland accident in 1980, killing 123 people [47], showed that accidents on oil platforms in rough seas far from the coastline, are potentially catastrophic. Increasing ship traffic due to larger areas free of ice, available for tourism and commercial shipping routes, leaves both

tourists and ship crews exposed in case of accidents. Incidents like this have only just been avoided on some occasions. Recently, the passengers of the cruise ship MS Explorer were evacuated to the ship's lifeboats in Antarctica in 2007. Fortunately, a Norwegian coastal steamer could rescue them all in calm weather [48]. The sinking of the passenger ferry Estonia on route between Tallinn and Stockholm in 1995 is an example of what can happen in worse weather conditions, even when responding ships and rescue helicopters are available at the site of accident shortly after the incident. 852 of the 989 passengers were killed, succumbing to drowning and hypothermia [49]. Of those who escaped the ship and awaited rescue in lifeboats, 1/3 were perished from hypothermia before the rescue helicopters arrived. The water temperature at the scene was 10-11°C, well above what victims of a capsize in Arctic waters would encounter [50]. Therefore, hypothermia is probably a killer also in shipwrecks in more tempered water. The most severe shipping accidents of modern times have not been in northern areas, but outside The Philippines and Senegal, killing thousands of people [51].

Drowning and hypothermia

On account of the described shipwrecks, among them Titanic were the majority of the victims probably died from hypothermia [31], it is possible that there are hidden numbers of hypothermic deaths in the drowning rates. Drowning is the 3rd leading cause of accidental death worldwide and it is estimated that 388 000 people died from drowning in 2004. Most of these accidents happened in low and middle-income countries, accounting for 96 % of fatal drownings. Especially China and India have high mortality rates and together stand for 43 % of drowning deaths worldwide [52].

Children

Many victims of drowning are children and in Bangladesh drowning accounts for 20 % of all deaths in children aged 1-4 years [52]. Smaller children have a larger body surface area compared to weight, thus they suffer hypothermia more quickly than adults. This might be one of the reasons why Children younger than 4 years have a better prognosis of drowning than older children [53]. If the body temperature of the drowning victim falls quickly, it might prevent neurological damage associated with hypoxia [54]. This was demonstrated in the good outcome of the Præstø Fjord accident [32]. In European waters, victims of drowning accidents are predominantly hypothermic, but low core temperatures are often associated with longer submersion and hypoxia. Therefore, core temperature is an unreliable indicator regarding the final prognosis of drowning victims [53]. This was shown by a study on cardiopulmonary bypass rewarming of children admitted to a Finnish pediatric hospital, due to hypothermia following submersion [55]. 8 of the 9 included patients died due to hypoxic brain injury, which indicates that the absolute majority of these patients are hypoxic before cooled to the level of neurological protection. The surviving girl was however submerged for as long as 45 min and also had the lowest recorded core temperature (18°C) in the study. Survival with good neurologic outcome in hypothermic children have been described after submersion for as long as 40 and 66 min in other case reports [56, 57]. This shows that resuscitation and rewarming of drowned children with the use of cardiopulmonary bypass can be meaningful even in the most severe cases and should be tried [6]. However, these children were all submerged in very cold water. Most drownings in children occur in more tempered water as in Bangladesh [52], thereby the victims are cooled at a slower rate. The protective effect of hypothermia in these drownings is dependent on that the victims manage to keep their airways free of water and breathe until cooled down to temperatures which gives cerebral protection against hypoxia. As immersion cooling is slower in such cases, these victims are subject to subacute hypothermia as opposed to acute hypothermia, which is more

likely to be the case in colder waters (defined as <20°C) [58]. Undoubtedly, most drownings among children are therefore primarily hypoxic and the most important changeable factor in decreasing deaths from drowning is to prevent these accidents from happening.

Fishermen, cold-shock and hypothermia

Drowning is an occupational hazard in some professions, e.g. for fishermen. The commercial fishing industry has one of the highest overall occupational mortality rates [59]. When looking at deaths due to fishing accidents in British Columbia over a 26-year period, Brooks et al. found that official records stated that 87 % of the victims drowned. After analyzing available data on these fatalities, the authors were only able to ascribe 10 % of the deaths to drowning, while 72.2 % of the cases didn´t have enough information to conclude on a cause of death. Of the remaining 17.8 %, the authors have described 10.8 % of the deaths as hypothermia or cold shock, indicating that hypothermia is an underestimated killer of these fishermen [60]. This is underlined by that at least 16 % of Alaskan fishermen, that died in accidents accounted for as drowning or hypothermia in the same period, used personal flotation devices [58]. Fishing is often carried out far from search and rescue services and searching for victims of fishing accidents is complicated by that first-aiders often arrive at the site of accident after several hours. The important difference between a hypothermic and a drowned victim is that hypothermic patients might survive for a long period before being rescued and brought to a hospital capable of rewarming with use of cardiopulmonary bypass. Rescuing immersed, hypothermic patients is therefore possible for a prolonged time when compared to submersed victims of hypoxic drowning. Differentiating victims of cold-shock and hypothermia is however difficult when immersion of the victim is not being witnessed, as cold-shock is described as immersion which kills within 2-3 min [60]. The pathophysiology causing cold-shock is not clear. Increased myocardial workload due to a fight and flight

response mediated by the sympathetic nervous system after sudden exposure to cold water may predispose cardiovascular incidents, especially in old and middle aged people [61]. Further, Shattock and Tipton proposed that the cold-shock was a consequence of an "autonomic conflict" between the parasympathetic diving reflex and the sympathetic cold shock response. The diving reflex is activated by excitation of vagal parasympathetic neurons innervating the heart, inhibition of central respiratory neurons and excitation of sympathetic vasoconstrictor neurons, giving bradycardia, apnea and vasoconstriction of peripheral arteries. The cold-shock response is sympathetically initiated tachyarrhythmia due to reflex patterns initiated by cutaneous thermoreceptors. The authors propose that when these two responses are activated at the same time due to sudden immersion in cold water, an autonomic conflict can be the result, leading to fatal arrhythmia [62]. Given that this theory is correct, cold-shock is very different from hypothermia, where arrhythmias are associated with lower core temperatures and are considered most common <28°C. When core temperatures in hypothermic victims decrease to levels lower than this, ventricular extra-systoles and atrioventricular blocks are common arrhythmias. When the victim reaches core temperatures below 20°C, asystole occurs [15]. Cardiac arrest at normothermia, after hypoxic drowning or cold-shock, induces cerebral ischemic injury over minutes. If temperature is reduced below 30°C before ischemia is induced, cerebral protection is substantial [54]. This is apparent in case-reports from Northern-Norway, which have proved that core temperatures down to 13,7°C can be survived and that hypothermic cardiac arrest is possible for up to 7 hours with a good neurological outcome [14, 63]. Thus, successful rewarming from accidental hypothermia can be possible several hours after the accident. Cases like these show that basic life support initiated at the site of accident, good search and rescue services and proper in-hospital procedures for treating accidental hypothermia can increase safety of people working in commercial fishing and offshore industries.

Trauma and hypothermia

In general, hypothermia in patients admitted to trauma centers seems common. In a study from Melbourne, Australia, which has a moderately temperate climate, 13 % of patients were hypothermic at admission. In these patients overall mortality reached 30 %, while the overall mortality for patients admitted to this trauma center was 9 % [64]. This is in accordance with findings from an American study [65], while other studies report that 5-37 % of trauma patients were hypothermic at admission [66, 67]. High mortality among hypothermic patients could be explained in two ways: 1) Low body temperatures worsens the pathophysiological condition associated with the primary trauma, 2) the primary traumas in patients admitted with hypothermia are more severe and that is why the patients cannot maintain a normal core temperature. Hypothermia was however found as an independent risk factor for mortality in the Melbourne-study, indicating that 2) cannot be the full explanation of higher mortality in hypothermic trauma patients [64].

5.5 Therapeutic hypothermia

The lowest temperature ever recorded in a surviving human was measured during experimental treatment of a woman with terminal ovarian cancer in 1958. With the intention to treat her cancer, Niazi and Lewis cooled her down to 9°C before successfully rewarming her [68]. After rewarming, she regained consciousness and had full cognitive recovery but died 38 days later do to complications of the cancer. Such whole body cooling to severe hypothermia for cancer treatment is obsolete, but severe hypothermia is still used for surgical procedures. Among these procedures are aortic arch surgery, where reduction in core temperature down to 15°C is regularly used [10] and surgery on the abdominal aorta where

hypothermia is associated with an increased demand for inotropic support [16]. Moderate therapeutic hypothermia is used for cerebral protection after cardiac arrest. Such treatment is carried out at temperatures (32-34°C) where both epinephrine (Epi) and isoprenaline have reduced inotropic effect in rats [19, 20]. Thus, in order to provide better guidelines for inotropic treatment of hypothermic patients, more knowledge about hypothermia-induced changes in pharmacology is needed.

5.6 Mortality

Rewarming from accidental hypothermia is a complicated procedure. MacLean and Emslie-Smith described a mortality rate up to 80 % dependent on rewarming methods [69], while it was reported to be 29 % in a more recent study [70]. Hypothermia is also an important complication in trauma patients, increasing the mortality rate. Ireland et al. found that 13 % of patients admitted to a trauma center were hypothermic and had a threefold increase in mortality compared to the normothermic patients [64]. In patients with burn injuries, hypothermia is also a common finding, which affected 40 % of such patients in Pittsburgh, US, giving a 2-fold increase in mortality [71]. Hypothermia is also an important factor in neonatal mortality, especially in low-income countries. This is a big problem, even in warm climates like the Sub-Saharan region of Africa where reported incidence of hypothermia in some regions exceed 60 % [72].

5.7 Hypothermia-induced cardiac dysfunction

The French surgeons Larrey and Moricheau-Beaupré were the first to describe circulatory collapse in hypothermic patients [22]. During the 200 years since these descriptions, several experiments have reproduced their findings. Dog experiments from the late 1940s and 50s

described decreased cardiac output (CO) during hypothermia and rewarming. Cooling dogs to a core temperature of 30°C and rewarming over 3h, showed a reduction in mean arterial pressure (MAP) and cardiac index (CI), while heart rate (HR) regained prehypothermic values after rewarming [73]. Prec et al. also found that the lowest recordings of CO did not occur at the lowest temperatures, but during rewarming [74]. Maclean and Emslie-Smith related the cardiac dysfunction to a sudden fall of peripheral pressure and lack of compensatory increase of CO 1977[69] and defined this circulatory collapse as a "rewarming shock" or "rewarming collapse". Subsequent research on the pathophysiology causing such hypothermia-induced cardiac dysfunction has been carried out in several animal models [15]. Insufficient oxygen supply has been ruled out as a contributor [75], while calcium overload is related to development of hypothermia-induced cardiac dysfunction [76, 77]. Also phosphorylation of cardiac troponin I (cTnI) is apparent after rewarming left ventricular papillary muscle from rat and is related to decreased force of contraction [78].

5.8 Treatment of accidental hypothermia

Guidelines for treating victims of accidental hypothermia have been issued both by the European Resuscitation Council [3] and the American Heart Association [5]. Although these guidelines use different classifications of hypothermia, they provide a general consensus on treatment of hypothermic patients. These patients are treated by the principle that "no one is dead until warm and dead". It is therefore recommended that victims of accidental hypothermic cardiac arrest should be treated with same chest compression and ventilation rates as for normothermic patients. In the local Northern-Norwegian guidelines it is recommended that a mechanical device is used for continuous chest compressions during transportation to hospital with core-rewarming facilities [6]. Use of cardioactive drugs is

recommended to be delayed until core temperature reach 30°C by both American and European guidelines [3, 5] and they also state that defibrillation might be challenging below the same temperature. The European guidelines recommend that after 3 unsuccessful defibrillation attempts further attempts should be delayed until core temperature is above 30°C.

Rewarming victims of accidental hypothermia can be achieved using several methods. In mild hypothermic patients, external rewarming is appropriate if the patient is awake and shivering. Active rewarming in the field should not delay transport to hospital with advanced rewarming techniques [3]. Preventing further cooling in such patients is best achieved combining an insulating layer and plastic [79]. However, in patients with severe hypothermia (<28°C / 30°C) and cardiac arrest both European and American guidelines agree that extracorporeal rewarming is the method of choice. It is therefore important that such patients are taken directly to medical centers with such rewarming facilities. This is also emphasized by Brown et al, who stated that the destination hospital should be contacted to ensure that ECMO or cardiopulmonary bypass is available for rewarming severely hypothermic patients [80]. As survival with full neurological recovery is reported after several hours of CPR [14], it is important that patients are taken directly to medical centers capable of extracorporeal rewarming without delay, even when the distance to such a center is considerable. This is illustrated by the recommendation of taking patients from Svalbard directly to the University hospital in Tromsø, a distance of 1000 km [6].

5.9 Pharmacological treatment during hypothermia

Guidelines for pharmacological treatment during rewarming from accidental hypothermia advise against using inotropic drugs below 30°C. When reaching temperatures above this, intervals for administering them should be increased [3, 5]. This is supported by studies finding that the function of liver enzymes important for drug elimination are impaired during hypothermia [81]. Several studies do however report that drugs with effect on the cardiovascular system are given during rewarming [82-84]. Only about 10 % of normothermic patients with acute heart failure receive the inotropic treatment [85]. The lack of consensus based guidelines results in different opinions on use of inotropic drugs in hypothermic cardiac arrest patients, even within the British health care system [86]. Several animal studies have been conducted to test effects of such drugs during hypothermia and rewarming, described in the following chapters.

5.9.1 Adrenergic receptor agonists

Adrenergic receptor agonists are used particularly for their effect on the cardiovascular system where the effects are mediated through G-protein coupled adrenergic receptors in myocardial and vascular tissue. Ahlquist was the first to describe how these receptors were divided into two main groups [87], named α- and β-receptors. Subgroups of these receptors have later been identified, separating them into $\alpha_{1\text{-}2}$ and $\beta_{1\text{-}3}$, which have a broad variety of effects in both the cardiovascular system and other organ systems The β_1-receptor is considered most important for inotropic effect and is also more numerous in the mammalian heart (75 %) than β_2 and β_3 [88], it will therefore be the most thoroughly discussed here. β_1-stimulation enhances heart muscle contraction, increase HR and enhances relaxation of myocardial tissue [89]. The effect of β_1-agonists is mediated through stimulation of adenylyl cyclase, which elevates cyclic adenosine monophosphate (cAMP), activating protein kinase A

(PKA), which phosphorylates several proteins. Among these are sarcolemmal calcium channels (increasing calcium influx) and phospholamban (increasing sarcoplasmic reticulum calcium pump rate). PKA also phosphorylates troponin I, which reduces calcium sensitivity of troponin C, a regulatory step in actin-myosin coupling [88]. β_2-stimulation can induce both a similar G-protein pathway as for β_1 or an inhibiting pathway in myocardial tissue, but most importantly gives vasodilation due to smooth muscle relaxation [90]. β_3-receptor stimulation has a negative inotropic effect, mediated by nitric oxide (NO), produced by NO synthase [88, 91] and gives dilatation of coronary arteries [92]. Both α_1- and α_2- receptors are further divided into three subgroups. Stimulation of all α_1-receptors will in general induce smooth muscle contraction, which gives vasoconstriction [93]. The α_2-receptor subgroups have diverse abilities. Due to this, stimulation of α_2-receptors has mixed effects on smooth muscle, leading to both vasodilation and vasoconstriction [94].

Epinephrine:

Epi is known to increase cardiac contraction and HR and either decrease (low-dose) or increase (high-dose) total peripheral resistance (TPR) in normothermic conditions [95]. This is conducted through non-selective binding to the major adrenergic receptors. However, these effects do not seem to be independent of temperature changes. Rubinstein found that Epi doses inducing vasodilation in normothermia would give increased TPR in hypothermia. He further claimed that the inotropic effect of Epi is reduced at 25°C [96]. Kondratiev et al. studied Epi administered during rewarming from 15°C in a rat model. This study showed that high doses (1.25 μg/min) of Epi, which increased stroke volume (SV) in normothermic animals, did not increase SV during rewarming from hypothermia. Further, a low dose (0.125 μg/min) of Epi, that decreased MAP in normothermic animals, failed to do this during rewarming, but increased CO [17]. Tveita and Sieck found the same dose-response

relationship also during cooling, where low-dose (0.125 μg/min) in contrast to high-dose (1.25 μg/min) Epi gave positive inotropic effects. However, these effects vanished during cooling to 28°C. After rewarming, only rats that had received saline during cooling showed prehypothermic hemodynamic responses to Epi [19]. Administering 1μg/min dosage of Epi during cooling also depress cardiac function during rewarming [18]. These results indicate that hypothermia has a severe impact on the function of β_1-receptor agonists. Hypothermia combined with β_1-adrenergic stimulation even impairs function of such drugs after rewarming. This relationship was also observed in two studies by Weiss et al. [97, 98]. Underlining the depressed inotropic function of Epi during rewarming from severe hypothermia, Rungatscher et al. showed Epi to be inferior to levosimendan in order to give inotropic effects in rats rewarmed on a cardiopulmonary bypass system [99].

Further indications that might advise against use of Epi during hypothermia are found in in vitro studies. Both SV and CO were depressed when administering Epi to the isolated rat heart at 28°C [100]. In such in vitro studies, hearts are subject to a calcium overload as a consequence of hypothermia [101]. This is also found in rat in vivo studies and the calcium overload is not reversed by rewarming [76, 77]. Schiffmann et al. demonstrated that Epi still had a depressive effect on SV and CO in the presence of additionally added calcium in the hypothermic heart. This is different from in normothermia, where Epi had increased inotropic effects in the presence of more calcium [100].

In normothermic patients, guidelines advise use of Epi during cardiopulmonary resuscitation after cardiac arrest. Recent studies questions whether Epi benefits these patients. Patients receiving Epi seems to gain return of spontaneous circulation more quickly, but it seems that these patients do not have a better long-term survival [102]. Effects of Epi during

resuscitation from cardiac arrest have also been tested in experimental animal models of hypothermia. In a hypothermic pig model, Epi increased coronary perfusion pressure, but also enhanced mixed venous acidosis. Return of spontaneous circulation was not more likely in pigs receiving Epi compared to placebo [103]. In dogs, Epi increased lethal temperature from 19.3 to 21.9°C and induced ventricular fibrillation in all animals [104]. In rats subjected to moderate hypothermia (32°C) after initiating ventricular fibrillation, a high dose infusion of Epi (20μg/kg) improved post resuscitation myocardial function. Normothermic rats subjected to the same procedure had negative effects of Epi [105]. These apparent positive effects of Epi in moderate hypothermia is different from the findings of Wira et al. who reviewed the literature of animal models of ventricular fibrillation in severe hypothermia. They found that intermediate and high-dose Epi gave no increase in return of spontaneous circulation, while low-dose Epi combined with amiodarone did [106]. However, return of spontaneous circulation was monitored over a maximum period of 60 min in these studies, and does not give information on long-time survival.

Norepinephrine

Effects of other catecholamines have also been tested in hypothermic conditions. Weiss et al. studied the effect of moderate hypothermia on response to infusion of norepinephrine. During normothermia, norepinephrine has higher affinity for α-receptors than Epi and the β_1-receptor affinity is thought to be equal, but it has little affinity for β_2-receptors. Infusion of norepinephrine therefore leads both to vasoconstriction of arterioles and induce increased cardiac contractility [107]. Cotton et al. demonstrated this inotropic effect in both normothermic controls and hypothermic dogs cooled to temperatures around 30°C [108]. Further, they described that although still positive, the inotropic effect of norepinephrine was decreased at low temperatures [109]. Cardiac inotropy was however measured by a strain

gauge arch sutured directly on the right ventricle in these studies, not giving information about SV or other hemodynamic parameters. When norepinephrine was administered in cats subjected to moderate hypothermia and rewarming, it decreased CO during hypothermia, but increased it at baseline before cooling and after rewarming. However, due to high standard deviation, the latter differences were not significant. The ability of norepinephrine to induce vasoconstriction seems to be the only feature intact during cooling in these cats, with consistent dose-related increase in MAP [98]. These findings indicate that α_1-receptors are intact during moderate hypothermia, while β_1-receptor functionality is altered. An apparent alteration of not only β_1- but also β_2-receptors function at low temperatures is also indicated in the findings of Kondratiev et al., who showed that only low-dose Epi is able to increase CO during rewarming from severe hypothermia and that Epi is unable to reduce MAP at these temperatures [17]. Intact β_2-receptor function has however been described during hypothermia. When norepinephrine and the non-selective β-receptor agonist isoprenaline were administered in patients cooled (28-32°C) using cardiopulmonary bypass, MAP decreased significantly during isoprenaline infusion, indicating an intact β_2-receptor response. At these temperatures, norepinephrine still possessed the ability to mediate vasoconstriction, as seen during normothermia [110]. Interestingly, Weiss et al. reported that norepinephrine, when administered after exposure to moderate hypothermia and rewarming, increased CO more than in cats kept normothermic [97]. This indicates that altered β_1-receptor function is reversible after rewarming from moderate hypothermia.

Isoprenaline

Isoprenaline is a non-selective β-receptor agonist. Studies looking exclusively at β-receptor stimulation often use isoprenaline on this background. Lauri et al. studied hemodynamic effects of isoprenaline before, during and after severe hypothermia (25°C) in dogs. During

hypothermia, no significant increase in inotropic effects were observed, but systemic vascular resistance was still decreased, indicating at least partly intact β_2-receptor response to stimulation [111]. This is also observed in patients cooled (32-28°C) using cardiopulmonary bypass [110]. Further, depressed β_1-mediated inotropy of isoprenaline was apparent in rat left atrial preparations at 28°C and 20°C, in contrast to at 35°C [112]. Underlining this is the findings that the inotropic response to isoprenaline and Epi was decreased in hypothermic rabbit atria at 23°C [113]. However, in isolated guinea pig hearts cooled to 27°C, isoprenaline still increased the contractility parameter LV dp/dt_{max} (the maximum rate of pressure change in the left ventricle), this was accompanied by a similar increase in HR, also mediated by β_1-receptor stimulation [114]. Isolated atria from guinea pig also had increased inotropic effects of isoprenaline at 25°C [115]. Reaching even lower temperatures, sustained ability of Epi and isoprenaline to increase contraction amplitude and rate was observed when cooling rabbit hearts to 22°C[116]. Inotropic effects of isoprenaline during cooling have further been investigated in rat in vivo experiments, testing several doses of isoprenaline during cooling to 24°C. Except for the highest dose (20ng/min), no effects of isoprenaline on SV or CO were seen during cooling to 24°C using concentrations that gave a dose-related inotropic response at 37°C. Although insignificant, some doses even seemed depressive on cardiac function. This change in response to β-receptor stimulation was also sustained after rewarming, when only the high dose of isoprenaline managed to elevate SV above baseline [20]. Due to the apparent hypothermia-induced disability of β-agonists to mediate positive inotropic effect when administering same doses as used during normothermia, it was important to investigate whether β-receptor sensitivity is intact at low core temperatures, as described in paper I.

5.9.2 Dopamine

Like Epi, norepinephrine and isoprenaline, dopamine is a catecholamine, working through G-protein coupled receptors in the heart. Different from adrenergic agonists, dopamine exerts its inotropic and vasoactive effects through dopamine receptors [117]. The use of dopamine as a vasopressor is recommended in the Up To Date accidental hypothermia guidelines [118]. This is supported by a better cardiovascular recovery in dogs, after core cooling to 25°C and subsequent rewarming [119]. Positive inotropic effects of dopamine were found in pigs core cooled to 30°C as well [120]. However, in surface cooled pigs cooled to 32°C [121] and 25°C [122] dopamine did not elevate CO. In the latter study, dopamine infusion at 25°C gave a four-fold increase in plasma concentrations compared to normothermia. In difference from β-adrenergic drugs, cardiovascular responses of dopamine were restored during rewarming [122]. The differences between animals subjected to core and surface cooling are somewhat comparable to differences between controlled therapeutic hypothermia and accidental hypothermia. These are two very different conditions, the first; a patient group cooled under heavy sedation and the latter; a situation where thermal receptors in the skin induce increased sympathetic activation during cooling. Based on these studies dopamine might be a safer option in patients subjected to therapeutic hypothermia than in victims of accidental hypothermia that are in need for inotropic support.

5.9.3 Phosphodiesterase 3 inhibitors

Phosphodiesterase 3 (PDE3) inhibitors increase cAMP by inhibiting phosphodiesterase 3 mediated breakdown. There are many inhibitors of various phosphodiesterase enzymes including caffeine, however, the inotropic drugs seem to be relatively selective for phosphodiesterase 3 inhibition [88]. By inhibiting this enzyme, these drugs avoid the G-

protein coupled adrenergic receptors (in particular β_1) when they exert their inotropic effects. By increasing cAMP, milrinone will induce PKA mediated phosphorylation of the same proteins as β_1-adrenergic drugs. This will increase calcium influx through phosphorylation of L-type calcium channels.

Milrinone

Different from in vivo cooling of hearts, cooling isolated myocardial cells gives increased force of contraction independent of inotropic drugs and at 25°C a 400-500 % increase in force of contraction is found. Bers describes that this must be an effect of rapidly increased intracellular calcium as cells are cooled [88]. Milrinone is a phosphodiesterase 3 inhibitor with inotropic effect due to its ability to increase cAMP. In isolated guinea pig trabecula, the inotropic effect of milrinone was abolished at 31°C and 34°C [123]. The lack of ability to give inotropic effect above baseline values was described as an effect of changed intracellular calcium levels during hypothermia. This is however different from the hypothermia-induced cardiac dysfunction observed in in vivo models of hypothermia and rewarming [15, 124], where cardiac dysfunction is reckoned to be related to cytosolic calcium overload apparent only after several hours of hypothermia and / or rewarming [76, 77]. Further, the inotropic effect of the β-agonist isoprenaline in vivo is depressed already when cooling rats to 34°C. Thus, decreased inotropic effect of β-agonists [20] in vivo, is most likely not due to calcium handling. A cellular model of hypothermia is therefore not an optimal model for studying the inotropic effects of different drugs during hypothermia. Tveita and Sieck stated the value of investigating the effects of milrinone in an in vivo model, when they found that milrinone, different from β-agonists, had sustained positive inotropic effects during cooling to 15°C [125]. Milrinone increases calcium influx through PKA mediated L-type calcium channel phosphorylation. Due to the reported calcium overload after several hours of hypothermia, it

was important to investigate whether milrinone infusion during rewarming could ameliorate hypothermia-induced cardiac dysfunction. This is described in paper I.

5.9.4 Calcium sensitizers

Calcium sensitizers possess positive inotropic abilities through binding the N-terminal of cardiac troponin C and increasing calcium affinity at high calcium levels [126].

Levosimendan

In hypothermic guinea pig trabecula, levosimendan was found to be the inotropic agent with best properties at 31°C and 34°C [123]. This was explained in relation with the calcium sensitizing features of levosimendan, giving an inotropic effect that could be independent of temperature related calcium changes. High doses of levosimendan also has a phosphodiesterase 3 inhibiting effect [127, 128], increasing cAMP. PDE3 inhibition has proven beneficial during cooling [125] and rewarming rats (paper I). Three studies have looked at the hemodynamic effects of levosimendan in deep hypothermic cardiac arrest in rat and pig, with use of cardiopulmonary bypass. The levosimendan dosage was the same in these studies, giving an infusion of 0.2μg/kg/min. The results did however differ as rats had positive inotropic effect of levosimendan compared to Epi after rewarming [99, 129], while levosimendan treated pigs only had a decreased intracranial pressure when compared to controls [130]. The positive effect of levosimendan when treating hypothermia-induced cardiac dysfunction is described in paper III.

6. Aims of the thesis

The motivation behind the present thesis was to find better pharmacologic treatment for victims of accidental hypothermia. Survival after severe exposure to low temperatures [63], prolonged hypothermic cardiac arrest [14] and submersion hypothermia [57] have all shown that survival is possible in extreme situations, but the mortality still remains high [70]. One of the major concerns related to rewarming of such patients is hypothermia-induced cardiac dysfunction [15], which have proven challenging to treat in pre-clinical studies using β-adrenergic drugs [17-20]. Guidelines for rewarming such patients do however include use of Epi after reaching core temperatures above 30°C [3, 5]. In order to find alternative inotropic treatment and obtain more knowledge about pharmacology in hypothermia, we wanted to investigate effects of inotropic drugs working through intracellular mechanisms to ameliorate hypothermia-induced cardiac dysfunction. Among the main questions discussed in this thesis are:

- What are the cardiovascular effects of Epi infusion before, during and after hypothermia?
- Are the impaired effects of β-adrenergic drugs during hypothermia caused by hypothermia-induced dysfunction in the β-receptor – PKA – cAMP pathway?
- Is inotropic treatment during rewarming from severe experimental hypothermia better achieved through PDE3 inhibition and calcium sensitizing than β-adrenergic receptor stimulation?

For this purpose we used an intact rat model developed in our lab in Tromsø. Important for monitoring hemodynamic function and thus the effects of the administered drugs, we used a

conductance catheter, measuring left ventricular (LV) pressure and volume. This method was crucial for paper II and III and also used for monitoring hemodynamic properties in paper I. In paper I we wanted to use two different methods for looking at hypothermia and rewarming-induced effects on the β-receptor – PKA – cAMP pathway. Improving treatment of victims of accidental hypothermia was the main aim of this thesis, but severe therapeutic hypothermia is used during some surgical procedures [10] and our results also have relevance in this setting. Further, comatose survivors of cardiac arrest are regularly cooled to temperatures (33°C) were β-adrenergic agonists have showed decreased inotropic effect [19, 20] in pre-clinical studies. Our findings could therefore be relevant also for these patients. Aims of each of the three papers are as follows:

6.1 Paper I

The aim of paper I was to establish whether deep hypothermia and rewarming affect the sensitivity of cardiac β-receptors and the ability of Epi to increase cAMP during normothermia, deep hypothermia and after rewarming. Further, we wanted to elucidate the relationship between hemodynamic responses of Epi and β-receptor integrity during hypothermia (15°C).

6.2 Paper II

The aim of paper II was to investigate whether the PDE3 inhibitor milrinone could ameliorate hypothermia-induced cardiac dysfunction during rewarming from severe experimental hypothermia in vivo. We wanted to describe the impact of milrinone on closely monitored hemodynamic function, using a conductance catheter, both during normothermic conditions and during rewarming.

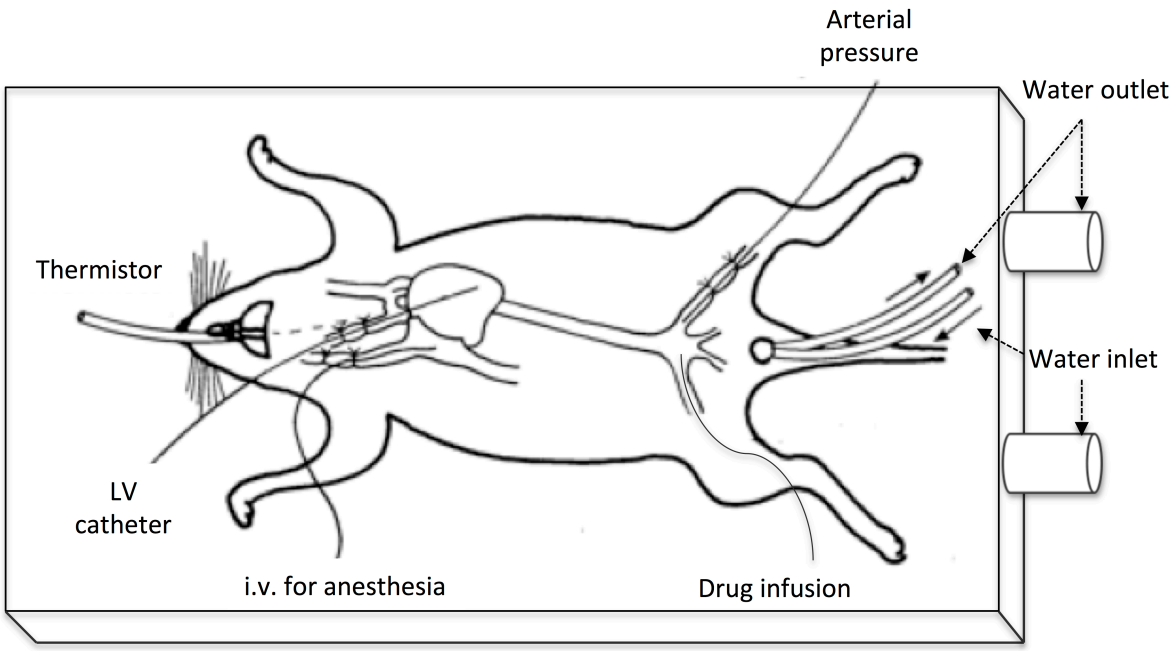
6.3 Paper III

The aim of paper III was to use the same model as in paper II to investigate the combined calcium sensitizing and PDE3 inhibiting effects of high-dose levosimendan. In addition to monitoring hemodynamic effects we also wanted to investigate cTnI phosphorylation, which has been proposed to be part of the pathophysiological picture initiating hypothermia-induced cardiac dysfunction. Further, we wanted to measure plasma cTnI as a marker of myocardial tissue damage.

7. Methodological considerations

7.1 Animal protocols (paper I, II, III)

In all studies, male Wistar rats (Charles River, Germany) were used. This strain was chosen based on considerable experience in our group using these rats in hypothermic experiments. Rats are well fitted for our purpose of studying long-lasting hypothermia, with maintained spontaneous cardiac activity throughout experiments, and the present rat model has been used in several studies in our laboratory [17-19, 75-77, 125, 131-134]. With minimal surgery this intact animal model of hypothermia provides excellent opportunities to investigate temperature dependent physiological effects on hemodynamic function and several other laboratory variables at low temperatures. The experimental protocol used in these studies, was approved by the Norwegian Animal Research Authority and the experiments were conducted on anesthetized rats according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18.III.1986).



7.2 Anesthesia (paper I, II, III)

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/hour pentobarbital sodium and 50 μg/kg/hour fentanyl through an intravenous line in the right jugular vein, extended to the right auricle. The infusion was maintained at all hours in normothermic animals. Infusion in hypothermic animals 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. We further monitored hemodynamics in all rats. Any changes in HR and MAP therefore helped us monitoring whether the animals were in distress and was used in concert with toe pinch after establishment of the LV conductance catheter and the pressure transducer in the femoral artery. Any signs of distress were thus monitored at all times, so that additional anesthesia could be provided if necessary. Toe pinch is a well-established method for testing the effects of analgesic drugs in rodents and has been extensively tested in rats [135] and is therefore used as the standard method for testing effect of anesthesia in the present rat model [17-19, 75-77, 125, 131-134].

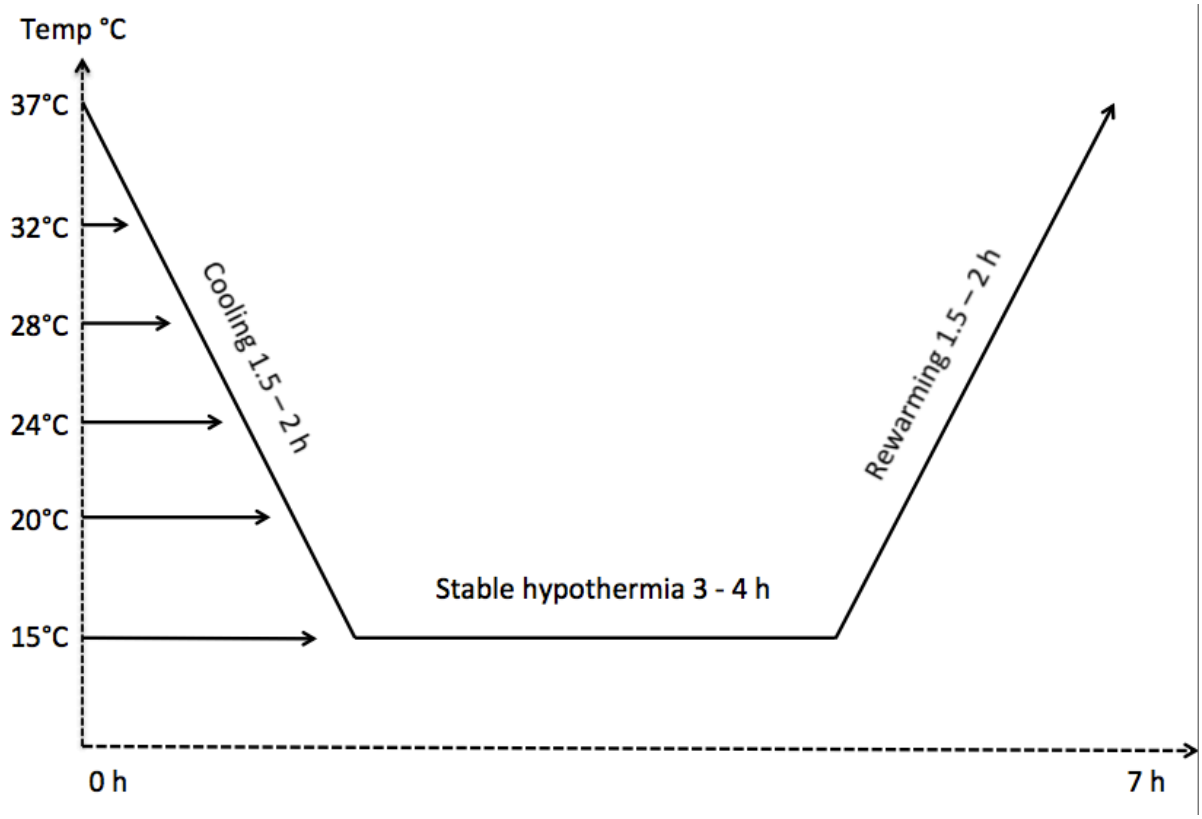
7.3 Respiratory Support (paper I, II, III)

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. Blood gases were measured in order to achieve normoventilation. Samples were drawn from the left femoral artery and all blood gases were analyzed at 37°C, using a commercially available blood gas analyzer (ABL 800 blood gas

analyzer, Radiometer, DK). According to Ashwood et al [136], we used the alpha-stat strategy and did therefore not correct pH and blood gas values for temperature in hypothermic animals.

7.4 Core Cooling and Rewarming (paper I, II, III)

Animals were cooled and rewarmed by circulating cold or warm water (Thermo stated water bath type RTE-110, Neslab Instruments, NH, US) 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. This technique for cooling and rewarming has been used extensively in our lab [17-19, 75-77, 125, 131-134] and provides control and fast change of core temperatures within a narrow range ($\pm$ 0.1°C). Core temperature was continuously monitored using a thermocouple wire positioned in the lowest part of esophagus, connected to a thermocouple controller (Thermalert Th-5, Bailey Instruments, UK). Cooling and rewarming of the animals each lasted 1.5h-2h, while the hypothermic period (15°C) lasted 3h-4h.

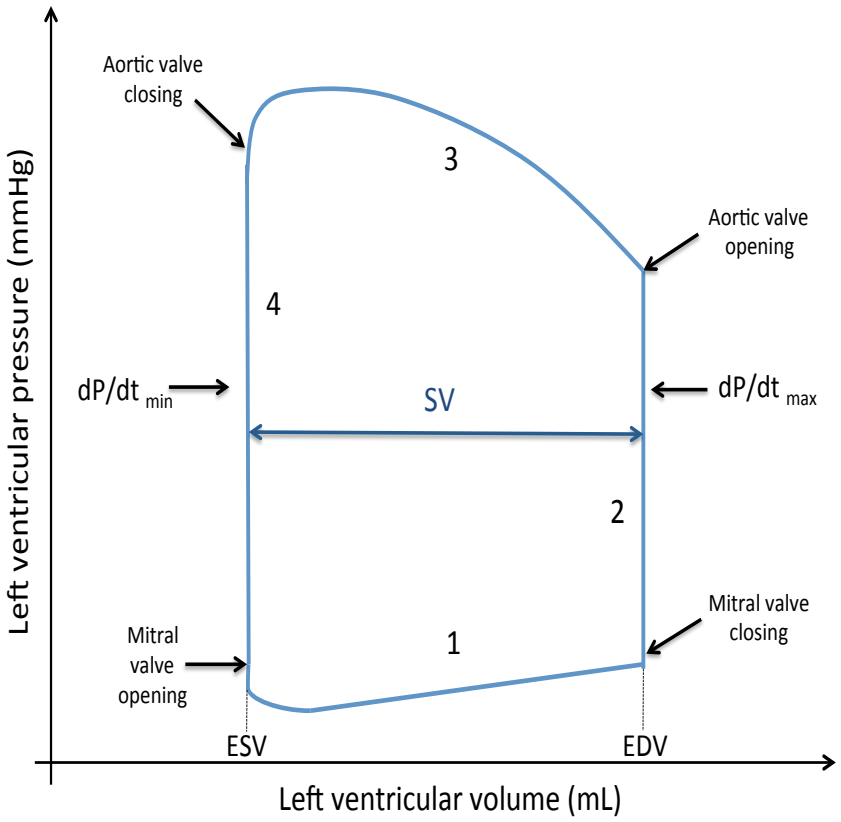


Temperature and time protocol of the experiments in the in vivo protocols (paper I,II and III).

7.5 Hemodynamic Measurements (paper I, II, III)

Hemodynamic variables were obtained using the Millar pressure–volume conductance catheter system (SPR-838, Millar Instruments Inc., TX, US). The miniaturized 2.0 French pressure–volume conductance catheter allowed for the assessment of *in vivo* LV mechanical function in rats [137]. 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. The measured conductance should be corrected for parallel conductance induced by the alternating current passing through the blood into the surrounding ventricular structures or inter-ventricular septum. A saline bolus injection method is generally used to measure parallel conductance at the end of experiments [138]. However, this method was not applied in this study due to the multiple

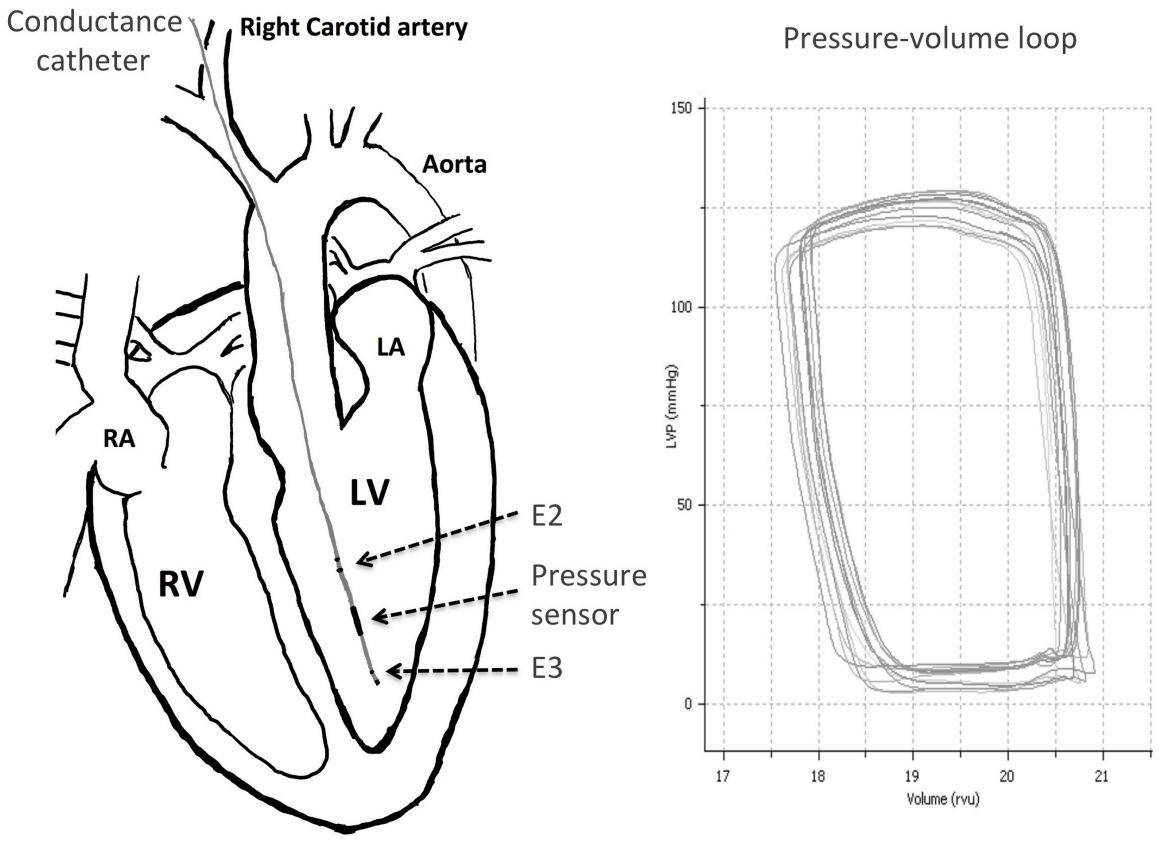
experimental temperatures (37, 32, 28, 24, 20, and 15 °C) of which measurements were taken. The viscosity of blood is affected by temperature. Due to the lack of calibration of each temperature by the saline bolus that would have a fatal effect on the animal, the volume measurements in these studies included parallel conductance (G_p) [139]. The pressure–conductance catheter was inserted into the left ventricle via the right carotid artery. In addition, MAP was measured using a pressure transducer connected to a fluid-filled catheter (22G) inserted into the left femoral artery. This MAP reflects peripheral vascular responses to cooling and rewarming.



Schematic drawing of a left ventricular pressure-volume loop: The area within the loop equals stroke work (SW), 1: Diastole, 2: Isolvolumetric contraction, 3: Systole, 4: Isovolumetric relaxation, SV: Stroke volume, ESV: End-systolic volume, EDV: End-diastolic

volume. dP/dt_{max}: Maximum rate of LV pressure change, dP/dt_{min}: Minimum rate of LV pressure change.

For a more accurate assessment of LV volume, the cuvette calibration was performed using insulator-type cuvettes of known diameter (2–7 mm) filled with heparin-treated blood. The volume measured by inserting the conductance catheter (SPR-838, Millar Instruments Inc., TX, US) into the volume cuvette was calculated by the following formula: the actual volume between electrodes (*E*2–*E*3) is $\pi r^2 L$ where *r* is radius of the cuvette and *L* (9 mm) the distance between *E*2 and *E*3. The volume cuvette containing heparin-treated blood was placed on the inside of a thermo-controlled water circulator so the temperature of the blood could be adjusted during the calibration. Considering temperature-dependent viscosity of blood, the volumes measured at our specific experimental temperatures (37, 32, 28, 24, 20, and 15 °C) were corrected using the cuvette calibration method according to Han et al. [20]. Linear regression at each temperature was run. Slopes and *y*-intercepts determined on linear regression at the different temperatures were applied to convert conductance units (relative volume unit: RVU, 1 RVU = 75 microsiemens) to true volume units (μl).



Cross section of the heart with placement of the conductance catheter within the left ventricle in the in vivo experiments, and a recorded trace of pressure-volume loops obtained from the conductance catheter. LA: Left atrium, LV: Left ventricle, RA: Right atrium, RV: Right ventricle, E2 and E3: Electrodes for conductance measurements, used to measure volume.

During cooling, myocardial irritability increases and risk of ventricular fibrillation rises [140]. Therefore we did not find it advisable to adjust the position of the conductance catheter during 3-4 h at 15°C. Due to this and failure to record proper volume signals in 6 animals (4 milrinone treated animals and 2 saline controls) at 15°C, recordings at 20°C during cooling and rewarming are used as hypothermic measurements in paper II.

7.6 Measurement of cTnI phosphorylation (paper III)

After successful rewarming, blood was removed by rapid flushing of sterile saline. The hearts were quickly isolated, left ventricles dissected out and flash frozen in liquid nitrogen [133]. Measurements of the cTnI phosphorylation (p-cTnI) level on PKA associated sites Ser23/24 was done with the use of Western blot. The tissue was homogenized with a standard cell lysis buffer (Cell signaling technology, MA, US) with 1mM PMSF (Sigma-Aldrich, MO, US). The protein level was measured with Lowry assay (Bio-Rad laboratories, CA, US). 45μG of protein was loaded in each well separated with SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad laboratories, CA, US). Transferred proteins on PVDF membranes were detected with specific antibodies for either total cTnI (Fitzgerald industries international, MA, US) or p-cTnI at Ser23/24 (Cell signaling technology, MA, US) and visualized by a chemo luminescent for detecting horseradish peroxidase (Bio-Rad laboratories, CA, US). The bands were quantitatively analyzed with molecular imaging software (v. 4.0.1 Kodak). The amount of phosphorylation is measured as phosphorylation rate (density of p-cTnI divided by cTnI).

7.7 Measurement of cTnI release (paper III)

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

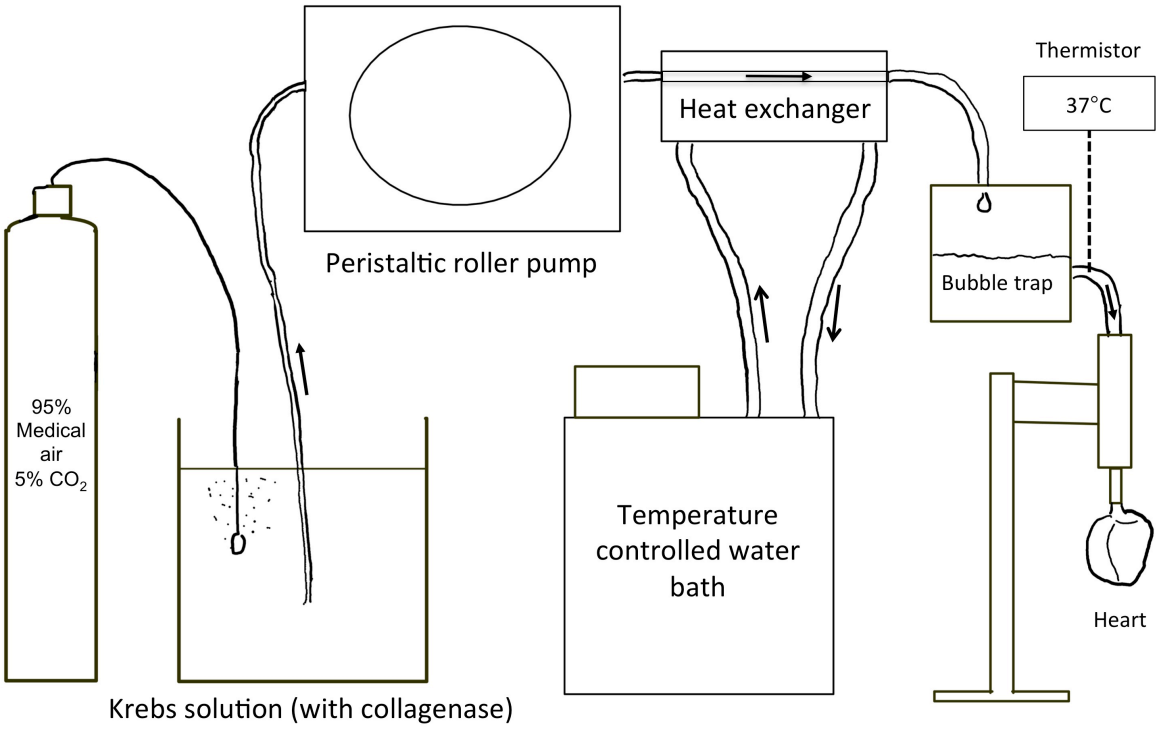
7.8 β-receptor measurements (paper I)

Rats were sacrificed by an intraperitoneal injection of pentobarbital sodium (220 mg/kg) and fentanyl (50 μg/kg). Hearts were rapidly excised and put in ice-cooled, oxygenated Krebs

solution before being connected to a Langendorff perfusion system. Initially a perfusion with Krebs solution was carried out over 5 min, before perfusing at 7 ml/min for 20 min with Krebs containing 0.6 mg/ml collagenase and 1 mg/ml albumin (collagenase solution) at 37°C. Hearts were then removed from the Langendorff system and sliced, before being put in Krebs solution containing 10 mg/ml albumin and 0.6 mg/ml calcium (calcium solution). While in the different solutions, heart preparations were always gassed with a 95% medical air-5% CO_2 gas mixture. Slices were held in calcium solution at 37°C on a shaking plate for 10 min, before they were removed and minced in collagenase solution. They were kept at 37°C for 10 min on the shaking plate, before being centrifuged prior to replacing collagenase solution gradually with calcium solution. Sedimentation of cells and replacement of calcium solution was finally carried out several times to discharge dead cells. In the final cell suspension, rod-shaped cells were counted as fraction of total cell number. This cell suspension was divided in 1 hypothermic group (15°C) and 1 normothermic group (37°C). β-receptors were equilibrated with 1nM of the hydrophilic radioactive marker [^{3}H]-CGP12177 (CGP) (Perkin Elmer, MA, US), binding β-receptors on the external surface of cell membranes. Addition of the lipophilic, non-selective β-receptor blocker propranolol (Actavis, Ireland) in increasing doses from 10^{-8}M to 10^{-5}M was carried out in both groups for displacement of CGP. As we aimed to examine binding properties of a β–receptor ligand in the in vitro experiment, not the cellular effects of β-receptor agonism or antagonism, propranolol was chosen according to earlier reports demonstrating its suitability when studying ligand-binding of β-receptors [141]. Incubation lasted ½ h, based on a pilot study not showing any differences with incubation over ½ - 2h at 37°C or 15°C.

After incubation, cell suspensions were washed through a 0.67 mm thick (pore size 2.7 μm) glass microfiber filter (Whatman, UK) in order to separate the cardiomyocytes from the buffer

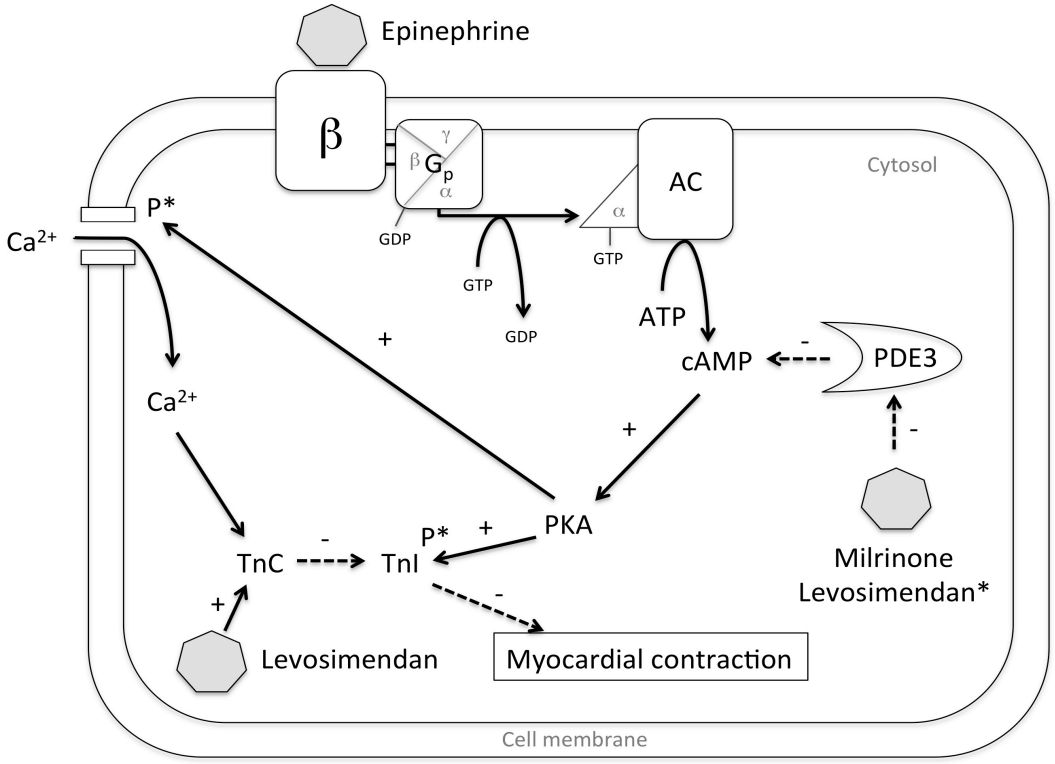
solution. Protein level in the cell suspension was determined using a Bradford protein assay (Bio-Rad Laboratories, CA, US). After washing, radioactivity in filters was measured using a liquid scintillation spectrometer (Model 1900 TR; Packard Instrument Company, IL, USA). Protein-corrected radioactivity was used to plot displacement of CGP with increasing concentrations of propranolol. The half maximal inhibitory concentration (IC50) was calculated according to Chou [142] and used as a measure of β-receptor sensitivity to propranolol.



Schematic drawing of the Langendorff perfusion system setup. Hearts were perfused with oxygenated Krebs solution and collagenase in order to isolate cells. The Langendorff setup allows retrograde perfusion of hearts against the closed aortic valves; this provides flow of oxygenated solution through the coronary arteries and distribution of collagenase throughout the heart.

7.9 Determination of cAMP levels (Paper I)

After finishing the temperature protocol, Epi groups were subjected to a 1.25μg/min Epi infusion. After 5 min, rats were euthanized and hearts were excised during ongoing infusion of Epi. Control animals underwent the same procedure without Epi infusion. The left ventricle was dissected free, freeze-clamped on liquid nitrogen and stored at -70°C. For measuring cardiac tissue cAMP levels in left ventricle samples, the tissue was pulverized and 50-100 mg was diluted in 1 ml, 0.1 M HCl and centrifuged. The supernatant was analyzed using a rat cAMP ELISA kit (Enzo Life Sciences, NY, US). Protein concentration in the supernatant was determined using a Bradford protein assay (Bio-Rad Laboratories, CA, US). Cardiac tissue cAMP levels were corrected according to protein level in each sample.



Overview over intracellular effects of epinephrine, milrinone and levosimendan. Levosimendan: High-dose levosimendan has both calcium sensitizing and phosphodiesterase 3 inhibiting effects. β: β-receptor, G_p: G-protein (with α, β and γ subunits), GTP:*

Guadenosine triphosphate, GDP: Guadenosine diphosphate, AC: Adenylate cyclase, ATP: Adenosine triphosphate, cAMP: Cyclic adenosine monophosphate, PDE3: Phosphodiesterase 3, PKA: Protein kinase A, P: Phosphorylation, Ca^{2+}: Calcium, TnC: Troponin C, TnI: Troponin I.*

7.10 Experimental protocols

All studies were performed in the animal research laboratory at UiT, the Arctic University of Norway, in Tromsø.

Paper I: 49 rats were assigned to in vitro β-receptor measurements or in vivo experiments measuring hemodynamic and cAMP response to Epi infusion at different temperatures. Animals in the hypothermic Epi group were core cooled to 15°C and maintained at this temperature for 4 h, before a 5 min infusion of 1.25 μg/min Epi administered through a catheter in the femoral vein at the end of experiments. This dose of Epi was selected according to other studies in the same model [17, 19]. Rewarmed Epi animals underwent the same protocol as the hypothermic Epi group but were rewarmed to 37°C prior to 5 min infusion of 1.25 μg/min Epi. In the normothermic Epi group animals were held at 37°C for 5 h, followed by 5 min, 1.25 μg/min Epi infusion. Control animals underwent the same surgical procedure and protocols as the Epi groups, without 5 min Epi infusion at the end of experiments.

Hemodynamic measurements: In the hypothermic groups, hemodynamic variables were measured at 37°C, 32°C, 28°C, 24°C and 20°C during cooling and rewarming. During stable hypothermia (15°C) the same variables were recorded every 60 min.

In vitro β-receptor assay: Animals selected for in vitro β-receptor measurements were sacrificed at the start of experiments. After isolation of cells, the cell suspension was incubated for ½ h at 37°C or 15°C before measurement of radioactivity.

cAMP measurements: cAMP values were measured in rewarmed, hypothermic or normothermic hearts, excised at the end of the experimental protocol.

Paper II: 20 rats were assigned into 3 groups. Animals in hypothermic groups were cooled to a core temperature of 15°C and maintained at this temperature for 3 h, before rewarming to 37°C. In the normothermic group, animals were held at 37°C for 5 h. Animals were given a 0.25 ml (25 μg) milrinone (0.1 ml 1 mg/ml Corotrop, Sanofi-Aventis, Paris, France, in 0.9 ml 0.9 % NaCl to give 0.1 mg/ml) bolus during 3 min after 2 h of hypothermia or 3 h of normothermia. This was followed by a continuous infusion of 1.2 ml/h (2 μg/min) given during the last hour of stable hypothermia (15°C) and during rewarming (hypothermic milrinone group) or during the last 2 h of normothermia (normothermic milrinone group). In the hypothermic control group, animals were given a 0.25 ml bolus dose of 0.9% NaCl after 2 h of hypothermia. This was followed by continuous infusion of 1.2 ml/h during the last hour of stable hypothermia (15°C) and during rewarming. Doses were chosen based on an earlier study from our group, testing milrinone during cooling [125].

Hemodynamic measurements: In the hypothermic groups, hemodynamic variables were measured at 37°C, 32°C, 28°C, 24°C and 20°C during cooling and rewarming. During stable hypothermia (15°C) the same variables were recorded every 60 min.

Paper III: 20 rats were assigned into 3 groups. Animals in hypothermic groups were cooled to a core temperature of 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. Levosimendan or

placebo-levosimendan was administered through an i.v. line in the left femoral vein, extended to the inferior caval vein. In high doses, levosimendan is reported to function as a PDE3 inhibitor as well as a calcium sensitizer [127, 128]. Based on the positive effects of milrinone [125], we therefore tested a high levosimendan dose, according to clinical studies [143]. Infusion was started after 3 h of normothermia or hypothermia. Initially, a 24 μg/kg dose of levosimendan (Simdax, Orion Corporation, Finland) or placebo-levosimendan was infused over a period of 10 minutes, followed by a continuous 0.6 μg/kg/min infusion during the last two hours of experiments. 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 4h, before rewarming to 37°C. In the normothermic group, animals were held at 37°C for 5h.

Hemodynamic measurements: In the hypothermic groups, hemodynamic variables were measured at 37°C, 32°C, 28°C, 24°C and 20°C during cooling and rewarming. During stable hypothermia (15°C) the same variables were recorded every 60 min.

cTnI phosphorylation: Phosphorylation of cTnI was measured in hearts excised from the hypothermic groups after rewarming.

Plasma cTnI-release: Release of plasma cTnI was measured in arterial blood drawn from the femoral artery at the end of experiments, after the last hemodynamic measurements and prior to euthanizing the rats.

7.11 Statistics

Results are presented as mean ± SEM. Rats in each experiment were allocated randomly using printed slips of paper that were put into a box and drawn after surgery to decide the experimental protocol. Changes from baseline in hemodynamic variables (study II and III) 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 hemodynamic variables (study II and III) and differences in cTnI phosphorylation (study III) between hypothermic groups at same temperatures were measured using a two-tailed, unpaired Student's t-test. Differences in cTnI release (study III) between groups were analyzed using one way repeated measures ANOVA on ranks. When significant differences were found, Dunn´s method was used to compare values between groups. Hemodynamic responses to 5 min Epi infusion within groups were measured using a paired t-test (study I). IC_{50} values for β-receptor binding of propranolol (study I) were compared using Mann-Whitney rank sum test, rather than an unpaired two-tailed t-test, as values were not normally distributed (Shapiro-Wilk test). Comparison of cAMP levels (study I) was analyzed by One-Way Analysis of Variance test followed by an all-pairwise multiple comparisons using Tukey's test. Differences were considered significant at $p < 0.05$.

8. Summary of results

8.1 Paper I

In paper I we wanted to elucidate the relationship between hemodynamic effects of Epi and β-receptor sensitivity in hypothermic rat hearts. For this purpose we did a combined in vivo and in vitro study of β-receptor function, testing the sensitivity of receptors to a displacer and increase of cAMP in presence of Epi. Both methods showed a significant increase in β-receptor sensitivity during hypothermia (15°C). The hemodynamic measurements during 5 min Epi infusion did however show negative inotropic effect at this temperature, with a significant decrease of SV and CO. We see this mainly as a consequence of vascular effects of Epi, recognized by increased TPR.

cAMP measurements

After 5 min infusion of 1,25 μg/min Epi, the protein corrected cAMP level in freeze-clamped LV heart tissue was increased in the normothermic and hypothermic groups, compared to untreated control rats undergoing the same protocol. The differences in cAMP levels were greatest in the hypothermic rats, while there was no statistical difference in cAMP levels between rewarmed rats receiving Epi and rewarmed control rats. cAMP levels were significantly higher in hypothermic Epi rats, compared to both normothermic and rewarmed Epi rats.

β-receptor sensitivity

Propranolol caused a concentration dependent displacement of CGP in both groups. Calculation of displacement values showed that the IC50 concentration of propranolol was 9

times higher in normothermic ($1.2 \bullet 10^{-7}$ M) than in hypothermic cells ($1.4 \bullet 10^{-8}$ M). Thus, in the normothermic cells a larger concentration of propranolol was needed to reduce the receptor specific binding of CGP. This shows a higher β-receptor sensitivity for ligand binding in hypothermic cells.

Hemodynamics

During 5min infusion of 1.25μg/min Epi, hemodynamic responses were recorded at 15°C in the hypothermic group and at 37°C in the rewarmed and normothermic groups. CO was reduced during Epi infusion in the hypothermic group, but increased in the rewarmed and normothermic groups. HR and MAP were increased in all groups. LV dP/dt_{max} was reduced in the hypothermic group and increased in both the normothermic and rewarmed groups, while dP/dt_{min} was reduced in all groups. TPR, LV end-systolic volume (LVESV) and LV end-diastolic pressure (LVEDP) were increased only in the hypothermic group. The minimum LV pressure (P_{min}) was increased in the hypothermic group and decreased in the rewarmed group. LV end-diastolic volume (LVEDV) and the isovolumic relaxation constant (tau) were unaltered during Epi infusion in all groups.

8.2 Paper II

In paper II we tested whether the PDE3 inhibitor milrinone could ameliorate cardiac dysfunction during rewarming from severe hypothermia. CO was significantly decreased in the saline group during rewarming, but returned to within pre-hypothermic levels in the group treated with milrinone, caused by positive effect on SV. In the saline group SV did not recover but HR recovered to within pre-hypothermic values in both groups. Although MAP was decreased in both groups after start of milrinone or saline infusion, TPR remained unchanged in the milrinone group and increased in the saline group. After rewarming, TPR

was increased in the saline group but not in the milrinone group, facilitated by a significant decrease in MAP seen in milrinone treated animals..

More detailed account on cardiovascular function

Normothermia: Compared to baseline values, CO, SV and LV dP/dt_{max} increased significantly throughout 120 min of milrinone infusion. Likewise, milrinone infusion decreased TPR significantly.

Stable hypothermia (20-15°C): Most indexes of hemodynamic function were significantly reduced from their prehypothermic values in both groups. Exceptions were LVEDV, which was increased and SV, which showed no difference from baseline. LVESV and TPR were only increased in the saline control group. When comparing the groups, MAP and LVEDP were significantly lower in milrinone animals than in rats receiving saline.

Rewarming: After rewarming saline controls, a statistically significant reduction in parameters of cardiac function had occurred. Posthypothermic values of CO, CI, SV, stroke work (SW), and $LVdP/dt_{max}$ were all reduced when compared to prehypothermic baseline values. Also LVSP and MAP were reduced after rewarming these animals. In contrast, TPR, LVESV and LVEDP were significantly elevated after rewarming. Different from saline controls, indexes of cardiac function: CO, CI, SV, SW and $LVdP/dt_{max}$ all returned to within prehypothermic baseline levels after rewarming the milrinone treated rats. Also LVESV and LVEDP returned to within prehypothermic levels, while LVEDV was significantly increased. In contrast, LVSP and MAP showed a significant drop when compared to baseline values. When comparing values after rewarming in the two hypothermic groups, CI was significantly higher in the milrinone group than in saline controls. In contrast, LVEDP, LVSP, TPR and MAP were significantly lower in rewarmed milrinone animals compared to in saline controls.

8.3 Paper III

In paper III we tested whether the combined calcium sensitizer and PDE3 inhibitor (in high doses, as used in the present study) levosimendan would ameliorate cardiac dysfunction during rewarming from severe hypothermia. During rewarming to 37°C, levosimendan treated animals regained pre-hypothermic contractility (PRSW) and CO, while HR was increased and contributed to the positive effect on CO together with regained levels of SV after rewarming of this group. In placebo treated animals, CO and PRSW were reduced and TPR increased.

Normothermia: Compared to baseline values, CO, HR, $LVdp/dt_{max}$, SV and SW increased significantly throughout 120 min of levosimendan infusion. In difference from the other measured parameters, TPR decreased during the two hours of levosimendan infusion.

Stable hypothermia: 1h after start of placebo or levosimendan infusions in the two groups, most indexes of hemodynamic function were significantly reduced from their prehypothermic values. Exceptions were LVEDP, LVEDV, LVESV and SV, which did not change from baseline. TPR was only increased in the placebo group.
TPR was significantly lower and HR higher in the levosimendan group compared to placebo group.

Rewarming: Typical of hypothermia-induced cardiac dysfunction CI, CO, SV, PRSW and SW showed a significant drop from baseline to post-hypothermic values in the placebo group. During rewarming TPR was maintained at a high level and showed a significant increase after rewarming compared to baseline. In the levosimendan group most variables returned to within pre-hypothermic baseline values. The minimum rate of LV pressure change ($LVdp/dt_{min}$) was

decreased after rewarming, while HR was increased. CO, CI, SV, HR, LVdp/dt_{max} and SW were significantly higher in the levosimendan group compared to placebo animals. TPR was significantly lower in the levosimendan group compared to placebo animals.

Phosphorylation of cTnI: After rewarming, Ser23/24 cTnI phosphorylation was significantly increased ($p < 0.05$) in the levosimendan group, when compared to placebo animals. The phosphorylation results are measured as a ratio between Ser23/34-cTnI phosphorylation and total cTnI protein.

cTnI release: cTnI in plasma samples taken at the end of experiments showed significantly elevated cTnI release in the hypothermic groups compared to the normothermic group, while no significant differences were found between the hypothermic placebo and levosimendan groups.

9. General Discussion

9.1 Effects of levosimendan and milrinone during rewarming from hypothermia

The present papers show development of hypothermia-induced cardiac dysfunction in rats given saline or placebo during rewarming from severe hypothermia. This condition has earlier been reported in several studies using the same rat model [17, 18, 75-77, 133]. Experimental treatment of such dysfunction has however proved challenging. Traditional β-adrenergic inotropes like adrenalin and isoprenaline does not have the same ability to exert positive inotropic function in cold hearts [17, 20] as during normothermic conditions. In paper II and III we show that by avoiding the β-receptor complex and target intracellular mechanisms for inotropic treatment, cardiac dysfunction can be ameliorated during rewarming. In milrinone and levosimendan experiments, our main finding was that drug treatment during rewarming restored CO to within pre-hypothermic levels. In milrinone animals this was achieved through restored SV, while no effect on HR was measured. In levosimendan treated animals, both SV and HR were increased compared to placebo-controls. The positive effect on CO in these animals was thus a combined effect of chronotropic and inotropic properties of levosimendan. These findings are different to the disability of drugs working through adrenergic β-receptors to mediate positive inotropic effects during hypothermia in pre-clinical studies [17-20]. Further, the infusion of milrinone (paper II) and levosimendan (paper III) throughout the whole rewarming period from severe hypothermia is different from the current guidelines on how to administer drugs during rewarming of hypothermic patients. Both the European and American guidelines state that drugs should not be used before temperatures above 30°C are reached [3, 5]. The positive hemodynamic outcome in treated rats (paper II and III) indicates that inotropic treatment during rewarming is beneficial, also prior to reaching 30°C. It is however evident that such treatment must be based on extensive knowledge about

hypothermia-induced pharmacology, as the potency of several inotropic drugs is altered by low core temperatures [17-20, 122]. Among the challenges when using traditional inotropes like Epi during hypothermia is increased afterload (paper I) [17-19]. Both milrinone and levosimendan are reported to be inodilators and thus have vasodilating as well as inotropic properties [144, 145]. During normothermic conditions both milrinone and levosimendan reduced TPR. This feature also applied to use of milrinone during hypothermic conditions as it proved to be a potent vasodilator. This probably contributed towards the positive outcome in treated rats, as TPR returned to within baseline values in contrast to in saline controls. This is in accordance with a study on patients with congestive heart failure, where the vasodilating properties of Milrinone contributed to the positive effect on LV pump function [146]. In the saline controls, LVESV was increased, accompanied by decreased SV and increased TPR. Milrinone treated rats had in contrast no increase in LVESV, but LVEDV was increased and contributed to recovery of SV to within baseline levels after rewarming. This is different from the acute congestion seen in paper I when administering Epi during hypothermia, resulting in decreased SV. Compared to milrinone, levosimendan did not have as pronounced vasodilating properties during rewarming, therefore it is likely that the combined inotropic effect of PDE3 inhibition and stabilizing of the cTnI-cTnC complex was sufficient to treat hypothermia-induced cardiac dysfunction alone in rats given levosimendan.

9.2 cTnI phosphorylation

Altered β-receptor function is only part of the complex physiological picture of severe hypothermia. Development of hypothermia-induced cardiac dysfunction, is more importantly related to calcium-overload and cTnI-phosphorylation on Ser23/24 sites [76-78]. Calcium overload is associated with myocardial contractile dysfunction [76, 77], while Ser 23/24 phosphorylation of cTnI is associated with negative inotropic effect [78]. Use of inotropic

drugs in the present studies during rewarming from severe hypothermia does not primarily target these known cellular mechanisms causing hypothermia-induced cardiac dysfunction. In fact both use of milrinone and high-dose levosimendan will increase calcium-influx through phosphorylation of L-type calcium channels and Ser23/24 phosphorylation of cTnI (paper III), through inhibition of cAMP breakdown. Based on that cTnI phosphorylation is induced by hypothermia in rats, it has been proposed that further pharmacological stimuli of the cAMP - PKA pathway will give a reduced inotropic effect during hypothermia [129]. In paper III, positive inotropic effect of levosimendan was seen in the presence of increased Ser23/24 cTnI phosphorylation compared to placebo animals, indirectly showing inhibited breakdown of cAMP through PDE3 inhibition. Different from our findings, the positive inotropic effects of levosimendan during rewarming have earlier been ascribed the ability to avoid stimulation of the cAMP - PKA pathway [129]. Our findings when using milrinone during rewarming in paper II and previously during cooling [125] does however show positive effects of stimulating the cAMP - PKA pathway during hypothermia through PDE3-inhibition. Levosimendan is also known to inhibit PDE3 in high doses as used in the present study [127, 128], where we found that levosimendan supported contractile function. Results from paper III therefore indicate that the potential negative inotropic effects of excessive cTnI phosphorylation after hypothermia and rewarming are less harmful than the positive inotropic effects of PDE3 inhibition. Although calcium overload and cTnI phosphorylation probably are parts of the pathophysiological picture initiating hypothermia-induced cardiac dysfunction, the positive effects of inhibited cAMP breakdown seems to ameliorate hypothermia-induced cardiac dysfunction as described in paper II and III. Paper I does however show that high increase of cAMP through β-receptor stimulation is not related with positive inotropic effects. Inhibition of breakdown of endogenous levels of cAMP during hypothermia (paper II, III) therefore seems to be a better strategy for providing inotropic

support than increase through β-receptor stimulation. In addition to PDE3 inhibition, the main pharmacologic action of high-dosage levosimendan is presumably calcium-sensitizing and thus stabilization of the cTnI-cTnC complex [126]. This effect was likely an important factor contributing to the positive inotropic effect of levosimendan in paper III.

9.3 Adrenergic receptor function in hypothermia

Interpreting results from studies conducted on adrenergic agonists during hypothermia is challenging because of different experimental conditions, but can give further indications about adrenergic receptor integrity in hypothermic cardiomyocytes. In this setting, there are big differences in studies conducted in vivo an in vitro. In vitro hearts lack autonomic control and are subjected to constant coronary perfusion rate, while single cells are subjected to experimental environments. The experimental findings of positive inotropic effect of isoprenaline in in vitro guinea pig hearts [114] is observed at same temperatures as Tveita and Sieck observed lacking inotropic effect of Epi [19]. Some authors even describe a β_1-receptor super sensitivity, when left atria and papillary muscle were stimulated by orciprenaline [147], observations consistent with our findings of increased cAMP in paper I. However, the apparent positive inotropic effects of Epi and isoprenaline in isolated guinea pig atria at 25°C [115] and rabbit hearts cooled as low as 22°C [116] is different from most in vivo studies. This might be due to experimental conditions and methods, as rabbit hearts were perfused at a constant flow rate and contractility was measured by oscilloscope instead of hemodynamic measurements. It could very well also be due to species dependent differences, but the decreased effect of isoprenaline in rabbit atria speaks against this [113]. Positive inotropic effects found in vitro, but not in vivo might also indicate that the vascular effects of Epi are important for providing the negative effects seen during hypothermic conditions in vivo (paper I). This does however not explain the lack of inotropic effect seen with use of

isoprenaline in vivo [20] and show that it is important to be careful when ascribing clinical value to results from in vitro studies on hypothermia-induced changes in pharmacology.

9.3.1 β-receptor

Despite differences in species and experimental conditions, the majority of studies on the β_1-receptor indicate that response to stimulation is depressed during hypothermia. In particular in vivo studies, which are most easily correlated to clinical settings show this relationship. Results from Epi studies might indicate that both β_1 and β_2-receptor function is altered by severe hypothermia. Positive inotropic effects of Epi disappears in rat at 28°C [19]. The critical temperature for β_2-receptors might be the same as or lower than for β_1, seen by possessed ability of isoprenaline to decrease MAP in patients at 28-32°C [110]. This is however different from cooled rats, where isoprenaline showed inability to decrease MAP at 33°C [20]. In these rats, some doses of isoprenaline even appeared to have a negative inotropic effect. This is hard to explain as a depressed β_1-receptor effect. However, in heart failure, down regulation of both β_1- and β_2-receptors is seen, while β_3-receptors lack this feature. β_3-receptor stimulation is associated with negative inotropic effect, which is normally masked by positive inotropic effect of β_1-receptor stimulation [88]. Thus, increased β_3-receptor stimulation could mediate negative inotropic effect of β-receptor stimulation in settings where β_1-receptors are down regulated. In paper I we did however not observe substantial down regulation of β_1-receptors, although Epi lacked inotropic effect during severe hypothermia (15°C). Rather than decreased β-receptor function, we found β-receptor sensitivity to be increased, both in isolated cardiomyocytes (IC50) and in vivo after administering Epi (increased cAMP). Substantial increase of TPR, LVEDP and P_{min} indicate

that α-receptor mediated increased vascular tone probably was an important contributor to the depressing effects of Epi on cardiac function. The reduced effects of isoprenaline in the same model [20] do however show decreased inotropic response to isolated β-receptor stimulation during hypothermia.

Mann et al. [148] found that high levels of cAMP in the cell can be cardiotoxic. This was related to a high increase in calcium-influx from the extracellular space through L-type calcium channels and a subsequent calcium overload. Lack of inotropic effect of the 4-fold increase in cAMP in the hypothermic Epi group (paper I) could therefore be related to a worsening of hypothermia-induced calcium overload [76, 77], mediated through PKA-phosphorylation of L-type calcium channels leading to increased calcium influx. Further, PKA-mediated phosphorylation of cTnI to un-physiological levels might be initiated by the substantial increase of cAMP. The altered effects of Epi observed at low core temperatures could therefore be due to a combined super-sensitivity of the β-receptor complex with a resultant increase of cAMP to unphysiological levels and intact or increased [149] vascular response, rather than a β-receptor dysfunction. An earlier study from our group supports this notion as low-dose Epi (0.125μg/min) demonstrated positive inotropic effects, in contrast to high-dose (1.25μg/min) Epi, during rewarming of rats from 15°C [17]. Conjoint with the present results, this indicates a reduced "therapeutic window" of Epi during hypothermia and rewarming, where elevated TPR and lack of positive inotropic effects dominate the hemodynamic response. Based on these results we believe that β-adrenergic stimulation is not the best option for inotropic treatment in hypothermic conditions.

9.3.2 α-receptor

α-Receptor function (in particular α_1) seems to be intact, due to increased systemic vascular resistance in response to Epi and norepinephrine in hypothermia. Hypothermic sensitivity of α-receptors was even found to be increased in human skin artery preparations [149]. This is consistent with our findings in paper I, where we found MAP to be increased to the same level in all groups. Relative to MAP values before start of Epi-infusion, however the increase of MAP in the hypothermic group was substantially higher (88 %) than in normothermic (26 %) and rewarmed (35 %) animals. This resulted in substantial increase of TPR during hypothermia, which had negative effects on cardiac function through congestion. This is shown by higher filling pressure (Pmin), which was not caused by a reduction of diastolic function (tau and dP/dt_{min}). Clinical use of this intact vasopressor effect can increase the chance for return of spontaneous circulation in hypothermic cardiac arrest, but does not appear to increase long-time survival [84, 103, 106].

9.4 Reported adverse effects of milrinone and levosimendan

Milrinone (paper II) and levosimendan (paper III) possess pharmacological properties giving them a great potential in treating hypothermia-induced cardiac dysfunction. Further use of these drugs for treating cardiac dysfunction also in human victims of accidental hypothermia has to be preceded by investigations in other species to confirm the same positive effects as in paper II and III. It is also important to investigate whether adverse effects like ventricular arrhythmias are associated with these drugs during rewarming. Concerns about the use of milrinone have already been raised in patients with chronic heart failure, where long term use have showed increased mortality [150]. Use of milrinone during or after surgery is also related to adverse cardiovascular effects. In children subjected to inotropic treatment with

milrinone after surgery for congenital heart disease, tachyarrhythmias were more common [151]. Use of milrinone in adult cardiac surgery is also associated with an unfavorable outcome, as it increases mortality in these patients compared to other inotropic drugs [152] and is an independent risk for atrial fibrillation [153]. Our model of hypothermia-induced cardiac dysfunction is a model of acute heart failure rather than chronic heart failure and some of the reported concerns related to use of milrinone might therefore not be relevant in this setting. In paper II we did not observe any indication of arrhythmias occurring during milrinone infusion. Further, a study using milrinone in rabbit hearts indicated anti-arrhythmic properties of this drug during moderate hypothermia [154]. Different from increased mortality related to use of milrinone after surgery or in chronic heart failure, use of milrinone in patients suffering from acute exacerbations of heart failure seems to be related with less complications [155]. Arrhythmias are however a problem related to milrinone used for inotropic support in acute heart failure, but this seems to apply only when the heart failure is of ischemic origin. In non-ischemic heart failure Milrinone treatment has been proven beneficial [155]. An important feature favoring milrinone in hypothermic conditions is thus that hypothermia-induced cardiac dysfunction is not related to ischemia [15]. In a study comparing milrinone and levosimendan in order to prevent low CO syndrome in infants after open-heart surgery, neither drug was found to give adverse effects and levosimendan had superior inotropic properties [156]. Levosimendan is a more novel drug than milrinone but based on published studies, it seems to have less adverse effects. In short term therapy of both ischemic and non-ischemic heart failure levosimendan had no effect on the incidence of arrhythmias [157]. Further, in a review of levosimendan used in a variety of critically ill patients mainly suffering from cardiac conditions and / or undergoing cardiac surgery, levosimendan was associated with reduced mortality [143]. Reduced mortality was also seen when comparing the effects of levosimendan with dobutamine in severe low-output heart

failure [158]. There has however been concerns about the risk for tachyarrhythmias in high-dose infusions of levosimendan [159]. In paper III we used a high dose infusion throughout rewarming. A possible explanation for the concerns related to high concentrations is the dose-dependent PDE3 inhibiting effect observed in this setting [127, 128]. It is therefore possible that levosimendan may have the same adverse effects as the PDE3 inhibitor milrinone in high doses. Many of the concerns related to milrinone use are however due to the increase in oxygen consumption. Hypothermia-induced heart failure differs from ischemic heart failure in that oxygen supply is higher than demand [75]. The positive effects of both milrinone and high-dose levosimendan in paper II and III might therefore be attributed both to the intracellular mechanisms of action and that sufficient oxygen supply prevents adverse effects of PDE3 inhibition.

9.5 Novel inotropic drugs

Based on the findings in the present thesis, cardiovascular support that avoids the β-receptor complex seems highly favorable over β-agonists in hypothermia (Paper I, II, III). Use of new inotropic drugs might help targeting pathophysiologic events in development of hypothermia-induced cardiac dysfunction directly or target mechanisms that will avoid enhancement of the pathophysiologic events like calcium overload or cTnI phosphorylation.

9.5.1 Cardiac myosin activators

Myosin activators have the advantage that they directly target reduced contractility, which is the main problem of systolic heart failure. In hypothermia this could be an advantage as it avoids increasing calcium and cTnI phosphorylation. Currently, one such drug has been developed and named omecamtiv mecarbil. This drug accelerates the rate-limiting step in

contraction, which is the transition from the weakly bound actin-myosin-ADP-Pi complex to the strongly bound actin-myosin-ADP complex [160]. A preclinical trial on dogs showed a 22 % increase in CO with no increase in oxygen consumption when using omecamtiv mecarbil, positive effects are also found in rats. The increased CO is however an effect of prolonged systolic ejection time, which could compromise coronary perfusion and diastolic filling of the left ventricle [160]. During hypothermia, oxygen supply is found to be sufficient [75] and it is shown that systolic, but not diastolic function is depressed after rewarming. Myosin activators might therefore have a potential in treatment of hypothermia-induced cardiac dysfunction during rewarming of hypothermic patients.

9.5.2 SERCA activators

Sarcoplasmic Ca^{2+} uptake pump (SERCA) activation increases calcium uptake to the sarcoplasmic reticulum (SR), which improves LV diastolic function and also systolic function through increasing the amount of calcium made available for contraction. Istaroxime is a novel drug with this ability that also has an inhibitory effect on sarcolemmal Na^+/K^+ ATPase, with resulting increase in intracellular calcium [161]. The hemodynamic properties of istaroxime have been investigated in heart-failure patients and it was found that it increased CI [162]. In hypothermic animals, calcium overload have however been found to be a probable event in development of hypothermia-induced cardiac dysfunction [77]. The sarcolemmal Na^+/K^+ ATPase inhibiting effect of istaroxime might therefore be unfavorable in hypothermia. Other molecules selectively targeting SERCA2a are under development and might be more promising [161].

9.5.3 Ryanodine receptor stabilizer

Calcium leak trough RyR is assosciated with altered calcium handling and decreased contractility in human heart failure [163]. The pathophysiological mechanism behind this is decreased amount of SR calcium available for contraction. Such calcium leak is also increasing the risk for ventricular arrhythmias [163]. If RyR calcium leak is a problem also in hypothermia-induced cardiac dysfunction, stabilization could be beneficial not only for inotropic support, but also as an anti-arrhythmic intervention. JTV519 is one of the developed compounds that target RyR. In addition to stabilization it also has an inhibitory effect on L-type calcium channels [163], which might be beneficial in calcium overloaded hypothermic hearts.

10. Final conclusions

The main objectives of the current thesis were to find improved treatment strategies for rewarming victims of accidental hypothermia and investigating hypothermia-induced effects on the β-receptor, – PKA, – cAMP system. We therefore studied the effect of milrinone and levosimendan during rewarming from severe hypothermia in order to ameliorate hypothermia-induced cardiac dysfunction. Further, we investigated the expression of β-receptors in normothermic and hypothermic cardiomyocytes and the increase in cAMP in response to Epi. Our main conclusions based on the results from our experiments are:

- Infusion of Epi during hypothermia leads to a reduction of SV and CO, while TPR is substantially increased, indicating a potent vascular effect of Epi at 15°C.

- Severe hypothermia (15°C) increases β-receptor sensitivity 9-fold in isolated cardiomyocytes and gives a 4-fold increase of cAMP in rats given Epi at the same temperature. We therefore believe that the negative inotropic effect of Epi at low core temperatures is related to vascular effects, rather than β-receptor dysfunction.

- Increased phosphorylation of cTnI, which in earlier studies has been related to hypothermia-induced cardiac dysfunction, did not prevent the positive outcome of levosimendan treatment during rewarming.

- Both milrinone and levosimendan are able to ameliorate cardiac dysfunction during rewarming from severe hypothermia in vivo. This is evident by that both drugs are able to prevent reduction in CO and SV after rewarming, when compared to pre-

hypothermic values. In milrinone treated animals this positive effect is seen in concert with reduced MAP, while in levosimendan treated animals positive inotropic effect was seen without simultaneous reduction of MAP. We therefore conclude that cytosolic strategies for treatment of hypothermia-induced cardiac dysfunction are superior to inotropic treatment through β-receptor stimulation.

11. References

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