The role of endogenous H2S production during hibernation and forced hypothermia
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Introduction
Hypothermia and its clinical applications

Hypothermia is a life-threatening condition in which core body temperature (Tb) drops below the desired temperature range maintained by thermoregulation. As a proper body temperature is required for normal metabolism and homeostasis, prolonged and severe hypothermia may lead to death (Fritsch, 1995; Jurkovich, 2007; Yoon et al., 2014). Hypothermia may be induced intentionally as a therapeutic approach to specific clinical situations or occur accidentally. Depending on its severity, hypothermia can be classified as mild (32-35 °C), moderate (28-32 °C), severe (20-28 °C) and deep (< 20 °C) (Darocha et al., 2014; Ding et al., 2014; Jarosz et al., 2014; Turtiainen et al., 2014). Intentional hypothermia (also called therapeutic hypothermia) is induced as a therapeutic intervention aimed at preserving cells or organs and maximizing their function. It is very often encountered in transplantation, during which the donor organ is preserved in a hypothermic solution during transportation prior to transplantation. Therapeutic hypothermia is also applied in major surgery, post myocardial infarction, and the intensive care unit. Several human and animal studies reported beneficial effects of therapeutic hypothermia by reducing complications and conferring neuroprotection in patients with traumatic brain injury, ischemic stroke and neonates with hypoxic-ischemic encephalopathy, and in patients with myocardial infarction, hence improving human survival (Marion et al., 1997; Margarit et al., 2000; Kozaki et al., 2002; Pokorny et al., 2000; van der Worp et al., 2007; Polderman, 2008; van der Worp et al., 2010; Faridar et al., 2011; Granja et al., 2011; Polderman and Andrews, 2011; Jomaa et al., 2013).

On the other hand, accidental hypothermia is unintentional drop in Tb below 35 °C as a result of unanticipated cold exposure (Yoshitomi et al., 1998; Udayaraj et al., 2004; Turtiainen et al., 2014; Yoon et al., 2014). It can be subclassified as acute (also called immersion hypothermia) as a result of sudden exposure to cold such as immersion in cold water or a person caught in a winter storm. There is also exhaustion accidental hypothermia in which the cold exposure is associated with lack of food and exhaustion such that the body’s thermoregulatory system is impaired and unable to generate heat. Chronic hypothermia is another type of accidental hypothermia mainly affecting the elderly, and it is often encountered over days or weeks (Guly, 2011). Since ancient times, death from accidental hypothermia has been recognised but the clinical syndrome of hypothermia was only recognized in the mid-20th century and only in extreme conditions such as in acute accidental hypothermia. In the United Kingdom, for example, hypothermia in less extreme conditions was only recognised in the 1960s (Guly, 2011).

1.1. Cellular mechanisms of hypothermic injury

While hypothermia extends organ life, there is mounting evidence that it also induces cellular injury. Strom and Suthanthiran (1996) reported that hypothermic organ preservation inhibits
hypothalamic enzymes, lowers pH and disrupts osmolarity, leading to phase transition of lipids and changes in membrane stability. Other authors also reported that exposure of organs ex vivo to hypothermic storage leads to marked disturbances in cellular ion homeostasis due to increase in membrane fluidity and permeability, and alters the function of membrane-bound enzymes (Hochachka, 1986; Belzer and Southard, 1988; Zachariassen, 1991). Hypothermia impairs proper functioning of ionic cell membrane pumps such as the Na+/K+-ATPase due to lack of ATP or excessive production of H+; which can contribute to disruption of cellular ion homeostasis (figure 1) (Priebe et al., 1996; Rolfe and Brown, 1997). This leads to an influx of Na+ and Cl− followed by an osmotic shift of water into the cell and efflux of K+, leading to cell swelling (Hochchka, 1986; Belzer and Southard, 1988; Marsh et al., 1989; Blankensteijn and Terpstra, 1991; Clavien et al., 1992; figure 1). Progressive cellular swelling is associated with necrotic cell death (Stahl andGattone, 1985; Oberbauer et al., 1999; Blankensteijn et al., 2003; Jani et al., 2011). In addition, cellular swelling leads to interstitial expansion and edema, capillary compression and poor tissue perfusion (Belzer and Southard, 1988). If the hypothermia-induced Na+ accumulation continues unabated, this increase will eventually result in membrane depolarization and rapid influx of Ca2+ (Hochachka, 1986). The increase in cytosolic Ca2+ is deleterious and might lead to cellular apoptosis (White and Somero, 1982; Hochachka, 1986).

Recent studies indicate that hypothermic storage of organs is characterized by impairment of mitochondrial function also results in depletion of adenosine triphosphate (ATP) and adenosine diphosphate (ADP), the major sources of cellular metabolic energy, due to reduction in the activity of mitochondrial enzymes (Boutilier, 2001; Salahudeen, 2004). This leads to a mismatch between ATP supply and demand. As levels of ATP decrease, this leads to failure of ATPases, followed by membrane depolarization and an uncontrolled influx of Ca2+ through voltage-gated Ca2+ channels. The rise in free cytosolic intracellular Ca2+ concentration is a major feature of cellular injury, and results in the activation of Ca2+-dependent phospholipases and proteases that further hasten the rate of membrane depolarization, leading to uncontrolled cellular swelling (figure 1) and, ultimately, to cell necrosis (Hochachka, 1986; Rolfe and Brand, 1996; Priebe et al., 1996; Rolfe and Brown, 1997; Plesnila et al., 2000). The Ca2+ overload leads to opening of mitochondrial permeability transition pore (MPTP), resulting in an increase in intracellular Ca2+, which increases further and more abruptly during rewarming (Piper et al., 1993; Salahudeen et al., 2003; Green and Kroemer, 2004).

A key feature of the hypothermia-induced mitochondrial swelling is the trigger of a number of pro-apoptotic events such as translocation of cytochrome c and increase in Bax-Bcl-2 ratio as well as increase in caspase-3 activity (Salahudeen et al., 2003; Green and Kroemer, 2004). Lactic acid generation during anaerobic metabolism due to hypothermia leads to
(intracellular) acidosis, which can result in lysosomal instability and might further impair mitochondrial function (Belzer and Southard, 1988). In summary, hypothermia induced impairment of ionic cell membrane pumps is also associated with mitochondrial dysfunction and activation of the mitochondrial apoptotic machinery.

1.2. Effects of hypothermia on organs

Hypothermia primarily affects all organs and organ systems. Recent studies indicate that hypothermia compromises the endothelial barrier function leading to further endothelial dysfunction (Jomaa et al., 2013). Hypothermia-induced damage negatively affects the vascular and barrier function of the endothelium by activating endothelial cells leading to expression of adhesion molecules which in turn enhances influx of leukocytes and thus, inflammation and tissue destruction (Kukan and Haddad, 2001; Scheinichen et al., 2003). In the transplantation setting, for example, this may lead to a decline in organ function and predispose the organ to functional impairment after transplantation, thereby jeopardizing a successful transplant. Transplantation of an injured allograft prior to implantation, may lead to both acute and chronic graft failure (Broman and Kallskog, 1995; Kukan and Haddad, 2001; Scheinichen et al., 2003). Thus, limiting organ injury by hypothermia during transplantation not only improves short-term graft survival, but also positively affects chronic rejection. Therefore, optimization of organ preservation might reduce the induction of graft injury during transplantation procedure and furthermore, might allow longer preservation times.

1.2.1. Kidney as a typical example of a vulnerable organ to hypothermia

In the renal system, hypothermic preservation of kidneys for transplantation, for example, is associated with an increase in preglomerular vasoconstriction leading to a decrease in renal blood flow (RBF) and reduced glomerular filtration rate (GFR). Renal function is eventually decreased during hypothermia due to the effect of the cold on the kidney metabolism itself. As the RBF decreases, renal vascular resistance (RVR) rises, leading to a further decrease in RBF and a subsequent decrease in GFR (Broman and Kallskog, 1995; Li et al., 2013). During hypothermia, renal oxygen consumption is more rapidly reduced relative to other organs such as the liver, heart, brain, skeletal muscle, and skin (Mori et al., 2011). At the ionic level, potassium moves into the cells as Tb drops and patients may experience hypokalemia (Polderman et al., 2001). Other imbalances that have been described during hypothermia include hypomagnesemia, hypocalcemia, and hypophosphatemia (Polderman et al., 2001; Arpino and Greer, 2008).

Studies have shown that reduction in Tb to 28°C is accompanied by decreases in RBF and GFR by 50% and an increase in renal vascular resistance in anesthetized rats (Broman and Kallskog, 1995; Li et al., 2013), and in other species including man (Boylan and Hong, 1966).
At these lowered temperatures, there is a corresponding reduction in the tubular secretion and reabsorption of ions and organic solutes along the nephron, as these are energy requiring processes and therefore temperature sensitive (Boylon and Hong, 1966). This, together with a decrease in tubular reabsorption of water, leads to hypothermia-induced natriuresis and diuresis. Thus, hypothermia leads to dehydration. The mechanisms for this cold-induced diuresis remain unclear. However, available data suggest that hypothermia-induced diuresis is an autoregulatory response of the kidney to a relative central hypervolemia induced by peripheral vasoconstriction (Chapman et al., 1975; Withey et al., 1976; Broman and Kallskog, 1995). Owing to a volume overload, the secretion and release of antidiuretic hormone (ADH) is suppressed (Broman et al., 1998). The subsequent hypothermia-induced diuresis decreases the blood volume so that a progressive hemoconcentration develops (Chapman et al., 1975). Hypothermia-induced diuresis is a major concern. For example, accidental hypothermia by cold water immersion has been shown to increase urinary output by 3.5-fold, and the subsequent decrease in body water may be a factor contributing to the ‘rewarming shock’ that occurs following active vasodilation induced by rewarming treatments (Cupples et al., 1980).

1.3. Reperfusion injury following hypothermia
Restoration of blood supply after hypothermic preservation or cold exposure of an organ or whole organism induces a cascade of events that further aggravates the hypothermia-induced injury. This key event is referred to as reperfusion injury (Perico et al., 2004; Tapuria et al., 2008; Zaouali et al., 2010). On reperfusion, oxygen becomes available and metabolism increases rapidly, resulting in a sudden production of reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals and hydrogen peroxide (Haugen and Nath, 1999). The cellular pathways to scavenge oxygen free-radicals are overwhelmed and cellular injury ensues. Thereby, restoration of blood supply results in oxygen supply in excess of the requirements, which (amongst others) leads to activation of macrophages in the vasculature and generation of ROS, which results in apoptotic cell death (Castaneda et al., 2003). Production of ROS leads to endothelial injury (Tapuria et al., 2008). Inflammatory cytokines and chemokines released during ROS generation, together with increased expression of adhesion molecules on the cell surface, attract neutrophils and monocytes, which in turn, release additional ROS and further potentiate the damage (Haugen and Nath, 1999; DeVries et al., 2003). Thus, reperfusion after hypothermic exposure of an organ or organism is associated with increased injury.

1.4. How nature deals with hypothermia and reperfusion: lessons from mammalian hibernators
During the hibernation season, often associated with winter, when food is scarce, hibernating
mammals cycle through phases of suppressed metabolism with low body temperature (torpor) that are interrupted intermittently by short periods of complete restoration of metabolism and body temperature (arousal) (Dausmann et al., 2004; Andrews, 2007; Bouma et al., 2010; Bouma et al., 2011; Talaei et al., 2011; figure 1). Torpor may occur daily with a reduction of body temperature to ~18-30°C for several hours (Geiser, 2004), or may be deep, during which body temperature of a typical hibernator might be reduced to temperatures as low as -2.9°C, lasting for several days to a month (depending on the ambient temperature and the species) (Andjus et al., 1964; Barnes, 1989; Kenagy et al., 1989; Hut et al., 2002; Heldmaier, 2004). Interestingly, available data from hibernating animals demonstrate that this repetitive cycles and the stress of torpor-arousal with a significantly reduced cardiac output and ventilation during torpor does not seem to cause significant organ damage in hibernators, as demonstrated by Zancanaro et al. (1999) and Talaei et al. (2012). Meanwhile, cooling and rewarming of organs of non-hibernating animals including humans generally results in substantial damage. Therefore, hibernation could represent a unique natural model of hypothermic organ preservation and rewarming in specific clinical situations such as in transplantation.

![Figure 1. Model of the hypothermic response in the cells of non-hibernating (A) and hibernating animals (B).](image)

(A) Hypothermic preservation impairs the Na+/K+-ATPase and also interrupts the normal balance between K⁺ efflux and the equivalent Na⁺ influx to the cell. This results in a net accumulation of Na⁺ and subsequent influx of Cl⁻ which creates a local increase intracellular osmolarity driving an inflow of water. Cellular and subcellular swelling ensues, followed by cellular edema. If left unabated, the accumulated intracellular [Na⁺] will eventually lead to membrane depolarization and subsequent opening of voltage-dependent Ca²⁺ channels and a rapid influx of Ca²⁺, which is deleterious to the cell. (B) Hibernating animals maintain cellular ion homeostasis in the cell during torpor. Therefore, cellular and subcellular swelling is absent.
1.5.0. Mechanisms of resistance against cooling-associated damage in hibernators

Whereas the Na⁺/K⁺-ATPase is impaired during hypothermic preservation of organs from non-hibernating animals including humans, Willis et al. (1969) observed that the activity of the Na⁺/K⁺-ATPase is fully preserved during \textit{ex vivo} cooling of organs taken from hibernating hamsters. In the kidney, for example, the number of Na⁺/K⁺-ATPase subunits and its hydrolytic activity remained unaffected in most segments of the nephron during deep torpor in the greater Egyptian jerboa (Bennis et al., 1995). Kamm et al. (1979) also reported that incubation of vascular smooth muscle cells from rats for 48 h at 7°C leads to a reduction in intracellular [K⁺] and a significant increase in cytosolic [Na⁺], thus suggesting dysfunction of Na⁺/K⁺-ATPase. In contrast, smooth muscle cells from hibernating ground squirrels were able to maintain their ionic integrity when incubated under the same conditions (Belzer and Southard, 1998). Wang et al. (1997) also observed increase in Ca²⁺ in cardiomyocytes of rats as temperature was lowered from 25°C to 7–12 °C. However, they observed a depressed Ca²⁺ influx in cardiac cells of the hibernating ground squirrel under the same condition, which evidently helped to prevent hypothermic Ca²⁺ overload and subsequently prevented cardiac injury. Thus, maintenance of the activity of Na⁺/K⁺-ATPase during torpor and arousal accounts for the reduced cell swelling and hence better cell survival in hibernating animals (Kamm et al., 1979; Wang et al., 1997).
1.5.1. Mitochondria in hibernating animals
Activation of specific anti-apoptotic pathways might allow hibernators to preclude cell death despite the cellular stresses of torpor and arousal that are known to activate pro-apoptotic proteins in non-hibernating animals (e.g. nutrient deprivation, low body temperature, ischemia/reperfusion, oxidative damage etc.). Jani et al. (2011) observed no difference in caspase-3 activity in the kidneys of hibernating and summer active thirteen-lined ground squirrels. The number of mitochondria in torpid dormice remained unchanged as compared to summer active animals (Soria-Milla and Coca-Garcia, 1986). Whereas the synthesis and activity of mitochondrial cytochrome c oxidase subunit 1 (COX1) and other genes are reported to be markedly decreased in warm ischemia/perfusion injury (IRI) of rat brain as well as in a cardiac model of IRI (Lesnefsky et al., 2004; Recay et al., 2009), protein expressions of COX1 and mitochondrial ATP synthase subunits 6 and 8 are significantly upregulated in the kidneys of thirteen-lined ground squirrels during torpor and return to euthermic levels upon arousal from torpor (Hittel and Storey, 2002; Smeek et al., 2011). The specific dynamics in COX1 and mitochondrial ATP synthase subunits in hibernators may thus confer mitochondrial tolerance and protection during hypothermia and reperfusion. Also, Carey et al. (2005) reported a larger increase in anti-apoptotic Bcl-xL expression (12-fold) relative to that of pro-apoptotic Bax (2-fold) in intestinal mucosa of 13-lined ground squirrels. This suggests that pro-apoptotic influences during hibernation are counteracted by increased expression of anti-apoptotic proteins, resulting in suppression, rather than activation of apoptotic pathway. Hence, it is seems likely that hibernating animals employ diverse mitochondrial anti-apoptotic mechanisms to counteract the pro-apoptotic machinery observed during hypothermic storage and reperfusion in non-hibernating animals.

1.5.2. Oxidative stress and antioxidant defense system during hibernation
Despite the repetitive cycling between torpor and arousal with significant changes in tissue oxygenation and oxygen consumption, hibernators do not show gross signs of organ damage. Likely, hibernators activate an effective antioxidant defense system to combat injurious effects of oxidative stress upon arousal. Several reports demonstrate increased activity of antioxidant enzymes, including glutathione peroxidase, superoxide dismutase (Buzadzic et al., 1990), peroxiredoxin 3/6 and glutathione reductase in organs from hibernating thirteen-lined ground squirrels (Jani et al., 2012). Other authors also reported increased levels of extracellular catalase in the blood of hamsters (Ohta et al. (2006)) and ascorbate in plasma from 13-lined ground squirrels during torpor (Drew et al., 2002). In addition, Jani et al. (2012) reported an increase in the cytoprotective enzyme heme oxygenase-1 in the kidneys of torpid 13-lined ground squirrels. Taken together, the increase in the levels of antioxidant enzymes during hibernation suggests that hibernators employ substantial antioxidant defenses to protect cells and tissues against the damaging effects of oxidative stress under conditions of
hypothermia, hypoperfusion and reperfusion.

1.6.0. Pharmacological considerations of hibernation
Several pharmacological compounds can induce an artificial and reversible torpor-like state in non-hibernating animals, a condition generally referred to as suspended animation. Pharmacologically reducing the demand for oxygen is a promising strategy to minimize unavoidable hypoxia-induced injury such as ischemia-reperfusion injury during transplantation and may hold an enormous promise in diverse fields, including transplantation and major surgeries. However, the induction of a suspended animation-like state by drugs is still a considerable challenge and is associated with a dramatically increased rate of cardiac arrest in non-hibernators (Zhang et al., 2006). It has been observed that during hibernation, erythrocyte and organs have significantly lower ATP levels (Doherty et al., 1993; English and Storey, 2000). In particular, the ATP level of erythrocytes from hibernating thirteen-lined ground squirrels is ~50% of the level observed in a euthermic state (Doherty et al., 1993).

Such a large decrease in ATP level in the erythrocyte would significantly compromise its function in regulating oxygen/carbon dioxide molecules necessary for maintaining high metabolic activity of the major organs, and therefore result in metabolic suppression of these organs. This implies that the metabolic pathways (e.g. anaerobic glycolysis, oxidative phosphorylation, fatty acid oxidation, etc.) that produce ATP are inhibited during hibernation. Under such conditions, it is highly possible that the metabolic rate of organs will slow down, and heat loss from the body to the environment will not be adequately controlled, hence hypothermia ensues. This reasoning is consistent with the hypothesis that decreased ATP production or utilization is involved in hibernating behavior (English and Storey, 2000; Heldmaier et al., 2004).

1.6.1. Hydrogen sulfide (H$_2$S) induces a reversible hibernation-like state
For several centuries, hydrogen sulfide (H$_2$S) was known for its toxicity with a characteristic rotten-egg smell. It is the second cause of gas-related mortalities in industrial accidents after carbon monoxide. However, H$_2$S can also be made at home by mixing bath additive and toilet detergent. In Japan, for example, Morii et al. (2008) reported 220 cases of home-made H$_2$S poisoning within three months with 208 deaths. Contrary to the widely known toxicity of H$_2$S, Kimura et al. (1996) discovered its biological importance as a signaling molecule. Since then, several researchers have reported the biological usefulness and therapeutic properties of H$_2$S. H$_2$S is a colorless, flammable and water-soluble gas. It is a specific and reversible inhibitor of cytochrome c oxidase, a key enzyme in the mitochondrial respiratory complex IV (Beauchamp et al., 1984). Inhibitors of the mitochondrial respiratory chain are toxic to mammals because they disrupt ATP production by oxidative phosphorylation. Inhibition of oxidative phosphorylation reversibly induces a state of profound hypometabolism (Padilla
and Roth, 2001; Nystul and Roth, 2004). H$_2$S is produced primarily by two cytosolic enzymes (cystathionine-β-synthase and cystathionine-γ-lyase), one mitochondrial enzyme (3-mecaptopyruvate sulfurtransferase) and as disclosed more recently by a peroxisomal enzyme (D-amino acid oxidase). At a low dose of 80 ppm, inhaled H$_2$S induced a hibernation-like state in mice for 6 h characterized by hypometabolism and consequent hypothermia at 15°C (Blackstone et al., 2005). No behavioral or functional differences were observed after recovery following the 6 h of exposure (Blackstone et al., 2005). Also, Beauchamp (1984) reported no long-term health effects of H$_2$S at the 80 ppm concentration. Johansson (1996) proposed that a Tb of 20°C in non-hibernating animals will lead to cardiac fibrillation. Moreover, the lowest Tb recorded in mice during fasting-induced torpor was about 23°C even when the ambient temperature was maintained at 5°C (Bouma et al., 2011). Thus, the ability to reversibly lower Tb to 15°C in a non-hibernating animal by administration of H$_2$S without major side-effects is a major step forward. It suggests that non-hibernating animals are fully capable of withstanding extreme hypometabolism and hypothermia in the presence of protective pharmacological agents.

1.6.2. 5′-Adenosine monophosphate (5′-AMP) as an inducer of a hibernation-like state

Another pharmacological compound that has been found recently to induce a torpor-like state in non-hibernating mammals is 5′-Adenosine monophosphate (5′-AMP), a metabolite of ATP hydrolysis. Injection of 5′-AMP induces profound and transient hypometabolism and hypothermia in rodents (Zhang et al., 2006; Zhang et al., 2009; Bouma et al., 2013; de Vrij et al., 2014). Also, high plasma levels of 5′-AMP were detected in fasting-induced torpid mice. All these studies suggest that 5′-AMP may be used to manage metabolic rate and Tb in humans during major surgery and trauma. The mechanisms for this 5′-AMP-induced hypometabolism and consequent hypothermia remain controversial. One hypothesis suggests that 5′-AMP induces a torpor-like state by activating adenosine A1 receptors (A1Rs) on temperatesensitive hypothalamic neurons, thereby suppressing the thermoregulatory responses that maintain Tb (Muzzi et al., 2012). Others hypothesized that uptake of 5′-AMP activates AMP-activated protein kinase (AMPK), a central enzyme involved in cellular energy homeostasis of which the downstream signaling pathways lead to decreased metabolism (Lindsley and Rutter, 2004; Lee, 2008; Melvin and Andrews, 2009). Thus, the induction of hypometabolism and subsequently hypothermia.

Taken together, induction of a hibernation-like state by pharmacological agents could be harnessed to clinical cases such as IRI encountered in transplantation and other clinical conditions. Thus, a full understanding of the mechanisms behind hibernation might lead to the development of pharmacological strategies to protect organs during several clinical cases including hypothermic storage for transplantation.
AIMS OF THE THESIS

The aim of this thesis is to identify the underlying protective mechanisms in mammalian hibernators against hypothermia-rewarming injury and to explore whether such mechanisms may benefit subjects during forced cooling and rewarming. To this end, we studied the effects of hypothermia-rewarming on neutrophil dynamics and the kidneys of hibernating and non-hibernating animals with or without administration of pharmacological compounds. In chapter 2, we investigated the mechanism by which neutrophilic granulocytes disappear from the circulation during hibernation in Syrian hamsters and forced hypothermia in rats. Thereafter, we demonstrated in chapter 3 the role of \( \text{H}_2\text{S} \) in the induction of natural hibernation and the protection against kidney injury. We described in detail activation of the \( \text{H}_2\text{S} \) system as a major underlying mechanism of renal preservation and protection during hibernation. Next, we explored pharmacological induction of a torpor-like state in natural hibernators using 5'-AMP and investigated the influence of 5'-AMP on the renal \( \text{H}_2\text{S} \) system (chapter 4). Chapter 5 focused on the beneficial effect of dopamine in protecting kidney from injury in an \textit{in vivo} rat model of deep hypothermia and rewarming, particularly addressing its effects on the renal \( \text{H}_2\text{S} \) system.
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