The role of endogenous H2S production during hibernation and forced hypothermia

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General Discussion & Future Perspectives

GENERAL DISCUSSION

Therapeutic hypothermia is widely used to reduce ischemic injury to cells, tissues and organs before transplantation, after cardiac arrest or a cerebrovascular accident and during major cardiac and brain surgery to minimize complications and improve survival of patients (Polderman, 2004). However, hypothermia is associated with a wide range of physiological changes that negatively affect cells, organs, organ systems and ultimately the whole organism. The negative effect of hypothermia is attributed mainly mediated to excessive production of reactive oxygen species (ROS) during rewarming, when blood and oxygen supply are restored to the hypothermic and hypoperfused tissue (Carden and Granger, 2000). Excessive ROS production leads to oxidative stress and subsequent cell injury and cell death (Carden and Granger, 2000). Interesting, hibernating animals undergo repetitive cycles of torpor (hypothermia) and arousal (rewarming) without signs of organ injury (Frerichs *et al.,* 1994; Kurtz *et al.,* 2006; Zancanaro *et al.,* 1999; Camici *et al.,* 2008). Bogren *et al.* (2014) also reported absence of systemic inflammation in hibernating arctic ground squirrel. Therefore, hibernators seem to be ideal organisms to study the natural resistance to hypothermia and rewarming as applied in specific clinical situations including organ transplantation, treatment of stroke and cardiac arrest. Recently, there are reports indicating a reduction in circulating leukocytes (leukopenia) during torpor (Bouma *et al.,* 2011).

To identify mechanisms that confer resistance to hypothermia, we studied (1) the mechanism underlying transient reduction in neutrophil number (neutropenia) during torpor and forced hypothermia in hibernating and non-hibernating animals, (2) the role of the H_2S pathway in natural hibernation/organ protection, (3) its role in pharmacologically induced hypothermia and (4) the effectiveness of interventions aimed at maintaining a patent H_2S pathway in *in vivo* hypothermia and rewarming in non-hibernating animals. Unraveling the molecular mechanisms underlying hibernation and organ protection may have major relevance for therapeutic hypothermia. Also, pharmacological metabolic suppression resembling natural hibernation may raise the possibility of safe procedures to allow for prolonged cardiac or neurosurgery, even under complete circulatory arrest.

In **chapter 1,** hypothermia and rewarming are introduced together with its therapeutic applications. Moreover, the negative effects of hypothermia and rewarming on cells and organs are discussed, using the kidney as a typical organ with high vulnerability to injury. This is followed by a comprehensive review on natural hibernation as a natural model for resistance to hypothermia/rewarming injury, which is characterized by repetitive cycle of torpor and arousal, and mechanisms of resistance against hypothermia/rewarming-induced damage including maintenance of the activity of Na^+/K^+-ATPase during torpor and arousal and activation of specific anti-apoptotic and antioxidant pathways. This section was followed

by a pharmacological consideration of hibernation and its potential applications in human medicine with specific reference to hydrogen sulfide (H_2S) and 5'-adenosine monophosphate (5'-AMP).

In **chapter 2**, we determined whether transient reduction in neutropenia during torpor (and hypothermia) in hibernating animals and forced hypothermia in non-hibernating animals is due to reversible margination or whether other mechanisms are involved, and how this might affect organ integrity. First, we demonstrate that neutrophils disappear from circulation during hypothermia, but are restored to normal levels following rewarming in both hibernating and non-hibernating animals. As the spleen is a major storage site of neutrophils, we surgically removed the spleen (splenectomy) to determine if neutropenia during torpidity is due to storage in the spleen and release into circulation following arousal. However, splenectomy, either prior to entering into hibernation or during torpor, had no effect on neutropenia as neutrophil numbers in splenectomized animals were similar to those in non-splenectomized animals, thereby ruling out the involvement of the spleen. We also reveal that the mechanism underlying this reversible neutropenia is unlikely to be due to apoptosis-and-replacement as the number of circulating immature neutrophils remained low during arousal, which rules out the involvement of bone marrow as the sole underlying mechanisms that governs neutropenia during hypothermia and torpor. Neutropenia during low body temperature seems to be due to the attachment of the neutrophils to the vessel wall (margination). By pretreatment with dexamethasone, an anti-inflammatory drug, we demonstrate the role of margination. Thus, indirect data suggest that margination is the mechanism underlying reversible clearance of circulating neutrophils during torpor and forced hypothermia. Potentially, this may be due to increased expression of adhesion molecules and integrins as margination is controlled by adhesion molecules on the neutrophils interacting with complementary receptors on the endothelium (Johnson *et al.,* 1995; Le Deist *et al.,* 1995; Hidax *et al.,* 1996; Kira *et al.,* 2005). However, direct evidence is lacking. Therefore, studies should be done to demonstrate the involvement of these adhesion molecules in margination. Since organ injury is aggravated by activation and recruitment of inflammatory mediators (Tapuria *et al.,* 2008), in which margination of neutrophils is an important early event, blocking margination of neutrophils (and other proinflammatory mediators) may reduce the degree of organ injury during clinical interventions requiring hypothermia and rewarming. Indeed, experimental blockade of margination reduced the extent of pulmonary injury following normothermic cardiopulmonary bypass in a sheep model (Friedman *et al.,* 1996). Interestingly, induction of hypothermia at 27.1°C in patients undergoing cardiopulmonary bypass surgery leads to the induction of a reversible leukopenia (Le Deist *et al.,* 1995), comparable with hibernating animals in torpor at 6.2°C (Bouma *et al.,* 2013) and hypothermic rats at 9.1°C (Shenaq *et al.,* 1986; Bouma *et al.,* 2013). Possibly, the increased resistance to ischemia/reperfusion injury and

hypothermic injury in hibernating animals (Lindell *et al.,* 2005; Kurtz *et al.,* 2006; Bogren *et al.,* 2014) prevents the occurrence of cellular injury and subsequent onset of inflammation, as might occur in specific clinical situations requiring hypothermia and rewarming. Thus, margination of neutrophils induced by hypothermia might be involved in the etiology of organ injury following hypothermia in humans and therefore, preventing margination might limit organ injury in specific clinical situations such as cardiopulmonary bypass.

To further unravel the mechanisms that underlie the induction of torpor and consequent organ protection during hibernation, we studied the H_2S system since exogenously administered H_2S inhibits leukocyte adherence to vascular endothelium (Fiorucci *et al.,* 2005), suppresses the expression of adhesion molecules on leukocytes (Fiorucci *et al.,* 2005; Zanardo *et al.,* 2006) and reduces LPS-induced accumulation of neutrophils in rat liver and lungs (Li *et al.,* 2007), leading to organ protection. Moreover, H_2S has been implicated in the induction of a hibernation-like state in non-hibernating rodents (Blackstone *et al.,* 2005; Blackstone and Roth, 2007; Bos *et al.,* 2009) without behavioural changes or organ injury after full recovery. Therefore, in **chapter 3,** we investigated the role of endogenous H_2S and its synthesizing enzymes (CBS, CSE and 3-MST) in regulating torpor-arousal cycle and organ protection during hibernation. We show that H_2S production seems to be of critical importance in the induction of torpor as blood levels of H_2S are substantially regulated during the hibernation cycle, with the highest values found during torpor. Moreover, the expression levels of all 3 H_2S-producing enzymes in kidney increased substantially during torpor phase. The involvement of H_2S in the induction of torpor is elucidated by administration of a non-specific inhibitor of H_2S production during torpor, which precludes a return into torpor. Next, we reveal that H_2S plays an essential role in protection of the kidneys throughout the torpor-arousal cycle, since pharmacological inhibition of the H_2S system during torpor did not only preclude re-entrance into torpor but also resulted in substantial renal injury characterized by neutrophil and macrophage infiltration as well as high levels of serum creatinine, KIM-1 protein and ROS formation. Moreover, winter euthermic hamsters show very low levels of blood H_2S and reduced expression of all renal H_2S-producing enzymes. In contrast to hibernating hamsters, winter euthermic hamsters displayed a substantial increase in serum creatinine, increased renal ROS production and, importantly, kidney damage, which was further aggravated following H_2S inhibition. Curiously, these phenomena were not observed during (late) arousal in hibernating animals, which are at similar body temperature (and presumably metabolic rate) as winter euthermic hamsters. Possibly, this difference is related to the duration of the housing under 5°C ambient temperature, lasting only about 4 days for arousals, whereas amounting several weeks in winter euthermic, exhausting defense mechanisms and provoking damage in the latter. However, other organs such as the brain, liver and the cardiovascular system, in which specific H_2S-producing enzymes are abundantly expressed, need to be

examined to confirm these findings. Our findings so far suggest that H_2S provides renal protection at high metabolic demand, possibly by maintaining adenosine triphosphate (ATP) production (Modis *et al.,* 2013; reviewed in Szabo *et al.,* 2014) during torpor/hypothermia. Unravelling the mechanisms that lead to induction of natural torpor and regulation of the torpor-arousal cycle, may have major clinical relevance for the prevention of acute organ injury upon therapeutic hypothermia and ischemia/reperfusion. Although the mechanisms by which H_2S induces hibernation-like state in smaller mammals are not completely understood, an attempt to reproduce a similar hibernation-like state in larger mammals failed (Haouzi *et al.,* 2008), and therefore H_2S might preclude therapeutic hypothermia or its therapeutic use should be cautioned. There are also reports that H_2S competes with oxygen and reversibly inhibits mitochondrial complex IV (Beauchamp *et al.,* 1984; Khan *et al.,* 1990), the terminal enzyme in the mitochondrial electron transport chain, thereby leading to hypometabolism and consequent hypothermia in small mammals. Since induction of a similar hypometabolic- and hypothermic state by H_2S failed in larger mammals, this poses the question of whether H_2S-induced hibernation-like state in smaller mammals exists in larger mammals. It might be that H_2S failed to inhibit the mitochondrial complex IV in these animals perhaps due to a smaller window of opportunity to compromise oxidative phosphorylation in larger mammals (and humans) (Szabo, 2007). It might also be that a higher concentration of H_2S and longer exposure time are required to induce such a hibernation-state in larger mammals. However, one possible drawback might be H_2S toxicity at a higher concentration and longer exposure time in larger mammals.

As H_2S seems to be a key player in induction of torpor and preserving renal integrity during torpor-arousal cycle in hamster, we hypothesized that pharmacological induction of torpor using 5'-AMP might occur through the H_2S pathway. 5'-AMP has been previously used in other studies to induce a hibernation-like state in non-hibernating rodents (Zhang *et al.,* 2006; Swoap *et al.,* 2007; Strijkstra *et al.,* 2012; Bouma *et al.,* 2013; de Vrij *et al.,* 2014). Therefore, in **chapter 4,** we induced pharmacologically induced hypothermia, resembling natural torpor by using 5'-AMP and investigated whether 5'-AMP influences the renal H_2S system in naturally hibernating animals and protects organs from hypothermic injury as observed in chapter 3. Our results show that 5'-AMP-induced hypothermia is independent of activation of the H_2S since inhibition of H_2S prior to and during 5'-AMP-induced hypothermia did not preclude hypothermia. However, 5'-AMP seems to activate the renal H_2S system and attenuated renal hypothermic injury. As 5'-AMP-induced hypothermia confers organ protection in non-hibernating species (Zhenyin *et al.* 2011; Miao *et al.,* 2012; Wang *et al.,* 2014), pharmacological inhibition of the H_2S system prior to and during 5'-AMP-induced hypothermia resulted in substantial renal injury, suggesting that H_2S plays a role in organ protection during 5'-AMP-induced hypothermia. Therefore, 5'-AMP might be an alternative

approach to induction of hypothermia in patients who require therapeutic hypothermia. This approach, however, is novel and may need further investigations.

The mechanisms underlying 5'-AMP-induced hypothermia are unclear. Previously, some studies reported that 5'-AMP induces a torpor-like state through dephosphorylation to adenosine, which activates adenosine receptors in the temperature-sensitive hypothalamic nucleus (Zhang *et al.,* 2006), thereby suppressing the thermoregulatory responses that maintain core body temperature. This subsequently results in a reduced cardiac output and thereby in hypothermia. However, there are reports that ATP and adenosine diphosphate (ADP) equally induce a torpor-like state, with even a much longer duration than 5'-AMP (Swoap *et al.,* 2007), thereby downplaying the involvement of adenosine. The mechanism by which these adenine nucleotides induce such a torpor-like state remains largely unknown. Whereas it is speculated that adenosine activates adenosine A_1 receptor in the sino-atrial and atrial-ventricular nodes of the heart, resulting in hyperpolarization of cardiomyocytes and consequent bradycardia as observed during the torpor-like state (Evans *et al.,* 1982; Pelleg *et al.,* 1987), we also demonstrated in chapter 4, that 5'-AMP-induced hypothermia seems to activate renal H_2S system, a system that induces hypometabolism (and hypothermia), and attenuated renal hypothermic injury. However, the mechanism by which 5'-AMP influences the renal H_2S system leading to reduced renal injury is unknown and needs to be explored.

The identification of organ protection by H_2S during natural hibernation and 5'-AMP-induced hypothermia has broken the ground for *in vivo* experiments in non-hibernating mammals. Therefore, in **chapter 5,** we mimicked the effects of hypothermia/rewarming during torpor-arousal by inducing forced hypothermia in rats (non-hibernating animals) under anesthesia with or without dopamine co-infusion followed by rewarming to normothermia and investigated the role of the renal H_2S system. Similar to previous data in cultured smooth muscle aortic cells (Talaei *et al.,* 2011), dopamine infusion in the whole rat increased blood H_2S level and maintained expression levels of all 3 H_2S-producing enzymes thereby precluding hypothermia/rewarming-induced renal injury. Nevertheless, most effects of dopamine were insensitive to blockade of H_2S production with AOAA, a non-specific inhibitor of H_2S enzymes (Asimakopoulou *et al.,* 2013), suggesting that H_2S does not play a part in the observed dopamine effects. However, it should be noted that of all the 3 H_2S-producing enzymes, dopamine infusion maintained the renal CBS expression, but did not maintain the renal expression of 3-MST and particularly of CSE in hypothermic and rewarmed rats treated with AOAA. This observation suggests that preservation of kidney integrity is most dependent on the maintenance of CBS expression, rather than CSE or 3-MST. Such a notion is in accord with previous data showing that selective down-regulation of CBS by siRNA abrogates the beneficial action of dopamine during hypothermia and rewarming (Talaei *et al.,* 2011).

In conclusion, we showed that regulation of the H_2S pathway is a crucial underlying mechanism in the induction of torpor and preservation of organ integrity during the physiological extremes of torpor and arousal. Also, the H_2S system seems to play a major role in attenuating renal hypothermic injury during pharmacologically induced torpor as it is activated by 5'-AMP. Finally, we demonstrated that the H_2S system is also essential in protecting organs (kidney) during forced hypothermia and rewarming in non-hibernating animals under anesthesia. Therefore, H_2S treatment could offer a novel therapeutic approach in treating clinical conditions, which require hypothermia and rewarming.

FUTURE PERSPECTIVES

While hibernators undergo repetitive cycles of torpor and arousal without signs of organ injury, a similar phenomenon in non-hibernating mammals including humans would be expected to result in apoptosis or necrosis, similar to what is observed following ischemia-reperfusion injury (IRI) due to interruption of cellular homeostasis. Although the underlying mechanisms of organ protection during torpor and arousal are not completely understood, hibernation could pave the way for *in vivo* experiments in non-hibernating animals and may lead to novel pharmacological strategies relevant to human medicine. This, therefore, raises the thought that humans can undergo a significant hypometabolic state analogous to natural hibernation. However, this might just be a science fiction as a search for pharmacological agents that induce hypometabolism, let alone *hibernation-on-demand*, in humans remains elusive. The recent discovery of pharmacological agents capable of inducing a safe hypometabolic, hypothermic state in small non-hibernating mammals with no apparent detrimental outcome was widely publicized as holding great promises and could be adopted as a routine clinical tool in the future. However, as discussed previously, recent reports indicate unsuccessful attempts to induce a similar hypometabolic, hypothermic state in large mammals (Haouzi *et al.,* 2008). Thus, the proposed idea of the therapeutic use of exogenously administered H_2S in human medicine should be reconsidered.

The most common clinical example of hypothermia and rewarming is encountered in organ transplantation, which is comparable to torpor and arousal (Green, 2000). So far there is no possible clinical approach to safely cool and rewarm organs without inflicting damage to the organs. There exists experimental evidence from *in vitro, ex vivo* and *in vivo* studies suggesting that dopamine protects against various pathologic stimuli during hypothermic storage (Schnuelle *et al.,* 2004; Yard *et al.,* 2004; Gottman *et al.,* 2006; Talaei *et al.,* 2011). Potential pathways by which dopamine is protective during ischemia/reperfusion-associated alterations comprise the reduction of proteolysis, a mechanism shown in cold-stored liver grafts (Koetting *et al.,* 2010), as well as the maintenance of vascular endothelial cell integrity through mitigation of ROS induced cell damage (Koetting *et al.,* 2010; Minor *et*

al., 2011). Therefore, addition of dopamine to hypothermic preservation solution in both static and machine perfusion may limit the unavoidable IRI during transplantation and might improve organ function after transplantation. Also, as a pharmacological intervention to maintain structural and functional integrity of an allograft between the time of harvest, transportation and final implantation into the recipient, inclusion of H_2S donor molecules and/or H_2S-releasing drugs to the preservation solution may improve standard methods of preparing for organ transplant, and may protect the graft from cold ischemic injury as well as reperfusion injury. Some groups have shown promising effects in experimental models in which exogenously administered H_2S mitigated renal graft IRI during transplantation and prolonged cold storage, improving early graft function and recipient survival in a clinically applicable model of renal transplantation (Hosgood SA, Nicholson ML, 2010; Lobb *et al.,* 2012). This, therefore, indicates a role in H_2S-based therapeutics in these settings.

Since the physiological benefits and therapeutic potentials of H_2S have been discovered by Hideo Kimura's group in 1996, there has been an explosive increase in the knowledge and properties of this rotten-egg smelling gaseous signaling molecule. Unlike nitric oxide (the first gaseous signaling molecule), whose introduction was met with skepticism by the scientific community, H_2S has been unreservedly accepted, and substantial efforts are currently being made to imbue it into the medical and pharmaceutical fields. Although research in the H_2S field is still in its infancy, a plethora of evidence indicates that H_2S has significant pharmacological and physiological benefits. As a young and rapidly growing field, there are problems and challenges that need to be addressed to enhance further H_2S research. Currently, there are no selective inhibitors of H_2S-producing enzymes and available inhibitors of H_2S synthesis have additional off-target effects (Asimakopoulou *et al.,* 2013). Moreover, there are no sustained and controlled-releasing H_2S donors that function in both *in vivo* and *in vitro* situations (Fiorucci and Santucci, 2011; Zhao *et al.,* 2011; Ali *et al.,* 2014), although a slow-releasing H_2S-generating compound has been newly synthesized (Robinson and Wray, 2012). In addition, finding an effective and quick method to measure H_2S concentration, especially in a non-invasive way is another challenge (Wu *et al.,* 2006). Unfortunately, there are no techniques with the sensitivity, selectivity, and real-time capability to measure intracellular H_2S (Wu *et al.,* 2006). Furthermore, H_2S has been implicated in specific diseases in which its concentration varies from one disease to another. In sepsis, for example, H_2S is overproduced while its level is insufficient in Alzheimer's disease (Gong *et al.,* 2011). This suggests that the mechanism controlling the actual concentration of H_2S in certain tissues could pose another problem in H_2S research. Taken together, H_2S seems to have considerable therapeutic potentials, and research in the coming years will very likely unravel several therapeutic feasibility of this gaseous signaling molecule. Moreover, to the best of our knowledge, we are the first to identify dopamine as a main modulator of endogenous H_2S

production and its synthesizing enzymes in preservation of kidney function and integrity in *in vivo* whole body cooling and rewarming, and therefore, could be implemented in specific clinical conditions which require cooling and rewarming.

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