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Molecular strategies used by hibernators: Potential therapeutic directions for ischemia reperfusion injury and preservation of human donor organs

E. Soo^{a,b}, A. Welch^a, C. Marsh^b, DB. McKay^{a,b,*}

^aScripps Research, Department of Immunology and Molecular Biology, 10550 North Torrey Pines Rd, La Jolla, CA, United States of America

^bScripps Clinic and Green Hospital, Department of Medicine and Surgery, 10660 North Torrey Pines Rd, La Jolla, CA, United States of America

1. Introduction

Hibernation and torpor are highly regulated physiological processes that allow animals to adapt and survive in extreme temperatures [1,2]. Animals that hibernate are naturally able to preserve organ function despite long periods of hypothermia and low blood flow. Many animals that hibernate reduce their body temperature to that of the ambient environment and drastically drop their metabolic rate for days to months through a physiologic process called torpor.

The length of torpor varies among different species, however, it is characterized in all animals by severe depression of body temperature and metabolic rate, and organ hypoxia. Torpor is not a steady-state, but is interspersed by short intervals where the body temperature and metabolic rate returns to normal, called interbout arousals [1,3]. Interbout arousals are one of the greatest mysteries of hibernation and they occur at different intervals depending on the hibernating animal [4–7].

The periodic resumption of normal metabolism and return to normothermia is energetically expensive and causes fluctuations in tissue perfusion [8,9]. The organs of hibernating animals experience multiple bouts of ischemia followed by reperfusion during torpor, yet remarkably their organs do not undergo ischemia reperfusion (IR) injury [9–11]. Understanding the molecular and cellular mechanisms that allow animals to preserve organ function during extreme shifts in tissue perfusion, as experienced by hibernators, will likely lead to drug targets to prevent IR injury in human organs.

The natural resistance to IR injury that hibernators experience persists outside of the hibernating season, and stable molecular adaptations to cellular stress have been demonstrated in many hibernators [1,3]. Many different cell types of hibernators, when

*Corresponding author at: Scripps Research, Department of Immunology and Molecular Biology, 10550 North Torrey Pines Rd, La Jolla, CA, United States of America. dmckay@scripps.edu (D.B. McKay).

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studied ex vivo, demonstrate an ability to preserve mitochondrial membrane potential, regulate ATP production and avoid cell death when exposed to hypoxic stress conditions [3,6,9,12]. The 13-lined ground squirrel, which is an important model for understanding the mechanisms of hibernation in vivo, has shown us that the organs of hibernators are remarkably resistant to either cold or warm IR injury [9]. The inherent protection against hypothermic and normothermic injury offers insight into molecular mechanisms that might be exploited to improve human organ preservation. This review focuses on the molecular changes underlying the physiology of torpor and describes potential therapeutic directions for preservation of donor organs prior to transplantation.

2. Methods

The comprehensive review of the literature was performed by systematic analysis of published literature to July 2019. The PubMed database (<https://www.ncbi.nlm.nih.gov/pubmed/>) was searched for variations of keywords: hibernation; molecular targets; ischemia reperfusion injury; hydrogen sulfide; AMP activated protein kinase; heme oxygenase; and opioid receptors. Publication review included cellular, animal and human studies.

3. Molecular strategies used by hibernating animals to preserve organs

Hibernators have evolved molecular strategies to suppress their metabolic processes in order to preserve their organs under extreme environmental conditions. Some molecular targets used naturally by hibernators have been tested experimentally in animals and humans. The best studied targets are hydrogen sulfide (H₂S), AMP activated protein kinase (AMPK), heme oxygenase-1 (HO-1) and the opioid receptors. These targets and their testing in preclinical and clinical models will be described below.

3.1. Hydrogen sulfide

Hydrogen sulfide (H₂S) is a naturally occurring water-soluble gas that mitigates hypoxic tissue injury in many experimental models. Endogenously, it acts as a messenger molecule, a so-called gasotransmitter, that has the characteristic odor of rotten eggs and has anti-apoptotic, anti-oxidative and anti-inflammatory effects.

By inhibiting cytochrome c oxidase, H₂S prevents oxidative phosphorylation and lowers the production of adenosine triphosphate (ATP) [13]. It has also been shown to induce angiogenesis, regulate neuronal activity, and to protect against ischemic injury in the heart, liver, kidney, lung and brain in preclinical studies [14–18].

H₂S is thought to play an important role in the regulation of hibernation and in organ protection during torpor and therefore has been extensively tested in animal models. Inhalation of H₂S reduces metabolism in non-hibernating animals and induces a hibernation-like state called suspended animation [19,20]. Other studies have shown that H₂S is involved in torpor by regulating neuronal activity [13,21,22]. In Syrian hamsters, H₂S levels were shown to increase in lung tissue during torpor and to decrease during arousal [20]. Other studies have found that H₂S does not induce hypothermia, but rather enhances the effects of hypoxia, which then leads to hypothermia in hibernating animals [23].

Several mechanisms have been proposed to explain how H₂S protects organs in hibernating animals (Fig. 1). One way is by directly protecting from cell death by binding to cytochrome C oxidase in the mitochondrial electron transport chain, which then decreases O₂ consumption and ATP production [21–23]. The cytoprotection is dose-dependent, and low concentrations (0.1–1 μM) have been shown to increase cellular ATP metabolism by increasing electron transport chain efficiency [21,22,24]. The role of H₂S in cytoprotection is not supported across all studies, however, as interspecies variabilities have been seen [25].

On a molecular level, H₂S protects against apoptosis by upregulating antiapoptotic proteins such as B cell lymphoma-2 (Bcl-2), BH3-interacting domain death agonist (BID), and downregulating proapoptotic genes such as Bcl-2-like protein (BAX), and caspase-3 [21,22,24]. In a murine study of myocardial ischemia, H₂S has been shown to directly block activation of the nucleotide-binding domain, leucine-rich-containing family pyrin domain-containing-3 (NLRP3) inflammasome and subsequent pyroptotic cell death [26]. H₂S also has a direct anti-inflammatory effect by inhibiting nuclear factor-kappa B, and MAPK signaling and by decreasing neutrophil/macrophage infiltration and decreasing proinflammatory cytokine expression in rodent and pig kidneys [21,24].

Delivery of H₂S has posed a problem for its therapeutic use. H₂S is a gas, and therefore it has been difficult to precisely control its concentrations in vivo [27]. Therefore, synthetic H₂S-releasing compounds have been developed. The synthetic compounds are generally divided into two types, H₂S donors that release H₂S as their only mechanisms of action and H₂S releasing hybrid drugs which have ancillary properties (refer to Wu, et al. for a comprehensive review of these different synthetic H₂S releasing compounds [28]).

Two H₂S donors have proven most successful in preclinical models (Fig. 1, table). The slow releasing diallyl trisulfide (DATS), coupled to nanoparticles such as mesoporous silica (MSN) or mesoporous iron oxide (MIONS) [29,30], and the mitochondria target (AP39) have shown promise in several experimental studies [21,22,29]. In rodents, addition of DATS-MSN to University of Wisconsin (UW) solution improved donor cardiac function and decreased inflammatory cytokine levels, mitochondrial swelling and apoptosis when compared to UW solution alone [29]. In a porcine model, addition of AP39 to UW solution improved renal tubular cell viability under conditions of static subnormothermic perfusion at 21 °C [31]. Another study in a rodent model, found the addition of AP39 to UW solution improved kidney graft function under conditions of prolonged cold storage [32].

3.2. AMP activated protein kinase (AMPK)

AMPK-dependent signaling plays a key role in the regulation of cellular energy and response to metabolic stressors. AMPK activators have become important drug targets for metabolic disorders such as diabetes mellitus and insulin resistance. Hibernating animals activate AMPK signaling during torpor leading to post-translational regulation of proteins that lower metabolic energy requirements [33]. Like H₂S, AMPK activators preserve metabolic and energy homeostasis and regulate oxidative stress, inflammation and apoptosis (Fig. 2) [33–37].

AMPK regulates protein synthesis, oxidative stress and autophagy by interacting with the kinase mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that exists in two forms; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [38,39]. mTORC1 is activated by growth factors and is inhibited by AMPK. Less is known about mTORC2 regulation although it is activated by growth factors and inhibited by mTORC1 [38,40]. Under hypoxic conditions, AMPK and mTOR act together to regulate autophagy. AMPK also regulates oxidative stress through heme oxygenase-1 (HO-1) activation via Nrf2, upregulation of manganese superoxide dismutase and catalase expression through forkhead box O1 (FoxO1) and suppression of ROS production [33].

AMPK has been proposed as a target for IR injury because activity of endogenous AMPK increases following ischemic injury in several organs, including the liver, kidney and heart [33,37,41,42]. It is widely accepted that AMPK has a positive role in IR injury because its activation preserves ATP levels by switching on pathways to produce ATP and shutting off pathways to prevent ATP consumption [43]. AMPK has been shown to protect against cellular malnutrition and oxygen deprivation in several models of IR injury. Kong et al. found that glycogen synthase kinase 3B inhibition activated AMPK, and subsequently inhibited mTOR, leading to increased autophagy during liver IR injury [44]. Inhibition of mTOR signaling resulted in increased autophagy and decreased inflammatory cytokine production, apoptosis and myocardial IR injury in rats [45].

In hibernating lemurs, AMPK stimulates fatty acid transporters that enhance lipid uptake during torpor [46]. During torpor, activated AMPK regulates protein synthesis by inhibiting mTOR [47–49]. Zhang et al. found that elevated AMPK levels correlated with reduced metabolic rates in the hearts of torpid lemurs [33]. AMPK signaling and posttranslational regulation of selected proteins may play crucial roles in the control of transcription and translation during daily torpor in hibernating animals.

Exogenous AMPK has been used in several experimental studies to promote tissue preservation (Fig. 2, table). The AMPK activator metformin and 5-amino-4-imidazolecarboxamide-ribose (AICAR), given within the first 15 min of reperfusion, decreased IR injury in rat hearts and kidneys [33] and protected from cardiac IR injury through, an AMPK-dependent mechanism [50], however others have found the protective mechanism is independent of AMPK [51]. Metformin has also been shown to protect against cerebral ischemia in an AMPK dependent manner [52–54], and against renal IR injury in a rat model [42,55]. The allosteric AMPK activator A769662 preserved cardiac muscle in a murine model of IR injury [56]. Rosiglitazone, a peroxisome proliferator-activated receptor (PPAR γ) agonist was also found to protect against murine ischemic cardiac injury by modifying AMPK activation [57]. Song et al. found that AMPK inhibition via the microRNA, miR-101, ameliorated hepatic IR injury and decreased autophagy [58], and Li et al. showed hepatocellular protection against IR injury [40].

3.3. Heme oxygenase-1

Heme oxygenase is an inducible enzyme that produces potent cytoprotective, anti-inflammatory and anti-apoptotic functions in most eukaryotes [59]. It exists in three isoforms; heme oxygenase—1 (HO-1), –2 (HO-2), and –3 (HO-3). HO-1 is a

transmembrane protein found in the endoplasmic reticulum, and HO-2 and HO-3 are heme-binding proteins [60]. Although HO-1 expression is low compared to HO-2 and 3, over-expression of HO-1 provides protection in conditions of stress [61,62].

Like H₂S and AMPK, HO-1 protects against oxidative stress (Fig. 3). Using a rat model, HO-1 activation was shown to reduce hepatic IR injury and inflammatory cytokine expression [63]. It has also been found to regulate macrophage polarization in mice and to protect against hepatic IR injury via alternatively activated macrophages (M2), which induce anti-inflammatory responses [64]. Another study examined myeloid HO-1 in murine liver IR injury models, cell culture systems, and human liver transplants and found reduced inflammation and liver damage through macrophage inhibition [65]. Similarly, myeloid HO-1 conferred protection against renal IR injury, and injection of hemin prior to surgery offered IR injury resistance in mice [66]. In a murine pancreas model, upregulation of HO-1 was also found to reduce neutrophil infiltration and lessen the severity of acute pancreatitis [67]. Other studies have found that HO-1 causes immature dendritic cells to differentiate into tolerogenic dendritic cells (DCs) [68].

Promising preclinical results have led to interest in the development of HO-1 targeting therapies (Fig. 3, table). Aminolevulinic acid (5-ALA) and sodium ferrous citrate (SFC) have been found to work together to induce HO-1 overexpression in animal models [69,70]. In murine kidneys, 5-ALA and SFC pretreatment increased HO-1 activity and subsequently increased CO production, which offered protection against renal IRI [69]. Similarly, the same treatment was found to reduce IR injury in fatty livers in mice by lowering ROS levels [69]. In humans, oral administration of 4-ALA induced HO-1 expression in PBMCs when given with iron [71]. Molecular hydrogen, has also been shown to raise HO-1 expression and lessen injury in rat lung tissue post transplantation [72]. Uto et al. found that a hydrogen-rich UW solution water bath upregulated HO-1 resulting in reduced oxidative stress and inflammation in a rat liver IR injury model [73].

3.4. Opioid receptors

Opioid receptors are activated in response to cellular stress and they are well-known to mediate adaptive and organ-specific cytoprotective responses in hibernating animals [74]. Opioid receptors are G_{i/o}-coupled protein receptors that exist in three isoforms: mu (μ)-opioid receptors (MOR), delta (δ)-opioid receptors (DOR), and kappa (κ)-opioid receptors (KOR) [75].

Activation of all three opioid receptor isoforms have been found to play a role during hibernation, however DORs are thought to be the most significant [76], and most relevant for studies of hibernation (Fig. 4). DOR activation is induced via [D-al², D-leu⁵]-enkephalin (DADLE). DADLE is an agonist that interacts specifically with DORs and has been associated with natural organ protection during hibernation in the brain, heart, lungs, liver, and kidneys [77–82]. DOR activation through DADLE was found to inhibit cellular apoptosis and increase autophagy in rat astrocytes, offering protection in ischemic brains (Fig. 4, table) [83]. DADLE treatment in a model of moderate hypothermia increased cell viability in primary rat neuronal cells [84]. Activation of DOR via DADLE in human umbilical cord-blood derived mesenchymal stem cells (MSCs) given prior to transplantation

increased MSC survival and anti-inflammatory potential [84]. Similarly, Deltorphin D, another DOR agonist, activated DORs and reduced cardiac reperfusion injury in isolated pig hearts [85].

Effectiveness of DADLE and DOR activation may rely on timing of administration [25]. DADLE has shown promise as a pre-conditioning treatment in rodents [78,86–88]. Pre-conditioning recreates an inherent hormesis response in which non-lethal metabolic stress yields both acute and delayed protection against more severe injury [3]. DOR and KOR activation have been linked to pre-conditioning processes in animal models [3,89]. The requirement for pretreatment however may limit the practical application of opioid agonists in the clinic [25,74].

DOR activation also has also been shown to have beneficial effects in post-conditioning treatments for IR injury [90]. In the heart, opioid receptor agonists administered immediately before or during reperfusion provided significant protection [74]. All three opioid receptor groups have been shown to provide cardiac protection from ischemic injury when administered in post-conditioning treatments in rodent models [91–93]. However, other studies have not found DADLE benefits when injected during ischemia or reperfusion in rodents [86,94,95]. These mixed results and the time-dependent nature of treatment are possible limitations for applicability in clinical practice.

4. Conclusion

Animals that hibernate have adapted highly regulated physiological processes that allow them to survive extreme changes in their environment. Hibernators preserve organ function despite long periods of organ hypoperfusion and multiple bursts of reperfusion. Intermittent hypoperfusion (ischemic conditioning) has been proposed as a way of organ protection, however while initially thought to be promising in many small studies, the benefit of ischemic conditioning has grown to be controversial [96,97]. The optimal methods to apply ischemic conditioning, to whom and in what time frame it should be applied are not yet known. Large studies however are ongoing [CONDI2 (NCT01857414) and ERIC-PPCI (NCT02342522)] to better assess the outcomes of ischemic preconditioning and it is hopeful that with greater study we will have a better idea of how to apply ischemic preconditioning and perhaps mimic strategies used by hibernators.

Several molecular strategies used by hibernators have been described in preclinical studies. The best studied strategies supported by preclinical animal studies are described in this review. However, at the time of this review, no single molecule, or combination of molecules, have been demonstrated to successfully induce and maintain hibernation in naturally hibernating mammals. Multiple screening methods have been used to monitor the expression of large gene sets in hibernation states, but this approach has been limited to single organs of tissues and therefore a complete picture has not been made to explain the whole-animal physiology of hibernation. A resource for better describing the transcriptome of hibernators can be found at (<https://d.umn.edu/~mhampton/GB18.html>). This interactive browser provides mRNA reads of over 14,000 genes. Statistical significance between any two activity states however are limited to specific tissues, not whole body. The 200

Mammals Project includes genome sequences of at least 15 hibernating mammals and this also serves as a promising resource for future analyses [98]. As the genomes of other hibernators are better defined, this approach will become a powerful means to identify genes that control hibernation in mammals.

Although questions for applicability remain, continued efforts to understand how hibernators naturally preserve their organ function under extreme conditions is likely to enhance our ability to prevent human ischemia reperfusion injury and to better preserve organs procured for transplantation. Several interesting studies have shown that organs of are resistant to liver and kidney damage and systemic inflammation after I/R relative to nonhibernators. The resistance to I/R injury in was not dependent on the ability to cool, but seemed to be related to altered metabolic and inflammatory responses in the organs of hibernators [99]. In a proteomics study of myocardial protein expression changes following experimental I/R in hibernating versus non-hibernating mammals, several prominent features of I/R injury were significantly reduced in the hibernator's heart; accompanied by differential expression of mitochondrial proteins [100]. Further studies are needed however to understand how to mimic the natural protection from I/R injury afforded to hibernators, as this would provide a transformative approach for patients at risk for I/R injury and for protection of donor organs procured for transplantation.

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Fig. 1.
Schematic of H₂S mechanisms of protection and table of preclinical studies supporting the use of slow releasing H₂S donors.

AMPK modulators successful in preclinical animal models			
Metformin	AICAR	A769662	rosiglitazone
<ul style="list-style-type: none"> • protects against rat cardiac IR injury ¹⁸ • protects against rat cerebral IR injury ⁶² • protects against rat renal IR injury ⁹⁸ 	<ul style="list-style-type: none"> • improved rat hepatic IR injury and mimicked effects of preconditioning and suppressed apoptosis of hepatocytes ¹⁰⁵ 	preserved cardiac muscle in rodent model of cardiac IR injury ^{66,106}	<ul style="list-style-type: none"> • reduced ischemic heart injury in mouse model of IRI ⁶⁷

Fig. 2. Schematic of AMPK mechanisms of protection and table of preclinical studies supporting the use of AMPK modulators.

HO-1 modulators successful in preclinical animal models	
5-ALA + iron	Molecular hydrogen (H ₂)
<ul style="list-style-type: none"> • protects against mouse renal IRI injury ⁷³ • reduces IR injury in livers of mice ⁷³ • induces HO-1 expression in PBMCs of humans ⁷⁵ 	<ul style="list-style-type: none"> • decreases rat lung injury after transplantation ⁷⁶ • decreases inflammation in rat liver IR injury model when given in cold UW solution ⁷⁷

Fig. 3.
Schematic of HO-1 mechanisms of protection and table of preclinical studies supporting the use of HO-1 modulators.

DOR agonists successful in preclinical animal models	
DADLE	Deltorphan D
<ul style="list-style-type: none"> • protection during hibernation in the brain, heart, lungs, liver and kidneys ^{79-82,107} • protects astrocytes in rat ischemic brain ⁴⁹ 	<ul style="list-style-type: none"> • reduces cardiac IR injury in isolated pig hearts ⁸⁶

Fig. 4. Schematic of opioid receptor mechanisms of protection and table of preclinical studies supporting the use of DOR agonists.