Translational research of hypothermia: therapeutic use in humans and pharmacological induction in experimental models

PhD Thesis

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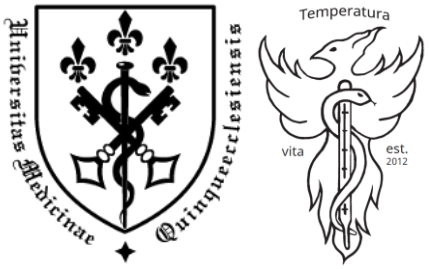

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INTRODUCTION

1. Hypothermia

Thermoregulation is a dynamic, homeostatic vital function of the autonomic nervous system in response to cold and heat stress, to maintain a stable, physiological temperature. Thermoregulatory physiology sustains health by keeping core body temperature (Tb) within the range of a degree or two of 37°C. In general, hypothermia is defined as a core body temperature of 35°C or less and it can be either induced (*i.e.*, therapeutic) or accidental. Guidelines distinguishing the depth or stages of hypothermia have differed between authors, and as a result there are various definitions within the published literature. The American Heart Association has arbitrarily adopted the definition of Polderman *et al*. (2009), which defines mild hypothermia as temperatures down to 34°C, while the hypothermia is moderate at 34-30°C and severe at <30°C (Polderman & Herold, 2009). In accidental cases, a single measurement of core temperature is often used to classify hypothermia as mild, moderate or severe. The treatment of patients with accidental hypothermia depends on the severity level, therefore, as our understanding of the pathophysiological mechanisms of hypothermia improves, it is important to have a general consensus on the stages so that treatment is appropriately targeted.

Therapeutic use of hypothermia has returned to the frontline in the prevention and in mitigation of neurologic impairment in different pathological conditions over the past decade. The application of hypothermia is considered as a successful therapeutic measure not just in neuro- or cardiac surgeries, but also in various (*e.g.*, traumatic) states causing brain injury or damage. Cooling the brain down to hypothermic temperatures is sufficient to reduce brain metabolic requirements to such an extent that blood flow can be completely interrupted. In recent years there has been growing interest into the beneficial effects of therapeutic hypothermia in different pathological conditions, such as ischaemic stroke and traumatic brain injury (TBI) (Basto & Lyden, 2018). In the former, the protective mechanisms of hypothermia affect the ischaemic cascade across several parallel pathways and it is therefore a condition where cooling may increase positive outcomes.

2. Therapeutic hypothermia in severe traumatic brain injury

TBI is recognized as a significant cause of mortality and morbidity predominantly in the young population (Yokobori & Yokota, 2016). Worldwide, TBI is estimated to affect 10 million people annually and nowadays it is one of the major causes of death and disability, posing a global health and financial burden for the society (Hyder *et al*., 2007). Among the leading causes of TBI are motor vehicle accidents in both more and less developed countries, most frequently involving young males (Hyder *et al*., 2007; Taylor *et al*., 2015). Mild head injuries may recover fully without any specific treatment, whereas severe injuries are often rapidly fatal or leave survivors with disabilities (Finfer & Cohen, 2001). In accordance with the poor outcomes, severe TBI constitutes a major health and socio-economic problem worldwide (Maas *et al*., 2008). Any potentially beneficial interventions should be implemented in order to mitigate the burden of TBI on the patients and on healthcare, as well as to improve the outcome of the disease. However, in recent years there has been growing evidence that therapeutic hypothermia is associated with unfavourable long-term outcomes when applied clinically (Crossley *et al*., 2014; Honeybul, 2016). This has been shown in both meta-analysis of previous studies (Shaefi *et al*., 2016) and in a large-scale clinical trial (Andrews et al., 2015b), which was prematurely terminated, because the clinical outcome did not improve in the hypothermia arm of the trial as compared to the normothermia arm. Many of the pathological mechanisms associated with TBI are temperature-sensitive (Stocchetti *et al*., 2017), suggesting that at a lower Tb the adverse processes can be decelerated and neuroprotective effects can be achieved. As a consequence, therapeutic hypothermia has been broadly investigated as a possible neuroprotective strategy to attenuate the harmful effects of severe TBI.

In animal models of TBI, the beneficial effects of therapeutic hypothermia have been shown repeatedly (Bramlett & Dietrich, 2012; Feng et al., 2010; Sahuquillo & Vilalta, 2007; Zhao *et al*., 2017), however different clinical studies provided contradictory results. It could be assumed that the high between-study variability in the statistical and clinical designs of the trials (e.g., randomization), inclusion criteria of patients, and the applied cooling protocols varied substantially among the studies, which differences altogether could be responsible for

the contradictory findings in the human studies. Indeed, high inter-study heterogeneity was reported in all of the performed meta-analyses so far (Crompton et al., 2017; Harris et al., 2002; Henderson et al., 2003; Li & Yang, 2014; McIntyre et al., 2003; Peterson et al., 2008; Zhu et al., 2016)

To examine and amalgamate the different findings and opinions, it was necessary to systematically review, categorize, and analyze the results, which is described in the first part of my dissertation. In addition to whether therapeutic hypothermia is beneficial or not, it was important to evaluate how it was implemented, since there are various physical devices and protocols for complete or partial body cooling in the clinical practice for this purpose.

In the clinical setting, the induction of hypothermia can be classified into: surface and internal cooling methods, whole-body, and localized cooling (Basto & Lyden, 2018). Whole-body cooling can be achieved by endovascular or surface methods, while localized cooling can be performed by endovascular infusions of cold saline or by focal cooling with an intracranially implanted device. Pharmacological strategies should be also mentioned among the hypothermia-inducing methods, but it must be noted that, as of today, the available thermopharmacological methods to induce a targeted and controlled drop in deep T_b are very limited, mostly due to the lack of a safe and usable substance for that reason in humans. Each method has advantages and problems that make them suitable for different situations (Basto & Lyden, 2018). The use of the thermopharmacological agents in combination with physical cooling can be an effective intervention to potentiate the molecular effects of therapeutic hypothermia.

Thermosensors, some of which belong to the group of temperature-activated TRP channels, play a key role in mediating alterations of deep T_b. Among the thermo-TRP channels, for the present work, the TRP ankyrin-1 (A1) ion channel has to be highlighted, which can be activated by many substances, also including H_2S, in addition to cold stimuli. The TRPA1 channel can be of crucial, and yet undiscovered, importance for the thermal action of H_2S, since TRPA1 channel-mediated effects of sulfide donors and polysulfides were identified in different experimental models. An extensive list of studies that describe TRPA1-

mediated effects of H_2S was recently collected by Pozsgai *et al* (2019), but thermoregulatory effects were not mentioned by the authors.

The diverse existence of H_2S-induced TRPA1 activation in different homeostatic processes may suggest that it could also be involved in thermoregulation. The investigation of the action mechanisms of H_2S-induced hypothermia constituted the basis of the second part of my dissertation.

AIMS

The ultimate goal of the present work was to evaluate the clinical importance of therapeutic hypothermia in human patients with TBI, and then to identify a potential pharmacological target for the induction of hypothermia in animal experiments. The main topics discussed in this thesis are as follows:

1. Therapeutic hypothermia was investigated repeatedly as a tool to improve the outcome of severe TBI, but previous clinical trials and meta-analyses found contradictory results. We aimed to determine the effectiveness of therapeutic whole-body hypothermia on the mortality of adult patients with severe TBI by using a novel approach of meta-analysis.
2. The cooling protocols of TBI patients widely differed among the studies, thus we aimed at developing a novel measure for the overall extent of the cooling. We calculated the integrated measure of therapeutic hypothermia from cooling parameters and introduced it as the cooling index (COIN), then we studied its relation to mortality in TBI.
3. H_2S has been shown in previous studies to cause hypothermia and hypometabolism in mice, however, the molecular target through which H_2S triggers its effects on deep T_b has remained unknown. We investigated the thermoeffector mechanisms of the hypothermic response to fast- (Na_2S) and slow-releasing (GYY4137) H_2S donors in C57BL/6 mice, and then tested whether their effects depend on the TRPA1 channel in *Trpa1* knockout (*Trpa1*$^{-/-}$) and wild-type (*Trpa1*$^{+/+}$) mice.

4. We also studied *Trpa1* expression in thermoregulation-related brain nuclei to explore the possible site of action for the hypothermic effect of H_2S.

MATERIALS AND METHODS

1. Analysis of human data

1.1. Data extraction

In our meta-analysis (Olah *et al*., 2018), we searched the PubMed, EMBASE, and Cochrane Library databases from inception to February 2017. The identified human studies were evaluated regarding statistical, clinical, and methodological designs to ensure inter-study homogeneity. We extracted data on TBI severity, T_b, mortality, and cooling parameters. Our meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (Moher *et al*., 2015); was based on the Participants, Intervention, Comparison, Outcome (PICO) model; and was registered with PROSPERO (CRD42017056535).

1.2. Data evaluation

The collected data were evaluated based on a statistical and a biomedical approach. Statistically, we assessed the randomization of the collected studies based on the authors' statements, Jadad analysis, and detailed evaluation of the randomization protocol described in the study. In the biomedical evaluation, we narrowed the list of eligible studies to those that dealt with the effects of therapeutic whole-body hypothermia compared with no temperature management, therefore, we excluded those articles, in which selective brain cooling or antipyretic drugs were used, or when patients with spontaneous (accidental) hypothermia were involved in the study. Eligible studies resulting from the statistical or the biomedical refinement or both were compared with the random effect model of meta-analysis for odds ratio (OR) as effect size. To assess which parameters of the cooling methods have the biggest

impact on the outcome of severe TBI, the studies were divided into subgroups based on target cooling temperature, cooling duration, and speed of rewarming.

1.3. Calculation of the cooling index

The influence of the used combinations of the three cooling parameters together was also assessed on the outcome of severe TBI. When all three parameters, *i.e.*, target cooling temperature, cooling duration, and speed of rewarming, were reported in the same study, we calculated the integrated measure of cooling and named it as the cooling index (COIN). The COIN represents the area between the T_b curve of cooled patients and a hypothetical horizontal line corresponding to a normal T_b of 36.5°C. The following formula was used: $COIN = \Delta T \times t + (\Delta T \times \Delta T/R)/2$, where "ΔT" is the difference between normal T_b (36.5°C) and the temperature reached at the end of cooling (in °C); ''t'' is hypothermia duration (in hours); and ''R'' is the rate of rewarming (in °C/h). For meta-analysis, the included studies were evenly distributed (N = 4) into subgroups with low (<160°C × h), moderate (160-200°C × h), and high (>200°C × h) COIN. From each study, we extracted the targeted cooling parameters (*viz.*, cooling temperature, cooling duration, and speed of rewarming), accounted for any deviations from or adjustments to them, and calculated COIN values.

After we originally introduced the COIN in 2018 (Olah *et al*., 2018), the results of a large, multicenter, randomized clinical trial (POLAR) were published, which seemed to contradict the benefits of therapeutic hypothermia in severe TBI (Cooper *et al*., 2018). In order to also account for the results from that trial, in our recent meta-analysis we also included the data from POLAR (Olah, Poto, *et al*., 2021).

1.4. Statistical analysis

The statistical analysis was performed according to the standard methods of meta-analysis. Publication bias was assessed with funnel plots by using the Duval and Tweedie trim and fill method and the Egger's test. Between-study heterogeneity was tested with Q homogeneity test and with I^2 statistical test. All analyses were performed using the Comprehensive Meta-Analysis software.

2. Experiments in animal models

2.1. Animals

In animal experiments (Olah, Rumbus, *et al*., 2021), male *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice (n = 18 and 14, respectively) and C57BL/6 mice (n = 77) were obtained from the Laboratory Animal Centre at the University of Pécs where they were bred and kept under standard pathogen-free conditions. All procedures were conducted under protocols approved by the Institutional Animal Use and Care Committee of the University of Pécs (registration no.: BA02/2000–6/2018, approved on 27 February 2018) and were in accordance with the directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC).

2.2. Surgeries

Mice were anesthetized with intraperitoneal (i.p.) administration of a ketamine-xylazine cocktail and received antibiotic protection intramuscularly.

For the intracerebroventricular (i.c.v.) substance administration, a 22-G steel guide cannula was implanted into the right lateral cerebral ventricle using a stereotaxic manipulator. In brief, a guide cannula was inserted through a small hole drilled in the skull 0.5 mm posterior from Bregma and 1.0 mm lateral from midline. The tip of the cannula was placed within the right lateral ventricle. The cannula was fixed to the supporting microscrews with dental cement and closed by a dummy cannula.

For the i.p. administration of substances, a polyethylene (PE)-50 catheter filled with pyrogen-free saline was implanted into the peritoneal cavity. The external end of the catheter was exteriorized at the nape and heat-sealed. The surgical wound was sutured in layers. The catheter was flushed with 0.1 mL of saline on the day after the surgery and every other day thereafter.

2.3. Experimental setups

Thermoregulatory experiments in unanesthetized mice were performed in either the thermocouple thermometry setup or the respirometry thermometry setup under subneutral conditions.

2.3.1. Thermocouple thermometry

The mice were placed in cylindrical confiners and equipped with copper-constantan thermocouples to measure colonic temperature. The colonic thermocouple was inserted 3 cm deep beyond the anal sphincter, fixed to the base of the tail with adhesive tape, and plugged into a data logger device connected to a computer. Mice in their confiners were then placed into a temperature-controlled incubator set to a T_a of 30°C, which is slightly below the thermoneutral zone for mice in this setup. When the mouse was pre-implanted with an i.c.v. cannula, a needle injector was fitted into the guide cannula and connected to a PE-50 extension, which was prefilled with a solution of Na_2S or GYY4137 or with saline. The injector needle protruded 1.0 mm beyond the tip of the guide cannula. The extension was passed through a port of the incubator and connected to a 10-µL syringe. When the mouse had an i.p. catheter, it was connected to a PE-50 extension, which was prefilled with the substance of interest and connected to a syringe placed in an infusion pump.

2.3.2. Respirometry thermometry

The respirometry setup was designed to characterize dose dependency of thermal effects and the involvement of metabolic rate inhibition in the thermoregulatory response to Na_2S and GYY4137 in C57BL/6 mice. The mice were equipped with thermocouples and PE-50 extensions the same way as in the experiments in the thermocouple thermometry setup. Then, the mice in their confiners were transferred to a Plexiglas chamber of the four-chamber open-circuit calorimeter integrated system. The chambers were sealed, submerged into a temperature-controlled water bath, and continuously ventilated with room air (200 mL/min). The fractional concentration of oxygen was measured in the air entering and exiting the

chamber, and the rate of oxygen consumption (VO_2) was calculated according to the manufacturer's instructions using the Oxymax Windows software.

2.3.3. Drugs and drug administration

Na_2S nonahydrate ($Na_2S \cdot 9H_2O$) was purchased from Sigma-Aldrich. For the i.p administration, the working solution (1.5 mg/mL) of Na_2S (or saline) was infused over 4 min (3.3 mL/kg) to deliver Na_2S at 5 mg/kg. For the i.c.v. administration of Na_2S (at doses of 0.5 and 1 mg/kg), the working solutions (5 and 10 mg/mL, respectively) of Na_2S (or saline) were infused (1 μL/min) over a 3 min period.

The slow-releasing H_2S donor GYY4137 was synthesized by our collaborators at the University of Exeter Medical School. A total dose of 3 mg/kg of GYY4137 was delivered i.c.v. Control mice were infused with saline.

2.4. Measurement of *Trpa1* mRNA expression

2.4.1. RNAscope in situ hybridization

For RNAscope studies 3 month-old male C57BL/6 mice (n = 4) were perfused as described above using 30 mL PBS followed by 100 mL of 4% paraformaldehyde in Millonig's phosphate buffer. Brains were postfixed for 24 h at room temperature, rinsed in PBS, dehydrated, and embedded in paraffin using standard procedures. 5 μm sections were cut using a sliding microtome. The RNAscope Multiplex Fluorescent Reagent Kit v2 was used according to the manufacturer's protocol. According to the atlas by Paxinos and Franklin (2001) fluorescent images of the medial preoptic area, dorsomedial hypothalamic area, as well as the lateral parabrachial nucleus and rostral raphe pallidus were acquired using an Olympus Fluoview FV-1000 laser scanning confocal microscope and Fluoview FV-1000S-IX81 image acquisition software system.

2.5. Data processing and analysis

Data on deep T_b, $\dot{V}O_2$, and blood flow intensity were analyzed through the application of two-way ANOVA followed by the Fisher's LSD post hoc test. Sigmaplot 11.0 software was used for statistical analyses.

RESULTS

1. The value of therapeutic hypothermia in human patients with severe traumatic brain injury

In our first meta-analysis (Olah *et al*., 2018), the literature search identified altogether 709 studies from the PubMed, EMBASE, and Cochrane databases. After enabling filters for human studies and English language and using additional filters (study types) 321 studies remained, which were screened for title and abstract for inclusion criteria. 273 articles were excluded because of insufficient data reporting or because children were studied. 48 studies were included in qualitative synthesis. A further 21 articles were excluded due to the lack of mortality data. 27 studies were included and pooled for quantitative synthesis. When we compared the effects of therapeutic hypothermia with no cooling by including all 27 identified studies in the meta-analysis, we did not find a significant difference in the OR for mortality between the groups. Importantly, the included studies were methodologically quite heterogeneous with regards to both statistical and clinical designs.

As a statistical approach to reduce heterogeneity, we analyzed separately those studies which were RCTs and evaluated the studies based on clinical and methodological designs. As result of the combined (statistical and physiological) evaluation of the studies identified by our literature search, 14 full-text publications (involving 1,786 adult patients with severe TBI; 896 in the therapeutic hypothermia group and 890 in the no cooling group), were included in the next steps of our analysis. All of them were RCTs, in which the exact cooling methods of the whole body (target temperature, cooling duration, and speed of rewarming) were reported, and the effect of therapeutic hypothermia on mortality was compared with patients without

temperature management in severe TBI. The homogeneity of the studies was verified by Egger's test, Q, and I^2 statistics, which showed no significant difference in inter-study variability. Meta-analysis of these studies revealed that therapeutic hypothermia significantly improved the outcome of severe TBI (OR = 0.675; p = 0.004).

In our next approach, we studied the integrated effect of the cooling parameters on the outcome of the disease. From the cooling parameters reported in the studies with medium and good level of randomization, the COIN was assessed by considering all three variables, *viz.*, target temperature, cooling duration, and speed of rewarming, in the formula. By calculating the COIN, we were able to compare the effect of the overall extent of hypothermia among studies which used different cooling parameters in their protocols. The OR in the subgroups with low (<160°C × h), moderate (160-200°C × h), and high (>200°C × h) COIN. Importantly, the only significant effect for an OR of less than 1, *i.e.*, when cooling was beneficial compared to no cooling, was observed in the subgroup of studies with high COIN (OR = 0.470; p = 0.035). These results suggest that in addition to the different independent contribution of each cooling parameter, the integrated measure of the magnitude and duration of therapeutic hypothermia (as indicated by the COIN) can play a decisive role in determining whether the applied cooling protocol will decrease the risk of death in patients with severe TBI.

In our later study (Olah, Poto, *et al.*, 2021), we extended our analysis based on COIN by including in it the data reported in the POLAR study. The COIN value would have been high in the POLAR study – if the targeted parameters were met. However, the targeted cooling parameters were reached in less than 50% of the patients receiving therapeutic hypothermia. In the hypothermia group, 85 patients (33%) were cooled for less than 48 hours; 27 patients (10%) never reached a deep T_b of 35°C; and 65 patients (27%) never reached 33°C (Cooper *et al.*, 2018). Hence, many patients in the POLAR study had a low level of COIN, and the overall level achieved in that study was only moderate. Based on the above, we included the POLAR data in the "Moderate" COIN subgroup of our meta-analysis. For all data, including POLAR, indicating that, overall, therapeutic hypothermia significantly decreased mortality in severe TBI. However, a significant decrease in OR (indicating a

beneficial effect of therapeutic hypothermia) was observed only in the “High” COIN subgroup: 0.470. The ORs in subgroups with “Low” or “Moderate” cooling intensity were 0.718 and 0.846, respectively.

2. The mechanisms of H_2S-induced hypothermia in animal experiments

2.1. Central administration of Na2S decreases deep body temperature in mice via inhibition of thermogenesis and induction of vasodilation in the skin

In response to Na_2S, the mice developed a decrease in deep T_b, which was more pronounced at the higher dose, whereas saline did not cause any effects. The effect of the treatment on T_b was significant for both the lower and the higher doses of Na_2S as compared to saline. At the 0.5 mg/kg dose of Na_2S, deep T_b was significantly lower than in saline-treated mice at 20, 170, and 180 min, while at the 1 mg/kg dose the drop in deep T_b was significant between 20–180 min compared to saline.

In the same experiments, we also measured the rate of oxygen consumption (VO_2), which was regarded as an indicator of non-shivering thermogenesis. Na_2S microinjection (3 μL, i.c.v) also significantly affected oxygen consumption. The Na_2S-induced hypothermia was brought about by a fall in VO_2, which changed with similar dynamics as deep T_b. Similar to T_b, the effect of the treatment on VO_2 was significant for both the lower and the higher doses of Na_2S as compared to saline. In a separate set of experiments, we also checked whether skin vasodilation – to increase heat loss – contributes to H_2S-induced hypothermia and we found that Na_2S administered centrally caused a marked cutaneous vasodilation in the back-skin of the mice, thereby indicating that increased heat dissipation also participates in the hypothermic response to H_2S.

The rapid, dose-dependent development of the hypothermic and hypometabolic response to centrally administered Na_2S suggests that the site of action for the released H_2S is located in the central nervous system. To test whether the hypothermic effect of Na_2S can be triggered from a peripheral site, next we studied the thermoregulatory response to the i.p.

administration of a high dose (5 mg/kg) of Na_2S. As expected, i.p. infusion of saline did not have any effect on deep T_b. In contrast with the i.c.v. administration, when the mice were infused with Na_2S i.p. their deep T_b did not differ significantly from that of saline-treated mice at any time points during the experiment even though a 10 times higher dose was delivered i.p. than the i.c.v. administered lower dose which caused hypothermia.

2.2. Central administration of GYY4137 decreases deep body temperature in mice

We also wanted to investigate whether the observed thermoregulatory effects of Na_2S can be triggered by GYY4137, which is a slow-releasing H_2S donor. When administered i.c.v. in the respirometry thermometry setup, GYY4137 (3 mg/kg) caused a marked hypothermia and hypometabolism as compared to saline treatment. Between the GYY4137-treated and saline-treated mice, deep T_b differed significantly at 80–100 min and 130–180 min, and the difference in VO_2 was significant at 20–30 min and 160–180 min.

2.3. The hypothermic response to Na2S and GYY4137 is attenuated in the absence of the transient receptor potential ankyrin-1 channel

Next, we investigated whether the TRPA1 channel is involved in the thermoregulatory responses to different H_2S donors in *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice. The i.c.v. administration of Na_2S (1 mg/kg) caused a sudden, pronounced drop in the colonic temperature and VO_2 of the *Trpa1*$^{+/+}$ mice. However, in the *Trpa1*$^{-/-}$ mice the hypothermic and hypometabolic effects of the same dose of Na_2S were markedly attenuated.

The i.c.v. administration of GYY4137 (3 mg/kg) caused a marked fall in the deep T_b of *Trpa1*$^{+/+}$ mice; however, the hypothermic response to the same dose of GYY4137 was significantly attenuated in *Trpa1*$^{-/-}$ mice. The colonic temperature of *Trpa1*$^{-/-}$ mice remained significantly higher than that of *Trpa1*$^{+/+}$ mice between 20 and 80 min post-GYY4137 administration.

2.4. *Trpa1* mRNA is modestly expressed in brain neurons within autonomic thermoeffector pathways

We studied the expression of the TRPA1 channel in thermoregulation-related brain structures. We used RT-qPCR to assess *Trpa1* mRNA in the hypothalamus of the mice, but we did not find any detectable amount. Then we used RNAscope in situ hybridization, a highly sensitive method that can detect transcripts as single molecules (Femino *et al*., 1998). We found detectable *Trpa1* mRNA transcripts in all of the studied thermoregulation-related nuclei, *viz.*, in the medial preoptic area, dorsomedial hypothalamic area, lateral parabrachial nucleus, and rostral raphe pallidus, although it should be noted that the number of the *Trpa1* transcripts was low.

DISCUSSION

During the past 20 years there were several clinical trials which compared therapeutic moderate hypothermia and normothermia in patients with severe TBI. In the majority of these trials there was no improvement of the outcome with the use of hypothermia, although there were subgroups of patients that may have benefited from hypothermia. Methodological differences prevented the direct comparison of these studies.

In the present thesis, we showed that whole-body cooling decreases the risk of death in patients with severe TBI by conducting meta-analysis of clinical trials (Olah *et al*., 2018), which were homogenous with regards to statistical, clinical, and methodological designs. With forest plot analysis of the cooling parameters we revealed that deeper and longer cooling and to a lesser extent reasonably fast rewarming are the most important to improve the outcome of severe TBI.

We introduced the COIN to assess the summed effect of the cooling, and showed that therapeutic hypothermia is beneficial in severe TBI only if the COIN is sufficiently high (Olah *et al*., 2018). The benefits of therapeutic hypothermia in severe TBI have been long debated. In 2018, the POLAR study (Cooper *et al*., 2018), a high-quality international trial, appeared to end the debate by showing that therapeutic hypothermia did not improve

mortality in severe TBI. However, the POLAR-based recommendation to abandon therapeutic hypothermia was challenged by different authors. We calculated the COIN for the POLAR study and ran a new meta-analysis (Olah, Poto, *et al*., 2021), which included the POLAR data and accounted for the cooling extent.

Our novel "POLARized" COIN analysis indicated that the results of POLAR strengthen our former conclusions about the beneficial effects of therapeutic hypothermia on death rate in severe TBI if COIN is sufficiently high. Although POLAR was a high-quality, international study, deviations from the protocol resulted in smaller extent of cooling than targeted, which could mask the benefits of therapeutic hypothermia in the overall cohort. It would be inevitable to separately analyze the outcomes in those patients who fully complied with the targeted cooling criteria and in those patients who did not. Until such result are available from POLAR or other high-quality trial(s), the idea of therapeutic hypothermia in severe TBI should not be abandoned.

The benefits of therapeutic hypothermia were also shown in the recent review by Moore *et al*. (2020). The authors applied the umbrella review methodology to several potentially low-value clinical practices and found that therapeutic hypothermia was the only one with evidence of benefit. However, the POLAR study was not included in any of the systemic reviews analyzed in the umbrella review by Moore *et al*. (2020). When the results of POLAR were translated into treatment guidelines prior to our POLARized meta-analysis (Chesnut *et al*., 2020; Hawryluk *et al*., 2019), the absence of an overall beneficial effect led to the recommendation to decrease the use of therapeutic hypothermia in severe TBI. However, some deviations from the cooling protocol occurred at different POLAR-participating centers and decreased the overall extent of cooling from "high" (targeted) to "moderate" (overall achieved) and even "low" (observed in many patients). This decrease in the COIN was likely to mask the benefits of therapeutic hypothermia in the overall cohort. Identification of the exact cooling protocol for a specified patient population would be in line with recent paradigms in the treatment of TBI, suggesting the need for targeted management of individuals or subsets of patients to improve the outcome (Sheriff & Hinson, 2015; Stocchetti *et al*., 2017).

In the second part of my work, we studied the mechanisms of H_2S-induced hypothermia in animal experiments (Olah, Rumbus, *et al*., 2021) and we concluded that fast- and slow-releasing H_2S donors cause hypothermia which is mediated by reduced thermogenesis and increased cutaneous vasodilation. The hypothermic and hypometabolic effects are triggered from the central nervous system and both of them are strongly attenuated in the absence of the TRPA1 channel. TRPA1 channels located on hypothalamic neurons within autonomic thermoeffector pathways can be suggested as the molecular targets for the H_2S-induced hypothermia.

Our aim was to find the molecular target responsible for the hypothermic response to H_2S. Temperature-sensitive members of the TRP channel superfamily can function as thermoreceptor elements in the thermoregulation system, but nonthermal activation of some of these TRP channels can also occur and contribute to the regulation of deep T_b independently from whether the given channel is a thermosensor or not, as it was discovered in case of TRPV1 (Garami *et al*., 2020; Romanovsky *et al*., 2009). With regards to an interaction between H_2S and thermosensitive TRP channels, strong evidence accumulated until present days for an action of H_2S on the TRPA1 channel in a vast number of different experimental models (Pozsgai, Bátai, *et al*., 2019), but whether TRPA1 also mediates the hypothermic effect of H_2S was unknown until now. In our work (Olah, Rumbus, *et al*., 2021), we studied the thermoregulatory response to H_2S donors (Na_2S and GYY4137) in the genetic absence of the TRPA1 channel by using *Trpa1*$^{-/-}$ mice. We showed that the hypothermic and the hypometabolic responses are both attenuated in *Trpa1*$^{-/-}$ mice as compared to their *Trpa1*$^{+/+}$ littermates. Our findings clearly indicate, for the first time to our knowledge, that hypothermia induced by either fast- or slow-releasing H_2S donors is mediated by the TRPA1 channel in unanesthetized mice.

CONCLUSIONS

In the present work, we used a novel approach to determine the efficacy of therapeutic hypothermia in TBI. In our meta-analysis, we carefully evaluated all studies identified by literature search based on statistical design, patient inclusion criteria, and the applied cooling protocol, thereby we identified studies which were homogenously designed from three aspects: statistically, clinically, and methodologically. Then, we conducted meta-analyses of these studies to evaluate the effects of therapeutic hypothermia as well as that of the individual parameters of the cooling protocol on the mortality rate of patients with severe TBI. We introduced the COIN, an integrated measure of therapeutic hypothermia calculated from three different cooling parameters, and studied its relation to mortality in severe TBI. In conclusion, including the POLAR study in our COIN-based meta-analysis suggests that the COIN should be flipped again to settle the dispute on the use of therapeutic hypothermia in severe TBI.

Seminal work by Blackstone *et al*. (2005), has re-established H_2S as a thermoregulatory mediator (Mancardi *et al*., 2009). Findings from the second part of my dissertation are consistent with the concept of H_2S-induced thermoregulatory effects are TRPA1-mediated. Furthermore, central H_2S effects induce hypothermia, in part, through inhibiting thermogenesis.

We demonstrated that administration of H_2S-donors, specifically Na_2S and GYY4137, (i) induce hypothermia in a dose-dependent fashion following i.c.v. administration; (ii) evoke hypothermia with different dynamics; (iii) do not induce significant thermoregulatory effects when delivered i.p.; and (iv) evoke the hypothermic response through the mediation of the TRPA1 channel. In sum, we showed that slow- and fast-releasing H_2S donors induce hypothermia through hypometabolism and cutaneous vasodilation in mice and that the hypothermic effect of H_2S is mediated by TRPA1 channels located in the brain, presumably on hypothalamic neurons within the autonomic thermoeffector pathways. Our findings highlight the importance of central TRPA1-mediated H_2S signaling in the thermoregulation system. In severe forms of systemic inflammation (*e.g*., septic shock), which is often

associated with hypothermia (Garami, Steiner, *et al*., 2018) and by enhanced production of H_2S (Bhatia & Gaddam, 2021; Whiteman & Winyard, 2011), the interaction between TRPA1 and H_2S can play a crucial role in the development of the response and, as perspective, may serve as a therapeutic target. Furthermore, the H_2S-induced activation of central TRPA1 channels may pave the road to the development of controlled induction of therapeutic hypothermia, but future research is needed to reveal the true thermopharmacological potential of the central TRPA1-H_2S interaction. A postulation that is also supported by Kwiatkoski *et al*., who reported that increased H_2S induces cryogenic effects during hypoxia (Kwiatkoski *et al*., 2012).

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Publications related to the subject of the thesis

- Number of publications related to the subject of the thesis: 3
- Number of publications not related to the subject of the thesis: 17
- Sum of all impact factors: 37.435
- Sum of impact factors from publications related to the topic of PhD thesis: 14.886
- All citations: 75
- Independent citations: 57

Publications related to the topic of the PhD thesis

Olah, E; Poto, L; Hegyi, P; Szabo, I; Hartmann, P; Solymar, M; Petervari, E; Balasko, M; Habon, T; Rumbus, Z; Tenk, J; Rostas, I; Weinberg, J; Romanovsky, A A; Garami A: Therapeutic whole-body hypothermia reduces death in severe traumatic brain injury if the cooling index is sufficiently high: Meta-analyses of the effect of single cooling parameters and their integrated measure. J Neurotrauma. 2018;35(20):2407-2417. **(IF: 3.754)**

Olah, E; Rumbus, Z; Kormos, V; Tekus, V; Pakai, E; Wilson, HV; Fekete, K; Solymar, M; Kelava, L; Keringer, P; Gaszner, B; Whiteman, M; Keeble, J; Pinter, E; Garami, A: The hypothermic effect of hydrogen sulfide is mediated by the transient receptor potential ankyrin-1 channel in mice. Pharmaceuticals. 2021;14(10): 992. **(IF 5.863)**

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Other publications, not related to the topic of the PhD thesis

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