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Neuroprotective mechanisms and translational potential of therapeutic hypothermia in the treatment of ischemic stroke

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Abstract
Stroke is a leading cause of disability and death, yet effective treatments for acute stroke has been very limited. Thus far, tissue plasminogen activator has been the only FDA-approved drug for thrombolytic treatment of ischemic stroke patients, yet its application is only applicable to less than 4–5% of stroke patients due to the narrow therapeutic window (< 4.5 hours after the onset of stroke) and the high risk of hemorrhagic transformation. Emerging evidence from basic and clinical studies has shown that therapeutic hypothermia, also known as targeted temperature management, can be a promising therapy for patients with different types of stroke. Moreover, the success in animal models using pharmacologically induced hypothermia (PIH) has gained increasing momentum for clinical translation of hypothermic therapy. This review provides an updated overview of the mechanisms and protective effects of therapeutic hypothermia, as well as the recent development and findings behind PIH treatment. It is expected that a safe and effective hypothermic therapy has a high translational potential for clinical treatment of patients with stroke and other CNS injuries.

Key Words: stroke; therapeutic hypothermia; drug-induced hypothermia; ischemia; cell death; inflammation

Introduction
Although recently stroke fell from the fourth to the fifth leading cause of human death in the United States, each year there are still approximately 750,000 individuals who suffer a new or recurrent stroke (ischemic or hemorrhagic) (Writing Group et al., 2016). About 610,000 of them encounter a primary stroke attack, and 185,000 have recurrent stroke events. A stroke attack occurs every 40 seconds, resulting in stroke-related deaths every 4 minutes in the United States. Due to the increasing aged population, the American Heart Association (AHA) has projected that healthcare costs associated with stroke would increase dramatically in the next 20 years according to the increased incidence and prevalence of stroke.

Despite tremendous advancements in understanding the pathogenesis and cellular/molecular mechanisms of stroke over the last few decades, thrombolytic therapy using tissue plasminogen activator (tPA) has been the only drug approved by the Food and Drug Administration (FDA) for treating acute ischemic stroke (Group, 1995). Although recent data show that the rates of tPA application has been increased between 2005 and 2009, still only < 4% of all stroke patients can benefit from the thrombolytic treatment. This is mainly due to the required early treatment and a high risk of hemorrhagic transformation associated with tPA applications (Adeoye et al., 2011). Thus, there is an urgent need to develop new therapies that have wider therapeutic windows and are more effective for more stroke patients (Shobha et al., 2011).

Over the past two decades, many neuroprotective drugs and treatments have failed the clinical translation from animal models to clinical practice due to the lack of efficacy and, in some cases, intolerable side-effects (Cheng et al., 2004). With the increasing understanding of the cellular and molecular injurious pathways and their interplays in ischemic cascades, it is now increasingly recognized that the conventional strategy of targeting a specific inhibitory or excitatory neuronal receptor or ion channel or a single signaling pathway/gene is far from enough to battle the overwhelming pathophysiologcral cascades that occur acutely, sub-acutely, and even chronically after a stroke attack. Thus, a global protection paradigm that covers different cell types including neuronal and non-neuronal cells and multiple signaling pathways is necessary to achieve clinically meaningful benefits for stroke patients.

Compelling evidence from pre-clinical research in animal models demonstrated marked protective effects of mild to moderate hypothermia (therapeutic hypothermia) against...
ischemic and hemorrhagic brain damage (Darwazeh and Yan, 2013; Chamorro et al., 2016). Therapeutic hypothermia ameliorates brain damage through inhibition of multiple pathways such as oxidative stress, inflammatory responses, metabolic disruption, and cell death signals (Katz et al., 2004; Choi et al., 2012). Furthermore, therapeutic hypothermia therapy improves functional outcomes in animal models of stroke and traumatic brain injury (TBI) (Polderman et al., 2002; Choi et al., 2012; Lee et al., 2014). In humans, multiple clinical trials involving both surface cooling and endovascular hypothermia have been effective in decreasing certain quantitative metrics that correspond with functional outcomes after TBI, including intracerebral pressure (ICP) and the mean diffusion-weighted imaging (DWI) lesion growth (Schwab et al., 1998a, b, 2001; De Georgia et al., 2004). In clinical practice, mild to moderate hypothermia (3–5°C reduction) is safe and has been used for the treatments of cardiac arrest and hypoxic-ischemic encephalopathy (Dae et al., 2003; Xiao et al., 2013). In fact, therapeutic hypothermia has been incorporated in the American Heart Association (AHA) guidelines for post-resuscitation care for more than 10 years (Sugerman and Abella, 2009). Thus, both pre-clinical and clinical evidence supports that therapeutic hypothermia has a promising potential to be an effective treatment for acute brain injury such as stroke and TBI (Schwab et al., 1998b, 2001; De Georgia et al., 2004; van der Worp et al., 2007; Torok et al., 2009; Kim et al., 2011; Yenari and Han, 2012; Lee et al., 2016a). Currently, there is no other experimental stroke therapy that has demonstrated such strong potential in both basic and clinical research, although challenges on the efficacy and the mechanism of action call for future and more specific investigations (Tahtir and Pabanej, 2016). This review will focus on emerging concepts in the protective mechanisms of therapeutic hypothermia for treating patients with stroke, as well as highlighting pharmacologically induced hypothermia (PIH), or drug-induced cooling treatments, that can be applied as an acute hypothermic treatment with some unique advantages.

**Mechanisms of Therapeutic Hypothermia against Ischemia-induced Brain Damage**

**Excitotoxicity**

Glutamate mediates excitatory synaptic transmission through the activation of ionotropic glutamate receptors that are sensitive to N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), or kainate in the nervous system. The excitatory transmission mediates normal information processing and neuronal plasticity (Won et al., 2002). It has been well established that excessive activation of glutamate receptors mediates the initial step in excitotoxicity. Upon an ischemic insult, the interruption of blood supply in the brain causes the deprivation of oxygen and glucose, leading to impaired energy metabolism (Choi, 1992; Dugan and Choi, 1994). Consequently, it increases glutamate release through membrane depolarization and the subsequent activation of the voltage gated Ca⁺⁺ channels (Choi, 1992; Dugan and Choi, 1994). Due to impaired energy synthesis, reuptake of glutamate is also interfered, which results in the excess accumulation of synaptic glutamate. The sustained over-activation of the ionotropic glutamate receptors leads to neuronal death, mostly due to necrotic cell death mechanism.

Recent reports have shown that therapeutic hypothermia prevents the accumulation or release of glutamate (Zhao et al., 2007a; Yenari and Han, 2012). Also, body temperature influences glutamate excitotoxicity during the acute phase of stroke, indicating that the release of these neurotransmitters is temperature dependent (Campos et al., 2012). Hypothermia can directly affect excitotoxicity through regulating the glutamate receptor 2 (Glur2) subunit of AMPA receptors (Colbourne et al., 2003). Hypothermia recovers the downregulation of Glur2 in hippocampal CA1 neurons after global cerebral ischemia in gerbils. After stroke, hypothermia significantly decreases brain glycine levels, which is needed to activate NMDA receptors and accelerate the function of NMDA receptors (Johnson and Ascher, 1987; Krivishvili, 2002). Additionally, hypothermia reduces the number of AMPA and NMDA receptors expressed on hippocampal neurons after stroke, which is associated with decreased infarct volume (Friedman et al., 2001; Li et al., 2011). Hypothermia also abates spreading depolarization that occurs after ischemic stroke by reducing the release of excitatory amino acids (EAA) (Nakashima and Todd, 1996).

**Oxidative stress**

Neurons are exposed to a minimum level of free radicals from both exogenous and endogenous sources in the normal condition (Dugan and Choi, 1994; Won et al., 2002). However, excess accumulation of reactive oxygen species leads to the damage of basic components for cell function and survival. Because the storage capacity of oxygen is limited in the brain, as well as there being a high probability of lipid peroxidation, neurons can be vulnerable to the change of free radical levels when oxygen supply is interrupted. Following stroke, increases in arachidonic acid, nitric oxide, glutamate, and over-activation of glutamate receptors rapidly develop in the ischemic tissue, which coincides with the production of free oxygen radicals such as superoxide (O₂⁻), peroxynitrite (NO₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH⁻), resulting in neuronal death (Dugan and Choi, 1994; Globus et al., 1995b; Yenari and Han, 2012). The production of free radicals after stroke is temperature-dependent, and the suppression of free radical production is linearly proportional to the decreased temperature (Globus et al., 1995a; Hall, 1997). As a result, hypothermia can significantly reduce the production of free radicals and maintain the endogenous antioxidant activity in injured cells (Globus et al., 1995a).

**Apoptosis**

Necrosis and apoptosis are two major forms of neuronal cell death after ischemic stroke. Necrosis is a form of cell injury where edema and cellular inflammatory responses occur,
leading to excitotoxic death. Apoptosis is the main type of programmed cell death caused by activation of a cascade of intracellular pathways. This cell death mechanism is regulated by the culmination of interactions between pro-apoptotic and anti-apoptotic signaling genes (Mattson, 2000). Hypoxia and ATP depletion trigger neurons to activate the apoptotic regulatory proteins and several cellular processes, which include mitochondria dysfunction, activation of caspase enzymes, acidosis, calcium imbalance, and other cellular energy metabolism disorders (Won et al., 2002; Xu et al., 2002). These apoptotic events are mostly responsible for some delayed and secondary brain injuries.

Experimental models of ischemia have shown that therapeutic hypothermia prevents neuronal apoptosis through decreasing p53 protein, a transcription factor which activates apoptosis and pro-apoptotic proteins, including Bak, Bax, and NAD depletion (Bargonetti and Manfredi, 2002; Choi et al., 2012; Lee et al., 2014). Hypothermia regulates the levels of apoptotic related genes such as B-cell lymphoma-2 (BCL-2), cytochrome C, and tumor necrosis factor (TNF) pathway genes (Bossemeyer-Pourie et al., 2000; Zhao et al., 2004, 2007b; Liu et al., 2008). After stroke, hypothermia stimulates anti-apoptotic proteins in the Bcl-2 family, causes the reduction of cytochrome c release into the cytosol, inhibits caspase activation, and thus enhances cell survival (Prakasa Babu et al., 2000; Zhao et al., 2004). Experimental animal models have shown that hypothermia has a beneficial effect on the dysfunction of ATP-dependent Na+/K+ pumps and Na+, K+, and Ca2+ channels and reduces the influx of calcium into the cells, abating neuronal damage (Siesjo et al., 1989; Hall, 1997).

Autophagy

Autophagy is normally a physiological catabolic process for nutrient recycling, involving degradation of damaged organelles and proteins. The process is tightly regulated by autophagy signaling pathways, and alterations in this process may lead to diseases or exacerbate damage under pathological conditions. In recent years, increased autophagy has been identified as one of the pathophysiological mechanism after ischemic stroke (Chen et al., 2014). We were one of the first groups to demonstrate that a suppressing effect of therapeutic hypothermia on autophagy contributes to the neuroprotection after ischemic stroke (Choi et al., 2012). In our investigation, autophagic activity was examined by the formation of microtubule-associated protein light chain 3 (LC3-II) and degradation levels of sequestosome 1/p62 in the penumbra region. Both autophagic factors were decreased by hypothermic treatment using the neurotensin receptor agonist ABS-201. LC3-labeled autophagosome formation and TUNEL/LC3/NeuN triple-labeled cells were also decreased by the treatment. At about the same time, a different group reported that ischemia and reperfusion stimulate cell autophagy and cause cell death, which can be attenuated by mild hypothermia (Cheng et al., 2013). More recent papers demonstrated that hypothermia can inhibit autophagic cell death after TBI and spinal cord injury (Jin et al., 2015, 2016; Seo et al., 2015). The mechanism underlying the anti-autophagy action of therapeutic hypothermia is an active area of current research.

Inflammation

Inflammatory mechanisms are activated after brain ischemia and act as important mediators in the pathogenesis of stroke-induced primary and secondary injuries (Vila et al., 2000; Gelderblom et al., 2009). Although a certain level of inflammation has beneficial effects required for tissue recovery and repairing, many reports have shown that inflammation is a major pathological mechanism underlying ischemic brain injury (Lakhan et al., 2009; Jin et al., 2010). During ischemia, inflammation is characterized by the production of pro-inflammatory cytokines such as TNF-α, interleukin-1β (IL-1β), IL-6, and anti-inflammatory cytokines such as IL-10, as well as the accumulation of neutrophils and the activation of microglia in the injured brain (Huang et al., 2006). Also, ischemia-mediated neuronal damage induces the synthesis and release of chemokines, such as monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and IP-10 (interferon-inducible protein), which can recruit microglia, monocytes, and neutrophils into the ischemic region (Rappert et al., 2004; Wang et al., 2008; Jiang et al., 2016).

Hypothermia reduces the expressions of this pro-inflammatory immune response such as TNF-α and IL-1β, but it also regulates the expression of some anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) (Matsui and Kakeda, 2008; Jiang et al., 2016; Lee et al., 2016b). Accumulated data suggest that MCP-1 and MIP-1α play crucial roles in ischemia-mediated cellular damage. Upregulation of MCP-1 and MIP-1α were observed after ischemia; while MCP-1 knockout mice show reduced infarct volume after ischemia (Che et al., 2001; Hughes et al., 2002; Wang et al., 2008; Strecker et al., 2013). In addition, MIP-1α injection exacerbated brain infarction but a broad-spectrum chemokine receptor antagonist using viral macrophage inflammatory protein-2 (vMIP-2), prevented neuronal damage from ischemic insults (Takami et al., 2001; Wang et al., 2008). Hypothermia attenuated the expression levels of chemokines such as MCP-1 and MIP-1α (Lee et al., 2016b). Recent reports have demonstrated that hypothermia prevented inflammation-mediated cellular damage through regulating both activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and Janus kinase (JAK) and signal transducer/activator of transcription pathway (STAT) signaling.

Microglia/macrophages are highly flexible cells with diverse phenotypes that are involved in the generation of distinct effector cells and functions (Murray and Wynn, 2011; Hu et al., 2012). Various cytokines by diverse stimuli lead to the development of M1 or M2 subtypes, which can express different levels of cell surface markers and secrete mediators such as scavenger receptors, chemokines, and cytokines. Recently, we showed that hypothermia shows...
Hypothermia prevents the activation of proteases responsible for degrading the extracellular matrix, such as the matrix metalloproteinases (MMPs) which are known to degrade tight-junction proteins, transport proteins, endothelial cells, astrocytes, and pericytes. Hypothermia prevents the activation of proteases responsible for degrading the extracellular matrix, such as the matrix metalloproteinases (MMPs) which are known to degrade tight-junction proteins, transport proteins, endothelial cells, astrocytes, and pericytes.

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Implication in Clinical Stage

Potential Therapeutic Strategy and

Invasive in the case of endovascular cooling. The long course of surface cooling often requires the concomitant use of anesthetics and paralytics in order to curb shivering, which are performed with endotracheal intubation and ventilation, which carry side effects, such as pulmonary infections (Hemmern and Lyden, 2007). The invasive nature of endovascular cooling can result in increased risk of infections and bleeding, and is not as universally applicable, due to the need for a skilled medical personnel to perform the catheter placement (Glushakova et al., 2016). In addition, physical cooling (PC) evokes shivering responses, which is a defensive metabolic adaptation to cold, as well as peripheral vasocon-
striction. This systemic response makes effective and accurate cooling difficult, and most often the cooling procedure has to be performed under general anesthesia or sedation reagents (Schwab et al., 1998a; Sessler, 2009). Another possible limitation of hypothermia is its association with systemic infections, including pneumonia and sepsis, although this side effect can be treatable (Hemmen et al., 2010; Geurts et al., 2014). Other potential side effects of hypothermic treatment includes hypothermia-induced diuresis, resulting in hypovolemia and electrolyte depletion, thrombocytopenia, and bradycardia (Schwab et al., 2001). Furthermore, while clinical trials have shown hypothermia to be a feasible treatment, there is conflicting data as to whether hypothermia can effectively improve functional outcomes (Krieger et al., 2001; Schwab et al., 2001; Polderman et al., 2002; De Georgia et al., 2004; Nielsen et al., 2013; Lyden et al., 2016). This is largely due to the plethora of parameters associated with TTM, aside from simply the temperature reduction. Even if patients undergo physical cooling to optimal temperatures of 33–35°C, other factors that could affect the outcomes include the severity, location and subtypes of the injury, the time of intervention, the speed/duration of the cooling treatment, and finally other pharmacological agents (e.g., anesthetics or paralytics) administered (Wan et al., 2014; Subramaniam et al., 2015). All of these factors contribute to the inconsistent findings, but they also provide tremendous potential for optimization of therapeutic hypothermia in order to ensure its efficacy.

Recent early pilot studies with clinical trials have provided modest evidence suggesting both the feasibility and efficacy of therapeutic hypothermia, especially when performed in conjunction with thrombolysis (Hemmen et al., 2010; Piironen et al., 2014). These studies have parlayed into a phase III multi-center randomized controlled trial, EuroHYP-1, and the results will have significant ramifications on the implementation of this clinical strategy (Worp et al., 2014). Nevertheless, considering the modest success associated with therapeutic hypothermia thus far, as well as the current ESO guidelines that discourage the use of current physical means of hypothermia to treat ischemic stroke, it is important to continue the exploration of other avenues that can induce hypothermia in safer and more efficient ways (Ntaios et al., 2015). This is important because it may be possible that the aforementioned limitations may be associated more or less with physical cooling, either with surface cooling or endovascular methods. Thus, increasing research attention has recently been drawn to pharmacological reagents that have shown cooling effects in animals and/or humans.

**Pharmacologically induced hypothermia**

Pharmacologically induced hypothermia (PIH), which targets the peripheral or central thermoregulatory mechanisms, has emerged as a more efficient and safer treatment for brain disorders (Choi et al., 2012; Wei et al., 2013; Cao et al., 2014; Lee et al., 2014). PIH allows for greater control of temperature changes necessary for TTM. More importantly, we have shown that a PIH therapy targeting the central thermoregulator may suppress external cooling-induced shivering and tachycardia and achieve more rapid and effective hypothermia (Feketa et al., 2013; Lee et al., 2016a). It is well known that shivering, as a physiological defense mechanism against body cooling, is a strong and troublesome reaction that is invariably coupled with physical cooling. This reaction often prevents effective and accurate control of hypothermic treatment. Normally, general anesthesia and/or sedation have to be applied to battle with shivering during physical cooling. PIH using hypothermic drugs acting at the central or peripheral thermoregulatory receptors/channels provides a mechanism-based strategy to eliminate cooling-associated shivering and other responses. This unique effect creates multimodal benefits, including the administration of hypothermic treatment in awake patients without the need for general anesthesia and the avoidance of a number of side effects seen with conventional physical cooling methods.

Currently, there are several major classes of pharmacological agents that are categorized based on their mechanisms of action and thermoregulation target: neurotensin, transient receptor potential vanilloid 1 (TRPV1), cannabinoid, opioid, thyroxine derivatives, dopamine, gas, and adenosine derivatives (Table 1). Other drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) that show mild TTM action have also been investigated in the context of therapeutic hypothermia but are not as effective for inducing and maintaining the minimum temperature reductions (Feigin et al., 2002).

Our group has developed the second generation of hypothermic compounds acting as a selective neurotensin receptor 1 (NTR1) agonist that can pass through the BBB and efficiently reduce the body and brain temperature in a dose-dependent manner (Choi et al., 2012). For example, HPI-201 and HPI-363 (also known as ABS-201, ABS-363) are NTR1 agonists acting at the hypothalamic thermoregulatory set point. It possesses a high affinity for human NTR1, exhibits BBB permeability, and effectively induces regulated hypothermia in rodents, resulting in protective effects and improved functional recovery after ischemic or hemorrhagic stroke or TBI in mice (Hadden et al., 2005; Choi et al., 2012; Wei et al., 2013; Lee et al., 2014, 2016a, b). Other groups showed that an agonist at transient receptor potential cation channel subfamily vanilloid member 1 (TRPV1) can decrease temperature via regulation of the peripheral temperature sensitive channel and confer neuroprotective effects after stroke in animals (Feketa et al., 2013; Cao et al., 2014).

Overall, PIH confers several significant advantages over physical cooling that allow for more feasible clinical implementation. These include the targeting of the thermoregulatory center in the hypothalamus in order to decrease the cold set point to reduce compensatory physiological responses to hypothermia. These effects, such as shivering, piloerection, and vasoconstriction, cause discomfort for the patients.
and reduces the efficacy of hypothermia (Díaz and Becker, 2010). Importantly, PIH and physical cooling are not mutually exclusive, and the best paradigm may ultimately be a combination therapy of the two in order to create a reliable regimen that uses a lower minimum pharmacological dose (to mitigate potential side effects) to induce hypothermia, but that also dulls the cold response (Muzzi et al., 2013; Liu et al., 2016). Preliminary data has already suggested that combination therapy can provide synergistic effects that augment the benefits of either treatment alone (Lee et al., 2016a).

Conclusion

Therapeutic hypothermia is one of the most promising therapies for neuroprotection against brain injuries such as stroke. The mechanism of action is multifaceted. Therapeutic hypothermia provides a global brain protection rather than targeting a single pathways or a single gene. Therapeutic hypothermia can regulate multiple pathways including excitotoxicity, oxidative stress, apoptosis, autophagy and promote regenerative activities. We expect that these actions can be utilized to show synergistic effects with other neuroprotective treatments such as ITP and help to develop combinatory stroke therapy for clinical treatments. To this end, PIH has been demonstrated to be an effective and more efficient hypothermic therapy that shows high feasibility and translational potential for clinical applications. Further study will be needed to have an in-depth understanding of the multiple mechanisms underlying therapeutic hypothermia and reinforced efforts are necessary to verify the efficacy of PIH compounds in large animals such as non-human primates. It is expected that safer and more effective hypothermic therapies may help develop more clinical treatments for stroke and other intractable brain disorders.

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