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Regulation of therapeutic hypothermia on inflammatory cytokines, microglia polarization, migration and functional recovery after ischemic stroke in mice

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ABSTRACT

Stroke is a leading threat to human life and health in the US and around the globe, while very few effective treatments are available for stroke patients. Preclinical and clinical studies have shown that therapeutic hypothermia (TH) is a potential treatment for stroke. Using novel neurotensin receptor 1 (NTR1) agonists, we have demonstrated pharmacologically induced hypothermia and protective effects against brain damages after ischemic stroke, hemorrhage stroke, and traumatic brain injury (TBI) in rodent models. To further characterize the mechanism of TH-induced brain protection, we examined the effect of TH (at ±33 °C for 6 h) induced by the NTR1 agonist HPI-201 or physical (ice/cold air) cooling on inflammatory responses after ischemic stroke in mice and oxygen glucose deprivation (OGD) in cortical neuronal cultures. Seven days after focal cortical ischemia, microglia activation in the penumbra reached a peak level, which was significantly attenuated by TH treatments commenced 30 min after stroke. The TH treatment decreased the expression of M1 type reactive factors including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-12, IL-23, and inducible nitric oxide synthase (iNOS) measured by RT-PCR and Western blot analyses. Meanwhile, TH treatments increased the expression of M2 type reactive factors including IL-10, Fizz1, Ym1, and arginase-1. In the ischemic brain and in cortical neuronal/BV2 microglia cultures subjected to OGD, TH attenuated the expression of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α), two key chemokines in the regulation of microglia activation and infiltration. Consistently, physical cooling during OGD significantly decreased microglia migration 16 h after OGD. Finally, TH improved functional recovery at 1, 3, and 7 days after stroke. This study reveals the first evidence for hypothermia mediated regulation on inflammatory factor expression, microglia polarization, migration and indicates that the anti-inflammatory effect is an important mechanism underlying the brain protective effects of a TH therapy.

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1. Introduction

Stroke is a leading cause of adult death and disability in the United States and around the globe (Go et al., 2014; Murphy et al., 2013). Despite advancements in understanding the pathogenesis and cellular, molecular mechanisms of stroke pathophysiology over the last few decades, thrombolytic therapy with tissue plasminogen activator (tPA) and endovascular recannulation are only FDA-approved clinical treatments for acute stroke. Due to the limited therapeutic window and hemorrhagic risk of tPA, only <4% of stroke patients can be benefited with the thrombolytic treatment (Shobha et al., 2011). Therefore, therapies that can benefit more stroke patients are urgently needed.

Abbreviations: ANOVA, analysis of variance; BBB, blood-brain barrier; CCA, common carotid artery; Iba1, ionized calcium binding adaptor molecule 1; IFN-γ, interferon gamma; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; IL-12, interleukin-12; IL-23, interleukin-23; iNOS, inducible nitric oxide synthase; IOS, intrinsic optical signals; IP-10, interferon-inducible protein; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; NT, neurotensin; NTR1, neurotensin receptor 1; PIH, pharmacologically induced hypothermia; TH, therapeutic hypothermia; TNF-α, tumor necrosis factor-α; TTC, 2,3,5-triphenyltetrazolium chloride; TTM, targeted temperature management.

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Therapeutic hypothermia (TH) has been incorporated in the American Heart Association guidelines for post-resuscitation care for more than 10 years (Sugerman and Abella, 2009). In clinical practice, mild to moderate hypothermia (3–5 °C reduction) is safe and has been used for the treatments of cardiac arrest and neonatal hypoxic-ischemic encephalopathy (Dae et al., 2003; Xiao et al., 2013). Growing evidence from preclinical and clinical studies shows that TH, also referred as targeted temperature management (TTM), is a potential effective treatment for stroke (Dietrich and Bramlett, 2010). Compelling evidence from pre-clinical research in animal models demonstrated marked protective effects against ischemic and hemorrhagic brain damage. TH therapy prevents brain damages through inhibition of multiple pathways such as oxidative stress, inflammatory responses, metabolic disruption, and cell death signals (Choi et al., 2012; Katz et al., 2004; Truettner et al., 2005). TH therapy improves functional outcomes in animal models and human patients of stroke and TBI (Choi et al., 2012; Lee et al., 2014; Polderman et al., 2002). However, hypothermia induction using physical cooling methods such as a cooling pad/blanket are generally slow to reach the target temperature (e.g. 2–8 h) in humans (Schwab et al., 1998). Although recent methods using intravenous heat-exchange or infusion have provided better and faster control of core temperature (Polderman et al., 2015), the forced cooling commonly triggers body defensive reactions such as shivering and vasoconstriction, making the cooling process and accurate temperature control very challenging. As a result, sedation of general anesthetics and/or muscle relaxants has to be applied, which increased the risk of adverse effects such as lung infection and coagulopathy (Oddo et al., 2016).

Alternatively, pharmacologically induced hypothermia (PIH) targets the brain thermoregulatory center and/or peripheral temperature sensors. This new approach may provide more efficient and safer cooling methods. Among the agents that can be used for PIH, neurotensin receptor 1 (NTR1) agonists are effective compounds that can achieve regulated reductions of body and brain temperatures (Fantegrossi et al., 2005). Our novel neurotensin derivatives such as HPI-201 (formally ABS-201) has the chemical structure of CH3-homolys-Arg-Pro-Tyr-*tert*-Leu-Leu-COOH and can cross the blood brain barrier (BBB). They show high affinity for human NTR1 and induce regulated hypothermia in a dose-dependent manner (Hadden et al., 2005; Orwig et al., 2009). Our group has demonstrated that acute and delayed administrations of HPI-201 and HPI-363 show marked protective effects against brain injury after ischemic and hemorrhagic strokes as well as traumatic brain injury (TBI) in adult and neonatal rodent models (Choi et al., 2012; Lee et al., 2014; Lee et al., 2016b; Wei et al., 2013). NRT1 agonists additionally show antinociceptive and antipsychotic actions (Guillemette et al., 2012; Mechanic et al., 2009). We have identified that the cooling action of the NRT1 compounds, but not their other pharmacological effects, is responsible for observed neuroprotection because when the body temperature is forced to stay at normal level after the drug administration its neuroprotective effect is eliminated (Choi et al., 2012; Gu et al., 2015; Lee et al., 2014; Wei et al., 2013). In addition, we verified that the tested NTR1 compounds do not alter basic physiological parameters including blood pressure, blood glucose, and blood pH, although heart beat increases (Choi et al., 2012; Lee et al., 2014). Therefore, these NTR1 agonists such as HPI-201 are suitable for the induction of TH and HPI-201 was tested in this investigation along with physical (ice/cold air) cooling. Our previous studies demonstrated that the pharmacological TH protects the brain through suppressing apoptosis, autophagy, and BBB damage (Choi et al., 2012; Lee et al., 2014; Wei et al., 2013). However, it is not entirely clear which pathological signaling/s and cellular mechanism/s may be regulated by TH induced by either pharmacological and physical means.

Inflammatory mechanisms are activated after brain ischemia and act as important mediators in the pathogenesis of secondary injury after stroke and TBI (Herz et al., 2014; Vila et al., 2000). Activated glial cells, infiltrated leukocytes, and release of pro-inflammatory cytokines can be detrimental in the ischemic brain and contribute to infarct formation (Sladojevic et al., 2014; Yenari et al., 2010). The inhibition of pro-inflammatory mediator production has been shown to prevent brain injury after stroke (Gelderblom et al., 2012). Microglia is a highly plastic cell with coexisting diverse phenotypes (polarization of M1 and M2) that can be beneficial or detrimental in response to specific microenvironment signals. M1 microglia are considered to be pro-inflammatory and secrete TNF-α, monocyte chemoattractant protein (CCL2/MCP-1) and inducible nitric oxide synthase (iNOS) (Murray and Wynn, 2011). They also express IL-1β, IL-18 and IL-23 through activation of the inflammasome (Ransohoff and Brown, 2012). M2 microglia is thought to be healing cells that are involved with neuroprotection and repair after injury. M2 activation is induced by Th2 cytokines IL-4, IL-13, IL-10, Fizz1, Ym1, or arginase-1, which can enhance the expression of scavenger receptors and pro-angiogenic factors, to have neuroprotective effects. These dual roles of microglia polarization are seen in stroke (Hu et al., 2012; Won et al., 2015). Whether TH, induced either by physical cooling or pharmacological reagents, may affect microglia activation/polarization has so far not been explored.

In the present study, we specifically tested the effect of TH therapies on inflammatory responses after a hypoxic/ischemic insult in cultured cells as well as in a stroke model of adult mice. Our data provide novel evidence that TH can suppress the expression of inflammatory factors, attenuate microglia activation, migration and promote functional recovery after stroke. It is suggested that comprehensive anti-inflammatory effects play a pivotal role in the brain protection achieved by a TH therapy after stroke.

2. Materials and methods

2.1. Chemicals

The synthesis of proprietary NTR1 agonist HPI-201 was performed using the procedures described previously (Hadden et al., 2005; Orwig et al., 2009). The full chemical structure of HPI-201 was provided in our previous report (Choi et al., 2012).

2.2. Animals and focal cerebral ischemic stroke model of mice

Adult male C57BL/6 mice (8–12 weeks, 22–28 g) were used in this study. The mice were housed in standard cages in 12-h light/12-h dark cycle in the Emory University animal facility where the room temperature was kept at 22 ± 1 °C. Food and water were provided ad libitum. Animals were randomly divided into sham control and experimental groups. In neuroprotection experiments, 6 mice were used in sham control and 12 mice in stroke or stroke plus treatment group. For the measurements of mRNA and protein levels, 3 and 5 animals were included in sham control and stroke/treatment groups, respectively. Immunohistochemical examinations used 7 or 8 mice in each group. In the study on microglia activation, each group contained 3–4 animals. For behavioral tests, 5 mice were tested as sham control and 12 mice were in each stroke/treatment group. In top scan and home cage measurements, 3–5 mice were included in each group.

Focal cerebral ischemic stroke targeting the right somatosensory cortex involving mainly the barrel cortex was induced by occlusion of distal branches of the middle cerebral artery (MCA), as described previously. Animals were anesthetized by 2% isoflurane in 100% Oxygen, followed by a maintenance dose of 1.5% isoflurane. The animal's head was held in a non-invasive holder between the palate and the bridge of the nose without interfering with breathing. A 10-mm incision was made midway between the right eye and ear. The underlying muscle was separated, and a 4-mm diameter circle was incised in the skull with a dental drill with a sterile round 1.5-mm bit to the inner layer of cranium; then the encircled bone was evulsed with a dental tool. The transparent dura was left intact. By videomicroscopy the barrel cortex is localized by intrinsic optical signals (IOS) during whisker stimulation (Wei et al., 1995). Three distal branches of the MCA enclosing the right

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