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Chemerin suppresses neuroinflammation and improves neurological recovery via CaMKK2/AMPK/Nrf2 pathway after germinal matrix hemorrhage in neonatal rats

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ABSTRACT

Chemerin, an adipokine, has been reported to reduce the production of pro-inflammatory cytokines and neutrophil infiltration. This study investigated the role of Chemerin and its natural receptor, ChemR23, as well as its downstream mediator calmodulin-dependent protein kinase kinase 2 (CAMKK2)/adenosine monophosphate-activated protein kinase (AMPK) /Nuclear factor erythroid 2-related factor 2 (Nrf2) following germinal matrix hemorrhage (GMH) in neonatal rats, with a specific focus on inflammation. GMH was induced by intraparenchymal injection of bacterial collagenase (0.3U) in P7 rat pups. The results demonstrated that human recombinant Chemerin (rh-Chemerin) improved neurological and morphological outcomes after GMH. Rh-Chemerin promoted accumulation and proliferation of M2 microglia in periventricular regions at 72 h. Rh-Chemerin increased phosphorylation of CAMKK2, AMPK and expression of Nrf2, and decreased IL-1beta, IL-6 and TNF-alpha levels. Selective inhibition of ChemR23/CAMKK2/AMPK signaling in microglia via intracerebroventricular delivery of liposome-encapsulated specific ChemR23 (Lipo-alpha-NETA), CAMKK2 (Lipo-STO-609) and AMPK (Lipo-Dorsomorphin) inhibitor increased the expression levels of IL-1beta, IL-6 and TNF- alpha, demonstrating that ChemR23/CAMKK2/AMPK signaling in microglia suppressed inflammatory response after GMH. Cumulatively, these data showed that rh-Chemerin ameliorated GMH-induced inflammatory response by promoting ChemR23/CAMKK2/AMPK/Nrf2 pathway, and M2 microglia may be a major mediator of this effect. Thus, rh-Chemerin can serve as a potential agent to reduce the inflammatory response following GMH.

1. Introduction

Germinal matrix hemorrhage (GMH) is a common cerebrovascular event that affects up to 20% of premature infants, and leads to severe long-term neurological and cognitive deficits, including cerebral palsy and mental retardation (Ballabh, 2014). After the hemorrhage occurs, the damage of brain tissue is amplified by the activation of inflammatory cascades, which are responsible for the further neurodegeneration (Brown and Neher, 2010; Chen et al., 2015; Tao et al., 2016; Zhou et al., 2014). Given the extended inflammation-induced tissue destruction, the suppression of inflammatory response could be a particularly important intervention to limit GMH-induced brain injury.

Microglia play critical roles in the immune response after GMH (Aronowski and Zhao, 2011). Traditionally, microglia was considered injurious in the past-hemorrhagic brain due to the production of inflammatory cytokines (Lan et al., 2017). However, emerging data show that microglia have a beneficial role in neonatal stroke and that depletion of microglia exacerbates neuroinflammation and brain injury in neonatal ischemic stroke (Chip et al., 2017; Fernandez-Lopez et al., 2016). Furthermore, preclinical studies show that regulation of the immune response after GMH involves an M1 to M2 phenotype transformation in microglia, and that promoting M2 polarization inhibits the expression of pro-inflammatory cytokines (Tao et al., 2016; Xu et al., 2015).

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Chemerin is synthesized as a 163-amino acid precursor, and released by several tissues, including immune cells, liver, and spleen (Mariani and Roncucci, 2015). This precursor is low biological activity, which needs further processing at the C terminus to be the active form (Kennedy and Davenport, 2018). During the tissue clearance in the terminal phases of acute response, serine or cysteine proteases released by both macrophages and apoptotic cells cleaved this precursor to generate anti-inflammatory and prophagocytic peptides (Mariani and Roncucci, 2015). Chemerin or Chemerin derived peptide has been demonstrated to inhibit the production of inflammatory cytokines in macrophages, reduce neutrophil recruitment and promote phagocytosis of apoptotic cells (Cash et al., 2013; Cash et al., 2010, 2008; Lin et al., 2017). Neutralization of endogenous chemerin exacerbates peritonitis, indicating the potent anti-inflammatory effect of endogenous Chemerin (Cash et al., 2008; Graham et al., 2009). Currently, three proteins have been identified as Chemerin receptors: the natural receptor, Chemerin Receptor 23 (ChemR23), as well as two other receptors, chemokine CC motif receptor-like 2 (CCRL2) and G protein-coupled receptor 1 (GPR1) (Mariani and Roncucci, 2015). Of note, Chemerin was unable to suppress proinflammatory cytokines and neutrophil infiltration in ChemR23(-/-) mice, suggesting that Chemerin exerts its anti-inflammatory effect primarily through ChemR23 (Cash et al., 2010, 2008). However, the effect of GMH on Chemerin/ChemR23 signaling in neonates and therapeutic benefits of Chemerin in an animal model of GMH remained unexplored.

The anti-inflammatory effects of Chemerin/ChemR23 signaling in brain hemorrhage are far from thoroughly understood. *In vitro* studies show that Chemerin potentiated the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) in endothelial cells and granulosa cells (Reverchon et al., 2012; Shen et al., 2013). Moreover, activation of calmodulin-dependent protein kinase kinase 2 (CAMKK2) reduced the transcription of pro-inflammatory cytokines and promoted the M2 polarization though the phosphorylation of AMPK in LPS-stimulated BV2 and primary microglial cells (Xu et al., 2015). Recent studies also revealed that nuclear factor erythroid 2-related factor 2 (Nrf2), one of the major downstream mediators of AMPK, contributed to hematoma clearance in brain hemorrhage (Zhao et al., 2015), suggesting that Nrf2 may modulate microglial polarization and function in hemorrhagic stroke (Lan et al., 2017).

Based on the above-mentioned evidence, we hypothesized that rh-Chemerin treatment would reduce pro-inflammatory cytokines, promote M2-like microglia polarization and alleviate neurological deficits in neonatal rat models of GMH and that these beneficial effects might be mediated by CAMKK2/AMPK/Nrf2 signaling. We also postulated that rh-Chemerin would reduce ventricular dilation by suppressing neutrophils infiltration in choroid plexus of the ventricle.

2. Methods

2.1. Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Loma Linda University. All studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals and complied with the ARRIVE guidelines. Two hundred and fifty-five P7 Sprague–Dawley neonatal pups (weight = 12–14 g, Harlan, Livermore, CA) were randomly subjected to either Sham (n = 32) or GMH (n = 223) group. All pups were kept in rooms with controlled temperature and 12-hour light/dark cycle, and given *ad libitum* access to food and water.

2.2. Germinal matrix hemorrhage (GMH) model

The general procedure for inducing GMH in unsexed P7 rats using collagenase infusion was performed as previously described (Lekic et al., 2015). In brief, pups were anesthetized with isoflurane (3.0% induction, 1.0–1.5% maintenance) on a stereotaxic frame. The skin was incised on the longitudinal plane to expose the bregma. A burr hole (1 mm) was drilled on the skull (1.6 mm lateral, 1.5 mm anterior to the bregma), and a 27-gauge needle was inserted (2.7 mm deep from the dura) for collagenase (0.3 unites of clostridial collagenase VII-S, Sigma-Aldrich, MO) infusion (3μl/3min) using a 10μl Hamilton syringe (Hamilton Co, Reno, NV, USA) guided by a microinfusion pump (Harvard Apparatus, Holliston, MA). The core temperature was maintained at 37 °C. After infusion, the needle was left in place for additional 10 min to prevent possible leakage and then was withdrawn at rate of 0.5 mm/min. After infusion, the pups were then placed back to the dams until sacrifice.

2.3. Drug administration

Recombinant human Chemerin protein (rh-Chemerin, Abcam) was dissolved in saline as described previously (Shi et al., 2017). Pups were randomly assigned to receive rh-Chemerin (3μg/kg/day, 9 μg/kg/day or 27 μg/kg/day) or saline via intraperitoneal or intranasal administration at 1 h post-GMH and then once daily for 3 days (short-term study) or 7 days (long-term study). The dosage and treatment regimen were based on previous studies (Brunetti et al., 2011; Flores et al., 2016; Shi et al., 2017; Zhao et al., 2014).

2.3.1. Intranasal administration of recombinant Chemerin

Intranasal administration was performed as previously described (Doyle et al., 2014; Rodriguez-Frutos et al., 2016; Topkoru et al., 2013). Under anesthesia, pups were administrated phosphate buffered saline (PBS) or recombinant Chemerin dissolved in PBS at 2 μl per drop every 2 min. A total volume of 6 μl was delivered into the bilateral nares (alternating nostrils at one time).

2.3.2. In vivo RNAi

Rat-derived ChemR23 siRNA (0.5 nmol/2μl, Life Technologies) or scramble siRNA (2μl, Life Technologies) was delivered via intracerebroventricular injection at 24 h prior to GMH induction (1.5 mm anterior, 1.5 mm lateral to the bregma and 1.7 mm deep on the ipsilateral ventricle) (Chen et al., 2017).

2.3.3. Liposomes administration

Liposomes (FormuMax) that contain a lipid fluorescent dye, Fluorescein DHPE (Lipo-DHPE), Alpha-NETA (ChemR23 specific inhibitor, Lipo-Alpha-NETA, Santa Cruz Biotechnology) (Graham et al., 2014), STO-609 (CAMKK2 specific inhibitor, Lipo-STO-609, Santa Cruz Biotechnology) (Cary et al., 2013) or Dorsomorphin (AMPK specific inhibitor, Lipo-Dorsomorphin, Santa Cruz Biotechnology) (Shi et al., 2017) were prepared according to the manufacturer's protocol. Intra-liposomal Alpha-NETA, STO-609 or Dorsomorphin concentration determined by microplate reader system (400 nm, SpectraMax i3x, Molecular Devices), was 6 μg/μl 1 μg/g (2μl) rat was intracerebroventricularly administered into contralateral ventricle of P6 rats of either sex.

2.4. Histological analysis

Pups were deeply anesthetized with isoflurane (≥5%), then transcardially perfused with ice-cold PBS followed by 10% formalin. Brains were post-fixed in 10% formalin overnight at 4 °C, cryoprotected in 30% sucrose in PBS at 4 °C for 72 h, snap frozen in

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