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Research Paper

CpG-ODN exerts a neuroprotective effect via the TLR9/pAMPK signaling pathway by activation of autophagy in a neonatal HIE rat model

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ABSTRACT

Hypoxic Ischemic Encephalopathy (HIE) is an injury caused to the brain due to prolonged lack of oxygen and blood supply which results in death or long-term disabilities. The main aim of this study was to investigate the role of Cytosine-phospho-guanine oligodeoxynucleotide (CpG-ODN) in autophagy after HIE.

Ten-day old (P10) rat pups underwent right common carotid artery ligation followed by 2.5 h of hypoxia as previously described by Rice-Vannucci. At 1 h post HIE, rats were intranasally administered with recombinant CpG-ODN. Time-course expression levels of endogenous key proteins, TLR9, pAMPK/AMPK, LC3II/I, and LAMP1 involved in CpG-ODN's protective effects were measured using western blot. Short (48 h) and long (4 w) term neurobehavior studies were performed using righting reflex, negative geotaxis, water maze, foot fault and Rota rod tests. Brain samples were collected after long term for histological analysis. Furthermore, to elucidate the pathway via which CpG-ODN confers protection, TLR9 and AMPK inhibitors were used. Time course results showed that the expression of TLR9, pAMPK/AMPK, LC3II/I, LAMP1 increased after HIE. Neurobehavioral studies showed that HIE induced a significant delay in development and resulted in cognitive and motor function deficits. However, CpG-ODN ameliorated HIE-induced outcomes and improved long term neurological deficits. In addition, CpG-ODN increased expression of pAMPK/AMPK, p-ULK1/ULK1, P-AMBRA1/AMBRA1, LC3II/I and LAMP1 while inhibition of TLR9 and AMPK reversed those effects. In summary, CpG-ODN increased HIE-induced autophagy and improved short and long term neurobehavioral outcomes which may be mediated by the TLR9/pAMPK signaling pathway after HIE.

1. Introduction

Hypoxic-ischemic encephalopathy (HIE) is a major cause of morbidity and mortality in infants (Vannucci et al., 1999). HIE affects the proper development of the immature brain and subsequently leads to long term disabilities such as epilepsy, mental retardation, cerebral palsy and behavioral difficulties (Vannucci et al., 1999). HIE causes delayed cell death via excitotoxicity, inflammation, and oxidative stress (McLean and Ferriero, 2004; Lorenz et al., 1998; Doycheva et al., 2013; Shetty, 2015).

Autophagy is a process that degrades cytoplasmic components as a result of stressful conditions (Mizushima, 2007). It is linked to cellular processes as diverse as cell survival, cell death, pathogen clearance and antigen presentation (Münz, 2009; Dalby et al., 2010; Levine et al., 2011). Several studies have reported that autophagy can be induced by hypoxia-ischemia and may preserve neurons during an ischemic event (Carloni et al., 2014; Carloni et al., 2010; Carloni et al., 2008). Therefore, promoting autophagy has attracted attention in the treatment of HIE.

TLR9 is an integral membrane receptor with an N-terminal ligand recognition domain, a single transmembrane helix, and a C-terminal cytoplasmic signaling domain. It is located intracellularly in endosomes and endoplasmic reticulum (Sanjuan et al., 2007). It has been shown to play a role after various injuries (Crack and Bray, 2007). Some studies

Abbreviations: HIE, Hypoxic-ischemic encephalopathy; CpG-ODN, Cytosine-phospho-guanine oligodeoxynucleotide

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have shown that number of positive cells of TLR9 significantly increased in the ischemic cortex at 24 h after ischemic stroke (Kumagai et al., 2008). There have been further reports that TLR9 mediated cellular protection by modulating energy metabolism in cardiomyocytes with activation of an AMP-activated kinase (AMPK) (Shintani et al., 2013; Shintani et al., 2014). Other papers have shown that AMPK can induce autophagy by activation of ULK1/2 while ULK1/2 further induced expression of AMBRA1. At last, the LC3 and LAMP1, autophagy markers, increased by activation of pULK1/2 and pAMBRA1 (Efeyan and Sabatini, 2010; Gwinn et al., 2008; Kim et al., 2008; Lee et al., 2010). Based on previous findings we hypothesized that TLR9 and its downstream signaling molecules (AMPK, ULK, AMBRA, LC3, and LAMP3) may be involved in neuronal protection after an ischemic event.

Cytosine-phospho-guanine oligodeoxynucleotide (CpG-ODN) is a TLR9 ligand, which has been used in human clinical trials to improve vaccines for cancer, allergy and infectious disease (Klinman et al., 2008). It has been reported to activate TLR9, improve cell survival, prevent cell apoptosis, attenuate cardiac dysfunction after I/R, ameliorate Alzheimer's disease-related pathophysiology and induce neuroprotection against ischemic injury (Dong et al., 2016; Cao et al., 2013; Gambuzza et al., 2014).

We hypothesized that treatment with CpG-ODN will promote autophagy via activation of the TLR9/AMPK/ULK/AMBRA pathway and hence provide neuroprotection and improve neurological function after HIE. Ten-day old unsexed Sprague-Dawley rat pups were used. This project can help elucidate CpG-ODN's protective effects and lead to new therapeutic strategies for HIE patients.

2. Material and methods

2.1. Animals

All experiments were approved by the Loma Linda University Institutional Animal Care and Use Committee. Animals were cared for per the guidelines of the committee. Sprague-Dawley rat mothers, with litters of 10–12 pups, were purchased from Harlan Labs (Livermore, CA). A total of 168 ten-day old unsexed Sprague-Dawley rat pups were used. All animals were randomly assigned to groups generated by excel. All researchers performing animal testing were blinded.

2.2. HIE model

HIE model was induced as previously described using the standard Rice-Vannucci Model (Vannucci et al., 1999; Chen et al., 2011). P10 pups of both sexes were used. An aseptic technique was used. After induction of anesthesia, the neck of the rats will be prepared and draped using standard sterile techniques. Next, a small midline neck incision on the anterior neck will be made. Using gentle blunt dissection, the right carotid artery will be isolated and gently separated from surrounding structures including the vagus nerve, trachea, and esophagus. The carotid artery will then be ligated (tied) and ligated. After the surgical procedure is completed the rats will be allowed to wake and recover for 1 h. After the 1 h recovery period, rats will be exposed to the hypoxia (low oxygen) using the standard published protocols of the Rice-Vannucci model. The flow rate will be monitored continuously in the chamber. After exposure to hypoxia, all animals will be monitored closely for any signs of distress, failure to thrive, infection, or serious disability. After hypoxia, animals will be assessed and returned to their mother. Pain during surgery and sample collection was controlled by anesthetizing the rats with 3–5% of isoflurane. To provide post procedural pain relief, buprenorphine was administered.

2.3. Experimental protocol

2.3.1. In experiment I

To determine the time course expression levels of endogenous TLR9, pAMPK, AMPK, LC3II/I, and LAMP1 after HIE, western blots analysis was performed using the ipsilateral/right hemisphere of each group for sham and 6,12, 24, 48, and 72 h after HIE. Seventy-two rats were randomly divided into Sham group (n = 6) and HIE + vehicle group (n = 30).

2.3.2. In experiment II

Double immunofluorescent staining was applied to characterize the col-localization of TLR9 with neuronal specific nuclear protein (NeuN) in sham and Vehicle groups at 48 h post HIE. In addition, double immunofluorescent staining was applied to the LC3 and LAMP1 to co-localize them on neurons. Sham group (n = 3), HIE + vehicle group (n = 3).

2.3.3. In experiment III

To evaluate the effects of intranasal administration of exogenous CpG-ODN after HIE. Rats were divided into five groups: Sham (n = 6), HIE + vehicle (n = 6), HIE + CpG-ODN (0.35 mg/kg) (n = 6), HIE + CpG-ODN (0.70 mg/kg) (n = 6), and HIE + CpG-ODN (1.40 mg/kg) (n = 6). Short term neurobehavioral outcomes were tested at 48 h post HI, after which pups were sacrificed and samples were used to analyze: percent infarct volume using triphenyl tetrazolium chloride monohydrate (TTC) staining. The samples for sham and HIE groups were shared from Experiment III.

2.3.4. In experiment IV

To assess the long-term effects of intranasal administration of exogenous CpG-ODN after HIE, neurobehavioral function was performed at 4 w post HIE. Rats were divided into sham (n = 12), HIE + vehicle (n = 12) and HIE + CpG-ODN (1.40 mg/kg) (n = 12).

2.3.5. In experiment V

Intranasal administration of iCpG-ODN or intraperitoneal administration of Dorsomorphin was performed to further clarify the essential role of TLR9 signaling pathway in the cortex of HIE. Western blot was applied to detect the expression of TLR9, pAMPK/AMPK, pULK1/ULK1, pAMBRA1/AMBRA1, LC3II/I, and LAMP1. Rats were divided into Sham (n = 6), HIE + vehicle = 6, HIE + CpG-ODN (n = 6), HIE + CpG-ODN + iCpG-ODN (inhibitor) (n = 6) and HIE + CpG-ODN + Dorsomorphin (n = 6).

2.4. Drug administration

CpG-ODN was purchased from InvivoGen and given intranasally. Three concentrations of CpG-ODN were tested: 0.35 mg/kg (low concentration, resuspend CpG-ODN with endotoxin-free water), 0.70 mg/kg (middle concentration) and 1.40 mg/kg (high concentration) (Se-Chan et al., 2014). Vehicle animals received equal volume of endotoxin-free water. We gave CpG-ODN at 1 h post HIE.

Intranasal administration was performed as follows: after being anesthetized with isoflurane the rat pups will be placed on their backs in an anesthesia chamber. Underneath the chamber, a heating pad will be used to maintain adequate temperatures. A rolled pad, held together with tape, will be placed under the necks of the pups. The necks will be kept flat and horizontal allowing the heads to be stable. This will ensure treatment reached brain instead of running down the back of the throat. For intranasal delivery, drops will be given every 2 min alternating the nares (1 μl to the left side and then 2 min later 1 μl to the right side, etc.). Each drop, 1 μl, is administered using a manual pipette. After treatment, the rat pups will be monitored until the pups are fully awake and exhibit normal breathing following which they will be returned to their dam.