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[GLY14]-HUMANIN REDUCES HISTOPATHOLOGY AND IMPROVES FUNCTIONAL OUTCOME AFTER TRAUMATIC BRAIN INJURY IN MICE

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Abstract—Humanin (HN) has been identified as an endogenous peptide that inhibited AD-relevant neuronal cell death. HNG, a variant of HN in which the 14th amino acid serine was replaced with glycine, can reduce infarct volume and improve neurological deficits after ischemia/reperfusion injury. In this study, we aimed to examine the neuroprotective effect of HNG on traumatic brain injury (TBI) in mice and explored whether the protective effect was associated with regulating apoptosis and autophagy. Compared to vehicle-treated groups, mice administered HNG intracerebroventricularly (i.c.v.) prior to TBI had decreased cells with plasmalemma permeability in the injured cortex and hippocampus (48 h, $P < 0.01$), reduced brain lesion volume (days 14 and 28, $P < 0.05$), improved motor performance (days 1–4, $P < 0.05$) and ameliorated performance in the Morris water maze test (days 11–13, $P < 0.05$) post TBI. Reduced lesion volume (day 14, $P < 0.05$) was also observed even when HNG was administered intraperitoneally (i.p.) at 1 h and 2 h post TBI, and minor amelioration in motor and Morris water maze test deficits was also observed. Immunoblotting results showed that HNG pretreatment (i.c.v.) reversed TBI-induced cleavage of cysteinyl aspartate-specific protease-3 and poly ADPribose-polymerase and decline of Bcl-2, suppressed LC3II, Beclin-1 and vacuolar sorting protein 34 activation and maintained p62 levels in the injured cortex and hippocampus post TBI (compared with vehicle). In conclusion, HNG treatment improved morphological and functional outcomes after TBI in mice and the protective effect of HNG against TBI may be associated with down-regulating apoptosis and autophagy.

Key words: traumatic brain injury, humanin, neuroprotection, apoptosis, autophagy.

INTRODUCTION

Traumatic brain injury (TBI), a term applied to brain injury caused by external physical trauma, is a serious and debilitating health problem that affect millions of people each year (Bramlett and Dietrich, 2004). TBI has long been thought to cause mechanical tissue destruction and secondary injury which included activation of excitatory amino acid receptor, Ca^{2+} overload, mitochondrial injury, production of oxyradical, caspase activation, and activation of inflammatory reaction (Jain, 2008; Zhang et al., 2012a). These enlisted secondary injury events after TBI finally caused neuronal cell apoptosis, necrosis, necroptosis, and autophagy (Clark et al., 2008; Liu et al., 2008). In the past several decades, the patho-physiological process of TBI was well advanced, however, effective therapies were rare. Search for neuroprotective agents, which can reduce injurious biochemical and molecular signal pathways or enhance the protective pathways, is still desperately needed and considered a promising strategy for TBI treatment (Jain, 2008).

Humanin (HN) is a newly identified 24-amino acid neuroprotective polypeptide which can suppress neuronal cell death induced by various Alzheimer disease-related insults such as familial Alzheimer disease proteins, antiamyloid-β precursor protein antibody, and neurotoxic Aβ peptides (Hashimoto et al., 2001a,b). Interestingly, [Gly14]-Humanin (HNG, replacement of the 14th amino acid serine with glycine), enhanced its neuroprotective activity to an extent of about 1000-fold greater than HN and was fully active at nanomolar concentrations (Hashimoto et al., 2001b; Xu et al., 2008; Niikura et al., 2011). A large number of *in vitro* studies demonstrated that HN suppressed Aβ-induced neuronal cell death in both F11 cells (Hashimoto et al., 2001b) and undifferentiated PC12 pheochromocytoma cells (Jin et al., 2010). Besides, more and more *in vitro* studies have revealed that HN and HNG inhibited non-AD-related neuronal cell death, including PC12 pheochromocytoma cell death induced by serum deprivation (Kariya et al., 2002), cell death of

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Abbreviations: caspase-3, cysteinyl aspartate-specific protease-3; EDTA, ethylenediaminetetraacetic acid; HNG, [Gly14]-Humanin; LC3, microtubule-associated protein-1A/1B light chain 3; PARP, poly ADPribose-polymerase; PI, propidium iodide; SDS, sodium dodecyl sulfate; TBI, traumatic brain injury; Vps34, vacuolar sorting protein 34.

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rat cortical neurons induced by soluble prion protein fragments (Sponne et al., 2004), HT22 and cortical neuronal death induced by ischemia and reperfusion injury (Xu et al., 2008; Zhao et al., 2011). In addition to *in vitro* assays, HN and HNG have also been proved to rescue memory impairment caused by Alzheimer's disease-related insults (Tajima et al., 2005; Miao et al., 2008; Yamada et al., 2008), recover learning and memory impairment in scopolamine-treated amnesic mice (Mamiya and Ukai, 2001), reduce infarct volume and improve neurological deficits after cerebral ischemia/reperfusion injury in mice (Xu et al., 2006), and reverse 3-quinuclidinyl benzilate-induced impairment of spatial memory in rats (Krejcova et al., 2004). As reported later, Niikura et al. (2011) discovered that intranasal HNG treatment ameliorated memory deficits in triple transgenic (3xTg-AD) mice. Recently, Zhang et al. (2012b) have found that HNG improved cognitive deficits and reduced amyloid pathology in middle-aged APPswe/PS1dE9 mice.

Despite the substantial literature on neuroprotective effects of HN and HNG on various neurologic disorders and injury, it was unknown whether HNG can protect against TBI. Thus, we designed a mouse TBI model to investigate the role of HNG on TBI and to explore HNG potential neuroprotective mechanisms through apoptotic and autophagic signal pathways.

EXPERIMENTAL PROCEDURES

Animals

Studies were performed using 10–12-week-old adult male CD-1 mice weighing 25–30 g. Mice were housed in a pathogen-free environment with 12-h day and night cycles. All experimental procedures were in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Soochow University. In all experiments, data were obtained by investigators blinded to the study group.

Mouse TBI model, experimental groups and drug administration

Mice were anesthetized with 4% chloral hydrate (0.4 mg/g) and mounted in a Kopf stereotactic apparatus (Luo et al., 2011) and a craniotomy was made using a portable drill and trephine over the left parieto-temporal cortex, and the bone flap was removed. Mice were then subjected to TBI in the left part of the brain using a weight-drop-controlled cortical impact device: a 40-g weight dropped from 20 cm onto a 2.5-mm-diameter footplate resting on the dura with a controlled depth of 1.0 mm, as described previously (Luo et al., 2011). The reproducibility and consistency of this TBI model were ensured by the accurate location, controlled hit pressure, injury depth, and hit duration. The craniotomy, which did not affect physiological parameters, was closed immediately after TBI (Luo et al., 2011). Animals were grouped into naive, sham + vehicle, sham + HNG, TBI + vehicle and TBI + HNG groups. In TBI + vehicle and TBI + HNG groups, animals were divided into several subgroups: pretreatment group (15 min before TBI) and posttreatment (1 h, 2 h, and 4 h after TBI) groups. HNG-treated groups were administered 0.1 μg of HNG (Sigma–Aldrich Corporation, St. Louis, MO, USA) in 5 μL of saline intraventricularly (i.c.v., coordinates 1 mm posterior, 1 mm lateral, 2.5 mm deep to bregma) before TBI or 1 μg in 100 μL of saline intraperitoneally (i.p.) post TBI (Xu et al., 2006). Vehicle-treated groups were administered saline 5 μL (i.c.v.) or 100 μL (i.p.). No operation or drug was applied to naive mice. Sham-injured mice received chloral hydrate anesthesia, drug by i.c.v. or i.p. injection of HNG or vehicle and craniotomy but not TBI. After injury, the scalp was sutured and mice returned to their cages to recover from anesthesia. The overall mortality rate was < 2%.

Assessment of propidium iodide (PI) labeling

To observe the effect of HNG on TBI-induced cell insults, mice (n = 10/group) were pretreated with HNG (i.c.v., 0.1 μg in 5 μL of saline) or saline as described above, and sacrificed at 48 h after TBI. Loss of plasmalemma integrity was evaluated by an intraperitoneal injection of PI (total volume: 200 μL, concentration: 0.4 mg/ml, Sigma–Aldrich Corporation, St. Louis, MO, USA) 1 h before sacrificing the animal (Whalen et al., 2008). The brain was quickly and carefully removed to keep the contusion region intact, rapidly frozen in liquid nitrogen vapor, and then coronally sectioned at 12-μm thickness with cryostat (CM1900, Leica, Bensheim, Germany). A series of sections were collected 200 μm apart from the anterior to posterior hippocampus from each brain (bregma −1.90 to −2.70). Sections were placed on gelatine-coated glass slides and stored at −80 °C. Cortical regions of interest were ×200 microscopic fields (1100 mm × 1100 mm) at the medial and lateral edges of the contusion, and one cortical field directly under the impact site; hippocampal regions were medial and mid-dentate gyrus, medial CA1, and the lateral most region of CA3, based on the spatial distribution of PI-positive cells after TBI (Whalen et al., 2008). From each brain, five ×200 cortical fields from five brain sections separated by at least 200 μm were chosen for analysis using a random number generator. For hippocampal cell counts, two adjacent ×200 dentate gyrus fields were counted in three different brain sections separated by at least 200 μm (six 200 fields per animal total), for CA1 and CA3 regions, one ×200 field encompassing approximately half of the entire anatomic region of interest was examined in three different brain sections, for a total of three ×200 fields per mouse. The mean number of positive cells was calculated by summing the cell count data from all the brain sections counted and dividing by the number of ×200 fields analyzed (Whalen et al., 2008).

Lesion volume measurements

According to a previous study with minor modification, lesion volume was detected at 14 and 28 days post TBI (You et al., 2008). To evaluate the effect of HNG on TBI-induced accumulation of cell loss, mice (n = 10/subgroup) were pretreated with HNG (i.c.v., 0.1 μg in 5 L of saline) or saline (i.c.v., 5 μL) prior to TBI or post-treated with HNG (i.p., 1 μg in 100 μL of saline) or saline (i.p., 100 μL) at 1 h, 2 h and 4 h post TBI, and sacrificed at 14 and 28 days post injury. The brains were removed, immediately fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), and then dehydrated in 15% and 30% sucrose in PBS. Frozen brain sections cut on a cryostat microtome (15 μm) were collected at 500-μm intervals and placed on gelatine-coated glass slides, air dried, and stained with Nissl's staining solution (Sigma–Aldrich Corporation, St. Louis, MO, USA). Tissue damage was determined by morphometric image analysis. The areas of the injured and non-injured hemisphere and cortex were determined using an image analysis system MCID, Imaging Research (St. Catharines, ON, Canada). The morphometric analyses involved computer-assisted delineation of the intact area of the ipsilateral hemisphere and the contralateral hemisphere. Lesion volume was calculated as previously