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Small Nogo-66-binding peptide promotes neurite outgrowth through RhoA inhibition after spinal cord injury

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ABSTRACT

Abortive regeneration in the adult mammalian central nervous system (CNS) is partially mediated through CNS myelin proteins, among which Nogo-A plays an important role. Nogo-66, which is located at the C-terminus of Nogo-A, inhibits axonal regrowth through the Nogo-66/NgR signalling pathway. In this study, two small peptides were tested in a neurite outgrowth assay and spinal cord injury (SCI) model to examine the effects of these molecules on the inhibition of Nogo-66/NgR signalling. PepIV was selected from a phage display peptide library as a Nogo-66 binding molecule. And PepII was synthesized as a potential NgR antagonist. The results indicated that PepIV and PepII decrease the mRNA levels of the small GTPase RhoA and partially neutralize CNS myelin inhibition to cultured cerebellar granule cells (CGCs). Moreover, treatment with both peptides was propitious to maintaining residual axons after SCI, thereby promoting regeneration and locomotion recovery. Because RhoA plays a role in stabilizing the cytoskeleton in growth cones and axons, enhanced neurite outgrowth might reflect a decrease in RhoA expression through PepIV and PepII treatment. Moreover, PepIV induced lower RhoA mRNA expression compared with PepII. Therefore, PepIV could block Nogo-66/NgR signalling and reduce RhoA mRNA level, and then contribute to neuronal survival and axonal regrowth after SCI, showing its ability to reverse CNS myelin inhibition to regeneration. Furthermore, selected small peptide might cover some unknown active sites on CNS myelin proteins, which could be potential targets for improving neurite outgrowth after injury.

1. Introduction

Some myelin proteins, such as Nogo-A, exhibit strong inhibition to regenerating axons in the central nervous system (CNS). In addition, growth cones collapse when contacting CNS myelin. The small GTPase RhoA has been implicated in this process (Gross et al., 2007), playing a potential role in abortive regeneration in the adult mammalian CNS after traumatic injury. Rho family proteins regulate gene transcription and actin cytoskeleton formation through multiple signalling pathways. Recent data have shown that RhoA controls neuronal responses in the Nogo/NgR signalling pathway, leading to actin cytoskeletal rearrangements, growth cone collapse and axonal outgrowth inhibition ()Teusch and Kiefer, 2006. Therefore, RhoA expression could reveal the status of regenerating axons.

Nogo-66, which is located at the C-terminus of Nogo-A, restrains neurite outgrowth by binding and transducing inhibitory signals through Nogo receptor (NgR) and its co-receptors (Fournier et al., 2001). Thus, interrupting the link between Nogo-66 and NgR might efficiently terminate CNS myelin inhibitory signalling transduction. Nogo extracellular peptide 1–40 residues (NEP1–40), a competitive antagonist of NgR derived from Nogo-66, partially reversed CNS myelin inhibition (GrandPré et al., 2002). Accordingly, other smaller peptides might exist and inhibit the Nogo-66/NgR signalling pathway.

In a previous study, we used phage display peptide (PhD) libraries in a screen against NEP1–35 to obtain Nogo-66 binding peptides (Deng et al., 2013), PepIV was identified after biopanning and in vitro test. In this study, according to GrandPré's et al. (2002) report that residues 1–33 of Nogo-66 are responsible for high affinity binding of Nogo-66 to NgR, PepII, a decapeptide covering residues 31–33 was synthesized as a potential NgR antagonist. To illustrate effects of PepIV and PepII on Nogo-66/NgR signalling pathway, we investigated RhoA mRNA levels in a neurite outgrowth assay. Furthermore, we examined the effect of these small peptides in a rat spinal cord hemi-section injury model. The results showed that PepIV and PepII both reduce RhoA mRNA expression and enhance neuronal neurite outgrowth on CNS myelin substrates.

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And there were significant differences between PepIV and PepII in their ability to reduce RhoA mRNA level. PepIV might function through binding multiple active sites on Nogo-66 and induce lower levels of RhoA mRNA. Moreover, PepIV promoted locomotion recovery after SCI, suggesting the potential application of small peptide in clinical treatment.

2. Materials and methods

2.1. Animals

Adult female Sprague Dawley (SD) rats, weighing 220–250 g, and postnatal Day 3 SD rats of both sexes were purchased from the Experimental Animal Center of Research Institute of Surgery in Third Military Medical University (TMMU). Animal breeding and care were performed according to the TMMU Animal Experiment Guidelines and all of the experiments were approved through the Animal Ethics Committee.

2.2. Neurite growth assay

PepIV (NH2-RRQTLSHQMRRP-COOH) and PepII (NH2-SFRIYKGVIQ-COOH) were synthesized and purified by HPLC to purity >90% (Huachen and CL, Xi'an). PepIV and PepII were dissolved in DMSO to make stock solution. Cerebellar granule cells (CGCs) were cultured in medium containing 1% DMSO (Deng et al., 2006). The neurite outgrowth assay was performed as previously described (Hu et al., 2005). Briefly, glass coverslips were precoated with 100 μg/ml poly-L-lysine and 1 μg/cm^2 myelin proteins. CGCs isolated from the cerebellar cortex of postnatal Day 3 rats were pre-incubated with PepIV or PepII at different concentrations for 2 min (min) before plating onto the coated coverslips. Neurite outgrowth was observed 24 h after plating. Fifty field views were randomly selected for each concentration. The digital images were recorded using an Olympus AC70 Image Collection system and analysed with Image-Pro Plus 5.0. Neurons separated well were selected (30 neurons for each treatment) and neurites twice as long as the neuron's diameter were recorded as valid data.

2.3. RT-PCR

Total RNA was extracted from the CGCs using TRIzol isolation reagent (Takara). The cDNA was amplified using the following primer sets: RhoA forward, 5′-GTAAGACATGCTTGCTCATA-3′, and RhoA reverse, 5′-CTCCGTCTTTGGTCTTTGCT-3′ (450-bp fragment), and β-actin forward, 5′-GGGACCTGACAGACTACCTC-3′, and β-actin reverse, 5′-GATGCCACAGGATTCCAT-3′ (275-bp fragment). The RT procedure was 30 °C for 10 min, 42 °C 30 min, 99 °C 5 min, 5 °C 60 s, one cycle. The PCR amplification procedure used in this study was as follow: pre-denature at 94 °C for 2 min, then 94 °C 30 s, 56 °C 30 s, 72 °C 60 s, for 35 cycles, finally extended at 72 °C for 5 min. Bio-Rad Gel imaging system was used to capture images. Bands were analysed using BioRad Quantity One software.

2.4. SCI surgery

Forty female SD rats were randomly divided into four groups, 12 rats for PepII, PepIV or 1% DMSO group, respectively, and four rats for normal control group. The rats were anesthetised with an intraperitoneal injection of 0.3% sodium pentobarbital. The dorsal hemi-section of the rat spinal cord was carried out as previously described (Dusart and Schwab, 1994). Briefly, a longitudinal cut was made along the midline of the back of the anesthetised rat and the dorsal regions of the vertebrate at T8-T10 were removed to expose the spinal cord. The dorsal two-thirds of the cord were transected using Vannas scissors (WPI). Before the muscle and skin were sutured, a blade was pulled back and forth at the injury site to ensure the transaction in the dorsal spinal cord. PepIV or PepII stocks were diluted into 0.4, 4 or 8 mg/ml using saline. For each rat, 10 μl of the peptide solution was absorbed using gelfoam at the injury site to mediate the diffusion of the peptides into the surrounding tissues. The 1% DMSO group just received 10 μl saline containing 1% DMSO. Animals were carefully fed and given Ampicillin (4×10^4 unit per rat) by intraperitoneal injection. Bladder extrusion was performed 2 times per day from Day 0 after surgery.

2.5. Behaviour test

After surgery, an independent examiner assessed the locomotor function of injured rats by recording the Basso, Beattie and Bresnehan (BBB) scores from Day 0 to Day 15 post-surgery (Basso et al., 1995).

2.6. Immunohistochemistry

Axons in spinal cord were visualised using a primary antibody against neurofilament 200 (NF200, Sigma) and a TRITC-conjugated secondary antibody. At Day 15 post-transection, animals were transcardially perfused with saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). The rat spinal cord was carefully removed and dehydrated in 30% sucrose in the same fixative solution and applied for cryostat-section. Parasagittal sections were 20 μm thick. The primary and secondary antibodies were applied to sections and PBS without primary antibody was used as a control. The fluorescence images were captured using an Olympus OX70 microscope. Two sections from each rat were picked for analysis and neurites in a fixed-area were counted. The density of NF200 positive axons in white matter at 0.4 cm rostral to the injury site was analyzed with Image-Pro Plus 5.0.

2.7. Statistic analysis

All data were analysed with SPSS 10.0. The One-way analysis of variance (One-way ANOVA) was used to compare the mean differences among each group, and least significant difference *t* test (LSD-*t* test) was used in further statistical method of multiple comparison.

3. Results

3.1. Small peptides promoted neurite outgrowth of CGCs on CNS myelin substrates

The effects of PepIV and PepII at multiple concentrations on neuronal neurite outgrowth were tested on coverslips precoated with CNS myelin substrates. Compared with 1% DMSO, PepIV and PepII promoted CGCs neurite outgrowth on coated coverslips ($p < 0.001$) (Fig. 1). Although there was no dramatic difference between PepIV and PepII in the reversal of CNS myelin inhibition, the results indicated that PepIV and PepII function in a concentration-dependent manner and partially neutralize CNS myelin inhibition in vitro.

3.2. Small peptides reduced neuronal RhoA mRNA levels

To verify whether the improvement of CGCs neurite outgrowth on CNS myelin was associated with Nogo-66/NgR signalling, we used RT-PCR to examine the mRNA levels of RhoA, which is a downstream molecule of this pathway. We observed that RhoA mRNA levels were reduced in response to PepIV- and PepII-induced neurite outgrowth (Fig. 2). Moreover, PepIV induced lower levels of