Neuroscience Letters 660 (2017) 34–38

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Artemin transiently increases iNOS expression in primary cultured trigeminal ganglion neurons

Haiqiong Shang[a,b], Yan Wang[a,d], Xiuhua Chao[a,b], Gaoying Sun[b,c], Xiaohui Bai[a,b], Lei Xu[a], Yuechen Han[a], Jianfeng Li[b,c], Haibo Wang[a,b,c], Zhaomin Fan[a,*]

[a] Department of Otolaryngology-Head and Neck Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, 250021, PR China
[b] Shandong Provincial Key Laboratory of Otology, Jinan, 250022, PR China
[c] Institute of Eye and ENT, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250022, PR China
[d] Department of Otolaryngology, People's Hospital of Rizhao, 276800, PR China

ARTICLE INFO

Keywords:
Artemin
Trigeminal ganglion
iNOS
GFRα3
TUJ-1

ABSTRACT

Artemin, a member of the glial cell line-derived neurotrophic factor family, is an important cytokine and a critical participant in trigeminal pain disorders such as tongue pain and migraine. However, the mechanisms underlying artemin's activity are largely unknown. In the present study, we used primary cultured trigeminal ganglion neurons (TGNs) to determine the effect of artemin on the expression of the inducible form of nitric oxide synthase (iNOS), which is released in response to painful and inflammatory stimuli. Following artemin treatment, western blot analysis showed that the protein level of iNOS was transiently elevated after artemin treatment for 15 min ($p < 0.05$). Immunofluorescence revealed that both the expressions of iNOS and GFRα3 were significantly up-regulated after artemin treatment for 15 min. In addition, iNOS expression induced by artemin was co-localized with GFRα3 and TUJ-1 in primary cultured TGNs, respectively. Our results indicate a previously unknown role of artemin in regulating iNOS expression in primary cultured TGNs, and regulation of iNOS might be involved in the mechanism through which artemin participates in the trigeminal pain pathway.

1. Introduction

Artemin, a member of the glial cell line-derived neurotrophic factor (GDNF) family ligands, exerts its effects by binding to the GDNF family receptor alpha 3 (GFRα3)/RET receptor complex [3,28]. The GDNF family ligands play critical roles in supporting the development and survival of various kinds of neurons in the peripheral and central nervous systems [2,12]. Recently, artemin has triggered considerable interest because it not only plays a protective role in nerve regeneration, but also contributes to inflammatory pain [13,21]. Peripheral inflammation produces pain by activating the peripheral terminals of primary sensory neurons, which in turn sensitize the central nervous system [15,33]. Previous studies revealed that overexpression of artemin in the tongue can cause oral sensitivity to chemical stimuli and can increase the sensitivity of trigeminal afferents [9,27]. In addition, artemin has been shown to be present in higher concentrations in the dura mater of rodent models of migraine [26]. The trigeminal pain pathway is considered to be a major pathway during migraine and tongue pain, but the exact mechanisms underlying how artemin is involved in the trigeminal pain pathway remain unknown.

Nitric oxide (NO) is one of the main mediators involved in inflammatory pain. As a free radical gasotransmitter, it functions as an important signaling molecule in numerous physiological processes. It is now known that overproduction of NO can have diverse effects such as inflammatory pain, nerve injury, and headache [16,30]. NO is derived from the conversion of L-arginine to L-citrulline by three different isoforms of the nitric oxide synthase (NOS) enzyme, including neuronal type I (nNOS), inducible type II (iNOS), and endothelial type III (eNOS) [1,17]. Among these three enzymes, nNOS and eNOS are constitutively expressed enzymes that are stimulated by increasing Ca^{2+} concentrations to regulate neural and vascular function, respectively. In contrast, iNOS, a Ca^{2+}-independent isoform that is often expressed in response to painful and inflammatory stimuli, is active for extended periods yielding high outputs of NO that modulate various kinds of inflammatory pain pathogeneses [7,29]. Furthermore, a recent study demonstrated that iNOS plays a key role in the trigeminal pain signaling pathway [6]. However, whether iNOS is involved in the mechanisms underlying the action of artemin on the trigeminal pain pathway remains unknown.

The present study was designed to determine the effect of artemin

* Corresponding author.
E-mail address: fanent@126.com (Z. Fan).

http://dx.doi.org/10.1016/j.neulet.2017.09.016
Received 27 April 2017; Received in revised form 8 September 2017; Accepted 8 September 2017
Available online 09 September 2017
0304-3940/

on modulating iNOS expression in primary cultured trigeminal ganglion neurons (TGNs) and to explore the underlying mechanisms behind this effect. Our results indicate that artemin up-regulates iNOS expression, and this might be the way in which artemin is involved in the trigeminal pain pathway.

2. Materials and methods

2.1. Experimental animals

The experiments were conducted in postnatal 3-day-old Wistar rats from the Animal Center of Shandong University (Jinan, China). The animal care and experimental protocols were approved by the Animal Care Committee of Shandong University, P.R. China.

2.2. Primary cultures of rat TGNs

After anesthesia by chloral hydrate, the postnatal rats were quickly decapitated. The temporal bones and brain halves were immediately removed, and the trigeminal ganglion tissues were separated from the cranial base under a microscope. The tissues were placed in PBS containing 0.125% trypsinase (Gibco, USA) for digestion (20 min, 37 °C). The cells were then mechanically dissociated with a Pasteur pipette in DMEM/F12 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 50 μg/ml ampicillin (Sigma-Aldrich, USA). In each set of western blot experiments, trigeminal ganglion neurons (TGNs) from 15 to 20 rats were plated in poly-L-lysine-coated 6-well plates (Corning, USA). For each immunofluorescence staining experiment, TGNs from 3 to 6 rats were inoculated in 4-well dishes (Greiner Bio-One, Germany) with 10-mm glass coverslips precoated with Cell Tak (BD Biosciences, USA). In order to prevent the division and proliferation of glial cells, once the TGNs adhered to the plastic or glass surfaces (about 3 h), the culture medium was changed to another one without fetal bovine serum. The TGNs were cultured in DMEM/F12 medium (Gibco, USA) supplemented with N2 (1:100 dilution, Invitrogen, USA), B27 (1:50 dilution, Invitrogen, USA), and 50 μg/ml ampicillin (Sigma-Aldrich, USA) for 2 days. The cultures were maintained at 37 °C, 5% CO_2, and 95% humidity.

2.3. Treatment of TGN cultures

In the artemin-treated groups, the TGNs were incubated with 10 ng/ml artemin (R & D Systems, USA) [25] for different times (15 min, 30 min, 1 h, 2 h, and 4 h) followed by the protein extraction procedure. TGNs in the control group were given the same volume of medium without artemin.

2.4. Protein extraction and western blot analysis

Total protein from the cultured TGNs was extracted by radioimmune precipitation in lysis buffer according to the manufacturer's protocols (Beyotime, China). The protein content of the samples was measured by means of the BCA protein assay kit (Beyotime, China). A total of 35 μg of each protein sample was denatured and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked in 5% skimmed dried milk for 1 h at room temperature. Next, the membranes were incubated with rabbit anti-iNOS (1:400 dilution, Abcam Systems, USA, ab3523) or mouse anti-β-actin (1:1500 dilution, ZhongShan Goldenbridge Biotechnology, China, TA-09) primary antibodies in TBST containing 3% fat-free dry milk for 1 h at room temperature and then overnight at 4 °C. After washing three times with TBST, the membranes were incubated with the secondary goat anti-rabbit IgG antibody (1:5000 dilution, Santa Cruz Biotechnology, USA) or goat anti-mouse IgG antibody (1:5000 dilution, Santa Cruz Biotechnology, USA) at room temperature for 1 h. Finally, the immunoblots were detected using an ECL kit (Santa Cruz Biotechnology, USA) and visualized after exposure on X-ray films. The relative optical density ratio was calculated with the Image J software by comparison with β-actin.

2.5. Immunofluorescence staining

After fixing in 4% paraformaldehyde for 30 min, the cultured cells were washed in 0.01 M PBS for 10 min and then blocked in PBS containing 0.3% Triton X-100 (Sigma, USA) and 10% heat-inactivated donkey serum (NQBB, USA) for 1 h at room temperature. Subsequently, the cells were incubated with primary antibodies including polyclonal rabbit anti-NeuN antibody (1:500 dilution, Millipore, USA, MAB377), mouse anti-TUJ-1 antibody (1:500 dilution, Neuromics, USA, MO15013), goat anti-GFRα3 antibody (1:50 dilution, R & D systems, USA, BAF2645) or rabbit anti-iNOS antibody (1:50 dilution, Abcam Systems, USA, ab3523) overnight at 4 °C. The next day, after being washed in PBS, samples were incubated with FITC-conjugated or TRITC-conjugated (1:1000 dilution, Invitrogen, USA) secondary antibody along with DAPI (1:800 dilution, Sigma-Aldrich, USA) in 0.1% Triton X-100 and 1% BSA in PBS at room temperature for 1 h. The samples were rinsed in PBS for 30 min and visualized with an inverted DMI 400CS confocal microscope (Leica, Germany).

2.6. Statistical analysis

The statistical analyses were performed using SPSS 17.0 software (SPSS Inc., USA). Data were presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. TGNs were identified by anti-NeuN and anti-TUJ-1 antibodies

After being cultured *in vitro* for 2 days, the TGNs were immunostained with the anti-NeuN and anti-TUJ-1 neuronal markers and were observed under a confocal microscope. As shown in Fig. 1, NeuN was expressed in the nuclei of neuronal cells and TUJ-1 was present in the neuronal cell body and neuronal axon. A total of 91.8 ± 2.7% of the trigeminal ganglion cells were NeuN-positive, and 87.9 ± 2.4% of the trigeminal ganglion cells were TUJ-1-positive.

3.2. iNOS protein expression was increased in cultured TGNs after artemin treatment

A western blot assay showed a band of iNOS immunoreactivity at about 135 kDa. The protein level of iNOS was significantly elevated in cultured TGNs after artemin treatment for 15 min compared to the control group ($n = 3$, $p < 0.01$) (Fig. 2). The protein levels of iNOS were then decreased and were similar to controls at 30 min, 1 h, 2 h, and 4 h (Fig. 2).

3.3. Immunofluorescence expression of iNOS was increased in TGNs following artemin treatment

Immunofluorescence staining was performed to detect the expression of iNOS in TGNs after artemin treatment for 15 min. The immunofluorescence signal confirmed that iNOS protein expression was increased in the artemin-treated group compared to the control group and showed that artemin-induced iNOS expression was co-localized with TUJ-1 in the TGNs (Fig. 3).