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Research article

Changes in nitric oxide synthase isoforms in the trigeminal ganglion of rat following chronic tooth pulp inflammation

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HIGHLIGHTS

- Chronic tooth pulp inflammation caused increase of iNOS and nNOS in the trigeminal ganglion.
- NOS isoforms were involved in the development of inflammatory pain.
- The mechanism of changes of NOS isoforms will help to develop therapeutic regimes for pain.

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ABSTRACT

Nitric oxide (NO) possibly plays an important role in the events resulting in hyperalgesia. NO synthase (NOS) is a key enzyme in the production of NO. Changes in NOS expression in primary sensory neurons may be involved in the persistent sensory abnormalities that can be induced by inflammation. To assess the possible roles of NOS in trigeminal sensory system, we studied changes in the expression of NOS isoforms in the trigeminal ganglion (TG) following chronic inflammation after pulp exposure (PX) in rats. The neurons innervating injured tooth in the TG were labeled by fluoro-gold (FG). Immunohistochemical staining was used to reveal the presence of NOS. The results showed that within the FG-labeled population, neuron counts revealed a significant increase in the proportion of NOS neurons following PX, in which the frequency of iNOS and nNOS-positive neurons started to increase at 3 and 7 day, respectively, and peaked at 28 day. There was no eNOS expression observed in the control group and PX-treated groups. The results demonstrate that PX-induced chronic pulpal inflammation results in significant increase of nNOS and iNOS in the TG. It suggests that nNOS and iNOS could be involved in mediation of peripheral processing of nociceptive information following chronic tooth pulp inflammation.

1. Introduction

Pain, especially chronic pain, in orofacial region is very common. Unfortunately, most orofacial chronic pain conditions are difficult to treat in a satisfactory manner [1]. The etiology and pathogenesis of chronic pain in orofacial disease need further investigation. The pulpal inflammation produces clinically toothache, hyperalgesia and/or allodynia. These symptoms are likely accompanied by peripheral and central changes in the trigeminal nerve system [2], by which the orofacial region is principally innervated. The cell bodies of most trigeminal primary afferents are located in the trigeminal ganglion (TG) [3]. The induced changes in the TG following peripheral injury or inflammation are likely to contribute to the orofacial chronic pain, which are thought to reflect a "peripheral sensitization", one of the mechanisms of pain [4].

NO is generated by three known isoforms of nitric oxide synthases (NOS), namely the neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3), and the inducible NOS (iNOS, NOS2). NOS1

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and NOS3 are constitutive and mainly expressed in neurons and endothelial cells, respectively [5]. NOS2 can be induced extensively under certain conditions in different cell types [5,6]. A large body of evidence indicates that NO/NOS is involved in a wide variety of physiological and pathological processes [7,8]. There is some evidence that NO/NOS plays an important role in inflammatory hyperalgesia at the spinal cord level [9,10]. Other studies have showed that NOS/NO may play a role in pathological pain states in trigeminal sensory system [11,12]. Recently, we have demonstrated that chronic pulpal inflammation results in significant increase of NO production in the TG cells by the use of NADPH-d histochemistry [13]. However, there is no information available on how the chronic inflammation alters the expression of all three NOS isoforms in the TG.

Dental injury models are favorable models, which can allow us to study interactions between nociceptive sensory neurons and dental tissue [14]. Type III dental injury model is induced by pulp exposure (PX) and infection, in which the extent of the inflammatory injury shifts over time [14]. Therefore, in the present study, we use type III dental injury model to examine the pattern and extent of NOS isoforms in the TG during the course of chronic tooth pulp inflammation by the use of retrograde tracing and immunohistochemistry.

2. Materials and methods

2.1. Animals

Thirty-five male Sprague-Dawley rats (220–260 g) were used for this study. All surgical and experimental procedures were conducted by the Animal Care Committee for the Care and Use of Laboratory Animals of Sun Yat-sen University.

2.2. Surgery

Retrograde labeling was performed to identify pulpal afferent neurons projecting to the injured tooth, which was described by Pan et al. [15] and used in our previously study [13]. Briefly, animals were anesthetized with Equithesin (0.35 ml/100 g, i.p.). The mesial and lingual surfaces of the right maxillary first molar were drilled a shallow cavity into the enamel with a high-speed dental drill with a miniature bur. A droplet (0.1 ml) of 5% solution of nerve tracer fluoro-gold (FG; Fluorochrome LLC, Denver, CO) was applied to the exposed dentin in the floor of each cavity. Then the cavities were sealed with a light-cured restorative material. After 4 weeks, each animal was again deeply anesthetized. A dental injury was applied to the occlusal surfaces of the right maxillary first molar, 3 pulp horns of which were slightly exposed. The injury cavities remained open for 1, 3, 7, 14, 21 and 28 days. Control animals were only anesthetized and not given any other treatment. Each group consisted of 5 rats.

2.3. Tissue preparation and immunohistochemistry

At the end of the fixed periods, the animals were transcardially perfused under deep anaesthesia (10% chloralhydrate, 0.35 ml/100 g, i.p.) with 4% paraformaldehyde in 0.1 M phosphate buffer. The right TGs were removed and postfixed in paraformaldehyde for 6 h, and embedded in paraffin. Horizontal sections (5 μm) were mounted serially on four series of slides. Before deparaffinization, FG pictures in TG were obtained with a fluorescence microscope (Zeiss, Axioskop 40, Germany), which were later compared with the immunohistochemical images. The slides were processed in xylene for deparaffinization. These sections were then rehydrated through a gradient of ethanol. After deparaffinization and rehydration, heat induced epitope retrieval was performed in 10 mM sodium citrate buffer pH 6.0 at a sub-boiling temperature for 20 min. The slide series were pretreated with 0.3% hydrogen peroxide to block endogenous peroxidase for 20 min. Nonspecific binding sites were blocked by 1% bovine serum albumin in 0.1 M PB. The sections were then incubated in a moisture chamber for 18 h at room temperature with mouse monoclonal anti-NOS (1:50), iNOS (1:50) or eNOS (1:50; Santa Cruz, CA, USA). The primary antibodies were localized by the avidin-biotin-peroxidase method (ABC kit, Vector Labs, UK) with biotinylated anti-mouse IgG. The final chromogenic reaction was achieved by incubation with DAB. The negative controls were performed by preabsorption of the primary antibodies with their respective haptens.

2.4. Quantification of NOS-immunoreactive profiles in the TG

After NOS immunohistochemistry, the regions containing FG-labeled neurons, which had been previously photographed under the fluorescence microscope, were investigated under light microscope (Zeiss, Axioskop 40, Germany) equipped with a CCD camera (Carl Zeiss, Hallbergmoos, Germany). Quantification of immunoreactive (IR) neurons was performed using image analysis software (Image-Pro Plus, Media Cybernetics, USA). The mean relative optical density (ROD) of IR product was quantified using a 255-level gray scale. The borderline of mean ROD between immunopositive and immunonegative neurons was set in the software. All neurons sectioned through their nucleus with mean ROD exceeding the borderline were counted as NOS-IR positive. Otherwise, the cell was considered as negative. The analysis was performed double-blinded. The frequencies of immunopositive neurons were calculated for each data group. In addition, we classified NOS-IR neurons by size as small (<30 μm), medium (30–40 μm), and large (>40 μm) in the TG. The number of positive cells was expressed as a percentage of total FG neurons. The chi-square test was used to compare the differences between groups regarding the proportions of NOS-IR positive neurons. Firstly overall differences in proportions between the groups were evaluated. Pairwise multiple comparisons were carried out between different pairs within the groups. The significance of statistical analyses was set at $p < 0.05$.

3. Results

A number of FG-labeled neurons were observed in the maxillary region of TG in control group and PX-treated groups (Fig. 1A and C). The labeled TG neurons had round or ovoid profiles of different sizes including small-sized, medium-sized and large-sized neurons (Fig. 1). Counts of FG-labeled neurons in each group have been pre-

Table 1
The number of NOS- immunoreactive neurons counted per number of Fluoro-gold neurons.

	Control	1d	3d	7d	14d	21d	28d
nNOS							
small-sized	10/48	11/57	9/54	22/55	24/52	26/49	33/65
medium-sized	9/58	12/53	6/50	16/57	17/61	16/51	18/59
large-sized	4/37	1/30	5/36	9/49	8/42	10/43	7/35
total	23/142	24/140	20/140	47/161	49/155	52/143	58/159
iNOS							
small-sized	2/50	1/38	14/60	21/57	31/50	33/43	31/44
medium-sized	1/48	2/41	12/46	13/63	29/53	42/57	36/47
large-sized	1/35	1/30	11/37	16/32	12/28	32/47	21/31
total	4/133	4/109	37/143	50/152	72/131	107/147	88/122

Based on cell size analysis NOS-immunoreactive neurons labeled FG were divided into three main subpopulations: small- (<30 μm), medium- (30–40 μm), and large-sized (>40 μm) ganglionic neurons. There are no eNOS-immunoreactive neurons double-labeled with FG in any animal.