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Masseter inflammation differentially regulates three nitric oxide synthases in the rat trigeminal subnucleus caudalis

Yang Hyun Chun [a], Q-Schick Auh [a], Jongseok Lee [b], Jin Y. Ro [a,b,*]

[a] Kyung Hee University, School of Dentistry, Department of Oral Medicine, 1 Hoegi Dong, Dongdaemun Gu, Seoul, Republic of Korea
[b] Department of Neural and Pain Sciences, University of Maryland Baltimore School of Dentistry, 650 W. Baltimore Street, Baltimore, MD 21201, USA

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ABSTRACT

Objective: The aim of the present study was to evaluate changes in expression levels of three nitric oxide synthases (NOSs), namely inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS), in the subnucleus caudalis of the trigeminal sensory nuclear complex (Vc) under experimental myositis conditions.

Design: Male Sprague Dawley rats were injected with an inflammatory agent, complete Freund's adjuvant (CFA), or capsaicin in the masseter muscle. The brainstem region containing the Vc was extracted at both immediate (30 and 60 min) and longer (1, 3, 7 days) time points to examine the changes in the three NOS protein levels via the Western blot technique. Subsequently, the RT-PCR experiments were carried out to verify the changes in iNOS mRNA.

Results: Following the injections of CFA, there were no significant changes in the level of the three NOS proteins at the immediate time points. However, there was a significant upregulation of iNOS mRNA and protein 3 days after CFA-induced inflammation. Neither nNOS nor eNOS showed significant changes in the protein level at any of the longer time points. Capsaicin injection in the masseter, which we recently reported to upregulate all three NOS at the immediate time points, did not result in significant changes at longer time points.

Conclusion: Acute and chronic muscle inflammation differentially modulates the expression of the three NOS in the Vc. These data suggest that the contribution of each NOS in craniofacial muscle pain processing under inflammatory conditions may be anticipated with distinct temporal profiles.

1. Introduction

Nitric oxide (NO) acts as a neurotransmitter or intercellular messenger molecule that contributes to pathological conditions including neuronal disorder in the brain, spinal cord injury, neuropathic pain and inflammatory pain.[1–3] Because NO has a short half-life and it does not act on membrane receptors, its signalling specificity has been studied at the synthesis level.[4] Three different isoforms of nitric oxide synthase: neuronal (nNOS), endothelial (eNOS), and inducible nitric oxide synthase (iNOS) differentially generate NO in a tissue type dependent manner.[5] The relative contribution of each NOS in nociceptive processing may vary during the progression of pathologic pain conditions since peripheral inflammation differentially modulates the transcriptional

* *Corresponding author at*: Department of Neural and Pain Sciences, University of Maryland Baltimore, School of Dentistry, 650 W. Baltimore St., Baltimore, MD 21201, USA. Tel.: +1 410 706 6027; fax: +1 410 706 0865.
E-mail address: jro@umaryland.edu (J.Y. Ro).
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regulation of the three NOSs in the spinal cord. For example, hindpaw inflammation induced by complete Freund's adjuvant (CFA) significantly upregulates nNOS in the spinal cord dorsal horn without affecting eNOS or iNOS.[6] There is a similar tendency for nNOS upregulation in the spinal cord dorsal horn following formalin-induced hindpaw inflammation.[7] Peripheral inflammation induced by zymosan, however, increases nNOS expression in neurons as well as iNOS expression in activated astrocytes.[8] Whilst the data implicating the role of each NOS in the spinal cord in specific pain conditions are accumulating, our knowledge about the relative contribution of each NOS in the trigeminal counterpart is limited.

Recently, we have shown that all three NOS proteins are significantly upregulated in the subnucleus caudalis of the trigeminal sensory nuclear complex (Vc) in the brainstem, the trigeminal homologue of the spinal cord dorsal horn, following acute masseter inflammation induced by capsaicin injection.[9] Blockade of each of the three isoforms of NOS in the Vc significantly attenuates the capsaicin-induced mechanical hypersensitivity, indicating that NO generated from all three NOSs is required for the development of mechanical hyperalgesia under the acute myositis condition.[9] Capsaicin is one of the few exogenous algogens that have been used to induce muscle pain in human studies. Acute capsaicin injection in the rat produces relatively quick onset of hyperalgesia and allodynia (1 min) that lasts up to an hour,[8] therefore, it is suitable for capsaicin to serve as an effective short-term inflammatory agent.

In the present study, we extended these observations by investigating the temporal profiles of the changes in the three NOSs in the Vc following muscle inflammation induced by CFA, which peaks between 1 and 3 days and lasts up to 14 days.[10] The assessment of NOS expression under different inflammatory conditions should contribute to our understanding on the relative involvement of each NOS in the development of pathological pain conditions through the course of inflammation.

2. Materials and methods

Male Sprague-Dawley (SD) rats weighing between 250 and 300 g were used. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were carried out under a University of Maryland approved Institutional Animal Care and Use Committee protocol.

Inflammation was induced with CFA (50 μl, Sigma; dissolved 1:1 in isotonic saline) and injected into the mid-region of one masseter muscle. In order to minimise discomfort, the rats were briefly anaesthetised with 1–2% isoflurane for all injection procedures. Brainstem blocks in the dorsolateral region of the Vc (3–6 mm caudal from obex) were harvested as described previously.[9,11] In order to assess the changes in NOS in a time course similar to those observed after capsaicin-induced masseter muscle inflammation, the Vc blocks were extracted 30 and 60 min following CFA injection in the masseter muscle. In separate groups of rats, the same brainstem blocks in the Vc were harvested 1, 3, and 7 days following CFA injection in the masseter muscle. Finally, in order to compare long-term effects of capsaicin on the expression of the three NOSs to those of CFA, we also harvested the brainstem blocks 1 and 3 days following the capsaicin injection as described previously.[9] The level of NOS expression was compared to that obtained from the same brainstem region in naïve rats, which received no injections.

Total proteins in the tissue sample were dissolved in radioimmunoprecipitation assay (RIPA) buffer. Protein samples of 20–40 μg were denatured in 1× loading buffer at 90 °C for 5 min. Denatured proteins were fractionated on a NuPAGE gel at a chosen concentration with running buffer containing sodium dodecyl sulfate (SDS). Fractionated proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane in a semi-dry system. The membranes were blocked with 5% milk in 1× PBS for 1 h at room temperature. The following primary antibodies were added: nNOS and eNOS (1:1000 mouse monoclonal; BD; San Diego, CA, Cat. No. 610308, Cat. No. 610296, respectively), and iNOS (1:1000; rabbit polyclonal; Stressgen; Victoria, BC, Cat. No. KAS-NO001). The bound primary antibodies were detected with a HRP-conjugated secondary antibody. The immunocomplex was visualised with enhanced chemiluminescence (ECL) reagents (Amersham Bio-science, Piscataway, NJ). Signals were recorded on X-ray film and scanned with Kodak Image Work Station for quantitative analysis. The protein level for each NOS was normalised to that of β-Actin in the same sample.

Total RNA was extracted from the same region of the Vc from naïve and CFA-treated rats (3 days post CFA injection) with Trizol (Invitrogen, CA, USA) and purified with an RNeasy kit (Qiagen Sciences, MD, USA) that included a DNase treatment to remove genomic DNA. Reverse transcription was carried out using the Superscript First Stand synthesis kit (Invitrogen, CA, USA). Superscript II (Invitrogen) was used to generate cDNA from 500 ng of RNA along with 2.5 ng of random primer per reaction. Real-time PCR analysis of cDNA equal to 100 ng RNA was performed on the Eppendorf Mastercycler Realplex 2.0. The primer pairs for iNOS mRNA were 5′-GCTACACTTCCAACGCAACA-3′ (sense primer) and 5′-ACAATCCACAACTCGCTCCA-3′ (antisense primer). The amount of iNOS mRNA was normalised to the GAPDH mRNA in the same sample. The primer pairs for detecting GAPDH mRNA were 5′-TCACCACCATGGAGAAGGC-3′ (sense primer) and 5′-GCTAAGCAGTTGGTGGTGCA-3′ (antisense primer). The cycling protocol used was 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 55 °C for 20 s and 68 °C for 30 s. Relative quantification of the iNOS mRNA was calculated by the comparative C_T method ($\Delta\Delta C_T$ method) between control and experimental groups.

The changes in the protein or mRNA level following inflammation were normalised to naïve and expressed as mean percent change ± standard error of the mean (SE). One-way ANOVA or Student's *t*-test was used to determine statistical differences in the expression levels between naïve and inflamed rats. Dunnett's multiple comparison tests were used for post hoc analysis. Each experimental or control group consisted of 4–5 animals. Thus a total of 58 rats were used for both Western blot and RT-PCR experiments. The significance level was set at $p < 0.05$ for all statistical analyses.