The Effect of Sumatriptan on Nitric Oxide Synthase Enzyme Production After Iatrogenic Inflammation in the Brain Stem of Adolescent Rats: A Randomized, Controlled, Experimental Study

Savas Demirpence, MD1; Semra Hiz Kurul, MD1; Müge Kiray, MD2; Kazim Tugyan, MD2; Osman Yilmaz, PhD3; and Galip Köse, MD1

1Department of Pediatrics, Dokuz Eylül University School of Medicine, Izmir, Turkey; 2Department of Histology-Embryology, Dokuz Eylül University School of Medicine, Izmir, Turkey; and 3Department of Laboratory Animal Sciences, Dokuz Eylül University School of Medicine, Izmir, Turkey

ABSTRACT

Background: Migraine is a common disabling disorder of childhood and adolescence. Despite advances in the understanding of migraine pathophysiology, treatment remains a challenge.

Objectives: The aims of this study were to investigate the production of nitric oxide synthase (NOS) enzymes in the brain stem of adolescent rats, using an experimental model of migraine, and the effect of sumatriptan pretreatment on the production of the NOS enzymes.

Methods: Male adolescent (aged ~2 months) Wistar rats were used in the study. The animals were anesthetized using pentobarbital. The trigeminovascular system was stimulated by injecting a proinflammatory molecule, carrageenan, into the cisterna magna of the anesthetized rats. The animals were divided into 3 groups of equal size: (1) the study group, in which the rats were treated with sumatriptan succinate 2 hours before intracisternal carrageenan injection; (2) the sham group, in which the rats were not administered intracisternal carrageenan injection or sumatriptan pretreatment; and (3) the control group, in which the rats were administered intracisternal carrageenan injection but were not pretreated with sumatriptan. In the control and study groups, the rats were euthanized using ether anesthesia 1 hour after intracisternal carrageenan injection. Rats in the sham group were euthanized 1 hour after intracisternal catheterization. Brain tissue was removed and endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) immunohistochemistry was performed.

Results: Twenty-one rats were randomized into 3 groups of 7. The mean values of the immunolabeling intensities for eNOS, nNOS, and iNOS enzymes in the brain stem were significantly lower in the sham group compared with the control group ($P = 0.001$, $P = 0.002$, and $P = 0.001$, respectively). The mean values of the immu-
nolabeling intensities of eNOS, nNOS, and iNOS in the brain stem were significantly lower in the study group compared with the control group \((P = 0.001, P = 0.025,\) and \(P = 0.005,\) respectively).

**Conclusions:** In this experimental model of migraine in adolescent rats, intracisternal injection of carrageenan was associated with a significant increase in the production of NOS enzymes in the brain stem. Pretreatment with sumatriptan was associated with a decrease in NOS production. (*Curr Ther Res Clin Exp.* 2009;70:129–135) © 2009 Excerpta Medica Inc.

**Key words:** migraine, adolescence, carrageenan, nitric oxide synthase, sumatriptan.

### INTRODUCTION

Migraine is a common disabling disorder (prevalence, 10%–20%) of childhood and adolescence.\(^1\) Despite advances in the understanding of migraine pathophysiology, treatment remains a challenge. Currently, treatments are classified as preventive or acute-attack therapies.\(^2\) Triptans have been used to treat migraines; however, these agents are not always effective.\(^3\) Novel therapeutic targets such as inhibitors of excitatory glutamatergic receptors and calcitonin gene-related peptide (CGRP) antagonists are currently being studied as a treatment alternative.\(^4\)

In recent years, studies have reported the role of nitric oxide (NO) in headache.\(^5\)–\(^7\) Experimental evidence suggests that NO participates in vasodilatation and activation of the trigeminovascular system. NO has been reported to play an active role in dural vasodilatation, thereby contributing to the pathogenesis of migraine.\(^6\),\(^7\) An infusion of NO was associated with migraine-like headaches for several hours in both control volunteers and headache patients who fulfilled the International Headache Society criteria for migraine.\(^8\) The role of NO synthase (NOS) enzymes after stimulation of the trigeminal sensory neurons is not fully understood. However, according to a search of MEDLINE using the terms *migraine, vascular headache, primary headache, adolescent animal,* and *adolescent rat,* almost all experimental studies in the field of primary headache research were performed in adult animals, and information is lacking about the pathophysiology of primary headaches, including migraine, in adolescent animals.

Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed in endothelial cells and neurons, respectively, and their expression is regulated at the transcriptional, translational, and posttranslational levels.\(^9\) Inducible NOS (iNOS) is the inducible isoform of NOS enzymes. The synthesis of iNOS is induced by endotoxin and by inflammatory cytokines in macrophages and many other cell types. A recent study reported iNOS expression in the central nervous system and in microglial cells.\(^10\)

The 5-HT\(_{1B/1D}\) serotonin receptor agonist, sumatriptan,\(^*\) is indicated for the treatment of acute migraine attacks.\(^5\) The therapeutic action of sumatriptan is thought to be consistent with neuronal inhibition. Sumatriptan has been reported to inhibit neu-

\*Trademark: Imigran\(^®\) (GlaxoSmithKline, Brentford, United Kingdom).
rogenic meningeal dural vasodilatation caused by electrical stimulation. This action was thought to occur via the inhibition of CGRP release from the trigeminal sensory fibers innervating the cranial blood vessels.\textsuperscript{3,11,12} Nitroglycerin-induced headache has been reported to respond to sumatriptan.\textsuperscript{13} It is unclear if sumatriptan has any effect on NOS enzymes.

The aims of this study were to investigate the production of NOS enzymes in the brain stem of adolescent rats, using an experimental model of migraine, and the effect of sumatriptan pretreatment on the production of the NOS enzymes.

**MATERIALS AND METHODS**

This study was approved by the ethics committee of Dokuz Eylül University School of Medicine, Izmir, Turkey.

Two-month-old male adolescent Wistar rats, provided by the Dokuz Eylül University School of Medicine, were used. The animals were housed in appropriate caging on a 12-hour light/dark cycle with free access to standard laboratory food and tap water. The animals were allowed to adjust to the housing facilities for $\geq 1$ week before the study was initiated.

The rats were divided randomly into 3 groups of equal size. In the study group, rats were anesthetized, catheterized, and treated with intraperitoneal (IP) sumatriptan succinate 0.3 mg/kg 2 hours before intracisternal carrageenan injection.\textsuperscript{14} In the sham group, the rats were anesthetized and catheterized but were not administered intracisternal carrageenan injection or sumatriptan pretreatment. Instead of sumatriptan, they received the same volume of IP saline. In the control group, rats were anesthetized, catheterized, and administered a 0.1-mL intracisternal carrageenan injection. Instead of sumatriptan, they received the same volume of IP saline.

**Animal Preparation**

The animals were anesthetized with IP pentobarbital 45 mg/kg. A midline skin incision was made from the occipital protuberance to the cervical area. The trigeminovascular system was stimulated using the proinflammatory substance carrageenan (1 mg dissolved in 0.1 mL of saline) in the study and control groups. The carrageenan solution was injected into the cisterna magna using a 27-gauge needle and tuberculin syringe.\textsuperscript{15} Animals in the sham group received no intracisternal injection. All rats were kept in a prone position until they were euthanized using ether anesthesia 1 hour after intracisternal catheterization or carrageenan injection.

**Tissue Processing, Histology, and Immunohistochemistry**

At the end of the experiment, brain tissue was removed from the rats. Tissue samples were fixed in 10% formalin in phosphate buffer, processed using routine histologic methods, and embedded in paraffin blocks. Sections were cut using a microtome (RM2255, Leica Microsystems GmbH, Wetzlar, Germany). eNOS, nNOS, and iNOS immunohistochemistry was performed using anti-eNOS (Genetex Inc., Irvine, California), anti-nNOS (Genetex Inc.), and anti-iNOS (StressGen Biotechnologies Corporation, San Diego, California) antibodies. Sections were deparaffinized in
xylene and rehydrated through a graded ethanol series. They were then treated with 2% trypsin at 37°C for 15 minutes. Sections were incubated in a solution of 3% hydrogen peroxide for 15 minutes to inhibit endogenous peroxidase activity. The sections were incubated overnight with anti-eNOS and anti-nNOS antibodies and then for another 30 minutes with biotinylated mouse secondary antibody. The bound secondary antibody was then amplified using an avidin:biotinylated enzyme complex kit (Vectastain ABC kit, Vector Laboratories, Burlingame, California). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.02% diaminobenzidine, and nuclei were counterstained with Harris hematoxylin. The sections were mounted onto lysine-coated slides. The images were analyzed using a computer-assisted image analyzer system consisting of a microscope (BX-50, Olympus, Tokyo, Japan) equipped with a high-resolution video camera (TK-890E, JVC, Tokyo, Japan). The immunolabeling scores were evaluated blindly. Immunolabeling intensity was graded as follows: 1 = mild; 2 = moderate; 3 = strong; and 4 = very strong.16

Statistical Analysis

All statistical analyses were performed using SPSS software version 15 (SPSS Inc., Chicago, Illinois). Results are presented as mean (SD). Group differences were analyzed using the Mann-Whitney U test. P < 0.05 was considered statistically significant.

RESULTS

Three groups of 7 rats (weight range, 66–115 g) were used to conduct the study. The figure shows the staining for eNOS, nNOS and iNOS by immunohistochemistry in the brain stem of the rats.

The mean (SD) immunolabeling intensities for eNOS, nNOS, and iNOS in the brain stem of the study group were 2.85 (0.37), 2.42 (0.53), and 2.57 (0.53), respectively; in the sham group they were 1.14 (0.37), 1.28 (0.48), and 1.28 (0.48); and in the control group they were 3.42 (0.53), 3.42 (0.78), and 3.71 (0.48) (Table). The mean immunolabeling intensities for eNOS, nNOS, and iNOS enzymes in the brain stem were statistically significantly lower in the sham group compared with the control group (P = 0.001, P = 0.002, and P = 0.001, respectively). The mean values of the immunolabeling intensities for eNOS, nNOS, and iNOS were statistically significantly lower in the study group compared with the control group (P = 0.001, P = 0.025, and P = 0.005, respectively).

DISCUSSION

Experimental models are helpful in advancing the understanding of the pathophysiology of primary headaches and in developing new therapeutic approaches.17 Iatrogenic chemical stimulation of the meninges has been used to produce head pain.17 Carrageenan, a sulfated polysaccharide, promotes inflammation by activating proinflammatory cells. It is used as an algesic agent in experimental studies.18 The experimental method we used in our study was reported previously by Nozaki et al.15 They injected carrageenan into the cisterna magna of anesthetized rats and reported that chemical stimulation of the meninges induced c-fos expression, a marker of nociception.
Table. The immunolabeling intensities of the nitric oxide synthase (NOS) enzymes in the brain stem of adolescent male Wistar rats (N = 21). Data are mean (SD).

<table>
<thead>
<tr>
<th>NOS</th>
<th>Study Group* (n = 7)</th>
<th>Sham Group† (n = 7)</th>
<th>Control Group‡ (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>2.85 (0.37)§</td>
<td>1.14 (0.37)§</td>
<td>3.42 (0.53)</td>
</tr>
<tr>
<td>nNOS</td>
<td>2.42 (0.53)‖</td>
<td>1.28 (0.48)‖</td>
<td>3.42 (0.78)</td>
</tr>
<tr>
<td>iNOS</td>
<td>2.57 (0.53)#</td>
<td>1.28 (0.48)§</td>
<td>3.71 (0.48)</td>
</tr>
</tbody>
</table>

eNOS = endothelial NOS; nNOS = neuronal NOS; iNOS = inducible NOS.

* Treated with sumatriptan succinate 2 hours before intracisternal carrageenan injection.
† Not administered intracisternal carrageenan injection or sumatriptan pretreatment.
‡ Administered intracisternal carrageenan injection but not pretreated with sumatriptan.
§ P = 0.001 versus control group.
‖ P = 0.025 versus control group.
¶ P = 0.002 versus control group.
# P = 0.005 versus control group.

Figure. Photomicrographs showing immunolabeling of endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) in the brain stem of adolescent rats. Significant positive immunolabeling was detectable in tissues from the control group, and slight immunolabeling was detectable in tissues from the study group (Harris hematoxylin stain; original magnification, 20X).
We applied this method to adolescent rats. Experimental studies of migraine pathophysiology have been performed in adult animals,\textsuperscript{8,10,15,17} and there are no data about the pathophysiology of primary headaches in adolescent animals. We propose that our findings in adolescent rats may be helpful in understanding the complex pathophysiology of primary headaches. Further studies are also needed to investigate the pathophysiology of primary headaches in adult and adolescent animals.

NO can induce headache in migraine patients and often triggers a delayed migraine.\textsuperscript{5} The initial headache is thought to be caused via direct action of the NO-cyclic guanosine monophosphate pathway that causes vasodilatation by vascular smooth muscle relaxation, while the delayed headache is likely to be a result of triggering trigeminovascular activation.\textsuperscript{6} It has been demonstrated that the increased nNOS activity in the trigeminal system causes CGRP release and dural vessel dilation.\textsuperscript{7} nNOS inhibitors were reported to partially inhibit neurogenic dural vasodilatation, while eNOS inhibitors were able to partially inhibit CGRP-induced dilatation.\textsuperscript{12} Our study did not show that sumatriptan was associated with the inhibition of neurogenic meningeal dural vasodilation. However, it found that intracisternal injection of carrageenan was associated with a statistically significant increase in the production of NOS enzymes in the brain stem of adolescent rats and that sumatriptan was associated with an attenuation of the increase in the NOS enzymes observed after the administration of carrageenan. These results indicate the participation of NOS enzymes in primary headache pathophysiology and support the opinion that specific NOS-enzyme inhibitors might serve as pharmacologic agents to treat primary headaches in the future.\textsuperscript{2}

A significant result of our study is that the increased NOS production in the brain stem might be antagonized by pretreatment with sumatriptan. These data suggest new therapeutic strategies for multitargeted combination drugs, including NOS inhibitors, to alleviate primary headache.

Although the experimental model in our study is useful for studying characteristics of trigeminal nociception, we did not conclude that the pathophysiology in this model is identical to the pathophysiology of human migraine headaches.

**CONCLUSIONS**

In this experimental model of migraine in adolescent rats, intracisternal injection of carrageenan was associated with a significant increase in the production of NOS enzymes in the brain stem. Pretreatment with sumatriptan was associated with a decrease in NOS production.

**REFERENCES**


**Address correspondence to:** Semra Hiz Kurul, MD, Department of Pediatrics, Division of Pediatric Neurology, Dokuz Eylül University School of Medicine, 35340 Inciralti, Izmir, Turkey. E-mail: semra.kurul@deu.edu.tr