대 한 체 질 인 류 학 회 지
Korean J Phys Anthropol
19(3): 215 ~ 222, 2006

Effects of Electroacupuncture on the Expression of Spinal Neuronal Nitric Oxide Synthase in Adjuvant-induced Arthritic Rat Model

Ji-Won Ryu, Young-Gi Gil[1], Byung-Tae Choi

Department of Anatomy, College of Oriental Medicine, Dong-Eui University
[1]*Department of Anatomy, Medical School, Kosin University*

To examine the effects of electroacupuncture (EA) on central nociceptive modulation, expressional changes of spinal neuronal nitric oxide synthase (nNOS) were investigated in complete Freund's adjuvant (CFA)-injected rats.

Inflammation was induced by an intraplantar injection of CFA into the hindpaw of male Sprague-Dawley rats. Bilateral EA stimulation at 2 Hz, 15 Hz and 120 Hz was applied at those acupoints corresponding to Zusanli and Sanyinjiao in man with 3-day intervals for 30 days. At 30 days after CFA-injection, effects of EA on nNOS expression were observed in the dorsal horn of the spinal cord using immunohistochemical methods.

The mean integrated optical density of nNOS immunoreaction was significantly increased in the dorsal horn throughout L1 to L5 lumbar segments in CFA-injected rats. The nNOS expression was attenuated in all regions of the dorsal horn by all types of EA. Especially, these reaction was markedly decreased in the superficial laminae and nucleus proprius of L1 and L3 lumbar segments by three types of EA, but a marked decrease in the neck of the dorsal horn was observed only in 2 Hz stimulation. The marked decrease of nNOS also showed in nucleus proprius and the neck of L5 lumbar segments in 2 Hz and 15 Hz EA stimulated rats.

It is concluded that EA treatment can attenuate chronic inflammatory process in CFA-injected rats through modulating expression of nNOS in the dorsal horn of the spinal cord.

Key words : Electroacupuncture, Nitric oxide synthase, Complete Freund's adjuvant, Spinal cord

Introduction

Nitric oxide (NO), an atypical transmitter, has many biological functions involved in vasodilation, neurotransmission, inflammation and cytotoxity. Previous studies have suggested that NO also plays an important and critical role in the development and maintenance of pain such as hyperalgesia within the nervous system (Cizkova et al. 2002, Tao et al. 2004). Localization studies have shown that nitric oxide synthase (NOS), the enzyme that synthesizes NO from L-arginine, is expressed in the central as well as peripheral nervous system (Vizzard et al. 1995). Considerable evidence has demonstrated that NO and its enzyme are involved in the central mechanisms of inflammatory hyperalgesia at the spinal cord level (Guhring et al. 2000, Hsieh 2005).

Peripheral inflammation induces increased expression of neural NOS (nNOS), one of the NOS family including inducible NOS and endothelial NOS, in the spinal cord (Guhring et al. 2000, Tao et al. 2004). The

Correspondence to : Byung-Tae Choi (Department of Anatomy, College of Oriental Medicine, Dong-Eui University)
E-mail : choibt@deu.ac.kr

contribution of NO to the processing of sustained nociceptive inputs at the level of spinal cord has been clearly illustrated by studies that used NOS inhibitors (Moore et al. 1991). The inhibition of NOS expressed in spinal cord tissue, predominantly nNOS, by the intrathecal injection of selective NOS inhibitor has been demonstrated to reduce pain behaviors in models of neuropathic and inflammatory pain (Li and Clark 2001). These results indicate NO and its synthesis enzyme NOS are involved in the development and maintenance of the pain.

Acupuncture, an important therapeutic technique in oriental medicine, has been clinically used to relieve acute and chronic inflammatory pain in patients (Ceccherelli 1999). The efficiency of electroacupuncture (EA) as a kind of acupuncture for producing analgesia is of increasing interest to western medicine (Wan et al. 2001). Although EA stimulations are know to have anti-hyperalgesia properties within the central nervous system, little is known about the anti-inflammatory effect of EA on expression of nNOS in the dorsal horn of the spinal cord. The intraplantar injection of complete Freund's adjuvant (CFA) in the hindpaw of rats is a commonly-used model to study chronic inflammatory pain caused by edema and hyperalgesia. In the present study, we investigated the anti-inflammatory effects of EA in CFA-injected rats, and observed distributional expression of immunoreaction for spinal nNOS in the dorsal horn of the spinal cord with and without EA stimulation.

Materials and Methods

1. Animals

Male Sprague-Dawley rats from 6 weeks of age, weighing approx. 120 g, were obtained from Hyochang Science, Daegu, Korea. Rats were housed under constant environmental conditions at 22℃ and a 12-hour dark-light cycle, and were fed a commercially obtained diet and allowed tap water *ad libitum* starting 2 weeks before and throughout the study. The experimental procedures were conducted under the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann 1983). Eight rats were used in each group.

2. CFA injection and EA stimulation

Rats were injected subcutaneously with 150 μL of CFA (1 mg *Mycobacterium tuberculosis* per 1 mL; Sigma, St. Louis, MO, USA) into the plantar surface of the left hindpaw. EA stimulation was performed according to Han et al. (1981). For EA stimulation at 2 Hz, 15 Hz and 120 Hz, rats were partially restrained in a plastic holder. Two stainless-steel needles with 0.25 mm diameter were inserted in each hindleg at those acupoints corresponding to Zusanli (ST36, 5 mm lateral to the anterior tubercle of the tibia) and Sanyinjiao (SP6, 3 mm proximal to the medial malleolus) in humans and were connected to an electric stimulator (SM-60 Saechang, Seoul, Korea). The intensity was set at 1 mA and was increased stepwise to 2 mA and 3 mA and each step lasted 10 min. EA stimulation was repeated with 3-day intervals for a total of 30 days after CFA-injection. Normal groups were injected with 150 μL of phosphate buffered saline (PBS, pH 7.4) only, and were not treated with EA.

3. Immunohistochemistry

Thirty days after CFA-injection, the rats were intracardially perfused under 10% chloral hydrate anesthesia (350 mg/kg i.p.) with saline and then 4% paraformaldehyde in PBS. The L1, L3 and L5 lumbar segments of spinal cords were removed, post-fixed in the same fixative for 4 h at 4℃, and immersed in 30% sucrose for 48 h at 4℃ for cryoprotection. Frozen sections (30 μm thick) were prepared and collected in 0.1 M phosphate buffer (PB, pH 7.4) to be processed immunohistochemically as free floating sections.

These sections were pre-incubated in 0.3% H_2O_2 for 15 min, rinsed roughly, then incubated in a blocking solution containing 3% normal goat serum and 0.3% Triton-X 100 in PBS for 30 min at room temp.

Sections were incubated for 16 h at 4˚C with a rabbit polyclonal anti-nNOS antibody (1 : 500, sc-1025, Santa Cruz Biotechnology, Santa Crus, CA, USA) diluted in PBS containing 0.3% Triton X-100. After being washed with PBS, sections were incubated with the secondary antibody, biotinylated anti-rabbit IgG for 30 min, followed by washing with PBS. Sections were further incubated with avidin-biotin-peroxidase complex kit (Vector, Burlingame, CA, USA) for 60 min at room temp. Diaminobenzidine substrate kit (Vector) to detect peroxidase was applied. As controls, treatment with primary and secondary antibodies was omitted.

4. Quantification

To quantify laminar expression, the ipsilateral dorsal horn of the spinal cord was divided into 3 regions: the superficial laminae (SDH, laminae I and II), the nucleus proprius (NP, laminae III and IV) and the neck of the dorsal horn (NECK, laminae V and VI). For the expression of nNOS, images of dorsal horns

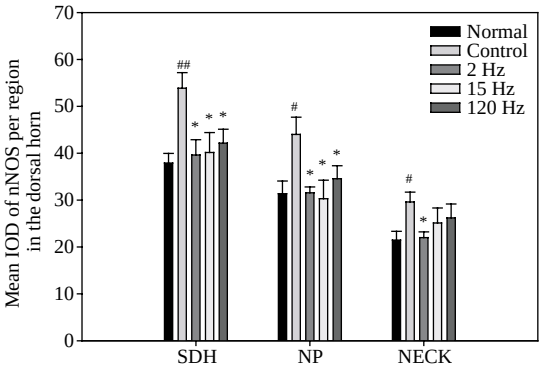


Fig. 2. Effects of EA at different frequencies on the mean IOD of nNOS immunostaining in the dorsal horn of the L3 lumbar segment in rat. Each bar indicates the means ± SEM (n=8). Note a marked decrease of the mean IOD in SDH and NP regions of the dorsal horn by all three types of EA stimulation. $^{\#\#}P<0.005$, $^{\#}P<0.05$ as compared with the normal group; $^{*}P<0.05$ as compared with the control group.

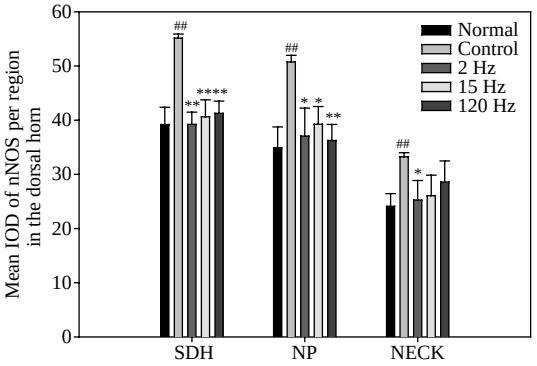


Fig. 1. Effects of EA at different frequencies on the mean IOD of nNOS immunostaining in the dorsal horn of the L1 lumbar segment in rat. Each bar indicates the means ± SEM (n=8). Note a marked decrease of the mean IOD in the SDH and NP of dorsal horn by all three types of EA stimulation. $^{\#\#}P<0.005$ as compared with the normal group; $^{*}P<0.05$ and $^{**}P<0.01$ as compared with the control group.

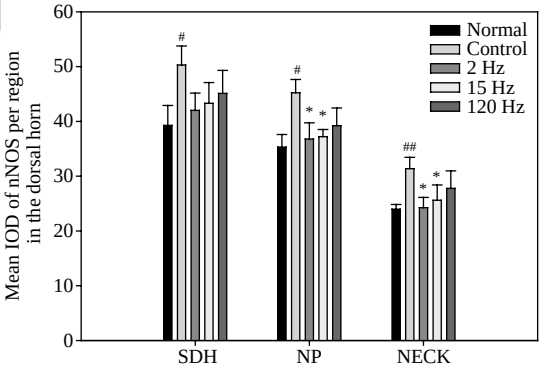


Fig. 3. Effects of EA at different frequencies on the mean IOD of nNOS immunostaining in the dorsal horn of the L5 lumbar segment in rat. Each bar indicates the means ± SEM (n=8). Note a marked decrease of the mean IOD in NP and NECK regions of the dorsal horn by 2 Hz and 15 Hz of EA stimulation. $^{\#\#}P<0.005$, $^{\#}P<0.05$ as compared with the normal group; $^{*}P<0.05$ as compared with the normal group.

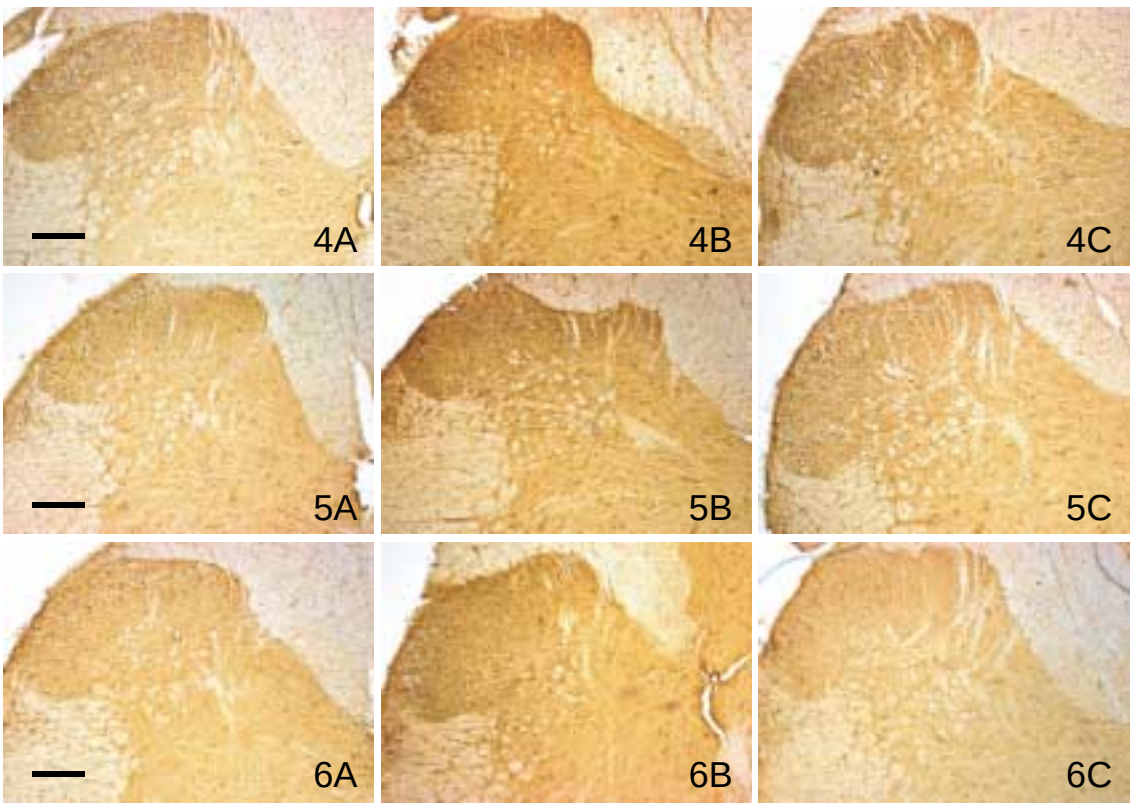
were captured at X100 magnification using a digital CCD camera (AxioCam, Carl Zeiss, Jena, Germany). The integrated optical density (IOD) of each entire region of the dorsal horn was measured automatically using Visus Image Analysis software (Foresthill Products Ista-Video TesT, Foresthill, CA, USA).

5. Data analysis

Data were expressed as mean±SEM. Calculations of means, standard errors and Student's *t*-test were made using SigmaPlot version 6.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statically significant.

Results

The expression of nNOS in the dorsal horn of the spinal cord with or without EA stimulation was determined to evaluate the activity of neurons in the spinal cord after induction of inflammation by CFA. The results of the present study on the nNOS expression are outlined in Figs. 1~6. Immunoreaction was observed throughout all laminae of dorsal horn, but was more intensive for nNOS observed in neurons of SDH and NP of the dorsal horn. At 30 days after CFA-injection, Mean IOD of nNOS in the CFA-treated groups were significantly increased in the whole dorsal horn as



Figs. 4-6. Photomicrographs of 30-μm-thick sections of the dorsal horn of lumbar L1 (Fig. 4), L3 (Fig. 5) and L5 (Fig. 6) lumbar segments showing nNOS expression at 30 days after CFA injection. A, normal group; B, CFA-injected control group; C, 2 Hz EA-stimulated group after CFA-injection. Note significant reduction of immunoreaction in the SDH and NP regions of the dorsal horn of the EA-stimulated group as compared with the control group. Bar, 200 μm.

compared with normal animal.

When the IOD of nNOS was measured in EA stimulated rats, EA with 2 Hz, 15 Hz and 120 Hz presented significant inhibitory effects. The mean IOD of nNOS in the SDH and NP regions of L1 and L3 level of the spinal cord was decreased by all types of EA stimulation examined compared with control. The EA stimulation with 2 Hz also attenuated this reaction in the NECK region of same level of spinal cord. As for mean IOD of nNOS in L5 level of spinal cord, both 2 Hz and 15 Hz EA stimulation showed a significant decrease of nNOS expression in the NP and NECK regions of the spinal cord, whereas there were no changes in SDH region.

Discussion

Administration of CFA induces both behavioral hyperalgesia and spinal neuronal hyperexcitability by inflammation at the intraplantar injection site. Our lab first observed inhibitory effects of EA on edema and mechanical thresholds in previous studies (Choi et al. 2005). EA stimulation of all frequencies produce a significant anti-edema and anti-hyperalgesic effect as compared with CFA-injected control rats. EA inhibits inflammatory edema and hyperalgesia by means of increased production of opioid peptides in the central nervous system (Ceccherelli et al. 1999, Han 2003). But EA-induced analgesia and anti-edema effects via nNOS has received relatively little attention.

NO played an important role in formalin-induced fos protein, the early response protein in neuron, which are widely used as markers of peripheral noxious stimulation in the spinal cord (Cao et al. 2005). Administration of NOS inhibitor diminishes hyperalgesia induced by the injury pain state in a rat model (Osborne and Coderre 1999). These results suggests that spinal nNOS plays a critical role in the development and maintenance of inflammatory hyperalgesia (Tao et al. 2004).

The goal of the present study was to observe expressional changes of spinal nNOS in CFA-injected rat due to EA stimulation. Immunocytochemical staining revealed a significant increase of nNOS-immunoreactive neuron after injury or trauma and in several pathologic states. The upregulation of nNOS has been observed in the dorsal horn of the spinal cord in the inflammatory animal models (Lam et al. 1996). Nerve injury such as tight spinal nerve ligation has been shown to evoke an increase of NOS expression and activity in dorsal root ganglion but a reduction in spinal cord neurons (Choi et al. 1996).

Consistent with previous studies of inflammatory pain models but not neuropathic pain models, similar nNOS expression was shown in the present study. The mean IOD of nNOS significantly increases in dorsal horn of the spinal cord, especially in SDH and NP, where nociceptive primary afferents terminate after CFA treatment. Luo et al. (1999) suggests that upregulation of nNOS may results from positive signals derived from increased ongoing afferent activity or active factors generated in the injured terminal.

Most nNOS-positive fiber of dorsal root ganglion terminates predominantly in the dorsal horn (Nunez et al. 1986). Immunohistochemical staining showed that nNOS-positive cells lie predominantly in SDH region where somatotopically defined unmyelinated nociceptive fiber afferents mainly terminate (Tao et al. 2004). The SDH region corresponds closely to spinal projections of small unmyelinated fibers containing substance P and enkephalins, which mediate pain and thermal input (Lam et al. 1996). With respect to the localization, nNOS was expressed widely in the spinal cord and are mainly distributed over the neurons in the SDH in the present study.

However, mean IOD of nNOS was significantly decreased following all kinds of EA stimulation examined in SDH and NP of L1 and L3 lumbar segments level as compared with controls. In the L5 lumbar

segments, NP and NECK regions showed a decreased IOD of nNOS in 2 Hz and 15 Hz EA stimulated rats. These results suggest that EA stimulation could be effective in alleviating symptoms of inflammatory pain. After ultrasound treatment, as the stimulation of peripheral nerves, also modulate and prevent the CFA-insult-induced increase in total and regional nNOS-positive neurons. Theses results show a pain-modulating effect of EA treatment on the spinal cord.

Our studies are also consistent with the observation that spinal NO production is necessary for increased synaptic efficacy following N-methyl-D-aspartate (NMDA) receptor activation in spinal cord (Haley et al. 1992). NO evoked by NMDA receptor activation enhances the release of excitatory amino acids including NMDA (Luo et al. 1999). These studies are consistent with previous observation that significant changes in NMDA responses and not in α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid responses may contribute to the inhibitory effects of EA on the development of a nociceptive response in CFA-injected rats (Choi et al. 2005).

Consequently, our results show that EA modulated nociceptive input and transmission at the spinal cord level by reducing expression of spinal nNOS. The nNOS participate in the anti-inflammatory effects of EA stimulation in chronic inflammatory processes. Significant altered nNOS expression in the dorsal spinal cord elicited by peripheral inflammation is also considered to be a marker of the activity of spinal cord neurons in chronic inflammation and anti-inflammatory effects of EA treatment.

References

Cao JL, Ding HL, He JH, Zhang LC, Duan SM, Zeng YM : The spinal nitric oxide involved in the inhibitory effect of midazolam on morphine-induced analgesia tolerance. Pharmacol Biochem Behav 80: 493-503, 2005.

Ceccherelli F, Gagliardi G, Visentin R, Sandona F, Casale R, Giron G : The effects of parachlorophenylalanine and naloxone on acupuncture and electroacupuncture modulation of capsaicin-induced neurogenic edema in the rat hind paw. A controlled blind study. Clin Exp Rheumatol 17: 655-662, 1999.

Choi BT, Kang J, Jo UB : Effects of electroacupuncture with different frequencies on spinal ionotropic glutamate receptor expression in complete Freund's adjuvant-injected rat. Acta Histochem 107: 67-76, 2005.

Choi Y, Raja SN, Moore LC, Tobin JR : Neuropathic pain in rats is associated with altered nitric oxide synthase activity in neural tissue. J Neurol Sci 138: 14-20, 1996.

Cizkova D, Lukacova N, Marsala M, Marsala J : Neuropathic pain is associated with alterations of nitric oxide synthase immunoreactivity and catalytic activity in dorsal root ganglia and spinal dorsal horn. Brain Res Bull 58: 161-171, 2002.

Guhring H, Gorig M, Ates M, Coste O, Zeilhofer HU, Pahl A, Rehse K, Brune K : Suppressed injury-induced rise in spinal prostaglandin E2 production and reduced early thermal hyperalgesia in iNOS-deficient mice. J Neurosci 20: 6714-6720, 2000.

Haley JE, Dickenson AH, Schachter M : Electrophysiological evidence for a role of nitric oxide in prolonged chemical nociception in the rat. Neuropharmacology 31: 251-258, 1992.

Han JS : Acupuncture: neuropeptide release produced by electrical stimulation of different frequencies. Trends Neurosci 26: 17-22, 2003.

Han JS, Li SJ, Tang J : Tolerance to electroacupuncture and its cross tolerance to morphine. Neuropharmacology 20: 593-596, 1981.

Hsieh YL : Reduction in induced pain by ultrasound may be caused by altered expression of spinal neuronal nitric oxide synthase-producing neurons. Arch Phys Med Rehabil 86: 1311-1317, 2005.

Lam W, Gundlach AL, Verberne AJ : Neuronal activation in the forebrain following electrical stimulation of the cuneiform nucleus in the rat: hypothalamic expression of c-fos and NGFI-A messenger RNA. Neuroscience 78: 1069-1085, 1997.

Li X, Clark JD : Spinal cord nitric oxide synthase and heme oxygenase limit morphine induced analgesia. Brain Res

Mol Brain Res 95: 96-102, 2001.

Luo ZD, Chaplan SR, Scott BP, Cizkova D, Calcutt NA, Yaksh TL : Neuronal nitric oxide synthase mRNA upregulation in rat sensory neurons after spinal nerve ligation: lack of a role in allodynia development. J Neurosci 19: 9201-9208, 1999.

Moore PK, Oluyomi AO, Babbedge RC, Wallace P, Hart SL : L-NG-nitro arginine methyl ester exhibits antinociceptive activity in the mouse. Br J Pharmacol 102: 198-202, 1991.

Nunez R, Gross GH, Sachs BD : Origin and central projections of rat dorsal penile nerve: possible direct projection to autonomic and somatic neurons by primary afferents of nonmuscle origin. J Comp Neurol 247: 417-429, 1986.

Osborne MG, Coderre TJ : Effects of intrathecal administration of nitric oxide synthase inhibitors on carrageenan-induced thermal hyperalgesia. Br J Pharmacol 126: 1840-1846, 1999.

Tao F, Tao YX, Zhao C, Dore S, Liaw WJ, Raja SN, Johns RA : Differential roles of neuronal and endothelial nitric oxide synthases during carrageenan-induced inflammatory hyperalgesia. Neuroscience 128: 421-430, 2004.

Vizzard MA, Erdman SL, de Groat WC : Increased expression of neuronal nitric oxide synthase in dorsal root ganglion neurons after systemic capsaicin administration. Neuroscience 67: 1-5, 1995.

Wan Y, Wilson SG, Han J, Mogil JS : The effect of genotype on sensitivity to electroacupuncture analgesia. Pain 91: 5-13, 2001.

Zimmermann M : Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16: 109-110, 1983.

Korean J Phys Anthropol
19(3): 215 ~ 222, 2006

Complete Freund's adjuvant 유발 관절염 모델 흰쥐의 척수 뒤뿔내 neuronal nitric oxide synthase 발현에 대한 전기침의 영향

류 지 원, 길 영 기[1], 최 병 태
동의대학교 한의과대학 해부학교실
[1]고신대학교 의과대학 해부학교실

간추림 : 전기침의 항부종 및 통각과민에 대한 효과와 척수내 nitric oxide (NO)의 관련성을 알아보기 위해 complete Freund's adjuvant (CFA)주사한 만성 관절염 모델을 사용하여 척수 뒤뿔내 neuronal nitric oxide synthase (nNOS)의 발현을 살펴 보았다.

흰쥐 오른쪽 발바닥에 CFA 주사한 후 족삼리와 삼음교에 해당하는 혈위를 2 Hz, 15 Hz 및 120 Hz 전기침을 30분간 3일 간격으로 30일 동안 처치하였다. CFA 주사 후 30일에 척수 중 제1번, 제3번 및 제5번 허리분절에 해당하는 분절의 nNOS발현을 면역조직화학적으로 검색하였다.

CFA를 주사한 흰쥐에서 척수 뒤뿔의 모든 부위에서 nNOS 발현이 현저히 증가하였으며 특히 superficial laminae (SDH, laminae I and II)에서의 증가가 현저하였다. 그러나 모든 전기침군에서 nNOS의 발현이 감소하였는데 특히 제1번과 제3번 허리분절의 SDH와 nucleus proprius (NP, laminae III and IV)부위에서 모든 전기침군이, neck of the dorsal horn (NECK, laminae V and VI)부위에서 2 Hz 전기침군이 유의성 있는 감소를 보여 주었다. 제5번 허리분절에서 NP와 NECK부위에서 2 Hz와 15 Hz 전기침이 유의성 있는 효과를 보여 주었다.

이로 보아 전기침은 척수 뒤뿔내 nNOS발현억제를 통한 통각과민 완화 효과를 나타내는 것을 알 수 있으며 통각유발과 NO관련성을 유추할 수 있으며 전기침의 항염증의 효과 검정에 있어 nNOS발현이 주요 표지로 이용가능 할 것이다.

찾아보기 낱말 : 전기침, Nitric oxide synthase, Complete Freund's adjuvant, 척수

교신저자 : 최병태 (동의대학교 한의과대학 해부학교실)
전자우편 : choibt@deu.ac.kr