

Effects of Ultrasound and Diclofenac Phonophoresis on Inflammatory Pain Relief: Suppression of Inducible Nitric Oxide Synthase in Arthritic Rats

Background and Purpose. The direct effects of ultrasound (US) and phonophoresis of a nonsteroidal anti-inflammatory drug (NSAID) on injured peripheral tissue have been widely investigated, but evidence concerning the effects of central spinal nociceptive modulation seems to be lacking. The purpose of this study was to investigate the peripheral influences of US and phonophoresis on the modulation of spinal inducible nitric oxide synthase (iNOS) expression elicited by hind paw stimulation with an ankle injection of complete Freund adjuvant (CFA). **Subjects and Methods.** Inflammatory arthritis was induced in 18 male Wistar rats with intra-articular tibiotarsal injections of CFA. Serial changes in inflammatory pain reactions, including hind-limb edema, and the locomotor activity of the arthritic animals were measured. Arthritic rats underwent US (n=6), diclofenac phonophoresis (n=6), or sham treatment (n=6) on the CFA-injected leg at 18 hours after injection. At 20 hours after injection, spinal inducible nitric oxide synthase-like immunoreactive (iNOS-LI) cells were examined. **Results.** Following the CFA injection, all animals' paw diameters and ankle circumferences ipsilateral to the injected leg were significantly increased compared with the values prior to injection. The rearing behavior of arthritic animals had improved significantly after US and diclofenac phonophoresis treatments. The mean total number (\pm SD) of iNOS-LI cells per section of segments L1 and L2 of the bilateral spinal cord of the sham treatment, US, and phonophoresis groups were 531.20 ± 6.11 , 124.20 ± 4.09 , and 114.80 ± 3.23 , respectively. The total numbers of iNOS-LI cells in rats treated with US and diclofenac phonophoresis were significantly smaller than in those receiving sham treatment. There were no significant differences in the total number of iNOS-LI cells ipsilateral to the injected leg between the US and diclofenac phonophoresis groups. **Discussion and Conclusion.** Ultrasound and phonophoresis treatments probably modulate and prevent the CFA-insult-induced increase in total and regional iNOS-LI neurons. Peripheral use of diclofenac phonophoresis offers little advantage over US alone in affecting the central mechanisms of nociception. The peripheral influences of US and phonophoresis on the central modulation of the spinal nociceptive processing system are important and may reflect the work being done through the neuroplasticity of spinal cord in response to peripheral input of US and phonophoresis. [Hsieh YL. Effects of ultrasound and diclofenac phonophoresis on inflammatory pain relief: suppression of inducible nitric oxide synthase in arthritic rats. *Phys Ther.* 2006;86:39–49.]

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Yueh-Ling Hsieh

Ultrasonography (US), a form of acoustic energy, is often used in physical therapy because of its deep-heat and pain-relieving effects.¹⁻³ When US enters the body, it can affect the cells and tissues through thermal and nonthermal mechanisms. Structures heated by US preferentially include fibrotic muscle, tendon sheaths, and major nerve roots.⁴ Evidence indicates, however, that where nonthermal mechanisms are thought to play a primary role in producing a therapeutically significant effect (ie, the stimulation of tissue regeneration), there will be increases in the rate of cell membrane permeability and diffusion, increases in intracellular calcium, and changes in the electrical activity of nervous tissue.^{1,5}

Phonophoresis is the migration of drug molecules through the skin under the US transducer.⁶ Its major advantages are (1) the introduction of medication to a local area without invasion of the skin and (2) the synergistic interaction of US and drugs.⁷ In physical therapy, phonophoresis with nonsteroidal anti-inflammatory drugs (NSAIDs) is commonly used to treat inflamed tissues.⁸ Physiologic and pharmacologic studies⁹⁻¹² have shown that hyperalgesia can be eliminated by treating an animal with a variety of drugs, including opioids and NSAIDs. Diclofenac, an NSAID with both anti-inflammatory and antinociceptive actions, is widely used in clinical practice, especially for painful and inflammatory rheumatic conditions and certain nonrheumatic conditions.¹³

Nitric oxide (NO) synthesized from L-arginine by the enzyme NO synthase (NOS) is a short-lived, gaseous free radical that serves as an atypical transmitter.¹⁴ Three isoforms of NOS—neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS)—have been identified, and each of them has been cloned.¹⁵ Many functions of NO in the central nervous system have been demonstrated, including a role in the processing of pain and inflammation.¹⁶⁻¹⁸ According to experimental arthritis models, overproduction of NO is associated with persistent inflammation¹⁹ and tissue destruction.²⁰ A considerable amount of evidence has demonstrated that NO and its enzyme are involved in the central mechanisms of inflammatory hyperalgesia at the spinal cord level.^{21,22} Inducible NOS is not normally expressed in

the resting neurons of the brain and spinal cord postnatally, but is synthesized upon cell activation when induced by stimuli.²³ The induction of iNOS has been implicated in the pathogenesis of inflammation.²⁴ Following injury or trauma and in several inflammatory pathologic states, iNOS expression can be induced in the brain and spinal cord.^{21,25,26} In this study, therefore, iNOS was used as a marker of inflammation and nociceptive processes in the spinal cord.

A number of good animal models representing human arthritis have been developed over the years.²⁷⁻²⁹ A chronic arthritic model created by intra-articular injection of complete Freund adjuvant (CFA) into a rat, is characterized by chronic inflammation and pain of the joint.^{30,31} The direct peripheral antinociceptive effects of US and NSAID phonophoresis in treating injured tissue have been widely investigated,^{4,8} but the possible effects of US and NSAID phonophoresis on the central modulation of inflammatory nociception are still unclear. The purpose of this study was to investigate the peripheral influences of US and phonophoresis on the modulation of spinal iNOS expression elicited by hind paw stimulation with an ankle injection of CFA. Based on the findings of the previous studies,^{16,23} I hypothesized that the US and phonophoresis would alter the increased numbers of spinal iNOS-containing cells induced by inflammation at the spinal levels.

Method

Experimental Animals

Experiments were performed on 18 male Wistar rats (weighing 250–300 g). Up to 4 animals were housed in a large cage with sawdust bedding and free access to food and water for at least 2 weeks before the experiments. They were kept in an animal house with 12-hour alternating light-dark cycles. Ethical guidelines of the International Association for Study of Pain in Animals were followed.³²

Induction of Arthritic Inflammation

Monoarthritis was induced according to the method described by Butler et al.³¹ The animals were put under brief halothane anesthesia and then the tibiotarsal joint of their right hind paw was injected with 50 μ L of CFA

YL Hsieh, PT, PhD, is Assistant Professor, Department of Physical Therapy, Hung-Kuang University, Taichung, 34, Chung-Chie Road, Sha Lu, Taichung 433, Taiwan, Republic of China (sherric@ms1.tcol.com.tw).

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(10 mg/mL *Mycobacterium tuberculosis*) (F5881).^{*} The monoarthritic animals were placed by themselves in clear acrylic restrainers (27 cm wide × 48 cm deep × 20 cm high), which allowed for their free movement, 18 hours before any experimentation in order to adjust to these conditions. Eighteen hours after CFA induction, all 18 arthritic animals were slightly anesthetized by ethyl ether inhalation and randomly divided into 3 groups of 6 animals each: the first group received US treatment, the second group received diclofenac phonophoresis, and the third group received a sham treatment, which consisted of placing a US apparatus on the hind limb but without delivering US output.

Evaluation of Inflammatory Pain Reactions

To assess the extent of the peripheral inflammation, 2 parameters of inflammatory edema were considered: the circumference and diameter of the ankle and paw ipsilateral and contralateral to CFA injection. These were measured with a tape and caliper 1 day prior to CFA injection and 18 hours afterward. Because acute treatment of US is ineffective in inhibiting edema development,³³ the data immediately after treatments were not collected in order to avoid unnecessary manipulations and noxious stimuli affecting iNOS expression.

The evolution of the inflammatory behavioral response was monitored by assessing the rearing behavior of the awake animal 1 day before CFA injection, 4 hours after CFA injection, and immediately after treatment. The locomotor activity tests of rats were performed by an experimenter who was unaware of group assignment. A Digiscan Animal Activity Monitor (Automated Digiscan, model RXYZCM 16)[†] was applied to monitor the rats' locomotion by using procedure previously established in my laboratory.³⁴ Animals were placed singly in a clear acrylic activity monitor cage (40 cm wide × 40 cm deep × 30.5 cm high). Infrared monitoring sensors were located every 2.54 cm along the perimeter (16 infrared beams along each side) and 4.5 cm above the floor. Two additional sets of 16 sensors were located 10.5 cm above the floor on opposite sides. Data were collected and analyzed by a Digiscan Analyzer (Accuscan Model CDA-8),[†] which in turn sent information to a computer where it was stored for future analysis. One of the activity variables, vertical activity (VA), which was calculated directly by the Digiscan Analyzer, was selected for further study.

The unanesthetized rats were not previously habituated to the cage to avoid accommodation and elicit more vertical exploratory locomotion after repeated expo-

ures. All behavioral monitoring was performed during the dark phase of the animals' light-dark cycle (ie, from 1900 to 2200 hours, in a dark room with a background noise below 30 dB). Each monitoring period lasted 30 minutes and the locomotor activity data were collected automatically.

Ultrasound and Diclofenac Phonophoresis Treatments

Eighteen hours after CFA induction, the animals were treated with US or diclofenac phonophoresis. The parameters of US chosen in this study were established previously in my laboratory.³⁵ The pulsed US was used to minimize heating.³⁶ Pulsed US (1 MHz, spatial average/temporal average intensity [I_{SATA}]=0.5 W/cm², 50% duty cycle) was applied for 5 minutes. These ratios resulted in sonication/nonsonication times of 2 milliseconds/2 milliseconds. Sonication was produced by using a commercially available device (US-3)[‡] with a treatment head of 1.7 cm in diameter and an effective radiating area of 0.75 cm². The US treatments were applied in a dynamic manner to the single hind limb ipsilateral to the CFA-injected leg. A dose of approximately 1.5 g of a standard coupling medium (Aquasonic 100)[§] was applied over the skin and used for the US, phonophoresis, and sham treatments.

The parameters for US also were those used for phonophoresis as recommended by Asano et al.³⁶ Transcutaneous diclofenac was delivered by using 0.4 g of 1% diclofenac diethylammonium gel (11.6 mg/g) (Voltaren Emulgel).^{||} This gel was first rubbed into the skin of the right hind limb, and a standard coupling medium was applied over the medication for phonophoresis. Simultaneously, the right hind limb in the other monoarthritic rat was used for the sham treatment, which was done without US output or diclofenac application.

Immunohistochemistry for iNOS

Two hours after US, diclofenac phonophoresis, or sham treatment, the rats were deeply anesthetized with inhaled ethyl ether. They then were intracardially perfused with 100 mL of 0.9% heparinized sodium chloride solution in 0.1 M phosphate-buffered saline (PBS, pH=7.4). This was followed by 500 mL of 4% paraformaldehyde diluted in 0.1 M PBS. The lumbar enlargement, including conus medullaris, was removed. These areas were postfixated for 6 hours in the same fixative, then immersed in 30% sucrose in 0.1 M PBS for 2 days at 4°C. Following these steps for postfixation and cryoprotection, serial coronal sections that were 30- μ m thick were cut on a freezing microtome. Every section was

^{*} Sigma-Aldrich, 3050 Spruce St, St Louis, MO 63103.

[†] AccuScan Instruments Inc (formerly Omnitech Electronics Inc), 5090 Trabue Rd, Columbus, OH 43228.

[‡] ITO Co, 1-23-15, Hakusan, Bunkyo-ku, Tokyo, Japan.

[§] Parker Laboratories, 286 Eldridge Rd, Fairfield, NJ 07004.

^{||} Ciba Specialty Chemicals, 5/F, No 261, Sec 3, Nanking East Rd, Taipei 105, Taiwan.

Table 1.Peripheral Edema, Ankle Circumference, and Paw Diameter Before and After Complete Freund Adjuvant (CFA) Injection^a

Group	Inflammatory Edema (mm)			
	Before Injection		18 Hours After Injection	
	Ankle	Paw	Ankle	Paw
Sham treatment	29.2±0.7/29.0±0.9	7.1±0.6/6.9±0.4	31.4±1.2 ^b /28.3±1.4	11.5±0.2 ^b /6.7±0.3
Ultrasound	28.9±0.8/28.6±1.1	6.9±0.8/6.7±0.3	31.0±1.1 ^b /28.8±1.2	11.1±0.8 ^b /7.1±0.7
Phonophoresis	28.4±1.0/28.8±1.2	7.1±0.5/7.1±0.6	31.2±1.2 ^b /28.8±0.9	11.4±0.8 ^b /6.9±0.5

^a Data are the ipsilateral/contralateral mean±SD.^b Significant compared with preinjection values, posttest Scheffé method ($P<.05$).

collected in wash/dilution PBS and processed as a free-floating section. To identify the iNOS-like immunoreactive (iNOS-LI) cells clearly, every fifth section was processed with thionin Nissl stain for the purposes of comparison.

Frozen sections of spinal cord were first mounted on slides coated with poly-L-lysine and then blocked in 10% normal goat serum (in PBS with 0.3% Triton X-100). They were subsequently exposed overnight at 4°C to rabbit polyclonal antibody against iNOS.[#] The next day, the primary antibody was removed, and the sections were washed and exposed to a secondary biotinylated goat anti-rabbit antibody** for 1 hour at room temperature. Next, the sections were washed, and then incubated with a streptavidin–horseradish peroxidase conjugate. In short, frozen sections were subjected to an avidin-biotin-peroxidase complex (ABC) reaction (Vectastain).^{††} Finally, cells containing the iNOS-ABC complex were visualized as brown precipitate yields by using 3,3'-diaminobenzidine (0.2 mg/mL) as a substrate.^{‡‡} All sections were air-dried, cleared in alcohol, and coverslipped. The locations of the iNOS-LI cells in the gray matter of the spinal cord were observed under a light microscope.

Data Analysis

To evaluate the extent of edema, paw diameter and ankle circumference were measured before and after CFA injection. Results are expressed as the mean±standard deviation (SD). Statistical analysis was performed using repeated-measures analyses of variance (ANOVAs) and Scheffé tests to compare the edema of the ankle and paw before and after CFA-induced inflammation. To analyze the behavioral locomotion of arthritic animals, total VA was calculated as the mean±standard error of the mean (SEM). A repeated-measures ANOVA and the Dunnett *t* test were used to

assess statistical significance. Analyses were conducted using SPSS for Windows, version 10.0.^{§§}

Tissue sections immunohistochemically stained with iNOS were examined under a light microscope at ×40 and ×400 to reconstruct the location of iNOS-LI cells in the gray matter of the spinal cord. Sections were first examined under dark field microscopy to determine the segmental level according to the method of Molander et al³⁷ and to assess the gray matter landmarks. The sections were then examined under light field microscopy at ×10 to localize the iNOS-LI cells. Labeled nuclei were counted using a camera lucida attachment. To study their laminar distribution, 4 regions were defined: superficial dorsal horn (laminae I–II, superficial), nucleus proprius (laminae III–IV), neck of the dorsal horn (laminae V–VI, deep laminae), and the ventral gray horn (laminae VII–X).

For each rat, 2 sets of analyses were made: (1) the total number of iNOS-LI cells in 20 randomly selected L1-L2 sections from each animal, and (2) the number of iNOS-LI cells per defined region of the spinal gray matter in these 20 sections. Differences in labeling for each treatment were assessed by performing an ANOVA and the Scheffé test. The investigator responsible for plotting and counting iNOS-LI cells was unaware of the experimental conditions of the animals. Results are expressed as the mean±SEM.

Results

Inflammatory Pain Reaction

Table 1 shows the significantly different extents of peripheral edema, paw diameters, and ankle circumferences before and 18 hours after intraplantar injection of CFA in each group with inflammation ($P<.05$, ANOVA). After CFA injection, all animals' paw diameters and ankle circumferences ipsilateral to the injected leg significantly increased compared with the values prior to

[#] Santa Cruz Biotechnology Inc, 2145 Delaware Ave, Santa Cruz, CA 95060.

** BioGenex, 4600 Norris Canyon Rd, San Ramon, CA 94583.

†† Vector Laboratories, 30 Ingold Rd, Burlingame, CA 94010.

‡‡ Pierce, 3747 N, Meridian Road, PO Box 117, Rockford, IL 61105.

§§ SPSS Inc, 233 S Wacker Dr, Chicago, IL 60606.

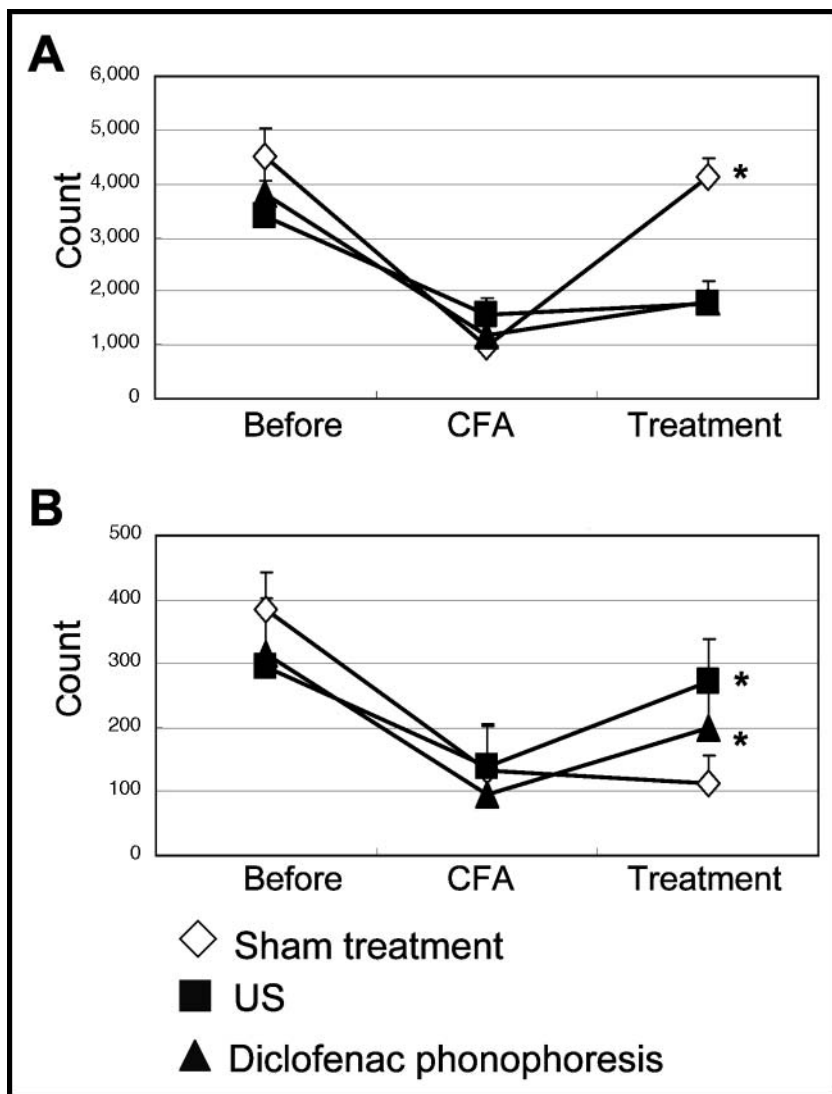


Figure 1. Serial changes in (A) horizontal activity and (B) vertical activity of rats 1 day before CFA injection (Before), 4 hours after injection of complete Freund adjuvant (CFA), and immediately after sham, ultrasound (US), or diclofenac phonophoresis treatments (Treatment). Asterisk indicates $P < .05$.

injection ($P < .05$, Scheffé test). The contralateral ankle circumference and paw diameter, however, were not significantly different from the dimensions before injection into the ipsilateral hind limb ($P > .05$, Scheffé test).

Figure 1 shows the evolution of inflammatory pain reaction. Before CFA injection, VA did not differ in each group ($P > .05$, ANOVA). Four hours after injection, VA decreased compared with preinjection the values in each group ($P < .05$, ANOVA). When animals that had inflammatory arthritis for 18 hours were treated with US and diclofenac phonophoresis, VA increased significantly ($P < .05$, Dunnett t test) compared with the values prior to treatment (4 hours after injection). At 18 hours of CFA-induced inflammation, VA did not change signifi-

cantly those arthritic animals that underwent the sham treatment ($P > .05$, ANOVA) compared with values before the treatment (4 hours after injection).

Lumbar Spinal Cord Samples

In the lumbar spinal cord of all CFA-induced arthritic animals, labeled cells were evident, with their dark brunet nuclei contrasting with the light-brown cytoplasm (Fig. 2).

Arthritic Inflammation Versus US and Diclofenac Phonophoresis

Intraplantar injection of CFA into the hind paw evoked abundant expression of iNOS in the ipsilateral and contralateral dorsal horns of the L1 and L2 segments of the spinal cord in the rats that underwent the sham treatment. Inducible NOS-LI cells were stained with immunohistochemistry to variable degrees, and all were considered for statistical analysis (Tab. 2 and Fig. 3). The mean total numbers of iNOS-LI cells per section of segments L1 and L2 of the bilateral spinal cord of sham treatment, US, and phonophoresis groups were 531.20 ± 6.11 , 124.20 ± 4.09 , and 114.80 ± 3.23 , respectively. The total numbers of bilateral spinal iNOS-LI cells were significantly different the sham treatment group and the US group and between the sham treatment group and the phonophoresis group ($P < .001$, ANOVA).

In the ipsilateral spinal cord, the mean total numbers of iNOS-LI cells for all laminae combined were 53.40 ± 3.23 and 65.00 ± 2.07 in the US and phonophoresis groups, respectively, which were significantly smaller than that of the sham treatment groups (317.60 ± 4.18 , Scheffé test, $P < .01$). There were no significant differences in the total number of iNOS cells ipsilateral to the injected leg between the US and diclofenac phonophoresis groups ($P > .05$, Scheffé test). On the contralateral side, the total numbers of labeled cells in the US and phonophoresis groups were 70.80 ± 5.03 and 49.80 ± 4.04 , respectively, which were significantly smaller than that of the sham treatment group (213.60 ± 2.56 , Scheffé test, $P < .01$). There was a significantly reduced number of iNOS-LI cells in contralateral spinal cord of the phonophoresis group than in the US group ($P < .05$, Scheffé test).

The spatial distribution of iNOS-LI cells observed in laminae I–II, III–IV, V–VI, and VII–X varied significantly

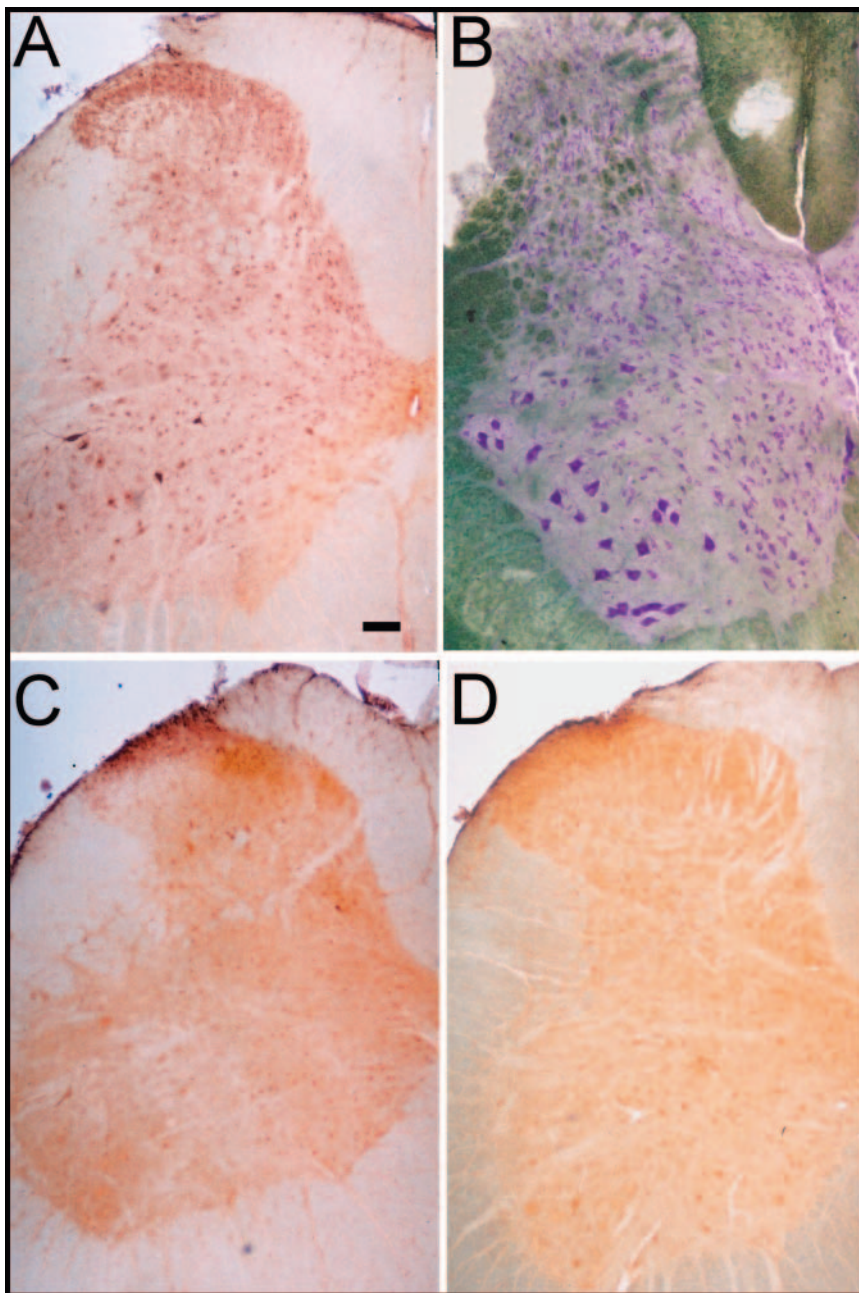


Figure 2. Photomicrographs illustrate inducible nitric oxide synthase-like immunoreactive (iNOS-LI) neurons in the lumbar spinal cord ipsilateral to the injected leg 18 hours after intraplantar injection of complete Freund adjuvant (CFA) and 2 hours after (A) sham treatment, (C) ultrasound, or (D) diclofenac phonophoresis. (B) Sample is processed with thionin Nissl stain to identify the location of iNOS-LI cells. Bar in lower right-hand corner of photomicrograph A indicates 0.1 mm.

among the sham treatment, US, and phonophoresis groups ($P < .05$, ANOVA). On the ipsilateral side in the sham-treatment group, the mean numbers of CFA-induced iNOS-LI were 82.80 ± 3.23 (26.1% of the total number of labeled cells) in laminae I-II, 49.40 ± 1.33 (15.6%) in laminae II-IV, 51.00 ± 2.10 (16.0%) in laminae V-VI, and 134.40 ± 2.84 (42.3%) in laminae VII-X.

After US and diclofenac phonophoresis, the mean numbers of CFA-induced iNOS-LI were 11.60 ± 0.51 (22.1% of total number of iNOS-LI cells) and 12.40 ± 1.21 (19.1% of the total number of iNOS-LI cells), respectively, in laminae I-II; 14.00 ± 1.87 (25.8%) and 10.40 ± 0.81 (16.2%) in laminae III-IV; 11.20 ± 0.58 (21.3%) and 15.60 ± 1.96 (23.9%) in laminae V-VI; and 16.60 ± 1.63 (30.9%) and 26.60 ± 2.36 (40.9%) in laminae VII-X, significantly smaller than the numbers in the sham-treated rats (all $P < .05$, Scheffé test). Laminal analysis revealed that spatial distribution of iNOS-LI cells showed no significant difference among laminae in the US and phonophoresis groups ($P > .05$), except in laminae VII-X ($P < .05$). In the contralateral spinal cord, significant differences were observed among the 3 groups in laminae I-II, III-IV, V-VI, and VII-X ($P < .05$, ANOVA). Spatial distributions and statistical differences in laminae I-II, III-IV, V-VI, and VII-X were similar to those of the ipsilateral side (Tab. 2). The sham treatment group had a significantly higher total number of labeled cells and cells in all laminae when compared with the other 2 groups ($P < .05$, Scheffé test).

Discussion

A previous study¹⁶ demonstrated an increase in iNOS expression in the spinal cord following the stimulus of peripheral inflammation. The results of my study confirm the previous finding¹⁶ of a substantial expression of iNOS in the bilateral lumbar spinal cord after right hind paw injection of CFA in control group that received sham treatment. Moreover, the increased spinal iNOS-LI cells in laminae I-II, III-IV, V-VI, and VII-X induced by CFA (noxious stimulus) were suppressed by US and diclofenac phonophoretic treatments.

After the induction of arthritis by injection of CFA, local inflammation is produced and the free nerve endings of nociceptors are activated. Not only do local inflammatory reactions, such as redness, edema, and heat, occur, but there also are high-frequency barrages of C-fiber discharges after this peripheral inflammation has begun. The high-frequency discharges of C fibers can result in the release of glutamate and

Table 2.

Expression of Inducible Nitric Oxide Synthase-Like Immunoreactivity (iNOS-LI) in the Lumbar Spinal Cord of Arthritic Rats After Sham, Ultrasound, and Phonophoresis Treatments^a

Group	Spinal Laminae				Sum
	I-II	III-IV	V-VI	VII-X	
Ipsilateral spinal cord from CFA ^b injection					
Sham treatment	82.80±3.23 (26.1%)	49.40±1.33 (15.6%)	51.00±2.10 (16.0%)	134.40±2.84 (42.3%)	317.60±4.18
Ultrasound	11.60±0.51 ^d (22.1%)	14.00±1.87 ^e (25.8%)	11.20±0.58 ^e (21.3%)	16.60±1.63 ^e (30.9%)	53.40 ± 3.23 ^e
Phonophoresis	12.40±1.21 ^d (19.1%)	10.40±0.81 ^e (16.2%)	15.60±1.96 ^e (23.9%)	26.60±2.36 ^{e,f} (40.9%)	65.00±2.07 ^e
<i>p</i> ^c	<.001	<.001	<.001	<.001	<.001
Contralateral spinal cord from CFA injection					
Sham treatment	78.00±2.85 (36.5%)	32.20±1.02 (15.1%)	34.20±3.92 (16.0%)	69.20±4.59 (32.5%)	213.60±2.56
Ultrasound	15.60±1.69 ^d (22.0%)	12.20±1.36 ^e (17.2%)	13.60±0.81 ^e (19.5%)	29.40±3.03 ^e (41.2%)	70.80±5.03 ^e
Phonophoresis	7.80±1.20 ^d (15.4%)	10.20±1.71 ^e (20.1%)	11.60±0.68 ^e (23.6%)	20.20±1.71 ^e (41.0%)	49.80±4.04 ^{e,f}
<i>p</i> ^c	<.001	<.001	<.001	<.001	<.001

^a Each value represents the average number of labeled neurons (±SEM) in each group for each set of laminae. Distributional percentage is shown in parentheses.

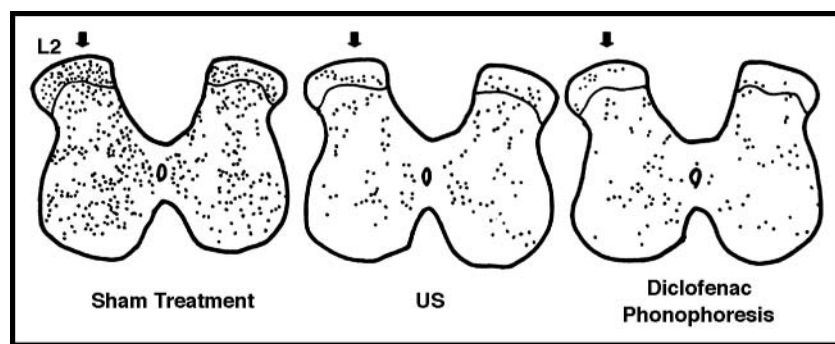
^b CFA=complete Freund adjuvant.

^c Analysis of variance was used to compare the iNOS-LI numbers among sham, ultrasound, and phonophoresis groups in each side of spinal cord.

^d Significant compared with sham group, posttest Scheffé method (*P*<.05).

^e Significant compared with sham group, posttest Scheffé method (*P*<.01).

^f Significant compared with ultrasound group, posttest Scheffé method (*P*<.05).

**Figure 3.**

Drawings illustrate the ventrodorsal extent of inducible nitric oxide synthase-like immunoreactive (iNOS-LI) cells in the second lumbar segment of 3 complete Freund adjuvant (CFA)-induced arthritic rats treated with sham treatment, ultrasound (US), or diclofenac phonophoresis. The left side of each image (arrows) is ipsilateral to the injected leg.

neurokinins from the central terminals of afferent fibers.³⁸ In a neurochemical and neurophysiological review article of rats with adjuvant arthritis,³⁹ the spinal cord level of this nonopioid peptide including substance P was observed to increase remarkably. Substance P concentrated in the laminae I and II of the spinal dorsal horn was suggested to play a role as a primary afferent transmitter mediating or facilitating the expression of nociceptive inputs. The results of a previous study⁴⁰ suggested that there may be an interaction between nerve terminals containing glutamate and substance P and that NO may directly participate in the regulation of synaptic transmission in substance P-containing nerve

terminals. The nociceptive effect of substance P was shown to be mediated by NO at the spinal levels.⁴¹ However, NO generated by *N*-methyl-D-aspartate (NMDA)-receptor activation could enhance pain transmission.³⁹ Thus, adjuvant arthritis is a model of inflammation in which sustained input in primary afferents can result in a production of NO. These changes may manifest as central sensitization and hyperalgesia.

The contribution of NO to the processing of sustained nociceptive inputs at the level of the spinal cord is clearly illustrated in studies of NOS inhibitors.⁴² These findings suggest that NOS-positive neurons

may be susceptible to nociceptive stimuli. When induced by appropriate stimuli, iNOS levels are 100 to 1,000 times that produced by nNOS.⁴³ Because iNOS generates such high levels of NO, its effects in the response to certain injuries of the central nervous system are potentially of great importance. Increased release of NO in the spinal cord has been associated with an enhanced excitability of dorsal horn neurons to nociceptive stimuli.¹⁶ The administration of a selective inhibitor of iNOS can result in dose-independent inhibition of zymosan-induced thermal hyperalgesia in the spinal cord.⁴⁴ Although the administration of an NOS antagonist does

not alter baseline nociceptive responses, it does reduce hyperalgesia and spontaneous nociceptive reflex induced by peripheral nerve damage or paw inflammation.⁴⁵

The expression of the neuronal iNOS has been characterized in numerous cell types as a consequence of inflammatory processes that follow infection, disease, or tissue damage.⁴⁶ In the adjuvant arthritis model, NO also has been shown to be involved in the development of inflammation.^{20,47} Inducible NOS has a closer relationship to the course of a peripheral inflammation than the other isoforms of NOS.¹⁶

Intraplantar CFA is associated with extensive inflammatory edema of the ankle and paw, as demonstrated by the significantly increased ankle circumference and paw diameter after injection. The contralateral ankle and paw were not significantly different to their preinjection condition, indicating that the intraplantar CFA injection produced localized peripheral inflammation. Unexpectedly, the data showed that the number of iNOS-LI cells also increased on the contralateral side, yet the increase was always higher on the ipsilateral side. Bilateral changes of metabolic glucose uptake by neuronal cells after ipsilateral injury stimulation also have been observed in sciatic nerve ligation and noxious thermal studies.⁴⁸ Increased glucose metabolic rates were observed on both sides, but higher on the ipsilateral side.⁴⁹

In addition, recent work⁵⁰ has demonstrated a bilateral increase in nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity in the rat lumbar spinal cord following unilateral hind paw inflammation. Because NADPH-diaphorase has been shown to co-localize with NOS,⁵¹ the result of that experiment supports those of my study. This bilateral increase in iNOS-LI cells suggests communication between the 2 sides of the spinal cord. In addition, the presence of pain perception may activate remote polysynaptic neural pathways that are important in pain adaptation behaviors leading to the bilateral increase in iNOS.

Wu et al⁵² first demonstrated that iNOS immunoreactivity appears in the ependymal cell layer around the central canal (laminae X) in the unilateral adjuvant arthritis model. This change occurred not only in the lumbar enlargement but also in the cervical enlargement; their observation suggests that induction might involve the entire spinal cord. In my study, distributions of iNOS-LI cells in the ipsilateral spinal cord of the arthritic animals receiving sham treatment mainly appeared in the laminae VII–X (42.3% of the total labeled cells) and largely confirmed the previously reported findings. In the sham-treated group, more than 49 iNOS-LI cells per section were demonstrated in 4

fairly well-defined regions: (1) the superficial layers of the dorsal horn (laminae I–II), (2) the nucleus proprius (laminae III–IV), (3) the deep layers of the dorsal horn (laminae V–VI), and (4) the ventral gray horn (laminae VII–X, pericentral area).

Inducible NOS also was demonstrated to be related to central sensitization, and its immunoreactivity continued to increase, at least during the development of arthritis induced by Freund adjuvant.⁵² In my study, the results are consistent with the observation that iNOS activity increases following noxious stimulus, and this finding suggests that NO is involved in nociceptive information processing. Following US or diclofenac phonophoretic treatment, the number of regional iNOS-LI cells decreased compared with the sham treatment values. Thus, US and diclofenac phonophoresis treatments modulated the CFA-insult-induced increase in total and regional iNOS-LI cells. Therefore, US and diclofenac phonophoretic therapies may have a modulating effect on inflammatory pain in the central nervous system may be suggested. This study demonstrated that US and phonophoresis act spinally in response to a peripheral CFA insult. Further study is required to determine whether this action is caused by reduced peripheral neural activity or by some other mechanism.

In my study, it was obvious that some of the labeled cells were neuronal (eg, the large motoneurons in the ventral horn), and it was equally apparent that many of them were not neuronal (compare sizes of nuclei with Nissl sections). The Nissl stain could mark the nuclei of both neurons and glia cells. Matching iNOS-LI positive cells, therefore, cannot distinguish whether iNOS positive cells are glia cells or neurons. Previous work^{16,23,53} has shown that iNOS in the spinal cord was expressed primarily in glia. At the level of the spinal cord, there is mounting evidence that glia within the dorsal horns create and maintain pathological pain. Spinal cord glia are implicated in exaggerated pain states elicited by subcutaneous inflammation.⁵⁴ These glia become activated in response to pathogens as well as substances released by both primary afferent terminals (eg, substance P) and pain transmission neurons (eg, NO). Once activated, these glia can release a variety of neuroactive substances, including NO and excitatory amino acid. In turn, these substances are capable of altering pain both by enhancing primary afferent resale of substance P and by increasing the excitability of pain transmission neurons.⁵⁴

Inflammatory pain states are reversed by blockade of the action of glia products, including proinflammatory cytokines and iNOS.⁵⁵ My laboratory has previously shown that the nNOS-LI neurons were abundant bilaterally in the L1 and L2 region of the spinal cord following

CFA-induced arthritis.⁵⁶ Moreover, the increases in CFA-induced spinal nNOS-LI neurons were suppressed by US treatment.⁵⁶ Similarly, in my study, the number of iNOS-LI cells was significantly higher after CFA insult than in those cells of rats treated with US and diclofenac phonophoresis. These findings of my study suggest that after peripheral inflammation, the iNOS expression, like glia cells, appears to be driven centrally because there was a global spinal cord response. Ultrasound and diclofenac phonophoresis may ameliorate the consequences of overwhelming NO generation through iNOS induction in glial cells following inflammation.

In my study, iNOS-LI cells around the central canal of the lumbar enlargement were more dense in rats treated with diclofenac phonophoresis than in those treated with US; however, there were no significant differences in the total number of iNOS cells ipsilateral to the injected leg between the US and diclofenac phonophoresis groups. The data did not reflect the augmentation of the positive synergistic action between diclofenac and US at the spinal level. A previous clinical study⁵⁷ also concluded that US results in decreased pain in these selected soft tissue injuries, but the addition of fluocinonide phonophoresis did not augment the benefits of US used alone. Ibuprofen phonophoresis was not superior to conventional US in patients with knee osteoarthritis.⁵⁸ Therefore, the results revealed that the suppression of increased iNOS-LI cells in arthritic rats treated with diclofenac phonophoresis was mainly attributed to US. Evidence suggests that systematic co-injection of NSAIDs, including ibuprofen and indomethacin, can reduce neuronal iNOS expression and cell death.⁵⁹ Central antinociceptive actions of NSAIDs have been reported to depend on the routes of administration, including intrathecal, intracerebroventricular, or systemic administration.⁶⁰ The effective dose of diclofenac required to alter further spinal iNOS expression was lower when exerted by phonophoresis than when exerted by systemic administration, which can probably explain this finding. The amount of diclofenac by phonophoresis that really reaches subcutaneous tissues warrants further study. Therefore, the antinociceptive action of peripherally local administration of diclofenac by phonophoresis did not probably take place at central levels. The peripheral use of diclofenac phonophoresis in this study offers little advantage over US alone in making an impact on the central mechanisms of nociception.

The measurement of VA can provide information about an animal's rearing behavior and is important in indexing the behavioral effects of various experimental manipulations.^{34,61} The most striking characteristic of the CFA-induced arthritis model is the position of the affected limb, which is flexed at the ankle, knee, and hip throughout more than 6 weeks.³¹ A previous study⁶²

demonstrated that the rearing behavior decreased following formalin-induced persistent pain in rats.⁶¹ In my study, there was a decrease in VA at 18 hours after CFA injection without treatment. These results are consistent with those of previous studies,^{62,63} which indicate that CFA-induced inflammation reduces rearing behavior. This reduced rearing behavior may be due to guarding induced by inflammatory pain arising from persistent noxious stimulation by CFA. The recovery of VA after treatment with US and diclofenac phonophoresis indicated that the increase in rearing behavior may have been due to increased activity of the rat's hind limb and suggests that the arthritic limb was less uncomfortable in bearing body weight while exploring the unfamiliar environment.

Despite frequent use of US and phonophoresis in the treatment of musculoskeletal disorders, firm evidence of their effectiveness remains uncertain. There seems to be little neurobiological evidence for the effectiveness of US therapy on a wide variety of disorders, including lateral epicondylitis, osteoarthritis, breast pain after childbirth, and traumatized perineum.^{64,65} Based on the findings of my study, the mechanism of US therapy is probably not governed by a universal peripheral thermal and nonthermal mechanism, but may be influenced by a centrally modulated mechanism at the spinal cord level. In addition, few studies have been done on the US management of neuropathic pain. Ultrasound therapy may provide a new approach in relieving neuropathic pain, including phantom pain and central pain, due to its central effect. Therefore, as our understanding of the complexities of the mechanism of US therapy increases, we are becoming more able to appropriately combine treatments to achieve not only improved pain relief but also improved function.

Conclusion

The present data provided evidence that the spinal expression of iNOS following US or diclofenac phonophoresis is decreased in monoarthritis compared with rats receiving sham treatment at the early phase of inflammation. This evidence may reflect the complexity of the transducing and suppressive transmitter systems involved in the central processing of ongoing pain and further support the view that there is a potentiation of central pain modulation of US in the presence of inflammation. Adjuvant arthritis in a rat constitutes the only laboratory animal model of chronic pain that has been validated to a significant extent.⁶⁶ Therefore, future work involving chronic inflammation and long-term intervention of US and diclofenac phonophoresis is needed to further assess the potential effects of US and phonophoresis on the central pain modulated mechanism.

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