Alterations in $N$-methyl-$d$-aspartate receptor subunits in primary sensory neurons following acid-induced esophagitis in cats

Bananri Banerjee, Bidyut K. Medda, Yue Zheng, Heather Miller, Adrian Miranda, Jyoti N. Sengupta, and Reza Shaker

Division of Gastroenterology and Hepatology and Division of Pediatric Gastroenterology, Hepatology and Nutrition, Medical College of Wisconsin, Milwaukee, Wisconsin

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Am J Physiol Gastrointest Liver Physiol 296: G66–G77, 2009. First published October 30, 2008; doi:10.1152/ajpgi.90419.2008.—The excitatory amino acid glutamate plays an important role in the development of neuronal sensitization and the ionotropic $N$-methyl-$d$-aspartate receptor (NMDAR) is one of the major receptors involved. The objective of this study was to use a cat model of gastroesophageal reflux disease (GERD) to investigate the expression of the NR1 and NR2A subunits of NMDAR in the vagal and spinal afferent fibers innervating the esophagus. Two groups of cats (Acid-7D and PBS-7D) received 0.1 N HCl (pH 1.2) or 0.1 M PBS (pH 7.4) infusion in the esophagus (1 ml/min for 30 min/day for 7 days), respectively. NR1 splice variants (both NH$_2$ and COOH terminals) and NR2A in the thoracic dorsal root ganglia (DRGs), nodose ganglia (NGs), and esophagus were evaluated by RT-PCR, Western blot, and immunohistochemistry. Acid produced marked inflammation and a significant increase in eosinophil peroxidase and myeloperoxidase contents compared with PBS-infused esophagus. The NR1–4 splice variant gene exhibited a significant upregulation in DRGs and esophagus after acid infusion. In DRGs, NGs, and esophagus, acid infusion resulted in significant upregulation of NR1 and downregulation of NR2A subunit gene expression. A significant increase in NR1 polypeptide expression was observed in DRGs and NGs from Acid-7D compared with control. In conclusion, long-term acid infusion in the cat esophagus resulted in ulcerative esophagitis and differential expressions of NR1 and NR2A subunits. It is possible that these changes may in part contribute to esophageal hypersensitivity observed in reflux esophagitis.

esophagus; dorsal root ganglia; nodose ganglia; esophagitis; NMDA receptor subunit

**NEURONAL SENSITIZATION** is an important underlying mechanism that can result in visceral hypersensitivity. For example, acid exposure alters primary sensory neurons innervating the gut and results in enhanced responsiveness of spinal neurons and gastrointestinal hypersensitivity (19, 22). Several molecular targets have been proposed as key players in the induction and maintenance of central and peripheral sensitization including the $N$-methyl-$d$-aspartate receptors (NMDAR), transient receptor potential vanilloid 1, acid-sensing ion channels, and TTX-resistant Na channels (1, 2, 5a, 16, 17, 20, 29). The NMDAR, in particular, are believed to be involved in synaptic transmission between primary afferents and dorsal horn neurons (16, 34), in addition to their participation in central sensitization in spinal neurons. These receptors have not only been shown to be present in the peripheral terminals of visceral primary afferents supplying the gastrointestinal tract and other hollow viscera, but their involvement in release of specific neurotransmitters during pain transmission at both the peripheral and central terminals of the sensory neurons has also been clearly documented (30).

The NMDAR are ionotropic glutamate receptors characterized by slow desensitization and high Ca$^{2+}$ permeability (13). They are composed of heteromeric combinations of NMDAR subunit 1 (NR1) and NMDAR 2 subunits (NR2A–D). Although the NR1 subunit is a glycine binding site and universally expressed in all NMDAR, the NR2 is the glutamate-binding subunit and is the major molecular determinants of the functional diversity. More recently, functional expression of NR2B-containing NMDAR have been predominantly been found on the soma of dorsal root ganglia, suggesting that NR2B subunits are of particular importance for pain perception (28).

Previous studies indicate that the NMDAR subunit composition vary not only with the type of insult but also with the duration of injury and inflammation (3, 4, 23). The differential expression of the NR1 component both developmentally and regionally affects many aspects of the channel’s function such as ligand binding, membrane expression, phosphorylation, and downstream signaling (7). The mRNA encoding the NR1 subunit is alternatively spliced to create eight possible splice variants by inclusion and exclusion of three exons: N1 (exon 5), C1 (exon 21), and C2 (exon 22). Recent studies have documented an increase in the expression of NR1 splice variants as a result of inflammation, further implicating the NMDAR in the development of hypersensitivity following an inflammatory process (10, 47).

Gastroesophageal reflux disease (GERD) is one of the most common gastrointestinal disorders in humans and can be associated with esophagitis and hypersensitivity. Fass and col-
leagues (14) have recently demonstrated a significant correlation between the chemosensitivity of primary afferents and reflux symptoms with the degree of endoscopically observed tissue injuries in GERD patients. A recent study in humans also demonstrates that acute acid induction results in esophageal hypersensitivity that could be prevented by intravenous administration of the NMDAR antagonist ketamine, further suggesting the involvement of NMDAR in gastroesophageal reflux-induced esophageal hypersensitivity (45). Although these receptors have been implicated in the development of hypersensitivity following acid exposure in humans, very little is known about the receptor expression and its subunit composition following chronic acid-induced esophagitis. Understanding these alterations may provide insight into the pathophysiology of GERD and potentially aid in targeting future pharmacological interventions to either prevent or treat pathological GERD.

The present study was undertaken to investigate alterations in the NMDAR subunit expression following acid-induced esophagitis in cats. The expression profiles of NR1 and NR1 splice variants (NR1-1, NR1-2, NR1-3, NR1-4, and NR1a/b) and NR2A subunit were evaluated in the thoracic dorsal root ganglia (DRGs, T1–T3), nodose ganglia (NGs), and the distal esophagus of cats following esophageal acid infusion (25, 46).

H&E staining of the esophageal tissues. An independent observer blinded to the groups determined the extent of mucosal damage in the esophagus. The criteria for macroscopic scoring included the presence of hyperemia, edema, erosions, ulcers, and intramural hemorrhage. The tissue sections of 5-μm thickness taken from the proximal, middle, and distal esophagus were stained with H&E and examined for different degrees of epithelial loss (e.g., splitting, erosion, or ulceration), reactive epithelial changes (e.g., basal hyperplasia and vascular alteration including edema, congestion, bleeding, vessel lesions) and inflammation (i.e., the presence of polymorphonuclear leukocytes, lymphocytes).

EPO staining of the esophageal tissues. Eosinophil infiltration was studied by microscopic examination after staining for EPO using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma). The sections were incubated initially with 10 mM cyanide buffer and rinsed in PBS, followed by incubation with peroxidase substrate DAB for 10 min at room temperature. The slides were rinsed thoroughly with water and coverslipped before examination under the microscope.

Esophageal EPO and MPO assay. To detect the degree of tissue inflammation, EPO and MPO contents of esophageal tissues from the acid-treated cats (Acid-7D) were assayed and compared with those of the PBS-treated cats (PBS-7D). MPO is a constitutive enzyme found in the intracellular granules of neutrophils and can be used as a marker for inflammation. Similarly, EPO is a marker for infiltrating eosinophils in the esophagus. The tissues were kept frozen (−70°C) until used for extraction. The tissue samples were placed in plastic test tubes (17 × 100 mm), and 1 ml of cetyltrimethylammonium chloride (0.5% in distilled water) was added to each sample and homogenized with a polytron tissue homogenizer for 15 s. The homogenates were centrifuged at 10,000 g for 10 min at 4°C and the clear supernatants were used for the assay. The extract was diluted 1/10 in 50 mM HEPES, pH 8.0 (EPO dilution buffer) or 10 mM citrate buffer pH 5.0 (MPO dilution buffer). For MPO assay, the substrate O-dianisidine (16.7 mg) was dissolved in 90 ml of distilled water followed by 10 ml of potassium phosphate buffer (pH 6.0), 120 mM resorcinol, and 50 μl of freshly prepared H2O2 (1%). An aliquot of 14 μl of each sample was used for the microtiter plate assay, and 200 μl of O-dianisidine solution was added to each well immediately prior to reading the plate. For EPO estimation, the substrate O-phenylene diamine (3 mM) was prepared in 50 mM HEPES, pH 8.0, along with 6 mM KBr and 8 mM H2O2. The appearance of the yellow color compound over time was measured with a spectrophotometer to determine the MPO and EPO contents of the tissues. The color development was measured as an absorbance at 460 nm for MPO over a period of 5 min. The MPO activity of the tissue samples was calculated on the basis of the change in absorbance per minute. The change in absorbance for 1 μmol H2O2 split is 1.13 × 10⁻² and the MPO content of the samples was calculated on the basis of the following formula: 1 unit of MPO = 1 μmol H2O2 split. For EPO content, the color development was measured at 490 nm and the intensity of the color development was directly proportional the EPO content of the tissues.

NR1 splice variant gene expression in the thoracic DRGs, NGs, and distal esophagus. RT-PCR analysis was performed in neuronal tissues (DRGs, NGs) and the distal esophagus to examine mRNA expression level of NMDAR subunits in experimental and control animals. We used semiquantitative RT-PCR analysis for studying the expression pattern of NR1 splice variants instead of real-time PCR because the sizes of the amplified products were larger, in the range of 200–400 bp (as designed based on the available rat sequences).

All protocols were approved by the Animal Care and Use Committee at the Medical College of Wisconsin and are in accordance with the International Association of Pain policies on the use of laboratory animals.

Surgery. Cats of either sex weighing 2–4 kg were anesthetized by intraperitoneal injection of ketamine (10 mg/kg) and xylazine (5 mg/kg). Prior to surgery, all animals received the antibiotic (Baytril, 5 mg/kg im) to prevent infection and following surgery daily for 7 days. A small incision was made in the ventral side of the neck to expose the cervical esophagus and an infusion catheter was implanted 4–5 cm below the upper esophageal sphincter. The other end of the catheter was externalized subcutaneously through the dorsal aspect of the neck and sealed with a plastic plug for later access. The incision was sutured in layers using 4-0 silk and animals were observed for 4–5 days. A small incision was made in the ventral side of the neck to extract the esophagus of cats following esophageal acid infusion (25, 46).

METHODS

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Animals in each group were euthanized with Nembutal (20 mg/kg ip), and the DRGs (T1–T3), NGs, and distal esophagus were removed. Total RNA was extracted by use of Aurum total RNA fatty and fibrous tissue kit from Bio-Rad (Hercules, CA). Each RNA preparation was subjected to DNase treatment to remove the contaminated genomic DNA and reverse transcription was performed by using SuperScript first-strand synthesis kit (Invitrogen, Carlsbad, CA). For each tissue sample, we carried out the RT reaction without the enzyme.
subjected both rat and human NR2A sequences to blast search against NR1 antibody for Western blots and immunohistochemistry that could recognize NMDA receptor subunits from both the species. We also show 90% sequence homology; therefore the sequences are highly conserved between these two species. Moreover, we have used human show 90% sequence homology; therefore the sequences are highly conserved between these two species. Moreover, we have used human NR2A primers from this conserved sequence regions. The primer sets have been designed using Beacon Designer program (Premier BioSoft International, Palo Alto, CA). Therefore, we examined the effect of chronic esophageal acid exposure on NR1 and NR2A subunit gene expression in cat and NR1 and NR2A primers designed for real-time PCR are shown in Table 1.

The PCR reactions were performed by use of iQ SYBR Green Supermix (Bio-Rad). We used 5 pmol forward and reverse primers and 2 μl cDNA from each tissue sample as template in a total of 25 μl of the reaction mixture. PCR reaction were carried out in 96-well microtiter plates, and the samples were incubated for 5 min at 95°C and were amplified for 45 cycles of 30 s at 95°C and 30 s at 57.4°C, followed by 40 s at 72°C, and 40 s at 72°C for final extension. The PCR setup was carried out in a single tube (target and reference genes were detected in separate reaction tubes). PCR products were electrophoresed in 1.5% agarose gel and viewed by ethidium bromide staining under UV light. The intensity of the bands on gel was determined by densitometric scanning.

Quantitative evaluation of NR1 and NR2A subunits gene expression in thoracic DRGs (T1–T3), NGs, and esophagus. We used real-time RT-PCR for accurate quantification of the target gene. The method was based on measurement of the PCR product during the amplification thermocycles by using SYBR Green fluorescent dye. A threshold for detection of the PCR product was set within the exponential interval of PCR amplification and was measured as Ct, the number of PCR cycles required to obtain this threshold. Initially we designed rat-specific NR2B subunit primers for quantitative estimation of NMDAR subunit expression in cat. However, except for NR2A none of the subunits, namely NR2B, NR2C, and NR2D, exhibited PCR amplification with use of cat brain stem cDNA, thereby indicating a distinct difference in NR2 subunit sequences between cat and rat. The NMDA receptor subunits NR1 and NR2A from both rat and human show 90% sequence homology; therefore the sequences are highly conserved between these two species. Moreover, we have used NR1 antibody for Western blots and immunohistochemistry that could recognize NMDA receptor subunits from both the species. We also subjected both rat and human NR2A sequences to blast search against the available cat gene sequences in the GenBank (the cat sequences by whole genome shotgun sequence), and we observed a significant sequence homology for NR2A with one gene from the cat database (*Felis catus* contig.148516). Cat NMDA subunit genes have not yet been characterized, but this high sequence homology indicates the possibility of conserved sequences between cat and other available sequences in the GenBank. For the present study we designed the NR2A primers from this conserved sequence regions. The primer sets have been designed using Beacon Designer program (Premier BioSoft International, Palo Alto, CA). Therefore, we examined the effect of chronic esophageal acid exposure on NR1 and NR2A subunit gene expression in cat and NR1 and NR2A primers designed for real-time PCR are shown in Table 1.

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### Table 1. RT-PCR primers for NR1 and NR1 splice variants

<table>
<thead>
<tr>
<th>NR1 Amplicon Outside COOH-Terminal Exons</th>
<th>Exons</th>
<th>Product</th>
</tr>
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<tbody>
<tr>
<td>NR1-1 forward</td>
<td>5′ GCCAGTTAAAGGCGCCATAA 3′</td>
<td>184 bp</td>
</tr>
<tr>
<td>NR1-1 reverse</td>
<td>5′ GTGGAGTTGAAGTGGTGC 3′</td>
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<tr>
<th><strong>NR1 splice variants</strong></th>
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<tbody>
<tr>
<td>Common forward primer</td>
<td>5′ GCCAGGTGCAGCAGGTCTCTC 3′</td>
<td></td>
</tr>
<tr>
<td>NR1-1 reverse</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td>+21,+22</td>
</tr>
<tr>
<td>NR1-2 reverse</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td>+21,+22</td>
</tr>
<tr>
<td>NR1-3 reverse</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td>+21,+22</td>
</tr>
<tr>
<td>NR1-4 reverse</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td>+21,+22</td>
</tr>
<tr>
<td>NR1 a/b forward</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td>a: −5</td>
</tr>
<tr>
<td>NR1 a/b reverse</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td>b: +5</td>
</tr>
<tr>
<td>rRNA F1 forward</td>
<td>5′ TCAAGAAGGAGAATGGGGA 3′</td>
<td></td>
</tr>
<tr>
<td>rRNA NR1 forward</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td></td>
</tr>
<tr>
<td>rRNA NR2 forward</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td></td>
</tr>
<tr>
<td>rRNA NR2 reverse</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
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<table>
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<tr>
<th><strong>Real-time PCR primers for NR1 and NR2A subunits</strong></th>
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<tbody>
<tr>
<td>RNR1-1 forward</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td></td>
</tr>
<tr>
<td>RNR1-1 reverse</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td></td>
</tr>
<tr>
<td>RNR2A forward</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
<td></td>
</tr>
<tr>
<td>RNR2A reverse</td>
<td>5′ GCCAGTTGCTGCTGCTGCTGCTGCT 3′</td>
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NMDA RECEPTOR SUBUNIT EXPRESSION IN ACID-INDUCED ESOPHAGITIS

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IL), followed by differential centrifugation first at 1,200 rpm for 30 min and then for another 30 min at 14,000 rpm. The membrane pellet was solubilized for 30 min on ice with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS containing protease and phosphatase inhibitors). The membrane extracts were centrifuged and assayed for protein content by the bicinchoninic acid (BCA) method (Thermo Fisher Scientific, Rockford, IL). Approximately 25 μg of brain tissue extracts from cat and rat and 50 μg of DRG and NG extracts from cat were electrophoresed on 8% SDS-PAGE and transferred onto nitrocellulose membrane. After transfer, the membrane was blocked with 5% nonfat milk and then probed with antibodies to NMDAR1 subunit (mouse anti-NMDAR1, 1:100, BD Biosciences, San Jose, CA). Protein bands were visualized by using a horseradish peroxidase-conjugated secondary antibody (Jackson ImmununoResearch, West Grove, PA) and an enhanced chemiluminescent detection system (Thermo Scientific). The relative changes in the intensity of NR1 subunit expression in various samples were normalized against the intensity of housekeeping gene β-actin for the same tissue sample by use of alpha imager 3400 software. For NR2A subunit protein analysis, two different antibodies from Alomone Labs (Jerusalem, Israel) and Santa Cruz Biotechnology (Santa Cruz, CA) were used. However, none of the antibodies showed specific reactivity with cat tissues; therefore, NR2A subunit protein analysis was not included in this study.

Immunostaining of NMDAR subunits in DRGs, NGs, and distal esophagus. Cats from both the groups (Acid-7D and PBS-7D) were deeply anesthetized with pentobarbital sodium (50 mg/kg ip) and the chest was opened by midsternal incision. Animals were perfused transcardially with cold phosphate buffer solution followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Bilateral thoracic (T1–T3) DRGs, NGs, and esophagi were collected and incubated in 4% paraformaldehyde overnight at 4°C. Tissue samples were cleaned of connective tissues and cryoprotected in 20% sucrose in 0.1 M PBS containing 0.5% Triton X-100, and 0.1% sodium azide for 60 min at room temperature. For double immunofluorescence staining with NR1 and substance P (SP) antibodies, sections were incubated with a mixture of rabbit anti-NR1 antibody (1:100; Alomone Labs) and guinea pig anti-SP antibody (1:50; Chemicon International, Temecula, CA) overnight at 4°C. The antibody dilution was carried out in PBS containing 3% NGS, 0.5% Triton X-100, and 0.1% azide (antibody diluent). After four washes with PBS containing 0.1% Triton X-100, 0.1% NGS, and 0.01% azide, sections were incubated at room temperature for 2 h with fluorescence-labeled secondary antibodies. Various secondary antibodies used were Alexa 488-conjugated goat anti-guinea pig antibody (1:500) and Alexa 568-conjugated goat anti-rabbit antibody (1:2,000). All the secondary antibodies used in this study were purchased from Molecular Probes (Invitrogen). The specificity of the primary antibodies was assessed by preincubating the antibody overnight at 4°C with the immunizing peptides (10 μM) prior to the application to the sections. Tubes containing exactly the same dilution of antibody but without any addition of peptides were incubated in parallel. The absorbed and nonabsorbed antibodies were incubated with the DRG and NG sections for 24 h at 4°C. Some sections in every run were also incubated with either PBS or normal rabbit serum instead of primary antibody and processed further following the protocol as mentioned above. For esophageal tissues, most of these antibodies exhibited a high background staining. The high background staining without specific immunoreactivity for the esophageal tissues could be due to low level of expression of NMDA receptor subunits in the esophageal tissues, and the immunostaining procedure used in this study may not be sensitive enough to detect the antibody bindings of the receptor subunits in the esophageal tissues. Therefore, no esophageal immunostaining was included in this study. Hence, NR1 immunostaining of DRG and NG tissues from the control and acid-treated groups was reported in this study.

Slides were examined under a fluorescence microscope (Nikon Eclipse 50i) using narrow band cubes for Alexa 488 (DM505, excitation filter 470–490, barrier filter 515–550 nm) and Alexa 568 (DM 568, excitation filter 540–560, barrier filter 575–645 nm). Images were captured with a Spot II high-resolution digital camera (Diagnostic Instruments, Sterling Heights, MI) and processed with the Adobe Photoshop program. Each color of double-labeled DRG/NG sections were digitally imaged at ×20 using filter sets for Alexa 488 (green) and Alexa 568 (red). To maintain the consistency of image capturing we used the same time of exposure, gain, and gamma adjustment for the control and the experimental samples for each fluorescence staining. We used the curve program of Adobe Photoshop (CS2) for the background adjustment to enable the counting of total number of cells. For selecting the cells for intensity measurement, we used two selection settings of different width and height for measuring the small-diameter and medium/large-diameter cells. Using these settings, we measured the integrated density of staining for individual cells. To define the cells with positive staining, we set up different cutoffs for integrated density for the small and medium/large-diameter cells based on the background using Image J program (NIH, Bethesda, MD).

The fluorescence images were converted into gray mode. Arbitrary grayscale units (in the range of 1–255) were assigned to quantify the intensity of staining of a given cell. Nonspecific background staining was measured in a similar way by selecting cells with staining below the threshold limit for the positively stained cells. Only cells containing a darker nucleus were selected, and intensity of staining was measured as integrated density for 10 random cells for each DRG and NG section. Three sections from PBS-treated controls and acid-infused experimental groups were used for estimating the intensity of staining and calculated as means ± SE.

RESULTS

Esophageal histology. H&E staining of tissues from the distal esophagus revealed hyperemia, edema, and superficial epithelial cell loss with isolated erosions, basal cell hyperplasia, and the presence of inflammatory cells in the Acid-7D group (Fig. 1A). In contrast there was no evidence of inflammation in the esophagus of the PBS-7D group (Fig. 1A). In the Acid-7D group, severe mucosal damage (high-grade esophagitis or ulcerative esophagitis) was characterized by extensive epithelial cell loss with erosion/ulceration, edema, and granulation tissue replacing the mucosa with extensive infiltration of neutrophils, eosinophils, and polymorphonuclear leukocytes and lymphocytes. To confirm eosinophilic infiltration in the esophagus, we stained the esophageal tissues for eosinophil peroxidase. An extensive eosinophilic infiltration was observed around the mucosa and submucosa in the acid-treated group (Fig. 1B).

Esophageal MPO and EPO assay. We examined the neutrophilic involvement in the esophagus of both groups by measuring the MPO activity in esophageal tissues. The esophagus of cats in the Acid-7D group demonstrated a significant increase in MPO content compared with PBS-7D (Fig. 1C; P < 0.01). These cats showed 35-fold increase in MPO units (35 ± 13.07 MPO units/mg tissue) compared with PBS-7D (Fig. 1C; 0.615 MPO units/mg tissue). Similarly, quantitative estimation of EPO as measured by ELISA absorbance at 490
nm showed a significant increase in the acid-treated group compared with PBS-treated controls (*P < 0.01, Fig. 1C).

NR1 splice variant gene expression in DRGs, NGs, and esophagus. To examine the specificity of amplified PCR products of cat cDNAs using rat specific NR1 splice variant primers, we carried out PCR reactions of these primer sets using cat brain stem cDNA as a template and compared amplified products with those obtained from the PCR with rat brain stem cDNA. With cat brain stem cDNA, distinct PCR amplified products were obtained for both NH2-terminal variants NR1-a and NR1-b and COOH-terminal variants NR1-1, NR1-3, and NR1-4. The sizes of the PCR products were comparable to that obtained from the PCR reaction with rat brain stem cDNA (Fig. 2) and also to the predicted sizes (Table 1). However, no PCR amplification was observed for NR1-2 in cat brain stem tissue, indicating either very poor expression of NR1-2 splice variant in cat or a gene sequence different from that of the rat sequence.

After evaluating the specificity of the amplified products, we used these NR1 primer sets for PCR amplification with cDNA preparations from DRGs (T3), NGs, and distal esophagus from PBS and acid-treated cats. Primers used for the amplification of six splice variants are shown in Table 1. In DRGs of PBS-infused cats, out of six NR1 splice variants, NR1-a (–exon 5)
and NR1-2 (−exon 21, +exon 22) failed to show amplification (Fig. 3A). The COOH-terminal splice variant NR1-4 showed the strongest expression. Acid infusion resulted in significant increase in the expression of NR1-b and NR1-4 variants (Fig. 3B, *P < 0.05, **P < 0.001 vs. PBS group). Interestingly, the NR1 mRNA expression (with primers designed outside the region of exons 5, 21, and 22) also significantly increased in DRGs from Acid-7D cats compared with PBS-7D (P < 0.05 vs. PBS). There appear to be distinct differences in the expression profile of NR1 splice variant mRNAs in NGs compared with DRGs (Fig. 4A). The major splice variants in NGs from PBS-7D cats were NR1-a, NR1-b, NR1-1, NR1-3, and NR1-4. No amplification was observed for NR1-2. Interestingly, acid exposure resulted in a significant increase of NR1 (P < 0.05 vs. PBS-infused cat) and a marked downregulation of NR1-4 splice variants compared with PBS control, although the difference was not statistically significant (Fig. 4B). In esophageal tissues from PBS-7D cats, we failed to detect the expression of any of the six splice variants and found only a very low expression of NR1 (Fig. 5A). However, in esophageal tissues...
from Acid-7D cats a significantly high expression was observed for NR1 and NR1-4 (Fig. 5B, \( P < 0.05 \) vs. PBS-7D).

Quantitative estimation of NR1 and NR2A subunit gene expression in NGs, DRGs, and esophagus. The quantitative NR1 mRNA expression in DRGs, NGs, and distal esophagus for PBS- and acid-treated cats as determined by real-time PCR is shown in Fig. 6A. For PBS-7D cats, the relative expression of NR1 was the highest in the NGs followed by DRGs and the lowest in the esophagus. The quantitative analysis of the expression profile of NR1 subunits in the DRGs, NGs, and esophagus exhibited a significant upregulation of NR1 subunits in these tissues after acid infusion (esophagus, \( P < 0.05 \); DRGs, \( P < 0.001 \); NGs, \( P < 0.001 \) vs. PBS-7D). The sizes of the amplified products were in the range of 200 to 100 bp (Table 1). In real-time PCR, the highest expression of NR2A subunit was observed in NGs followed by esophagus and DRG, respectively (Fig. 6B). However, in the acid-treated group, a significant downregulation of NR2A subunit gene expression was observed in esophagus and DRG (Fig. 6B, \( P < 0.05 \) vs. PBS-7D).

Western blot analysis of NR1 subunit polypeptide expression in NGs and DRGs. In Western blot, we analyzed two protein extracts each from both rat and cat brain stems using rat specific NR1 antibody (Fig. 7A). We have used a monoclonal antibody raised against NMDA-NR1 subunit and generated by using a fusion protein encoding NMDA-NR1 amino acid residues 660 – 811, representing the intracellular loop between the transmembrane region III and IV not encompassing any splice variant regions. A specific polypeptide band of 120 kDa was identified in cat brain stem, and the size was comparable to the expression pattern in rat tissue extracts, thereby indicating the sequence homology between cat and rat NR1 subunits of NMDAR. By use of this rabbit anti-NR1 antibody, a significant increase in polypeptide level of the NR1 subunit was detected in DRGs and NGs from acid-treated group compared with PBS controls (Fig. 7, B and C, \( P < 0.05 \)).

Immunohistochemical analysis of NR1 immunoreactivities in NGs and DRGs. Immunohistochemical analysis of NR1 staining demonstrated a significant increase in the intensity of NR1 expression in DRGs from Acid-7D cats compared with PBS-7D (Figs. 8A and 9A). The majority of these NR1 expressed cells also showed a high expression of SP indicating NR1 expression was mostly in SP expressing peptidergic C fibers. In DRGs, immunostaining with NR1 antibody preabsorbed with blocking peptide failed to show significant immunoreactivities, indicating the specificity of the reaction (Fig. 8B). The immunostaining using normal rabbit serum as primary antibody also failed to show any specific immunoreactivity in DRG sections (Fig. 8B). In NGs, a marked increase in the intensity of NR1 staining was observed after esophageal acid exposure in Acid-7D group compared with control; however, this was not statistically significant (Figs. 8C and 9B). Unlike DRGs, less than 50% of the NR1 expressing cells in NGs exhibited coexpression of SP, indicating the involvement of other neurotransmitters or cell types in NMDAR-mediated signaling in NGs (Fig. 8C).

DISCUSSION

We have shown in the present study that repetitive acid exposure in cats results in tissue damage and inflammatory cell infiltration in the esophagus. This model mimics the findings reported in humans with high-grade esophagitis characterized by severe epithelial injury with erosions, ulceration, and infiltration of polymorphonuclear cells (neutrophil, eosinophils, and lymphocytes). This inflammation is confined primarily in
Our results also demonstrated a significant increase in the NR1 subunit of the NMDA gene receptor and overall down-regulation of NR2A mRNA in the DRGs, NGs, and esophagus in cats following esophageal acid exposure. These findings are in accordance with our Western blot and immunohistochemical data exhibiting a significant increase in the intensity of NR1 protein immunoreactivity in the DRGs and NGs in the Acid-7D group compared with PBS-7D. To our knowledge this is the first study demonstrating alterations in NMDAR subunit expression as a result of acid-induced esophagitis.

Clinical observations suggest that acid exposure and acid-induced esophagitis may sensitize primary sensory neurons leading to wind-up or sensitization of secondary neurons in the spinal cord and/or brain stem (11, 32, 35, 38, 39). In humans, secondary allodynia of the chest wall and proximal esophagus has been reported following acute acid infusion in the distal esophagus. Furthermore, this hypersensitivity is attenuated by intravenous administration of the NMDAR antagonist ketamine, suggesting that the NMDAR plays an important role in the development of acid-induced hypersensitivity (45).

Several immunohistochemical and molecular studies have investigated NMDAR expression in DRG cell bodies and in vagal afferent neurons projecting from the stomach and duodenum (6, 8, 26, 29, 40). In rats, for example, experimental colitis with TNBS (trinitrobenzene sulfonic acid) results in NMDAR subunit phosphorylation and upregulation in the colon and DRGs (25). Since TNBS-induced colitis results in hypersensitivity even after the inflammation has resolved, it can be speculated that these alterations in NMDAR play an important role in hypersensitivity following inflammation (33).

Clinical observations suggest that acid exposure and acid-induced esophagitis may sensitize primary sensory neurons leading to wind-up or sensitization of secondary neurons in the spinal cord and/or brain stem (11, 32, 35, 38, 39). In humans, secondary allodynia of the chest wall and proximal esophagus has been reported following acute acid infusion in the distal esophagus. Furthermore, this hypersensitivity is attenuated by intravenous administration of the NMDAR antagonist ketamine, suggesting that the NMDAR plays an important role in the development of acid-induced hypersensitivity (45). Although studies have documented that ketamine attenuates pain by modulating ionic influx through the NMDA channel and reducing NR1 phosphorylation (42), it requires frequent repeated administration of the drug to maintain analgesia. This is primarily due to short-acting nature and rapid metabolism of the drug by hepatic microsomal enzymes (41). In the present study, ketamine was used in combination with xylazine acutely only one time to induce anesthesia during surgical procedure (see METHODS). It is very unlikely that such acute use of the drug will leave a long-lasting effect on expressions of NR1 and NR2 subunits. Despite the fact that both experimental (i.e., chronic acid infused) and control (i.e., PBS infused) cats received ketamine and xylazine as anesthetics during the surgical procedure, the expressions of NMDAR subunits are significantly different in acid-treated cats, suggesting that altered expression observed is due to acid and not the influence of ketamine used during surgery.
Fig. 8. Photomicrograph of immunoreactivities of NR1 subunit and substance P (SP) in the thoracic T3 DRG and NG. A: immunostained sections (20 μm) of DRG from acid- and PBS-treated cats are represented at left and merged images of NR1 and SP stained sections are shown at right. The majority (>90%) NR1-positive cells exhibited high expression of SP in the acid-treated cats compared with PBS-treated cats. Arrows indicate some of the small- and medium-diameter cells coexpressing NR1 and SP. B: in DRG, immunostaining with blocking peptide preabsorbed NR1 antibody failed to show significant immunoreactivities, indicating the specificity of the reaction (left). The immunostaining using normal rabbit serum as primary antibody also failed to show any specific immunoreactivities (right). C: immunostained sections (20 μm) of NG from acid- and PBS-treated cats are represented at left and merged images of NR1 and SP stained sections are shown at right. In NGs, <50% of NR1-positive cells exhibited SP immunostaining. Arrowheads indicate the NR1-positive cells not expressing SP and arrows indicate cells coexpressing NR1 and SP. Scale bar, 100 μm.
In the NGs, the NH2-terminal splice variant NR1-a (between rats and cats. We have demonstrated the expression of NMDAR subunit expression in visceral organs has been documented in recent studies. Following carrageenan-induced hind paw inflammation, NR1 serine phosphorylation and NR2B suppression has been observed in the dorsal horn neurons (3). The long-lasting decrease in NR2B expression in this injury model is in sharp contrast to the findings in the TNBS-induced colitis model, in which inflammation resulted in upregulation and persistent phosphorylation of NR2 subunit expression in the DRG neurons (25). In the present study, esophagitis following acid infusion resulted in significant upregulation of NR1 protein and mRNA with a significant downregulation of NR2A mRNA in the DRGs, NGs, and esophagus. These data indicate that NMDAR are highly dynamic in their subunit composition during the development, maintenance, and recovery from a pathological condition.

This differential subunit expression pattern of NMDAR is also an important factor in regulating function and neuronal plasticity under pathological conditions. For example, rapid calcium-mediated signaling through NR1/NR2A in contrast to slower signaling through NR1/NR2B may activate different downstream signaling and gene expression patterns under various pathological conditions. Therefore, experimental findings of the NMDAR expression and activation in various animal models of inflammation need careful interpretation and may be important for the development of targeted pharmacological inventions.

Overexpression of N1 and C1 splices and serine phosphorylation within the C1 cassette have been observed 14 days following inflammation of the rat colon with TNBS. In contrast, rats without inflammation fail to show expression of N1 and C1 splice variants (10, 47). In the present study, 7 days of acid infusion resulted in the expression of NR1-4 splice variants in esophageal tissues, whereas PBS-treated cats failed to show any NR1 splice variant. It is likely that in the esophagus, NMDAR have lower levels of constitutively expressed NR1 subunits and alternative splicing under normal physiological states with a subsequent upregulation of NR1 splice variants in the pathological state.

In a recent study, repeated long-term application of an NMDAR agonist resulted in a rapid loss of functional NR1-NR2A channels at the surface of HEK-293 cells (44). This use-dependent receptor downregulation was prevented by application of tyrosine phosphatase inhibitors, indicating that a tyrosine-based signaling mechanism is involved in NMDAR subunit modulation and expression. An overall downregulation of NR2A subunit in the present study is in accordance with previously reported suppression of NR2 subunits in rats following carrageenan-induced hind paw inflammation (3).

Since currently there is no reliable, reproducible, and quantifiable animal model to measure the esophageal pain, the major limitation of the present study is the absence of behavioral testing to determine the esophageal hypersensitivity following repeated acid infusion. However, on the basis of previously reported human psychophysical studies and electrophysiological recordings from spinal and brain stem neurons of cats it can be speculated that acid exposure results in altered esophageal sensations (15, 31, 32, 35, 38, 39). Human study has also documented that NMDAR antagonist ketamine prevents esophageal hypersensitivity following acute infusion of acid (45). Therefore, our present findings indicate that altered NMDAR expression following acid exposure may contribute to the esophageal hypersensitivity.
GRANTS

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