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Peripheral inhibition of glutaminase reduces carrageenan-induced Fos expression in the superficial dorsal horn of the rat

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Abstract

In inflamed tissue, the levels of the excitatory amino acid glutamate are increased. Glutamate sensitizes peripheral axons of primary afferent neurons during inflammation leading to decreased firing threshold and hyperexcitability. One proposed source of glutamate is the primary afferent. Antagonizing glutamate receptors on peripheral axons of primary afferents during inflammation provides analgesia in animals and humans. The enzyme glutaminase is used by primary sensory neurons to convert glutamine to glutamate, and peripheral inhibition of glutaminase with 6-diazo-5-oxo-L-norleucine (DON) provides long-lasting analgesia during inflammation. In this study, we measured the effects of glutaminase inhibition on carrageenan-induced spinal Fos expression. Rats were given intraplantar injections of carrageenan and treated locally with either vehicle or DON. After 3 h of inflammation, hind paw swelling and spinal expression of Fos were examined. CellProfiler was used to automate Fos nuclei counting in five laminar groupings in the spinal cord (I–II, III–IV, V–VI, VII–IX, X). Carrageenan increased hind paw thickness by ~70% and spinal Fos expression in superficial (I–II) and deep (V–VI) laminae by 10-fold and 5-fold, respectively. Treatment with DON reduced hind paw swelling by ~13% and suppressed Fos expression in the laminae I–II by ~54%, but not the deep laminae. Our results further support the notion of glutamate as a peripheral inflammatory mediator and indicate that glutaminase should be considered as a novel therapeutic target for treatment of inflammatory pain.

Keywords

Glutamate; Inflammatory pain; Primary afferents; DON; *c-fos*

Exogenous glutamate excites and sensitizes peripheral axons of primary afferent neurons [18], and it is generally accepted that glutamate is involved in the sensitization of primary afferents during inflammation [7] through both ionotropic [9,14,41] and metabotropic [3] glutamate receptors. The source of endogenous glutamate that interacts with primary afferents is not clear, although a probable candidate is the peripheral terminal. Antidromic stimulation of the sciatic nerve increases glutamate concentrations in peripheral tissues, indicating a neural source of glutamate [15,24]. Furthermore, increases in peripheral glutamate concentration after capsaicin stimulation are attenuated by local morphine [24],

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which suggests that glutamate is released by heat sensing (TRPV1) nociceptors. Increased glutamate concentrations resulting from inflammation [28,34] can be diminished by nerve block [28], which again suggests the primary afferent as the source of glutamate. Lastly, peripheral terminals of primary afferent neurons are immunoreactive for vesicular glutamate transporters [4] and have varicosities that contain small clear vesicles akin to those that contain glutamate in the central nervous system [26]. These studies, therefore, support the idea of glutamate autostimulation of nociceptive afferent fibers.

Injection of carrageenan into the plantar surface of the rat hind paw increases the expression of *c-fos* mRNA [17] and Fos protein [21] in the lumbar spinal cord. Many analgesics attenuate the Fos increase induced by carrageenan, indicating that Fos expression is a measure of activation in pain pathways and can serve as means of quantitating analgesia [13]. For example, non-steroidal anti-inflammatory drugs (NSAIDs) decrease the formation of inflammatory mediators that can sensitize primary afferents [35] by inhibiting the enzyme cyclooxygenase (COX). Peripheral inhibition of COX suppresses carrageenan-induced Fos expression [5]. If glutamate is to be considered among the molecules that sensitize primary afferent fibers during inflammation, then a similar approach would be to target the enzyme that synthesizes neuro-transmitter glutamate from the precursor glutamine, glutaminase (GLS). We have previously shown that the irreversible GLS inhibitor, 6-diazo-5-oxo-L-norleucine (DON), provides long term analgesia in a dose dependent manner [29] and that GLS expression is increased [20] in the complete Freund's adjuvant (CFA) monoarthritis model. In the present study, we use spinal Fos expression to quantify the analgesic effect of DON on carrageenan-induced hind paw inflammation. Our goal is to determine if the glutamate that sensitizes peripheral terminals is produced by GLS during inflammation.

Sprague-Dawley rats ($n = 15$; 200–300 g) were housed on a 12 h light:12 h dark cycle and given free access to food and water. Procedures were conducted according to guidelines from the National Institutes of Health [33] and were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee. All appropriate efforts were made to minimize discomfort and the number of animals used in this study.

To test the effects of DON on carrageenan-evoked spinal Fos expression, rats ($n = 6$) were injected with 10 μ mol/25 μ L DON (Sigma) in 0.01 M sodium phosphate buffered 0.9% (w/v) saline (PBS) in the right hind paw one day before being injected with 10 μ mol DON in 100 μ L of 1% (w/v) λ -carrageenan (Sigma) diluted in PBS. This dose of DON was chosen based on previous behavioral experiments [29]. Controls consisted of rats injected with vehicle (PBS) and carrageenan ($n = 5$) in the same manner and naïve rats ($n = 4$). Three hours after carrageenan injection, hind paw metatarsal thickness was measured with calipers (Mitutoyo) to the nearest 0.05 mm. All rats were anesthetized with 2.5% (w/v) Avertin and xylazine and transcardially perfused with 75 mL PBS followed by 325 mL 4% formaldehyde in 0.1 M sodium phosphate buffer. L4 spinal cord segments were post fixed for 4 h at 4 °C and cryoprotected in 30% sucrose (w/v) for two days. Serial 20 μ m frozen sections were thaw mounted to slides. After blocking in 5% (v/v) NGS diluted in PBS with 0.25% Triton X-100 (PBS-T), sections were incubated overnight at 4 °C in rabbit anti-Fos (Ab-5 Calbiochem) diluted 1:8000 in blocking solution. Sections were rinsed and incubated for 2 h in

biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted to 1.0 µg/mL in PBS-T, followed by rinsing and incubation for 1 h in Vectastain ABC reagent (Vector Laboratories). Tissue sections were incubated for 5 min in 3,3'-diaminobenzidine tetrahydrochloride (Sigma) diluted to 10 mg/24 mL in TBS with 0.5% hydrogen peroxide. The reaction was stopped by rinsing in PBS before the sections were dehydrated and cleared in an alcohol series and xylenes, respectively. Coverslips were apposed with Permount (Fisher).

Ten sections from each rat were photographed using the 4× objective on a BX51 microscope (Olympus) with a SPOT RT740 camera (Diagnostic Instruments). Laminal groupings I–II, III–IV, V–VI, VII–IX, and X [30] on the side ipsilateral to hind paw injection were traced manually for each section with a Cintiq 21UX interactive pen display (Wacom) in ImageJ (NIH). Traced laminal groupings were made into binary mask images with white laminal groupings on a black background. Fos-positive nuclei were identified automatically and counted in each image by CellProfiler (Broad Institute Imaging Platform) [10] using the binary mask images to obtain Fos-positive nuclei counts per laminal grouping. The CellProfiler pipeline used is summarized in Table 1.

Hind paw thickness measurements were compared with a two-way ANOVA with the fixed factors being hind paw (contralateral or ipsilateral to injection) and treatment groups (naïve, PBS, DON). Numbers of Fos-positive nuclei also were compared with two-way ANOVA but with fixed factors being laminal groups (I–II, III–IV, V–VI, VII–IX, and X) and treatment groups. Multiple comparisons were made with Bonferroni post-tests. *P* values less than 0.05 were considered significant. Values are reported as mean ± standard error of the mean.

Intraplantar carrageenan injection elicited an inflammatory response that caused significant swelling (~70% increase) of the metatarsal thickness in the ipsilateral hind paw (8.4 ± 0.4 mm) versus the naïve rats (4.9 ± 0.1 mm; Fig. 1). Pretreatment and co-injection with DON, the GLS inhibitor, had a small but significant effect (~13% decrease) on carrageenan mediated hind paw swelling (7.3 ± 0.3 mm), but it did not reduce hind paw thickness to that of naïve rats. Automated counting of Fos nuclei per laminal grouping provided a strictly objective criteria for identifying nuclei labeled for Fos that was consistent for all images analyzed. After laminal groupings were traced on all images, CellProfiler took an average of 14.1 s to process each image through the pipeline (Table 1). Naïve rats had less than five Fos nuclei per laminal grouping. Carrageenan significantly increased the number of Fos nuclei in ipsilateral laminae I–II (23 ± 5) and laminae V–VI (28 ± 4), without significantly affecting the number of Fos nuclei in other laminae (Figs. 2 and 3). Pretreatment and co-injection with DON significantly decreased (~54%) the number of Fos nuclei in ipsilateral laminae I–II (11 ± 1), but not laminae V–VI (22 ± 3), as compared to PBS treated rats. There were still significantly more Fos nuclei in laminae I–II and laminae V–VI in DON treated rats than in naïve rats.

Peripheral processes of primary afferent neurons have been implicated as the source of elevated glutamate released during inflammation [7]. We hypothesized that inhibiting the enzyme GLS at the site of inflammation would suppress carrageenan-induced Fos expression in spinal cord. Our finding that peripheral DON injection was able to suppress Fos expression in spinal cord supports the long-lasting analgesic effects of DON observed in

the CFA inflammation model [29]. Antagonism of NMDA glutamate receptors systemically suppresses spinal Fos expression during peripheral inflammation throughout all laminar groupings in spinal cord, even ventral horn, but does not affect hind paw edema [11]. Such findings indicate that effects of systemic NMDA antagonism are mediated primarily by acting on the central nervous system. Peripheral NMDA antagonism also suppresses spinal Fos expression by ~35% during inflammation [39], which indicates glutamate sensitization of primary afferents at least partially accounts for increased spinal Fos expression. In addition to glutamate, intraplantar antagonism of two other sensitizing factors, the enzyme COX [5] and the bradykinin B2 receptor [6], also reduces carrageenan-induced spinal Fos expression. Peripheral COX inhibition with the highest dose of flurbiprofen resulted in a $52 \pm 5\%$ suppression of the increase in Fos expression in laminae I–II and a $62 \pm 2\%$ suppression in laminae V–VI [5]. Antagonism of the B2 receptor with the highest dose of HOE140 resulted in a $51 \pm 6\%$ suppression of the increase in Fos expression in laminae I–II and a $48 \pm 7\%$ suppression in laminae V–VI [6]. The highest doses of flurbiprofen and HOE140 also decreased hind paw edema by $67 \pm 7\%$ and $41 \pm 3\%$, respectively. COX is already the target of many analgesics, and bradykinin antagonism is being considered as a new analgesic strategy [12,27]. The finding that GLS inhibition has similar effects on Fos expression in laminae I–II to COX and bradykinin inhibition supports glutamate as a sensitizer of primary afferents and suggests GLS as a potential therapeutic target for inflammatory pain. The smaller anti-inflammatory effect of DON as compared to flurbiprofen and HOE140 may account for the lack of a significant effect on Fos suppression in laminae V–VI. Although much evidence points toward the primary afferent as the source of increased glutamate during inflammation, other peripheral cell types cannot be ruled out. Keratinocytes [32], fibroblasts [37], and white blood cells [31] all have GLS and may have a role in producing glutamate that acts on primary afferent fibers.

Primary afferent nociceptors have an efferent role during inflammation [36]. Following transection of the sciatic nerve or neonatal destruction of capsaicin sensing DRG neurons, plasma extravasation during inflammation is markedly attenuated [23]. The component of inflammation contributed by the primary afferent has been called neurogenic inflammation, and it is presumed to be mediated by release of the neuropeptides substance P and calcitonin gene related peptide resulting in vasodilatation and edema [1,16]. Exogenous glutamate causes overt nociception and swelling that can be blocked by antagonizing substance P [2]. In this study, a 13% decrease in hind paw edema was observed after DON inhibition of carrageenan inflammation. We hypothesize that decreasing the concentration of glutamate with DON may suppress the amount of substance P released, decreasing the neurogenic component of inflammation.

Antagonism of glutamate receptors at the periphery provides analgesia during inflammatory pain models [3,14,19,22]. Such analgesic effects have been attributed to glutamate receptors on the primary afferents themselves [8,9,25,38]. Exogenous glutamate injection causes pain and vasomotor responses in humans as well, and glutamate receptor antagonists can provide analgesia [40]. In light of this information, our study further indicates that antagonism of glutamate at the periphery is a viable pain therapy and we propose the enzyme GLS as novel therapeutic target.

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Fig. 1. Effects of peripheral GLS inhibition on carrageen induced hind paw swelling. Intraplantar carrageenan injection caused a unilateral inflammation of the hind paw. Swelling was slightly reduced (13%) by DON treatment, but not brought to normal.

Fig. 2. Representative images of spinal Fos immunohistochemistry after carrageenan-induced inflammation. Carrageenan injection with PBS treatment induced Fos expression in superficial (I–II) and deep laminae (V–VI) of the ipsilateral spinal cord dorsal horn (top). Inhibition of GLS with DON decreased the number of carrageenan-induced Fos nuclei in superficial laminae (54%) without affecting the number of Fos nuclei in the deep layers (bottom).

Fig. 3.

Effects of peripheral GLS inhibition on carrageen induced Fos expression. Automated counting of Fos nuclei for five laminar groups revealed significant increases in Fos expression in the superficial (I–II) and deep (V–VI) dorsal horn after carrageenan injection with PBS treatment. Treatment with DON significantly reduced Fos expression in the superficial (54%) but not the deep laminae. * $P < 0.01$, ** $P < 0.001$.

Table 1

Summary of pipeline used in CellProfiler for automated detection and counting of Fos nuclei in images of spinal cord sections. Manual identification of laminar groupings was required prior to using the CellProfiler software. Each module listed has additional parameters that are set to specify in detail how to handle the data.

Module	Description
LoadImages	Loads image pairs (binary mask of laminae and original)
IdentifyPrimAutomatic	Identifies laminae as objects from binary mask image
InvertIntensity	Inverts intensity of original so Fos nuclei are white
ApplyThreshold	Sets all gray values below a certain intensity to black
IdentifyPrimAutomatic	Identifies Fos nuclei by shape and intensity
MeasureObjectAreaShape	Obtains area measurements for laminae and nuclei
MeasureObjectIntensity	Obtains intensity measurements for laminae and nuclei
Relate	Counts number of Fos nuclei within each laminar grouping
ExportToExcel	Exports all measurement data to Excel spreadsheet