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Biglycan Inhibits Capsaicin-Induced Substance P Release by Cultured Dorsal Root Ganglion Neurons

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Abstract

Objective—To examine the inhibitory effects of biglycan on substance P release from cultured sensory neurons in response to capsaicin.

Study Design—In vitro study of cultured primary sensory neurons from the rabbit dorsal root ganglion (DRG). We interrogated the culture system function with capsaicin. Biglycan is an important structural component of the intervertebral disc that may regulate growth factors and inflammatory mediators. We tested the hypothesis that biglycan inhibits substance P release in response to capsaicin.

Results—The DRG cultures were shown to contain both neurons and astrocytes by immunostaining using antibodies recognizing neuron and glial cell markers. Cultured DRG cells respond to capsaicin in a dose- and time-dependent manner (capsaicin dose ranges from 5 to 500μM; stimulation time ranges from 0 to 60 minutes). The neurons preincubated with biglycan released 27% less substance P compared with neurons without biglycan (n=4, p=0.036).

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Conclusion—We have established a rabbit dorsal root ganglion (DRG) cell culture system which contains both sensory neurons and the supporting astrocytes. Biglycan, an inhibitor of substance P release by DRG cultures, may serve as an ingredient in intradiscal injectable to reduce back pain.

BACKGROUND

Intervertebral disc (IVD) pathology is believed to be responsible for a large proportion of back pain.^{1, 2} The notion of discogenic pain has been supported by some clinical evidence,³ and is further supported by delineating the neurotransmission pathway from the IVD to the nervous system (Figure 1). In a normal annulus fibrosus (AF), free nerve endings are found only in the outermost layers.⁴⁻⁶ The nerve endings give rise to the sinuvertebral nerve, which travels to the dorsal root ganglion (DRG).⁵ Subsequent to receiving peripheral sensory stimulation, central branches of the sensory neurons convey sensory impulses through dorsal roots to the dorsal horn of the spinal cord (Figure 1). The development of fissures in the AF may lead to vascular tissue and nerve ending invasion of the normally avascular and aneural tissue.^{7, 8}

An important point is to identify molecules that may stimulate the nerve endings in the IVDs and the source of these molecules. It known that IVD cells are capable of producing a complex mixture of neurotransmitters, cytokines, and other inflammatory mediators that may stimulate nerve endings in the IVD.^{9, 10} Pain information from these nerve endings could be transmitted via small-diameter sensory axons to the cell bodies of primary sensory neurons in the DRG (Figure 1). The glial cells (both astrocytes and microglia) may play a critical role in pain modulation, possibly by secreting soluble factors and/or interacting directly with the neurons.¹¹ Because of the essential role of both neurons and glial cells in pain generation and maintenance, we have designed an *in vitro* neuron and glial cell co-culture system as an *in vitro* model for pain generation. An *in vitro* cell culture system would allow screening of multiple molecules that may modulate pain generation, and elucidate the molecular mechanisms of action.

To interrogate the function of cultured DRG cells, capsaicin is used to stimulate the culture system. Capsaicin is a substance extracted from hot pepper that has been used as a topical analgesic for centuries. Its receptor, the transient receptor potential cation channel subfamily V member 1 (TrpV1) receptor, is a trans-membrane receptor-ion channel complex that provides integrated responses to temperature, pH, and endogenous lipids.^{12, 13} Binding of substance P to its receptor, TrpV1, results in the release of substance P. Substance P, an undecapeptide (a peptide composed of a chain of 11 amino acid residues) is known to modulate chronic pain and inflammation.¹ In addition to its known function, substance P was selected as a marker to measure neuronal response because the methodology to measure its concentration is well established.

Biglycan is a small leucine-rich proteoglycan (SLRP) known to be expressed at higher levels in normal appearing human IVD tissues from older adults compared with those from younger individuals.¹⁵ Biglycan is an important structural component of the IVD. Emerging evidence suggests that it regulates growth factors [e.g., transforming growth factor (TGF)- β] and inflammatory mediators [e.g., tumor necrosis factor (TNF)- α], by binding directly to

these factors, with low affinity.¹⁶ Although biglycan does not fall into the traditional cytokine inhibitor category, it has been shown to modulate inflammatory responses.¹⁷ In this study, we examined the effects of biglycan on substance P release by cultured DRG cells in response to capsaicin.

MATERIALS AND METHODS

Rabbit dorsal root ganglion (DRG) cell culture

Young adult New Zealand white rabbits (10-week-old, Myrtle's Rabbitry, TN, USA) were used according to Rush University Institutional Animal Care and Use Committee (IACUC) guidelines. Shortly after euthanasia, DRGs were dissected and cells seeded using methods modified from those of Skoff et al. and Steinhoff et al.^{18, 19} Specifically, thoracic and lumbar DRGs were isolated and pooled. The DRGs were rinsed with Hanks' balanced salt solution (HBSS), minced, and incubated with phosphate buffered saline (PBS) containing 0.1% collagenase (Roche, Germany), 0.01% DNase I (Sigma, MO, USA) and 0.05% trypsin (Gibco, CA, USA) for 30 min at 37°C in a humidified chamber with 5% CO₂, with intermittent trituration to break up the tissues. The tissues were then rinsed twice with HBSS and centrifuged at 300g for 5 min. Cells were then suspended in DMEM/F12 medium (Mediatech Inc., VA, USA) containing 2% fetal bovine serum, 2mM L-glutamine, 1mg/ml penicillin/streptomycin, and seeded in 24-well plates coated with poly-D-lysine (BD Biosciences, NJ, USA) at a concentration of 0.5×10^5 cells/cm², with 1ml medium per well. The cells were further cultured for 4–5 days before treatments.

Immunostaining for neurons and astrocytes

To further verify the DRG neurons in the culture system, immunocytochemical staining was performed. Primary antibodies included mouse anti- β_3 tubulin (Millipore, MA, USA) as the neuronal marker and rabbit anti-glial fibrillary acidic protein (GFAP, Millipore) as the marker for astrocytes. Specifically, DRG cells cultured on poly-D-lysine coated chamber slides were fixed with 4% paraformaldehyde for 20 min at room temperature and rinsed three times with PBS. After blocking with 5% goat serum in PBS at room temperature for 1h, cells were then incubated with primary antibodies at 4°C overnight. Subsequently, goat anti-rabbit rhodamine-conjugated antibody (Millipore) and goat anti-mouse FITC-conjugated antibody (Millipore) secondary antibodies were applied for 1 hour at room temperature. Following several washes in PBS, slides were dehydrated through graded alcohol (50%, 70%, 95%, 100%), cleared in xylene, and coverslips were applied. Cells double-labeled with the fluorescent antibodies were examined by a Fluoview Laser Confocal system equipped with an Olympus microscope and an argon/krypton laser. The images were obtained by dual-channel confocal laser scanning, using appropriate emission filters: for rhodamine, excitation at 568 nm and a BA585IF filter; for FITC, excitation at 488 nm and two emission filters: BA510IF and BA550RIF.

DRG cell treatments with capsaicin and biglycan

First, to determine the optimal time of DRG cell response, cultured DRG cells were incubated with capsaicin at 25 μ molar (M) in concentration. Immediately after the addition of capsaicin and 5 minutes to 60 minutes after treatments, 100 μ l of culture medium was

collected at regular intervals. Substance P released by the primary sensory neurons derived from cultured DRGs was quantified by ELISA, using a substance P ELISA kit (Cayman Chemical Co., Ann Arbor, MI).

To determine the dose-dependent response of the cultures to capsaicin, the DRG cells were treated with capsaicin ranging from 5 μ M to 500 μ M in concentration. Culture medium was collected 5 minutes after capsaicin treatments, and substance P in the medium was quantified by ELISA.

To determine the effects of biglycan on capsaicin-induced substance P release by the DRG cells, the cells were preincubated with biglycan (Sigma-Aldrich, St. Louis, MO) at 4.4 μ g/ml for 30 minutes. The culture was subsequently exposed to 25 μ M capsaicin for 15 minutes. The culture supernatant was then collected for substance P quantification by ELISA.

Statistics

Comparisons between treatments were performed using Analysis of Variance (ANOVA) followed by Student's t-test. $P < 0.05$ was considered significant.

RESULTS

Primary rabbit dorsal root ganglion (DRG) cell cultures contain both primary sensory neurons and astrocytes

We have characterized the DRG culture system by immunostaining for both neuronal and astrocyte markers. DRG cultures were immunostained with an antibody specific for class III β -tubulin, a neuronal specific protein.²⁰ The neurons are shown in green because secondary antibody is conjugated to FITC (Figure 2A&2D). The stained neurons display the typical pseudounipolar morphology, with one axon springing from the soma and then further branching out, clearly visible under high magnification (Figure 2D).

The cultured cells were further immunostained with an antibody specific for glial fibrillary acidic protein (GFAP, Millipore), an intermediate filament found in glial cells.²¹ The GFAP staining was detected with a secondary antibody conjugated to rhodamine, and is thus shown in red (Figure 2B). The characteristic star-shaped appearance of the astrocyte is seen, with multiple long processes extending from the soma. Overlay of panels A and B demonstrates that neurons coexist with astrocytes (Panel C). Taken together, these data confirm that these DRG cell cultures contained both primary sensory neurons and astrocytes.

Cultured DRG cells released Substance P in a time-dependent manner following Capsaicin stimulation

To confirm that the cultured DRG cells functioned properly, the cultures were stimulated with capsaicin, a substance extracted from hot pepper known to trigger substance P release by binding to the heat sensitive calcium channel.¹³

First, in order to determine at what time point media should be collected after stimulation in subsequent experiments, a time course for Substance P release was established (Figure 3). Medium containing 25 μ M of capsaicin was added to the cultured rabbit DRG cells.

Immediately (0 minutes) or 5, 15, 30 or 60 minutes following the addition of medium containing capsaicin, an aliquot of conditioned media was collected for Substance P quantification by ELISA. The relative amount of Substance P released in response to capsaicin was calculated based on time 0 values (medium withdrawn immediately after addition of capsaicin). We found that Substance P was released by the cultured DRG cells after 5 minutes, and increased linearly over the 1-hour study period (Figure 3). Thus in subsequent experiments, we quantified substance P 5 or 15 minutes after initial exposure of the cells to capsaicin.

Cultured DRG cells release Substance P in response to capsaicin in a dose-dependent manner

To establish an optimal capsaicin concentration to use in future experiments, capsaicin concentrations ranging from 5 μ M to 500 μ M were added to the cultures, and Substance P concentrations in the media were determined 5 minutes following capsaicin addition. Substance P fold changes were calculated based on values in cultures without capsaicin treatment. Substance P release into the culture media was dose-dependent (Figure 4). Specifically, stimulating the cultured DRG cells with 500 μ M of capsaicin resulted in a 13.7 fold increase in Substance P released into the culture media compared with controls without capsaicin treatment (n=6, p=0.0251). Cells stimulated with 100 μ M of capsaicin secreted 10.6 times the amount of Substance P as did control cells (n=3, p=0.0140). Cells stimulated with 25 μ M of capsaicin released 4.6 times the amount of Substance P as did untreated controls (n=7; p=0.0245; Figure 4).

Biglycan attenuates Substance P release by Primary DRG cells cultured in vitro

To examine the effects of biglycan on neuronal responses to capsaicin stimulation, DRG cells were preincubated with biglycan for 30 minutes. DRG cultures were then stimulated with 25 μ M capsaicin for 15 minutes, and Substance P released into the media was quantified. There was a 29% decrease in substance P released by the DRG cells preincubated with biglycan followed by capsaicin stimulation, compared with the capsaicin only group (n=4, p=0.0363; Figure 5). Controls were incubated with biglycan, without capsaicin stimulation. Experiments were repeated four times with different animals. Each assay was performed in duplicate.

DISCUSSION

Here we have set up an in vitro DRG culture that contains both primary sensory neurons and astrocytes. This system responded to capsaicin, a calcium channel agonist, by releasing substance P. We have also confirmed that the cultured DRG cells responded to capsaicin in a dose- and time-dependent manner. A similar culture system has been used to test cultured DRG cell inflammatory responses to TNF- α by PCR, although release of neurotransmitters was not tested in that study.²² In another study, a DRG culture system was used to examine neuroinflammation.²³ These studies confirm the validity of the *in vitro* DRG culture system, which should be useful for examining potential pain-generating factors.

We further observed that biglycan inhibited substance P release by cultured sensory neurons in response to capsaicin stimulation. It is not entirely surprising that biglycan, a small proteoglycan with a core protein containing leucine-rich repeats (LRRs), interacted with capsaicin. LRR-containing receptors are known to regulate plant development, symbiosis and defense.²⁴ In animals, Toll-like receptors (TLRs) contain LRRs in their ectodomains,²⁵ which interact with extracellular ligands.²⁶ Signals transduced by TLRs likely play a role in animal development and host defense.²⁴ Finally, crystal structure analysis of biglycan reveals a pocket near the N-terminal disulfide knot in biglycan (cysteine residues 27 linked to 33, and 31 linked to 40),²⁷ which potentially sequesters capsaicin. Potentially informative studies might include in vitro binding assays of capsaicin with biglycan, and molecular modeling to identify binding sites involved in this putative intermolecular interaction.

In summary, we have established a rabbit DRG cell culture system which contains both sensory neurons and the supporting astrocytes. Cultured DRG cells respond to capsaicin in a dose- and time-dependent manner. Biglycan, a small Leucine-rich repeat proteoglycan (SLRP), inhibited capsaicin-induced substance P release by cultured DRG cells.

Physical Medicine and Rehabilitation specialists (physiatrists) often diagnose and evaluate patients with back or joint pain. Molecules naturally occurring in the intervertebral discs and joint cartilage such as biglycan may regulate inflammation and modulate pain perception. Increase of these molecules may be achieved by stimulating their production by native tissues, or introducing the molecules exogenously by injection.

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AUTHORS' CONTRIBUTIONS AND ACKNOWLEDGEMENTS

PS performed rabbit dissections, cell culture, and ELISA; EC performed confocal microscopy; YZ and HSA are responsible for conceptual design, data interpretation, and securing funding. YZ has access to all data collected. All authors critically reviewed and approved the manuscript. The authors gratefully thank Dr. Brett Lenart and Dr. Dean Hartley for helpful discussions about rabbit neuron cultures, and Dr. Marion Young for insightful discussions on biglycan. The authors thank Martin Heyworth, MD, for critically editing the manuscript.

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Figure 1. Neurotransmission pathway from the Dorsal Root Ganglion (DRG) to the spinal cord

Figure 2. Cultured primary rabbit Dorsal Root Ganglion (DRG) cells

A: Sensory neurons immunostained for β -III tubulin, a neuron-specific marker; **B:** astrocytes stained for glial fibrillary acidic protein (GFAP); **C:** overlay of A & B; **D:** image of one sensory neuron showing typical pseudounipolar morphology.

Figure 3. Substance P released by cultured Dorsal Root Ganglion (DRG) cells incubated with 25μM capsaicin increased linearly over time (normalized to no-capsaicin controls; *P<0.05).

Figure 4. Substance P released into the culture media in response to stimulation with various concentrations of capsaicin ranging between 5 μ M and 500 μ M (normalized to no-capsaicin controls; *P<0.05).

Figure 5.
Substance P released by the cultured Dorsal Root Ganglion (DRG) cells is decreased by pre-incubation with biglycan (BGN; Controls were incubated with BGN with no capsaicin; * $p < 0.05$).