Original Article

The Discovery of Stannin in Rat Dorsal Root Ganglia Using an Integrated Proteohistological Approach

Potential Role in Inflammatory Hyperalgesia

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Abstract

The use of proteomic analysis to discover proteins (previously identified or unknown) in a tissue sample is a valuable tool. However, there is a limit to the extent one can validate a discovery with any single technology. In an effort to obviate this inherent constraint and to add value and dimension to protein profiling, we have coupled the information obtained through proteomic techniques with the validation provided by *in situ* hybridization and immunohistochemistry techniques. This approach can be illustrated by our efforts in the discovery of stannin in rat dorsal root ganglia (DRG). In this study, we initially used the Ciphergen ProteinChip® to perform protein profiling on the DRG of rats in a carrageenan-induced paw inflammation study. In an effort to discover new potential targets in inflammatory pain models, we profiled many potential peaks unique to the ipsilateral DRG of interest. One protein, found to bind to a hydrophobic chip at a molecular mass of 9500 Dalton, was preliminarily identified as stannin. To confirm its identification, we performed *in situ* hybridization and immunohistochemistry on the source DRG tissue to investigate the presence of stannin mRNA and protein expression, respectively. In addition to confirming the presence of stannin in these DRGs, we observed the upregulation of stannin in the DRGs over the course of carrageenan-induced inflammation, suggesting a possible role of stannin in inflammatory hyperalgesia. Taken together, these results illustrate the synergistic benefits of coupling proteomic and histochemical techniques in identifying and validating targets and biomarkers for drug discovery.

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Introduction

Proteomic technology is used to identify and characterize a large set of proteins in an effort to provide information concerning location, abundance, modification, and protein-protein interactions. A relatively novel proteomic tool is the Ciphergen ProteinChip®, which is based on surface-enhanced laser desorption ionization mass spectrometry *(1)*. This technology makes use of the preferential adherence of peptides and proteins to selective surfaces based on chromatography, such as hydrophobic (H4), weak cationic exchange (WCX), strong anionic exchange (SAX), and metal binding (IMAC). By manipulating specific chip surfaces, buffers, and conditions, certain proteins bind to the chips while others are washed away. This selectivity allows for a semiquantitative measurement of the relative amounts of each protein from sample to sample. The information gathered from the chips consists of molecular weight, protein characteristics (p*I* or hydrophobicity), and relative expression of the protein. Using a database of protein characteristics, these parameters can be used to determine potential identifications for proteins present in a biological sample *(2)*. However, the data obtained from this proteomic tool require validation and further characterization. Because of the limited amount of sample, rather than attempting to isolate and identify the protein following ProteinChip analysis, we used *in situ* hybridization and immunohistochemistry to positively identify the expression of mRNA coding for a protein of interest. *In situ* hybridization uses probes that are developed to hybridize with the mRNA from the original cells or tissue. With this method, binding of the probe to sections of the tissue confirms the expression of message coding for the protein of interest. Furthermore, *in situ* hybridization

establishes whether there is a necessity for the production of an antibody to directly test for the proteins of interest. Immunohistochemical techniques can then be used to determine the cellular presence and relative degree of expression of the protein of interest.

In an effort to discover novel targets, we have used this unique integration of functional proteomics, *in situ* hybridization, and histochemical techniques to study a well-characterized model of inflammation and inflammatory pain that involves the injection of carrageenan into the hind paw of rats. Carrageenan causes an increase in paw volume, commonly known as edema, and an exacerbated sensitivity or hyperalgesia to thermal and mechanical stimuli *(3)*. Paw inflammation provokes changes in neuronal gene regulation and protein expression both at the dorsal root ganglia (DRG) and spinal cord levels *(4–6)*.

We used the Ciphergen ProteinChip to perform initial protein profiling on lumbar DRG from rats in a carrageenan-induced inflammation study. Using the integration of proteomic and histology techniques, we have identified the expression of stannin in the DRG of rats in the carrageenan-induced inflammation model. Stannin was first described in trimethyltin (TMT)-sensitive neuronal cell populations and may play a role in TMT toxicity *(7,8)*. In addition, antisense oligonucleotides generated against stannin were shown to inhibit TMT-induced apoptosis in primary neuronal cell cultures *(9)*. We are currently exploring possible roles for stannin in inflammatory and neuropathic diseases, as well as applying immunohistochemical techniques to characterize stannin in a number of different tissues for its potential role in other diseases.

Materials and Methods

Carrageenan Paw Hyperalgesia Model

Male Sprague-Dawley rats (Charles River Labs) weighing 350–450 g were used for this

Table 1
Experimental Design of Carrageenan Study

Treatment group	Injection	Duration	Behaviors	Fresh frozen for protein chip (n)	Perfusion fixation for ISH/IHC (n)
Naïve	None	24 h*	BL,1,4,24 h	2	4
CAR 1 h	Carrageenan	1 h	BL, 1 h	–	3
CAR 4 h	Carrageenan	4 h	BL, 1,4 h	2	3
CAR 24 h	Carrageenan	24 h	BL, 1,4,24 h	2	3

*Synchronized with 1-h ($n = 3$) and 24 h ($n = 3$) injected animals.
BL, baseline; CAR, carrageenan; ISH, *in situ* hybridization; IHC, immuno-histochemistry; *n*, number of rats.

study in accordance with approved Institutional Animal Care and Use Committee protocol procedures. Rats were maintained under a 12-h on/12-h off light cycle and allowed free access to food and water throughout the study. Animals were randomly assigned to treatment groups according to the design below (Table 1). Baseline thermal response latencies to radiant heat stimulation were obtained for each hind paw using methods described previously (UCSD device [10,11]). Carrageenan (1%, Sigma) was injected into the plantar aspect of the left hind paw in a volume of 100 µL. Thermal response latencies were reassessed at 1, 4, and 24 h following carrageenan injection up to the time of DRG tissue harvest as prescribed in Table 1. Lumbar (L4–L6) DRGs ipsilateral to the injection site were removed by laminectomy following CO_2 asphyxiation and flash frozen on dry ice for ProteinChip analysis. Identical DRG samples were harvested for *in situ* hybridization and immunohistochemical analysis from a separate matched cohort of rats that were perfused (transcardiac) with PBS followed by a 4% formalin solution.

Lysate Preparation

Tissue lysates were prepared using a Triton lysis buffer (50 m*M* Tris, pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA, 0.2% Triton-X-100 with Complete® protease inhibitors, Pierce). The DRG tissue was incubated with the buffer for 10 min, dounce homogenized (20 strokes), and centrifuged at 20,000*g* for 10 min at 4°C. The supernatant was collected and applied to the protein chip surfaces.

ProteinChip Arrays

The four surfaces used in these experiments were H4, WCX2, SAX2, and IMAC-Cu. ProteinChip Arrays were equilibrated, bound, and washed with buffers specific for each surface (H4: 50 m*M* HEPES, pH 7.0, 10% acetonitrile; WCX2: 0.1 *M* Na acetate, pH 4.0; SAX2: 0.1 *M* Tris, pH 9.0; IMAC-Cu: 0.1 *M* HEPES, pH 7.0, NaCl 0.5 *M*.). Chips were equilibrated for 5 min at room temperature. Total protein (20 µg) was incubated on the chip for 45 min at room temperature followed by two 5-min washes with equilibration buffer and one wash with a 1:100 dilution of the specific buffer. Samples were read on a PBS IIc with a high mass of 200 kDa, mass optimization of 3–10 kDa, a laser intensity of 250, and a detector sensitivity of 8. The information gathered from the chips consisted of molecular weight, binding characteristics (p*I* or hydrophobicity), and relative expression of the protein. Using these parameters, we identified potential candidate proteins.

Table 2
Stannin Sense and Antisense Probes and Peptide for Antibody (Ab) Production

Antisense	CCCCCAGGGCCGCGATGGCAATGAGGATGACAATGACTGT
Sense	ACAGTCATTGTCATCCTCATTGCCATCGCGGCCCTGGGGG
Peptide	RLQRISQSEDEESC

In Situ *Hybridization*

Paraffin-embedded DRG tissue sections were treated with pepsin for 10 min. Slides were hybridized in probe diluent containing digoxigenin-labeled stannin oligoprobes (Table 2) (30 μg) at 37°C for 18 h. Slides were then washed with 2× SSC at 42°C for 5 min followed by 0.1× SSC at 42°C for 5 min. Digoxigenin was detected using peroxidase-labeled anti-digoxigenin antibody (Boehringer Mannheim) at a 1:150 dilution. The peroxidase-labeled antibody was detected using two 5-min incubations of diaminobenzidine (DAB).

Immunohistochemistry

Briefly, tissue sections on microscopic slides were dewaxed and rehydrated. Slides were microwaved in Target buffer (Dako). For heating accuracy, a constant number of slides were heated in the slide rack regardless of the number of slides with tissues on them. After antigen retrieval, the slides were cooled, placed in phosphate-buffered saline (PBS, pH 7.4), and treated with 3.0% H_2O_2 for 10 min at room temperature. All incubations (30 min each) and washes were performed at room temperature. Normal blocking serum (Vector Labs) was placed on all slides for 10 min. Following a brief rinse in PBS, sections were treated with polyclonal anti-stannin primary antibody. Slides were washed in PBS and treated with goat anti-rabbit biotinylated secondary antibodies (Vector Labs). Slides were again washed with PBS, followed by the addition of the avidin-biotin-horseradish peroxidase complex reagent (Vector Labs). All slides were washed and treated with 3,3′-DAB

(Biomeda) two times for 5 min, rinsed with distilled water, and counterstained with hematoxylin.

Databases

Determination of candidate protein identification was performed using the TagIdent database *(12)*. Molecular weights with an error of 0.2% and p*I* values of 7.00 +/− 10.00 were entered into the SwissProt database, which yielded a list of proteins matching the entered parameters. The proteins that matched species, organ, and disease model system were retained as potential identifications.

Results

Carrageenan-Induced Inflammatory Hyperalgesia

Thermal response latencies were recorded from all animals remaining in the study at each time-point. Figure 1 depicts the time-course of hyperalgesia development following intraplantar injection of carrageenan. Note that the carrageenan-induced reduction in response latency is prominent by 1 h, maximal by 4 h, and returned to baseline by 24 h postinjection. Uninjected animals failed to show marked changes in response latencies over the time-course.

Proteomics: Discovery of Stannin in the DRG of Carrageenan-Treated Rats

To identify proteins important in inflammatory pain, we examined the ipsilateral L4–6 DRG from rats following a carrageenan footpad injection study. DRG lysates from control
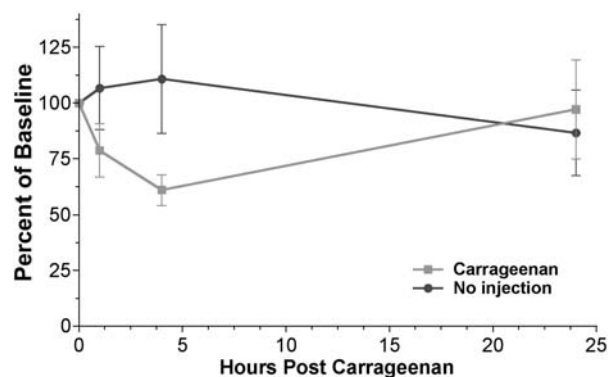
Fig. 1. Time-course of radiant thermal hyperalgesia development following intraplantar injection of carrageenan. Note that the carrageenan-induced reduction in response latency is prominent by 1 h, maximal by 4 h, and returned to baseline by 24 h postinjection. Uninjected animals failed to show marked changes in response latencies over the time-course.

animals and carrageenan-injected animals 24 h after injection were tested for protein expression on four protein chip surfaces. The differential protein expression is shown in Fig. 2. Using the automatic peak detection software provided with the Ciphergen system with the detection level set at the median point, the most abundant protein peaks were detected in the control and 24 h samples (peak map, Fig. 2). A differential peak map (Fig. 2) indicated that the most prominent change was a 9500-Dalton protein on the H4 chip that was upregulated in the carrageenan-treated group (24 h) compared with the control group. When the sensitivity of the automatic detection was increased, several other proteins were found to be differentially expressed. Two of these proteins (10.2 kDa and 11.7 kDa) binding to the IMAC chip were also increased in the 24 h treated carrageenan group (Fig. 2). We selected the 9500-Dalton peak as a target for further analysis, it being the most abundant change. Because of the very small size of the tissue sample and relative low abundance of the protein, we chose not to attempt the isolation

of the protein for identification by mass spectrometry. Instead, using the Tag-Ident database-searching program, we searched the Swiss-Prot database for proteins with a molecular mass of approx 9500 Dalton. Of the 16 proteins obtained from the search, we preliminarily identified the protein as stannin based on size, sample species, hydrophobicity (ability to bind to a hydrophobic surface), and tissue specificity.

In Situ: *Verification of Stannin mRNA Expression in Rat DRG*

Having potentially identified the presence of stannin in the DRGs associated with carrageenan-induced inflammation, we wished to confirm this observation and further evaluate the expression and tissue distribution of stannin. Because of the very small size of the tissue sample and relative low abundance of the protein, we used *in situ* hybridization techniques to positively identify the expression of stannin mRNA in the DRG tissue samples. *In situ* probes were designed to specifically hybridize to the stannin mRNA. Sense and antisense digoxigenin-labeled oligoprobes were synthesized (Table 2). The oligoprobes for stannin were hybridized to normal human tissue as a control. Hybridization of the antisense probe was observed in neurons and kidney (Fig. 3). The sense probe was unable to bind to the tissue samples, indicating specificity of the probes under the hybridization conditions (Fig. 3). *In situ* analysis using the stannin probes was also performed on DRG tissue sections from both rats treated with carrageenan and control rats, thus paralleling those tissues used for lysates in the original protein analysis. We observed binding of the antisense probe to sections of DRG tissue, confirming the expression of stannin. Figure 4 shows the expression of stannin in DRG tissue following carrageenan treatment. We found that the level of mRNA for stannin was upregulated in the DRG tissue at 1 h poststimulation with carrageenan. Similar
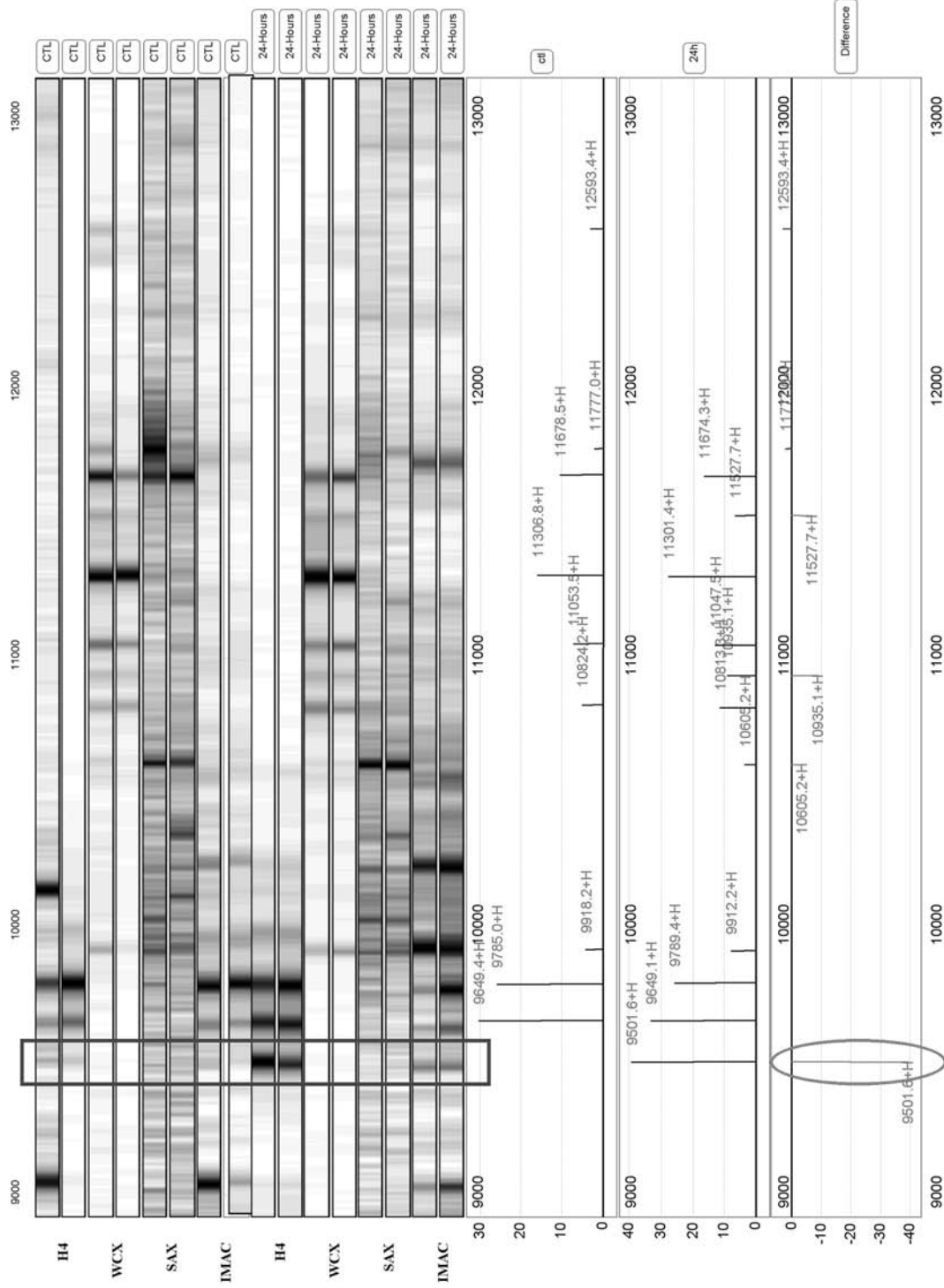
Fig. 2. Protein profiling of carrageenan-treated animals. Virtual gel representation of the protein expression differences on hydrophobic (H4), weak cation (WCX), strong anion (SAX), and copper (IMAC-Cu) Ciphergen chip surfaces for both the control (CTL) and 24-h post-carrageenan treatment (24-Hours). The peak maps show the protein expression of control (CTL) and 24-h post-carrageenan-injected animals (24-h) in the 9000- to 13,000-m/z range. These peaks are compared in the differential expression map (Difference), which shows that the expression of a 9501-kDa peak was upregulated in the 24-h post-carrageenan-injected group.
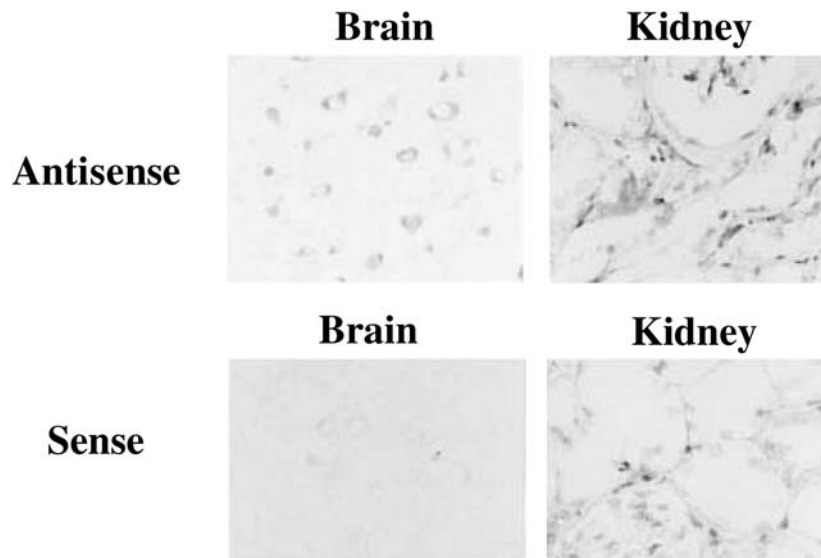
Fig. 3. *In situ* hybridization of known stannin expressing tissue. Antisense probe specific for stannin mRNA labeled both kidney tubules and neurons in the brain. The control sense probe did not label the same tissues.

results were seen at 4 h poststimulation and to a lesser degree at 24 h poststimulation.

Immunohistochemistry: Confirmation of Stannin Protein Expression in Rat DRG

A polyclonal antibody to stannin was produced to confirm the presence of stannin in DRG tissue sections by antibody analysis. We found that protein levels of stannin were increased in DRG sections from animals 4 h and 24 h after injection with carrageenan over those that did not receive carrageenan injections (Fig. 5). Taken together, these observations show that stannin is present and the expression of this protein in the DRG is altered in this rat model of inflammatory pain.

Conclusions

Injection of inflammatory agents into the footpad in rats leads to edema and an enhanced sensitivity to evoked stimuli, thereby making such models useful for the evaluation of anti-inflammatory and analgesic therapies.

Not only are local mediators within the paw involved in the inflammatory response, but also neurons innervating the injected dermatome undergo profound changes in protein expression *(4–6)*. We sought to discern protein expression differences between naïve and inflamed rats to discover potential indicators of the disease state and to provide new insights into the pathogenesis of inflammatory pain. Using a combination of protein and nucleic acid techniques, we have shown the upregulation of stannin in the DRG in response to carrageenan-induced inflammation. Stannin was first described in TMT-sensitive cell populations and has been implicated in playing a role in TMT toxicity *(7,8)*. Our identification of stannin in tissues by *in situ* analysis is in agreement with previous reports showing the presence of stannin transcripts in the spleen, kidney, and brain *(13)*. In addition, antisense oligonucleotides generated against stannin were shown to inhibit TMT-induced apoptosis in primary neuronal cell cultures *(9)*. Although the role of stannin in the action of neurotoxicants has been described, it is unknown if
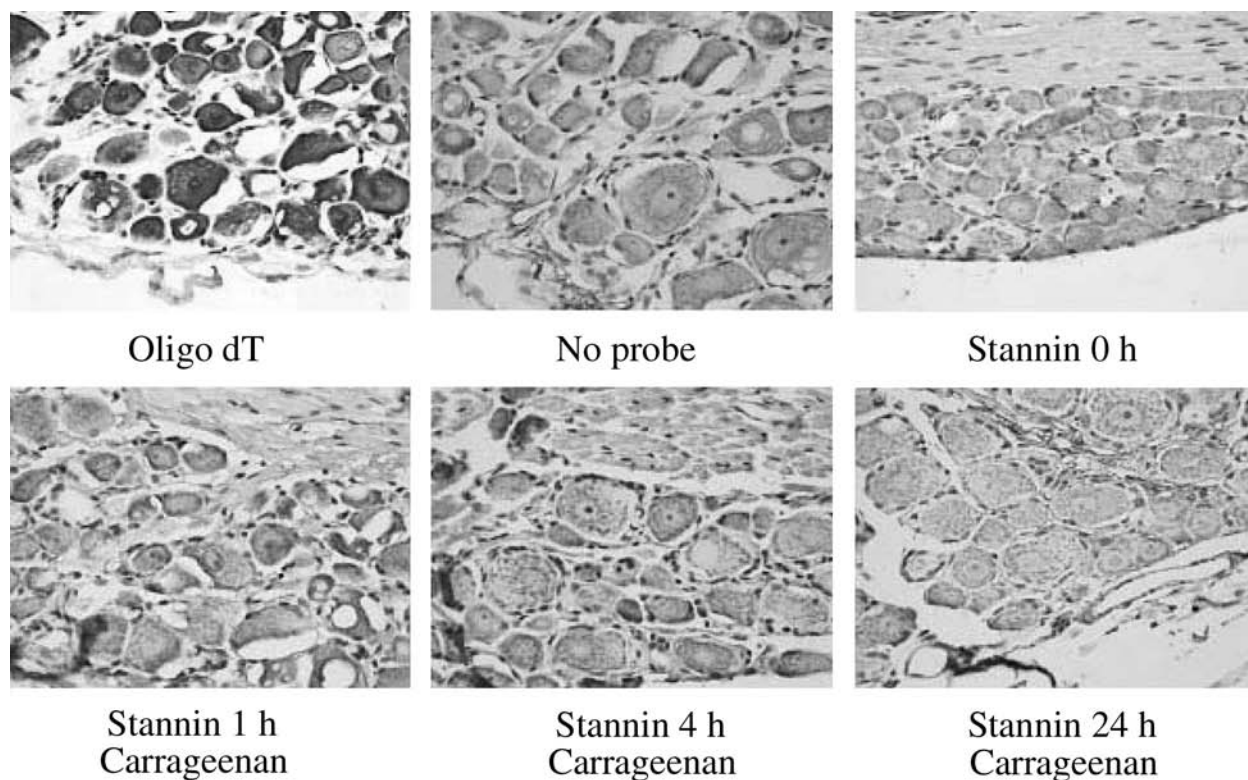
Fig. 4. Stannin mRNA expression in dorsal root ganglia (DRG) after carrageenan injection. Stannin mRNA levels were determined by *in situ* hybridization with a stannin-specific probe. Stannin mRNA expression was increased in DRG 1 h after carrageenan injection into the footpad as compared with control animals.

stannin plays a similar role in neuronal damage by other mechanisms.

This is the first time that the expression of stannin has been demonstrated in DRG and shown to be upregulated in a model of inflammatory pain. We found that the level of stannin mRNA was upregulated in the DRG tissue after 1 h and 4 h of stimulation with carrageenan. This temporal expression of stannin mRNA corresponds with the time-course of protein expression, 4 h to 24 h following carrageenan treatment, that was observed with the protein profiling on the Ciphergen ProteinChip® and confirmed using immunohistochemistry.

The carrageenan model is well established and accepted for the study of inflammatory pain. Carrageenan and other inflammatory agents are known to activate tumor necrosis factor (TNF)-α, which in turn initiates a cascade of inflammatory cytokines, ultimately leading to neuronal sensitization and hyperalgesia *(14)*. Interestingly, TNF-α has been shown to induce the expression of stannin mRNA in vascular endothelial cells *(15)*. Recently it was shown that TNF-α induces stannin production in neurons and that this production of stannin protects cells from TNF-α-induced apoptosis (unpublished data, personal communication, Dr. Brian Reese). It follows that increased expression of stannin may be a protective mechanism used by neurons to prevent damage or ameliorate changes induced by excessive local levels of TNF-α.

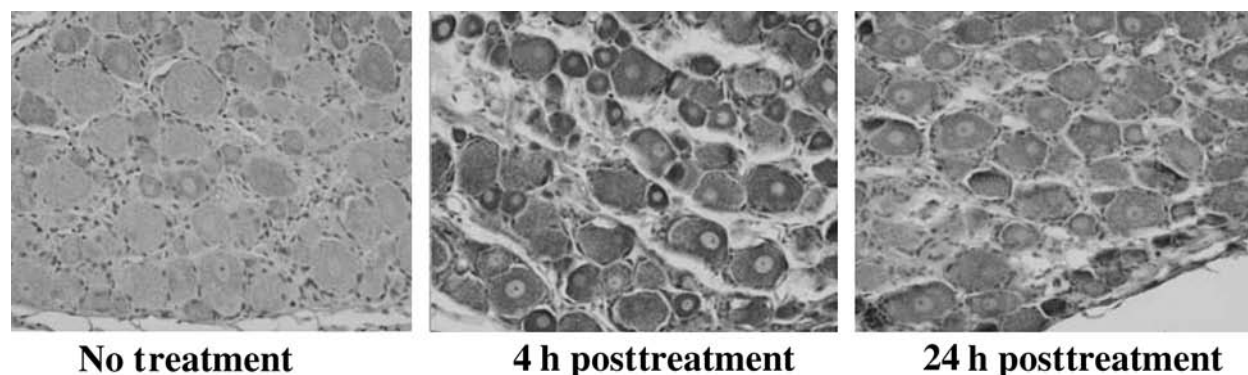| No treatment | 4 h posttreatment | 24 h posttreatment |

Fig. 5. Stannin protein expression in dorsal root ganglion is increased by carrageenan treatment. Stannin protein expression was determined by labeling with an anti-stannin polyclonal antibody. Stannin protein levels were increased 24 h post-carrageenan injection as compared with control animals.

On the basis of the tissue localization and the previously observed ability of TNF-α to induce stannin *(15)*, we are exploring a potential role for stannin in inflammatory pain.

In conclusion, we examined DRGs treated with carrageenan in an attempt to further delineate a mechanism for the exaggerated pain response experienced by patients with inflammatory hyperalgesia. Our initial protein profiling experiments determined the differential pattern of a 9.5-kDa protein using mass spectral analysis. Preliminary informatics suggested this protein to be stannin. To validate this observation, we used functional histological assays to show the presence and upregulation of stannin in these DRGs. Our data generated from the combination of these two technologies suggest that the stannin may play a role in the neural mechanisms associated with inflammatory pain. The sequential application of the techniques described in the current study show that it is possible to exploit the sensitive protein profiling capabilities of a proteomic tool through subsequent confirmation and validation using histological tools. This example, which is currently being applied to the analysis of other peaks in this study as well as many other investigations, presents a powerful combinatorial approach to enhance the initial discovery of biomarkers and the identification of proteins that are present in samples of interest.

Acknowledgments

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