Published in final edited form as:

Pain. 2010 May ; 149(2): 243–253. doi:10.1016/j.pain.2010.02.008.

Peripheral inflammation induces tumor necrosis factor dependent AMPA receptor trafficking and Akt phosphorylation in spinal cord in addition to pain behavior

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Abstract

In the present study, intraplantar carrageenan induced increased mechanical allodynia, phosphorylation of PKB/Akt and GluR1 ser 845 (PKA site) as well as GluR1, but not GluR2 movement into neuronal membranes. This change in membrane GluR1/GluR2 ratio is indicative of Ca⁺⁺ permeable AMPA receptor insertion. Pain behavior was reduced and biochemical changes blocked by spinal pretreatment, but not post-treatment, with a tumor necrosis factor (TNF) antagonist, Etanercept (100μg). Pain behavior was also reduced by spinal inhibition of phosphatidylinositol 3-kinase (PI-3K) (wortmannin; 1 and 5μg) and LY294002; 50 and 100μg) and Akt (Akt inhibitor IV; 3μg). Phosphorylated Akt was found exclusively in neurons in grey matter and in oligodendrocytes in white matter. Interestingly, this increase was seen first in superficial dorsal horn and α-motor neurons (peak 45 min) and later (peak 2 h post-injection) in deep dorsal horn neurons. Akt and GluR1 phosphorylation, AMPA receptor trafficking and mechanical allodynia were all TNF dependent. Whether phosphorylation of Akt and GluR1 are in series or in parallel or upstream of pain behavior remains to be determined. Certainly, TNF mediated GluR1 trafficking appears to play a major role in inflammatory pain and TNF mediated effects such as these could represent a path by which glia contribute to neuronal sensitization (spinal LTP) and pathological pain.

Keywords

GluR1; GluR2; Carrageenan; Rat; PI-3K; TNF

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CONFLICT OF INTEREST

None of the authors has a conflict of interest with the contents of this paper.

Introduction

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine released from glia [13;38] known to increase neuronal excitability through a variety of post-transcriptional mechanisms [26;53], including changes in neuronal α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. These receptors are composed of up to four subunits, GluR1–GluR4; those without GluR2 subunits are Ca^{++} permeable (Ca^{++} -perm) [4;23] and frequently participate in synaptic strengthening [1;25]. Under basal conditions, immunostaining for GluR1 and GluR2 is prominent throughout the superficial dorsal horn [5], with GluR2 being found at virtually all AMPAR puncta [50]. Both subunits are found in deeper laminae, but with lower density, significantly, GluR1 increases in this region following dorsal rhizotomy [5]. It has been suggested that in naïve rats, GluR1 staining is more highly associated with GABAergic neurons [30]. In experimental systems where GluR subunits are quantified, increases in Ca^{++} -perm AMPAR are expressed as an increased GluR1 or GluR4/GluR2 ratio.

In hippocampal neurons and α -motor neurons, TNF increases plasma membrane concentration of GluR1 containing, Ca^{++} -perm AMPAR within minutes [3;18;43]. As yet, no connection has been made between spinal TNF and Ca^{++} -perm AMPAR in dorsal horn. However, spinal Ca^{++} -perm AMPAR contribute to hyperalgesia [22;28;49;55] and multiple peripheral insults increase Ca^{++} -perm AMPAR in dorsal horn cells [20;45;47], including nociceptive projection neurons [29;31;62].

While the initiating stimulus resulting in increased AMPAR trafficking and membrane Ca^{++} -perm AMPAR in dorsal horn is still not determined, some of the intervening steps have been demonstrated. There is a strong evidence implicating phosphatidylinositol 3-kinase (PI-3K) [20;47]. Antagonism of Akt/PKB a downstream mediator of PI-3K has similar anti-hyperalgesic effects [57]. Although, as Akt activates nuclear-factor-kappa B and through it cyclooxygenase 2 [9], the anti-hyperalgesic effects of Akt inhibitors may be mediated through this or another spinal transduction pathway. Interestingly, PI-3K is also required for AMPA receptor insertion in hippocampal neurons during long term potentiation (LTP) [35]. Another requirement for AMPA receptor insertion during hippocampal LTP is phosphorylation of GluR1 at ser 845 by protein kinase A (PKA) [1;15;33]. Dorsal horn activation of PKA leading to P-GluR1 ser 845 occurs following intradermal capsaicin and spinal antagonism of PKA is sufficient to block capsaicin induced hyperalgesia [16;17]. Roles for P-Akt, PKA or P-GluR1 in mediating TNF triggered AMPAR trafficking have not been addressed in any system.

This study demonstrated that intraplantar carrageenan induces pain behavior, insertion of GluR1, but not GluR2 into neuronal membranes and phosphorylation of Akt, and GluR1 ser 845 within the dorsal horn. Spinal TNF antagonism not only reduced carrageenan induced mechano-allodynia but, most importantly, blocked trafficking of GluR subunits and changes in P-Akt and P-GluR1 ser 845. Antagonists to PI-3K and Akt confirmed their involvement in hyperalgesia and immunohistochemistry demonstrated P-Akt in neurons. Our results point to TNF as a necessary mediator in the development of AMPA receptor trafficking and pain behavior following inflammation and a potential mechanism of glial to neuronal communication. Furthermore, we identify phosphorylation of both Akt and GluR1 ser 845 as steps along TNF initiated nociceptive pathways.

Materials and Methods

Animals and intrathecal (i.t.) catheter implantation

Male Holtzman rats (Harlan Industries, Indianapolis, IN, USA) weighing 250–300g were housed on a 12-h light/ 12-h dark cycle and controlled temperature with free access to food and water. Efforts were made to minimize animal discomfort and reduce numbers of animals used. All experiments were carried out according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the Institutional Animal Care and Use Committee of the University of California, San Diego approved this study protocol.

For catheter implantation, a polyethylene-5 (PE-5) catheter was inserted into the subarachnoid space under isoflurane (4% for induction, 2% for maintenance) anesthesia. The catheter was passed 8.5 cm caudally to the level of the lumbar enlargement through an incision in the atlanto-occipital membrane. The external part of the catheter, which connected with PE-10 catheter, was tunneled subcutaneously to exit at the top of the head. The skin was closed with 3-0 silk sutures. After surgery, rats were housed in individual cages. Rats received a 5 mL subcutaneous injection of Lactated Ringer's solution (Baxter HealthCare Corporation, Deerfield, IL, USA) containing carprofen (5 mg/kg; Pfizer Animal Health, New York, NY, USA) immediately after surgery and again on the following day. After recovery from anesthesia, any rats with motor or postural deficits (less than 5%) were immediately sacrificed with inhalation of carbon dioxide. Experiments were performed a minimum of 6 days after surgery.

Carrageenan-induced inflammation

Carrageenan (degraded λ -Carrageenan, Wako Pure Chemical Industries, Japan) was dissolved in saline to form a 2% solution and stored at room temperature for 24 hrs; 100 μ l of the solution was then injected subcutaneously into the center of the left hind paw under light isoflurane anesthesia using a 30 g needle (Behavioral and Western blot studies). For time course and membrane subcellular fractionation studies (Western blots) and immunohistochemical studies, carrageenan injection was bilateral.

Behavioral testing

Animals were acclimated to the testing room for 60 min (30 min in room, 30 in individual Plexiglas test chambers with wire mesh floors). Mechanical allodynia was assessed with von Frey filaments (Stoelting, Wood Dale, IL, USA) having buckling forces between 0.41 and 15.2 g. The paradigm was based on the up-down test [8] to obtain the 50% probability withdrawal threshold. Filaments were applied perpendicularly to the plantar surface of hindpaw through the wire mesh floor with the filament being bent slightly. Each application was maintained for 6 seconds or until the animal withdrew the hindpaw. Rapid lifting or licking of the hind paw was regarded as a positive response. Intrathecal drug administration and intraplantar carrageenan injection were performed after obtaining baseline thresholds for both hindpaws. Any rat with a basal paw withdrawal threshold below 10 g on either paw was excluded from the study. After carrageenan injection, withdrawal thresholds were examined for a 4-hour period at 1-hour intervals. All testing was performed by an experimenter who was blinded to the contents of the intrathecal injection.

Western Blots—Based on preliminary time course studies, we examined trafficking of GluR1 and GluR2 into and out of the plasma membrane and cytosolic compartments of the cells 1 h after intraplantar carrageenan. We also measured phosphorylation of Akt at the ser 473 and thr 308 residues and of GluR1 at ser 845 in whole cell homogenates of dorsal spinal cord tissue at 1 and 2 h after paw injection with carrageenan. As these substrates were all

altered by carrageenan injection, we examined the ability of spinal pretreatment with Etanercept (100 µg) to block evoked changes.

Subcellular Membrane Fractionation and Detection of GluR1 and GluR2 subunits: At designated time points after carrageenan injection, the animal was deeply anesthetized with isoflurane, decapitated and the spinal cord was extruded with cold saline. After dissecting a 1 cm length of lumbar enlargement (L2–L5), the dorsal quadrant ipsilateral to the carrageenan injection was harvested and immediately frozen with dry ice and stored at -70°C . For initial processing, tissue was homogenized in buffer (10 mM Tris-HCl, 5mM EDTA, 300mM sucrose, protease and phosphatase inhibitors; pH 7.5). Homogenates were centrifuged (8000 rpm for 10 min at 4°C) and the resulting supernatant was re-centrifuged (15,000 rpm for 60 min at 4°C) to obtain supernatant containing a crude cytosolic fraction and a pellet containing a crude membrane fraction [27] adapted from [6;20]. A solubilizing buffer (50mM Tris-HCl, 2mM EDTA, 150mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.5% saponin and 0.1% SDS, pH8.0) was added to the cytosolic fraction until its final concentration was 10%. The pellet was re-suspended in the solubilizing buffer. Pellet (crude membrane) and supernatant (cytosolic) fractions were then separately sonicated, vortexed, ice cooled and stored at -70°C . Protein concentrations were determined and samples were run on gels as above, however, a pan-cadherin, a plasma membrane marker, (Novus Biologicals, Inc., Littleton, CO, USA) was used as the loading control for the membrane fractions. Controls have been performed showing that there was no pan-cadherin in the cytoplasmic fraction and that endosomal markers such as EEA-1 were located predominantly in the cytoplasmic fraction (not shown) [27]. EEA-1 is present in newly endocytosed (early) endosomes, while other markers such as Rab4 are present on recycling or late endosomes [14] and both types are concentrated in the cytoplasmic fraction. Gels of both the membrane and cytoplasmic fractions were probed with rabbit anti-GluR1 and anti-GluR2 (both 1:1000, Millipore).

Whole cell homogenates: Tissue was obtained as for standard Western blots above. Spinal tissue was homogenized in extraction buffer containing protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA), 0.5 % Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 3 % sodium dodecyl sulfate (SDS). The homogenate was centrifuged at 14,000 rpm for 15 min at 4°C , and the supernatant was used for Western immunoblotting.

The protein concentration of the supernatant was determined using a bicinchoninic acid (BCA) kit (Pierce Biotechnology Inc., Rockford, IL, USA). Equivalent amounts (20 µg) of protein from each sample was loaded into a Nu-PAGE 4–12 % Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in Tris-HCl buffer containing 0.1% Tween 20, pH 7.4 (TBS-T) for 1 hour at room temperature and then incubated overnight at 4°C with phospho-primary antibodies. These included rabbit anti-P-Akt ser 473 (1:1000) and rabbit anti-P-Akt thr 308 (1:2000; both from Cell Signalling Technology, Danvers, MA), and rabbit anti-P-GluR1 ser 845 (1:1000; Millipore, Temecula, CA). The membrane was washed with TBS-T and then incubated with goat anti-rabbit HRP (horseradish peroxidase)-linked secondary antibody (Cell Signalling) for 1 hour on the next day. After incubation the membrane was exposed to SuperSignal West Femto substrate (Pierce Biotechnology, Inc.) to enhance the signal. Following exposure to X-ray film, membranes were stripped and reprocessed for one more protein of interest and then for β -actin (mouse anti- β -actin, 1:10,000, Sigma) as a loading control. Immunoblots were scanned and densitometric analysis performed using ImageQuant (Amersham Biosciences, Piscataway, NJ, USA). Immunoblot density was normalized to controls run on the same gel.

Drugs

Etanercept (30, 100, 300 µg; TNF antagonist, Amgen, Thousand Oaks, CA, USA), Wortmannin (0.3, 1.0, 5.0 µg; phosphatidylinositol 3-kinase (PI-3K) inhibitor, m.wt. 428.4, Sigma), LY294002 (50, 100 µg; 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, PI-3K inhibitor, m.wt. 307.4, Sigma), and Akt inhibitor IV (0.6, 3.0 µg, m.wt. 614.6, Sigma) were used as pretreatments. Etanercept was dissolved in sterile isotonic saline; Wortmannin and Akt Inhibitor IV were dissolved in 5% DMSO/95% saline and LY294002 was dissolved in a vehicle consisting of 5% DMSO, 2.5% EtOH and 92.5% saline. The vehicle of each drug was used as its control. Etanercept was usually administered 1 hour before the carrageenan injection, however, in one experiment Etanercept (100 µg) was given 90 min after carrageenan injection as a test for its post-treatment efficacy. All other agents were usually given immediately before the intraplantar injection, but due to the short half-life of wortmannin, we administered a second shot in one experimental paradigm 2 hour after carrageenan to see if we could extend the duration of the anti-allodynia. All drugs were administered through the intrathecal catheter in a volume of 10 µl followed by a 10 µl saline flush to clear the catheter.

Immunohistochemistry

Following carrageenan injection to the paws, rats (without intrathecal catheters) were deeply anesthetized with isoflurane and transcardially perfused with room temperature heparinized 0.9% saline containing phosphatase inhibitors (Sigma) followed by chilled 4% paraformaldehyde in 0.1 M phosphate buffer. Time points were chosen at either 0 (no injection/naïve control) or 0.75, 1.3, 2 or 3 h post paw carrageenan. Spinal cords were removed and post-fixed in perfusate for 6 hs and transferred, first to 20% sucrose for 12–24 hs and then to 30% sucrose until they sank for cryoprotection. Tissue was kept at 4°C. The fixed lumbar enlargements were embedded in O.C.T. compound (Tissue-Tek, Torrance, CA, USA) snap frozen, and transverse sections (20 µm) from L2-S1 were cut on a Leica CM 1800 cryostat. Sections were mounted on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA, USA) and double labeled with rabbit anti-P-Akt ser 473 (1:200; Cell Signalling, Danvers, MA, USA) and the cell markers mouse anti-Neu N (neurons, 1:500; Millipore, Temecula, CA, USA), OX-42 (microglia, 1:100; BioSource International, Camarillo, CA, USA), mouse anti-glia fibrillary acidic protein (GFAP) (astrocytes, 1:500; Chemicon) and mouse anti-APC (oligodendrocytes, 1:500; Oncogene Research, Boston, MA, USA) to confirm cellular location of the enzymes. At least four random (non sequential) sections were taken from L4 and L5 as well as from segments rostral and caudal to the principle paw projection area. Reported results were observed in a minimum of four animals under each condition and clearly immunopositive cells were counted, under blinded conditions, within the boundaries of laminae I–III, lamina IV, lamina V and the ventral horn. Cells were counted only if there was a clearly visible nucleus. Ventral horn cells had a minimum somal diameter of 25 µm and thus, were presumptive α -motor neurons. Binding sites were visualized with species matched goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1:500; Invitrogen, Carlsbad, CA, USA) or goat anti-mouse antibody conjugated with Alexa Fluor 594 (1:500, Invitrogen). Equivalent dilutions of normal rabbit or mouse IgG were substituted for primary antibodies as a control for non-specific staining. Bilateral images were captured with a fluorescence microscope (Olympus, Melville, NY, USA) at 10–60X. To confirm antibody co-localization, confocal images were acquired with a Leica TCS SP2 confocal system; single optical sections of 0.3–0.4 µm thickness were taken and images processed with Adobe Photoshop software.

Statistical analysis

All data were expressed as mean $\pm$ S.E.M. Time courses and area under the curve (AUC) of von Frey test and data from Western blots were analyzed using one-way analysis of variance

(ANOVA) followed by Tukey's multiple comparison tests (GraphPad Prism 4.0, San Diego, CA, USA). Number of P-Akt positive stained cells across time points was also analyzed using ANOVA, while comparison of number of positive cells in different spinal areas in the same animals was performed using a paired t-test. A value of $P < 0.05$ was considered as statistically significant.

Results

Pretreatment with a TNF antagonist reduces mechanical allodynia

Groups did not differ with regard to baseline paw withdrawal thresholds prior to drug injection. Rats injected with i.t. saline 1 h prior to intraplantar injection showed a pronounced carrageenan-induced mechanical allodynia that lasted for the entire 4 h observation period. Spinal pretreatment with Etanercept resulted in a dose dependent decrease in magnitude of the allodynia over the first 3 h (Fig. 1A). The treatment effect was significant for the 100 and 300 μg groups ($p \leq 0.01$ and 0.05, respectively) as indicated by the AUC for the entire observation period (Fig 1B). These data indicate that TNF is necessary for full manifestation of carrageenan-induced allodynia, but the less than complete antagonism indicates that other triggers are also involved. Posttreatment with 100 μg Etanercept was totally without benefit. This is similar to the pattern seen with intrathecal administration of 5 μg Joro spider toxin, an antagonist to the Ca^{2+} permeable AMPAr, where early treatment caused a robust almost complete blockade of allodynia and post-treatment was no different than saline [55].

Pretreatment with PI-3K antagonists reduces mechanical allodynia

Groups did not differ with regard to baseline paw withdrawal thresholds prior to drug injection. Following i.t. pretreatment with 5% DMSO, carrageenan injection induced a drop in withdrawal thresholds similar to, but not quite as steep, what was observed after saline pretreatment (Figure 2A). Thresholds remained significantly lower than baseline ($p \leq 0.01$) for the entire observation period and were no different than saline injected animals in previous experiments indicating a minimal anti-allodynic effect of the vehicle. Intrathecal pretreatment with 0.3 μg (0.07 μM) of wortmannin, a PI-3K antagonist resulted in post-carrageenan thresholds no different than vehicle, however, raising the wortmannin dose to 1 or 5 μg (0.23 or 1.17 μM) resulted in a dose dependent blockade of the allodynia for the first 2 h after injection, withdrawal thresholds then fell and approached those of the vehicle treated animals (Figure 2 A and D). As wortmannin is known to have a short half-life, we administered a second dose (1 μg) after 2 h to see if the period of anti-allodynia could be extended. However, post-treatment was without effect on the latter timepoints and thresholds continued to drop at this time (data not shown).

We then examined the effect of pretreatment with the more specific PI-3K antagonist, LY294002. Due to problems with solubility in 10% DMSO, we used a vehicle consisting of 5%DMSO+ 2.5% EtOH. Pretreatment with this vehicle delayed and reduced the carrageenan-induced allodynia making it harder to assess the anti-allodynic effect of the drug. However, thresholds were higher than vehicle following administration of the 50 and 100 μg (16.27 and 32.53 μM) doses (Figure 2B) and the area under the curve (AUC) indicated a significant blockade of the allodynia at these doses examined over the full 4 hour period (Figure 2E).

Pretreatment with Akt antagonist reduces later portion of mechanical allodynia

As PI-3K is upstream of Akt phosphorylation, we also used Akt inhibitor IV as a pretreatment to determine whether it was also potentially involved in the carrageenan-induced hyperalgesia. The low dose (0.6 μg (0.1 μM)) was without any effect compared to

vehicle, however, the higher dose (3 μ g (0.49 μ M)) was successful in reducing the allodynia (Figure 2 C and F). Interestingly, unlike the two PI-3K inhibitors and Etanercept, the beneficial effect was only seen in the latter half of the observation period.

Carrageenan induces TNF dependent trafficking of GluR1, but not GluR2 into membrane fractions of tissue homogenate

Preliminary time course studies were performed assaying the membrane enriched fractions to examine carrageenan-evoked changes in AMPA receptor trafficking. Membrane GluR1 was determined in tissue from animals with no paw injury and 0.5, 1, 2, 3 and 4 h after bilateral intraplantar injection of carrageenan. The time course was similar to that seen for P-Akt with increased levels at 1 and 2 h post-injection that were not different from each other (data not shown).

In addition to causing a TNF dependent increase in P-Akt and P-GluR1 ser 845, intraplantar carrageenan also elicited a TNF dependent increase in total GluR1 ($p \leq 0.05$; $N=3-4$) in membrane fractions of dorsal spinal cord homogenates ipsilateral to the paw injection (Figure 3A). Total GluR1 in cytoplasmic fractions, which in our preparation contains the majority of the endosomes, from the same tissue had a marked tendency to decrease in samples obtained from carrageenan-injected animals, but this was not significant. We believe that these data indicate a movement of late endosomes containing GluR1 into the plasma membrane. Surprisingly, when the same gels were stripped and re-probed, neither the membrane nor the cytoplasmic fraction showed a carrageenan-induced change in GluR2 (Figure 3B).

Carrageenan induces phosphorylation of Akt at both the ser 473 and thr 308 residues: P-Akt ser473 is TNF dependent

Separate preliminary time course studies were performed on carrageenan evoked responses to pick optimal time points for later studies; in these, P-Akt ser 473 in whole cell homogenates were determined in animals with no paw injury and 1, 2, 3 and 4 h after bilateral intraplantar injection of carrageenan. Bilateral carrageenan injected into the hindpaws of animals without intrathecal catheters elicited a clear two fold increase in P-Akt at the ser 473 residue that developed within 1 h and remained elevated through at least the second hour and fell back to basal levels by the third hour. The histogram in Figure 4A illustrates the time course ($N=4-7$ rats/time point) and shows representative blots taken from a single gel. Based on these data, we performed experiments in animals with i.t. catheters with unilateral paw injection and harvested tissue one and two h post injection. Unilateral intraplantar carrageenan injection, preceded by i.t. vehicle, consistently induced an increase in P-Akt ser 473 compared to control; this was true for tissue harvested 1 ($p \leq 0.05$; $N=3$ /gp; data not shown) and 2 ($p \leq 0.05$; $N=5-7$; Figure 4B) h after carrageenan injection. There was a tendency for the carrageenan-induced increase to be smaller than that seen after bilateral injection, however, this did not reach significance. Some gels were stripped and re-probed for P-Akt thr 308 and showed a similar pattern; unilateral injections of carrageenan combined with i.t. saline pretreatment induced an increase in P-Akt at both the thr 308 and ser 473 residues compared to control ($p \leq 0.05$; $N=3-4$), however in many cases, blots for P-Akt thr 308 had multiple bands and high background making them hard to clearly measure. We examined single gels in which we had probed for both P-Akt ser 473 and P-Akt thr 308 and in which both had given us clean results and plotted blot densities for phosphorylation sites to determine if they were correlated, i.e., does the amount of phosphorylation between the ser and thr sites co-vary (Figure 4C). Pearson correlation analysis indicated a significant linear relationship between the phosphorylation at the 2 sites ($p \leq 0.02$).

The carrageenan-induced increase in P-Akt ser 473 at both time points (1 and 2 h) was completely prevented by i.t. Etanercept pretreatment (Figure 4B, only shown for 2 h time point). This is consistent with the hypothesis that Akt and most likely PI-3K are downstream of TNF receptor activation.

In naïve animals, very few dorsal horn cells of any type (none in the superficial laminae and less than 4/section in laminae IV and V combined) were positive for P-Akt (Figure 5A and F). Given the strong peak of P-Akt induced at 2 hrs post carrageenan observed with Western blotting, we first perfused animals at that time (Figure 5D). Unlike previous studies which saw the preponderance of P-Akt in the superficial dorsal horn after peripheral injection of nociceptive substances [47; 57], we observed P-Akt predominantly in lateral lamina V neurons (22.3 ± 1.6 /dorsal horn) with a smaller number of stained neurons in lamina IV (4.4 ± 0.9 /dorsal horn; Figure 5F). In these neurons, P-Akt staining appeared to be confined to the cytoplasm and was not observed in nuclei, but did extend into the dendrites (Figure 5H). Some of the stained neurons were large pyramidal shaped cells with dorsally extending dendrites and are likely to be nociceptive projection neurons (Figures 5D and H). Only rare neurons in the superficial dorsal horn stained for P-Akt at this time point (0.1 ± 0.1 /dorsal horn). These data prompted us to examine an earlier time period. When the experiment was repeated with perfusion at 0.75 h post-carrageenan, we observed a total shift in the P-Akt staining pattern (Figure 5B). At this earlier time, P-Akt was elevated in neurons in the superficial dorsal horn (13.2 ± 1.2 /dorsal horn) compared to naïve ($p \leq 0.0001$), but the number of lamina V neurons positive for P-Akt had not yet started to increase. Each section at this timepoint contained one or more large stained marginal cells that draped medio-laterally over lamina I (Figures 5 B and G). Examination of intermediate and later time points indicated an early peak in P-Akt neurons in superficial laminae at 0.75 h or earlier that was no longer different than naïve by 1.3 h post-injection (Figure 5C). In lamina V, the number of immunoreactive neurons was first increased over naïve at 1.3 h ($p \leq 0.01$) and the peak was significantly later than that of the lamina I peak at 2 h post injection with a fall off of immunoreactive neurons earlier and later (Figures 5C and E, respectively). There were more P-Akt neurons in lamina V than IV only at the 2 and 3 h points, $p \leq 0.001$ and 0.01 , respectively (paired t-test).

When we examined more rostral sections from L2 spinal cord, at 2 h after carrageenan injection, P-Akt staining resembled that seen in naïve tissue of L4/5 with no positive neurons in the superficial dorsal horn and only a few scattered in the deeper laminae (not shown). Similar results were observed when we looked at tissue from more caudal segments (L6).

Cellular location of carrageenan-induced P-Akt

Co-staining with cell specific markers indicated that while P-Akt was found extensively in neurons, it did not co-stain with markers for astrocytes (GFAP), microglia (Ox-42) (Figure 6 B and C) or oligodendrocytes (APC, not shown) within the grey matter. Lack of co-localization was observed under naïve conditions as well as 0.75 and 2 h post-injection. There was, however, extensive co-localization with APC within the dorsal columns (Figure 6A) and other white matter tracts including the dorsolateral and lateral funiculi at 0.75 h (not shown and 2 h post carrageenan. Little co-localization was observed in naïve animals. We recently reported this same pattern, co-localization with APC exclusively in white matter, for P-p38 staining in oligodendrocytes after a burn injury [54].

Time course of carrageenan-induced P-Akt staining in ventral horn

Surprisingly, intraplantar carrageenan also induced an increase in the number of α -motor neurons positive for P-Akt (Figure 7 A–C). Numbers of P-Akt stained neurons was

extremely low in naïve animals, however 0.75 h following carrageenan injection numbers increased and fell again by the 2 h post injection time (Figure 7D). Comparison of the time course of P-Akt occurrence in motor neurons with that of the dorsal horn neurons shows a strikingly similar time course to that seen for neurons in the superficial dorsal horn, but not those in laminae IV and V. This argues against motor neurons being activated by a substance diffusing from dorsal horn and instead suggests that the afferent input entering the superficial dorsal horn and resultant nocifensive flexion responses triggers the activation and perhaps sensitization of α -motor neurons. As yet, our data do not indicate if carrageenan-induced effects on motor neuron are mediated via local release of TNF, however, TNF does elicit an increase in Ca^{++} permeable AMPA receptors on motor neurons [18]. Importantly, these data indicate that enhanced motor output (behavioral responses or activity in motor nerves) following peripheral inflammation may be a function of sensitization of α -motor neurons as well as sensitization of nociceptive sensory pathways.

Carrageenan induces TNF dependent phosphorylation of GluR1 at ser 845

Unilateral carrageenan injection preceded by i.t. saline resulted in a more than two fold increase in phosphorylation of the GluR1 AMPAR subunit at ser 845 (PKA site) compared to control. This increase was also completely prevented by pretreatment with Etanercept (Figure 8) indicating a dependence of GluR1 phosphorylation via PKA on TNF.

Discussion

In the present study, intraplantar carrageenan induced an increase in P-Akt, P-GluR1 ser 845 and insertion of GluR1, but not GluR2 into membrane fractions of dorsal spinal cord homogenates. This change in the membrane GluR1/GluR2 ratio is consistent with Ca^{++} perm AMPA receptor insertion into plasma membranes as well as increased AMPA receptor density. Spinal TNF was necessary for all of these events to occur as i.t. pretreatment with Etanercept, a TNF antagonist blocked all three of these outcome markers. Importantly, spinal Etanercept also reduced peripheral inflammation-induced mechanical allodynia. Spinal antagonists to PI-3K and Akt also reduced carrageenan-induced pain behavior albeit with different time courses. It is significant that, in our hands, none of the antagonists employed resulted in complete, or close to complete, blockade of mechanical allodynia. This is unlike what we have observed after administration of Ca^{2+} perm AMPAR antagonists [55].

Previous work demonstrated that peripheral inflammation and nociceptive stimulation can induce insertion of Ca^{++} permeable AMPA receptors into plasma membranes [20;29;45;46;62]. Interestingly, in animal models where separate measurements of GluR1 and GluR2 were employed, GluR1 was shown to increase in acute models such as capsaicin and formalin injection with no substantial change in GluR2 [20;47]. In contrast, following intraplantar injection of complete Freund's adjuvant (CFA), which takes days rather than minutes to hours to develop, the opposite was observed and membrane GluR2 decreased with no change in GluR1 [29;45;46]. We sampled at 1 and 2 hrs after carrageenan, and accordingly our results follow the more 'acute pattern'. Previous studies of hippocampal neurons demonstrated that TNF induced exocytosis of GluR1-containing AMPAR from intracellular stores [56]. Microinjection of TNF into the ventral horn or spinal cord injury shows similar results in α -motor neurons [18]. In addition, spinal inhibition of protein exocytosis with Brefeldin-A blocks acute nociceptive stimulus induced GluR1 trafficking into membranes [20]. Taken together, these data support the hypothesis that acute increases in Ca^{++} permeable AMPA receptors occur through membrane insertion of preassembled GluR1, but not GluR2 containing AMPA receptors. It is unknown to what extent the same or different triggering mechanisms contribute to the increase in membrane GluR1 and the decrease in membrane GluR2 overlap before the final insertion or removal of the receptor, but it seems that TNF is necessary to trigger GluR1 insertion under 'acute' conditions.

Spinal TNF antagonism was also sufficient to reduce thermal hyperalgesia for days following CFA injection [42]. However, since daily treatment began prior to CFA injection it may be that these data also reflect acute antagonism. Interestingly, in both the CFA/thermal hyperalgesia study [42] and our study, which used mechanical allodynia as an outcome, blockade of pain behavior was not complete. One potentially confounding factor is presence of the spinal catheters, as they may produce spinal glial activation [12] which, in turn could enhance carrageenan-evoked release of TNF. While this is possible, carrageenan-induced release of spinal TNF in the absence of spinal catheterization [19] suggests that it is only the magnitude of our observations that might be influenced and not the observations themselves.

Increases in Ca^{++} perm AMPA receptors, in both acute and more chronic models, contributes to spinal sensitization and pain behavior. This parallels hippocampal studies where insertion of AMPAR from intracellular pools to plasma membrane resulting in increases of AMPAR density and/or number of Ca^{++} -perm AMPAR is required for long term potentiation (LTP) [10;66]. Under basal conditions, membrane insertion of GluR1 containing complexes is slow and is balanced by an efflux out of the membrane, however, the insertion rate increases following increased neural activity [34]. Spinal LTP-like mechanisms are thought to contribute to spinal sensitization, in part due to glial-neuronal interactions [24;52;65]. As TNF, acting through TNFR1 receptors, induces insertion of Ca^{++} permeable AMPA receptors into hippocampal pyramidal neurons [2;56] and TNF has more recently been shown to induce insertion of GluR1 into synaptic membrane of motor neurons, we postulated that it might induce insertion of Ca^{++} perm AMPAR into dorsal horn neurons. The Western blot data directly support this hypothesis and the behavioral data are in agreement with a role for spinal TNF in paw carrageenan-elicited pain behavior. Spinal TNF is thought to arise in great part from glial activation [13;32] and infiltrating macrophages [63] although the spinal meninges are also a likely TNF source [64]. While TNF frequently acts in an autocrine fashion, contributing to glial activation including activation of p38 in microglia after injury [36;58], we propose that it also acts directly on neurons via surface receptors to increase AMPA signaling. Thus, TNF could be an important mediator of glial to neuronal communication.

Intraplantar carrageenan induced a prolonged increase in P-Akt, presumably mediated via PI-3K activation, which was blocked by TNF antagonism. Spinal antagonists to both PI-3K and Akt reduced the carrageenan-induced pain behavior, albeit with different time courses. A causal link for PI-3K between peripheral tissue injury and GluR1 membrane insertion has been demonstrated in other models [20;47]. However, this is the first study to demonstrate that this pathway is initiated by TNF. Not only do our data demonstrate that antagonism of spinal TNF reduces peripheral inflammation induced pain behavior, it also blocks inflammation-induced phosphorylation of Akt, trafficking of GluR1 into membranes and phosphorylation of GluR1 at ser 845.

TNFR1 has been shown to constitutively form a complex with PI-3K in a variety of cell types and TNFR1 activation elicits a time dependent increase in P-Akt activity [48]. This may occur via crosstalk within caveolae or other lipid rafts as has recently been shown in endothelial cells [11]. Alternatively, TNF binding to TNFR1 has been shown to produce sphingosine 1-phosphate via activation of sphingosine kinase and sphingosine 1-phosphate activates PI-3K and Akt [44]. Linkage between TNFR2 and PI-3K activation has been demonstrated in cortical neurons [37]. Downstream, PI-3K forms complexes with AMPAR subunits GluR1 and GluR2, and activation of PI-3K within the complex appears to be necessary for insertion of AMPAR into plasma membranes in at least some models of hippocampal LTP [35]. Similarly, the chemokine receptor CXCR2 is also coupled to the

PI-3K system [60] and elicits a PKA mediated phosphorylation of GluR1 ser 845 in HEK cells and in hippocampal neurons [7].

The intracellular coupling of PKA with its various substrates within distinct subcellular compartments is tightly regulated through association with A kinase anchoring proteins known as AKAPs [59]. Protein kinase A is upstream of Akt in many systems and phosphorylation at both the ser and thr sites is triggered by forskolin [39;40]. While it is not known if this is the same PKA isoform needed to phosphorylate GluR1, localization of PKA and Akt on the same anchoring protein allows us to hypothesize that the reverse action takes place and Akt activates PKA. Alternatively, PKA activation could be Akt independent, as PDK-1, which is also downstream of PI-3K can directly phosphorylate PKA and some isoforms of PKC [21;61].

Activation of P-Akt in superficial dorsal horn has been seen as early as 5 min after intraplantar formalin injection [47]. This is the peak time of primary afferent C fiber activity [51]. Activation of peripheral C fibers peaks within 0.3 h following intraplantar formalin and remains at this level for at least 1.3 h [41]. Thus, while it is possible that sampling prior to 0.75 h would unmask an earlier peak in superficial dorsal horn, we feel that our data are in agreement with that of Pezet and colleagues. The time difference between the early appearance of P-Akt in superficial dorsal horn and motor horn and the later appearance in deep dorsal horn neurons, which is a minimum of 1 h or more, is perplexing and suggests that the cascade leading to P-Akt differs in the different laminae. The two peaks that we observed with the immunohistochemical results roughly correspond to the 1 and 2 h post-injection times where we observed increased P-Akt in our Western blots. At 3 h post injection, neither the Western blots nor the number of stained neurons in any laminae was different from naïve. Importantly, both the 1 and 2 h Western blot peaks were blocked by spinal Etanercept pretreatment indicating that Akt activation in both laminae I and V neurons was triggered directly or indirectly by TNF. One intriguing possibility is that Akt phosphorylation in lamina V is downstream of activity in lamina I.

In summary, paw carrageenan induces pain behavior, phosphorylation of Akt and GluR1 and GluR1 trafficking into membranes. These outcomes are all blocked by spinal pretreatment with a TNF antagonist. Pain behavior is also blocked by spinal inhibition of PI-3K and Akt. While these factors are all TNF dependent, and are also likely to be PI-3K dependent, whether phosphorylation of Akt and GluR1 are in series or in parallel remains to be determined. Certainly, TNF mediated GluR1 trafficking appears to play a major role in inflammatory pain and TNF mediated effects such as these could represent a path by which glia contribute to neuronal sensitization (spinal LTP) and pathological pain.

Acknowledgments

Grant information: This work was supported by NIH R01NS048563 (L.S.S.); R21DA021654 (C.I.S., LSS) and NIH T35HL07479 (AB)

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Figure 1. Intrathecal Etanercept (TNF antagonist) dose-dependently reduced mechanical allodynia. Rats were pretreated with intrathecal Etanercept or saline 1 hour prior to carrageenan injection into paw. A, Time course; B, Area under the curve (AUC). C. Rats post-treated (90 min) with 100 µg etanercept * $p < 0.05$, # $p < 0.01$, significantly different from vehicle. $n = 6-7$ rats/group

Figure 2.

Inhibitors of PI3K/Akt pathway reduced mechanical allodynia evoked by carrageenan. Carrageenan was injected into the paw just after the intrathecal injection of wortmannin (PI3K inhibitor), LY294002 (PI3K inhibitor), Akt inhibitor IV (Akt inhibitor), or vehicle. Vehicle was 5% DMSO for wortmannin and Akt inhibitor IV and 2.5% EtOH and 5% DMSO for LY294002. A–C, Time course; D–F, Area under the curve (AUC).

* $p < 0.05$, # $p < 0.01$, + $p < 0.001$, significantly different from vehicle. $n = 7$ rats/group

Figure 3 (A.)

Figure 3 (B.) **Figure 3.**

Intraplantar carrageenan induced a TNF dependent increase in GluR1, but not GluR2 in membrane fractions of cellular homogenates. Histograms illustrate that intraplantar carrageenan induced an increase in GluR1 in membrane fractions 1 h after intraplantar injection. GluR2 in the same fractions showed no change in levels compared to controls. In cytoplasmic fractions obtained from the same tissue, GluR1 showed a marked tendency to decrease, but it did not reach significance. Spinal pretreatment with Etanercept (100 μ g) blocked the carrageenan induced GluR1 trafficking indicating the TNF dependence of the process.

Figure 4.

Intraplantar carrageenan induced a time dependent increase in P-Akt in dorsal spinal cord. A. Level of P-Akt increased at 1 and 2 h after bilateral carrageenan injections. Representative P-Akt blots along with β -actin loading controls are illustrated. + is a P-Akt positive loading control. Animals did not have i.t. catheters. B. Histogram illustrates P-Akt response 2 h after a unilateral carrageenan injection. This increase in P-Akt was blocked by spinal pretreatment with Etanercept (ETA, 100mg) 1 hour prior to carrageenan injection. Equivalent increases and ETA antagonism were observed 1 h after carrageenan. * $p < 0.05$, significantly different from naïve or control. $n = 4-7$ rats/group. C. Graph illustrates covariance between Akt phosphorylated at ser 473 and thr 308 in the same samples run on the

same membrane. Samples were obtained from spinal saline-injected animals with and without paw carrageenan. Samples were collected 2 h after the paw injection time. Axes are in arbitrary units.

Figure 5.

Representative sections double labeled for NeuN (red) and P-Akt (green). Panels A–E were taken from animals perfused at 0, 0.75, 1.3, 2 and 3 h respectively, after bilateral carrageenan injection into the paws. F. Histogram illustrating time course of P-Akt within various regions of the dorsal horn. Neurons positive for P-Akt and NeuN were counted by a blinded observer, all counted neurons had a visible nucleus. Counts are averages of 4 separate sections/animal. Counts are of 1 hemisection. N=4 animals/group for all bars. Panel G is an enlargement of the lamina I neuron indicated by the arrowhead in panel B (0.75 h). Neuron has the large, flat morphology typical of a lamina I projection neuron. Panel H is an enlargement of lamina V neurons indicated by the arrowhead in panel D (2.0 h). P-Akt

appeared to be confined to the cytosol and in many cases to extend into the dendrites. Size bar = 100 μm panels A–E and 20 μm panels F and G

Figure 6.

Animals were perfused 2 h after carrageenan injection and sections stained for A. APC, B. GFAP or C. OX-42 (red) to identify oligodendrocytes, astrocytes and microglia, respectively. Sections were all double stained for P-Akt (green). A. Oligodendrocytes in dorsal columns were frequently double labeled for P-Akt. This was also true for other white matter tract areas, including the lateral funiculus (not shown). However, there was no co-localization within the grey matter. Lack of co-localization was observed under naïve conditions as well as 0.75 and 2 h post-injection. B. Although there was plentiful P-Akt in the deeper lateral dorsal horn, it did not co-localize with GFAP. This was true for all areas of the spinal cord and 0.75 h post-injection. WM = white matter C. Although there was

plentiful P-Akt in the deeper lateral dorsal horn, it did not co-localize with OX-42. This was true for all areas of the spinal cord and at 0.75 h post-injection. Size bar = 50 μ m, magnification was the same in all 3 panels.

Figure 7.

Representative sections double labeled for NeuN (red) and P-Akt (green). Micrographs were obtained from ventral horn using the same animals as in Figure 6. Panels A–C were taken from animals perfused at 0, 0.75 and 2 h respectively, after bilateral carrageenan injection into the paws. Panel D illustrates the time course of P-Akt within the -motor neurons. All counted neurons had a visible nucleus, α -motor neurons had a diameter greater than 25 μ m across. Size bar = 100 μ m,

Figure 8.

Intraplantar carrageenan induced an ipsilateral increase in P-GluR1 ser 845 at 1 (illustrated) and 2 (not shown) h after a single intraplantar carrageenan injection. Representative P-GluR1 ser845 blots along with β -actin loading controls are also illustrated. This increase in P-GluR2 ser 845 was blocked by spinal pretreatment with Etanercept (ETA, 100 μ g) 1 hour prior to carrageenan injection. * $p < 0.05$, significantly different from naïve or control. $n = 3-4$ rats/group