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Prooxidant-induced c-Src/nuclear factor kappa B-coupled signaling in sensory ganglia mediates cutaneous hyperalgesia

O. J. Igwe

Division of Pharmacology & Toxicology, School of Pharmacy, University of Missouri-Kansas City, USA

Abstract

Background—Persistent pain resulting from peripheral injury/inflammation is associated with altered sensitivity to cutaneous stimuli, which can manifest as hyperalgesia. The role of oxidant stress in the development, progression and maintenance of hyperalgesia is still not understood. Furthermore, there appears to be a relationship between c-Src kinase in the pain pathway and oxidative stress.

Methods—We have used a novel prooxidant inflammatory pain model that involves potassium peroxychromate (PPC), a unique prooxidant that produces the same reactants as activated phagocytes. This model was used to investigate the role of oxidant-activated c-Src in mediating hyperalgesia. We compared the effects of PP2 [a Src family kinase inhibitor] and c-Src siRNA on behavioral hyperalgesia with sodium stibogluconate (SSG) [a non-receptor tyrosine phosphatase inhibitor] and AG 1478 [a receptor tyrosine kinase inhibitor].

Results—PP2 and c-Src siRNA attenuated PPC-induced thermal hyperalgesia, while SSG enhanced it. AG 1478 had no effect. PP2 decreased the levels of IL-1 β , c-Src/inhibitory kappa B kinase complex formed and prostaglandin E₂ produced in the DRG ipsilateral to the inflamed paw, while SSG increased the levels of these parameters. c-Src siRNA decreased Src expression and activity in the DRG ipsilateral to the inflamed paw.

Conclusions—These results confirm that prooxidant-activated c-Src plays a role in initiating and maintaining hyperalgesia by regulating a stimulus-response coupling between the inflamed tissue and the DRG in the pain pathway. Our data also suggest that oxidant-induced dysregulation of c-Src/NF κ B coupling may contribute to our understanding of the transition from acute to chronic dysfunctional pain state seen in many human diseases.

1. Introduction

The processing of pain occurs in an integrated matrix throughout the neuroaxis and on at least three levels - the periphery, spinal and supraspinal sites. Many aspects of the mechanisms of pain initiation and maintenance in the periphery are still not well understood. It may require activation of multiple intracellular pathways including c-Src non-receptor tyrosine kinases (nRTKases) (Igwe, 2003; Jin et al., 2004) with associated non-receptor protein tyrosine phosphatases (nRTPases) (Zhang et al., 2000; Peng et al., 2012) and transcription factors (e. g., NF κ B) that couple neuronal and non-neuronal messengers to synaptic plasticity (Kalia et al., 2004).

Corresponding author : Orisa J. Igwe, Ph.D., 2464 Charlotte Street, HSB #2247, University of Missouri-Kansas City, Kansas City, MO 64108-2741, Tel #: 816-235-1996, Fax #: 816-235-1776, igweo@umkc.edu.

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Oxidative/nitrosative stress (ONS) induced by reactive oxygen species (ROS) [which also encompasses reactive nitrogen species] is an important initiating factor in many diseases with little or no effective treatment (Halliwell, 1994; Halliwell and Cross, 1994). ROS play a critical role in persistent pain of different origins (Chung, 2004; Kim et al., 2004; Salvemini et al., 2006), and serve as functional messenger molecules in central sensitization, which is an underlying mechanism for chronic pain (Gao and Ji, 2009; Lee et al., 2007; Schwartz et al., 2008). The mechanisms that regulate the role of ROS in pain signaling are still poorly understood.

ROS can activate c-Src, which functions in the PNS and CNS as a co-transducer of mitogenic signals (Zhao et al., 2000; Leu and Maa, 2003). c-Src regulates vascular permeability and responses to inflammation by recruiting and activating immune cells in tissues (Okutani et al., 2006), but its role in prooxidant-induced hyperalgesia is still not understood. Furthermore, c-Src acts as a redox sensor in NF κ B activation (Fan et al., 2003), and can regulate ROS produced *in vivo* by NADPH oxidase (Gianni et al., 2008).

Diverse signals activate NF κ B to play a role in regulating the promoter regions of many genes. A key pathway in the regulation of NF κ B activation is its nuclear translocation (Balwin, 1996) after release from the I κ B kinase α/β (IKK α/β) (Häcker and Karin, 2006; Sun, 2012) to which it is bound in the cytosol. Regulation of NF κ B activation is usually achieved by phosphorylation of I κ B α on serine residues mediated by I κ B α kinases (Hinz et al., 2012). As a primary regulator of stress response *in vivo* (Mercurio and Manning, 1999), it is not clear how ONS affects NF κ B activation. Changes in NF κ B activation may involve tyrosine phosphorylation of IKK α/β by c-Src to favor enhanced coupling of c-Src to IKK α/β (Singh et al., 1996; Fan et al., 2003). Phosphorylation of I κ B α/β at the tyrosine moiety might activate NF κ B for a long time to delay its ubiquitin-dependent degradation (Imbert et al., 1996; Xiao, 2004) and enhance synthesis of proinflammatory mediators. However, the potential contribution of Src-IKK α/β coupling in NF κ B activation mediated by peripheral prooxidants has not been examined.

The purpose of the present studies was two-fold: first, to examine the role of c-Src in peripheral prooxidant-induced hyperalgesia; and second, to determine the specificity of modulating c-Src expression and activity on the levels of IL-1 β , c-Src-IKK α/β complex formation, and prostaglandin E2 production in the sensory ganglia ipsilateral to the inflamed site.

2. Materials and Methods

2.1 Animals and drugs

Pathogen-free male Sprague-Dawley rats (13–15 weeks old and 195–300 g body weight; Harlan, Indianapolis, IN, USA), maintained in a facility approved by the Association for Accreditation of Laboratory Animal Centers, were used in all experiments. All animal procedures followed the U.S. National Institutes of Health (NIH) Guidelines on Laboratory Animal Welfare and the International Association for the Study of Pain as reviewed and approved by the UMKC Institutional Animal Care and Use Committee (IACUC).

Potassium peroxychromate (PPC), a unique prooxidant that decays *in vivo* to produce the same reactants (e.g., ROS, hydroxyl radicals) as activated phagocytes (Miesel et al., 1995a), was synthesized in the laboratory according to a previously described method (Miesel et al., 1995b). It was fully characterized by elemental and infrared analyses and shown to be 98.9 % pure.

A non-receptor Src-family tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) (Salazar and Rozengurt, 1999; Severgnini et al., 2005), its inactive control PP3, and AG 1478 (a receptor tyrosine kinase inhibitor) (Ward et al., 1994), were purchased from Tocris Biosciences (Bristol, UK). Sodium stibogluconate (SSG), a robust inhibitor of non-receptor tyrosine phosphatases (nRTPs) [Pathak and Yi, 2001], and c-Src-specific siRNA duplexes and their mis-sense (MS) control siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNA pools were covalently modified to increase their stability and enhance their performance *in vivo* (Duxbury et al., 2004).

2.2 Drug treatments and measurement of behavioral hyperalgesia

Peripheral inflammation was induced by intraplantar (itp) injection of PPC in the left hind paw. Under isoflurane anesthesia, different groups of rats were given a single itp injection of PPC at 250 pmol/rat in a volume of 100 μ l of normal saline (NS) or NS alone via a Hamilton syringe coupled to a 30-gauge needle. Drug agents PP2, PP3, AG1475, and SSG were dissolved in 60 % propylene glycol (PPG)/40 % NS (PPG/NS). To examine the specificity of c-Src-mediated signaling in PPC-induced hyperalgesia, different groups of animals were treated with equimolar amount of each drug (50 μ moles in 200 μ l of vehicle) by ip injection 1h before PPC and once daily thereafter for 3-d. Because thermal hyperalgesia was more robust at 72 h compared to 24 h after PPC, animals were killed and biochemical parameters were measured in this set of experiments at 72 h only.

Under isoflurane anesthesia, separate groups of rats were also treated with c-Src-specific siRNA duplexes or their mis-sense (MS) control siRNAs that were previously validated *in vitro* with a high confidence of Src knockdown efficiency (Duxbury et al., 2004). The siRNA duplexes were dissolved in a 5:1 (v/v) ratio of sterile 5 % glucose [RNase-free] and ExGenTM *in vivo* transfection reagent (Thermo Scientific) to facilitate cellular uptake and delivery of double stranded RNA. A single itp injection of either c-Src siRNA or the mis-sense (MS) Src siRNA duplexes (450 pmoles in 50 μ l of the vehicle) was made in the left hind paw 5-h before itp PPC in the same paw. Animals were killed in this set of experiments to obtain tissue for biochemical assays at 48-h after PPC.

Thermal hyperalgesia was measured in a blinded fashion as thermal withdrawal latency (TWL). Testing was based on the thermal stimulus paradigm of Hargreaves et al., 1988, using a calibrated Plantar Analgesia Instrument (Stoelting, IL) as described previously (Igwe O, 2006).

2.3 Procurement of DRG and tissue preparation used in biochemical assays

Animals were anesthetized with isoflurane, and killed by decapitation and exsanguination 1 h after behavioral testing, and 48 h or 72 h after PPC. The L4 –L6 and cervical (C) 8 DRG corresponding to the ipsi- and contra-lateral itp PPC-injected paw were dissected on ice, rinsed in ice-cold 50 mM Tris (pH7.4) containing 0.16 mg/ml heparin to remove any red blood cells and clots, and snap frozen in liquid nitrogen. Pooled frozen DRG tissue was homogenized in ice-cold Tissue Protein Extraction and Lysis Buffer (Tissue PE LBTM) (G-Biosciences, St Louis, MO, USA) supplemented with a cocktail of protease inhibitors. DRG homogenates were centrifuged at 20,000- $\times$ g at 4 °C for 30 min. The resulting supernatants were divided into aliquots and either frozen at –80 °C for later use, or used immediately as appropriate in different biochemical assays. Protein levels were determined with a Micro BCA kit (Pierce/Endogen).

Aliquots of the supernatant were lyophilized and stored at –70° C until used to quantify levels of IL-1 β with rat-specific ELISA (Thermo Fisher Scientific, Rockford, IL) in a 96-

well format with Microplate reader (Bio-Tek, Inc., Winooski, VT). The activity of c-Src was determined by immunocomplex kinase assay as described previously (Igwe, 2003).

2.4 Assay for PGE₂ levels as prostaglandin E metabolites (PGEM)

Aliquots of the DRG supernatants were used to quantify prostaglandin E₂ (PGE₂) as PGEM. The homogenate was treated with ethanol, vortexed briefly, incubated on ice for 5 min and centrifuged at 3,000× g for 10 min to remove precipitated proteins. The supernatant was adjusted to pH 4.0, and fractionated on activated C-18 SPE cartridges (Cayman Chemical, Ann Arbor, MI, USA). After several washes, PGEM was eluted with ethyl acetate [EA] containing 1 % methanol and stored at -20° C for later analysis. Before final analysis, EA was evaporated completely in vacuo with A160 Speed Vac concentrator. Sample residues were re-dissolved in enzyme immunoassay (EIA) buffer, vortexed and derivatized completely by overnight incubation at 37 ° C in Carbonate Buffer provided in the EIA kit (Cayman). After dilution in Phosphate and EIA buffers, the derivative was used to quantify PGEM in the samples in a 96-well plate format pre-coated with anti-rabbit PGEM IgG mAb. Samples were analyzed in duplicate with a sensitivity of less than 2 pg/ml for PGEM. The amount of PGEM was determined from a standard curve generated with PGEM supplied in the kit and derivatized under the same conditions as the samples.

2.5 Co-immunoprecipitation (co-IP)/immunoblot (IB) analysis of c-Src-IKK α / β -coupled complex

Portions of DRG homogenates with added activated sodium orthovanadate and sodium fluoride (at 1 mM each) were used to determine the levels of c-Src-IKK α / β complex formed (by co-IP and IB) and β -actin (by IB for loading control). Dynabeads® co-IP kit and DynaMag™-2 magnet were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). The kit was used for covalent coupling of mouse anti-Src, clone GD11 (EMD Millipore, Billerica, MA) onto the surface of the beads for subsequent co-IP with anti-IKK α / β (Santa Cruz Biotechnology Inc., Santa Cruz, CA) according to the manufacturer's instructions. Captured proteins and protein complexes were separated from each 250 mg aliquots of total protein derived from DRG homogenates using the DynaMag™-2 on the basis of the magnetic properties of Dynabeads. Protein complexes bound to the beads were washed twice with wash buffer containing 0.02 % Tween 20, eluted with the elution buffer supplied with the kit and used in IB.

c-Src-IKK α / β complexes formed in the tissue after different treatments was denatured by incubation at 90°C for 15 min for IB analysis. Sample aliquots (15 μ l with 20 μ g of total protein) containing the protein complex were electrophoresed, fractionated for 35–50 min on 4–12% Bis-Tris NuPAGE gels in MOPS running buffer and electrotransferred onto PVDF membranes. Membranes were blocked by a standard IB protocol for 1h at room temperature (RT). c-Src-IKK α / β complex formed was identified by blotting with anti-IKK α / β pAb (Santa Cruz) at 1:1000 dilution. After washing in Tris-buffered saline-5% Tween 20 (TBS-T) containing 5% dry milk, blots were incubated for 1 h at room temperature with pre-adsorbed goat anti-rabbit IKK α / β IgG secondary antibody conjugated to HRP at 1:5000 dilution in TBS-T. Blots were visualized with ECL system (Amersham Biosciences). The molecular size of c-Src-IKK α / β complex with anti-Src as co-immunoprecipitate was estimated with Cruz molecular weight marker-compatible secondary antibodies. To measure β -actin levels in the IB, we used aliquots of total tissue supernatants from the same DRG homogenates used in the co-IP/IB experiments and containing equal amounts of proteins (5 μ g). Membrane blots were incubated with anti- β -actin pAb raised in rabbit at 1:5000 dilution under the same conditions used to identify the Src-IKK α / β complex formed. Equal amounts of protein were loaded on each lane and verified by comparing them to the band intensity of β -actin. Optical density (OD) signals were quantified by densitometry.

2.6 Statistical Analysis

GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistical data analysis. Quantitative data are presented as mean $\pm$ SEM, and were analyzed by 2-way analysis of variance (ANOVA) followed by Fisher's post hoc test. Significance was assigned at $P = 0.05$ %.

3. Results

3.1 c-Src inhibition attenuated thermal hyperalgesia induced by PPC

A single itp PPC in the rat hind paw produced localized inflammation characterized by erythema, edema and hyperalgesia. Thermal withdrawal latencies (TWL), as a measure of pain sensitivity, did not differ for either of the hind paws before PPC in all test groups. Injection (ip) of PP2, SSG or AG 1478 alone had no effect on TWLs either (data not shown) demonstrating that these agents are ineffective in the absence of an inflammatory stimulus (Fig. 1A). A significant decrease in TWL occurred in the paw injected with [Drug vehicle/PPC] ($p = 0.01$) compared to control group or the contralateral, un-injected paw of the experimental groups. Whereas PP2 attenuated PPC-induced heat hyperalgesia, SSG enhanced it compared with [Drug vehicle/PPC] group ($p = 0.01$). We had previously established (data not shown) that the latency between the first ip dose of PP2 followed by PPC and the earliest manifestation of anti-nociception was ~ 3 h. Equimolar AG 1478 did not affect PPC-induced hyperalgesia on the injected paw, or the fore paws at 72 h post PPC.

Itp c-Src siRNA in the same paw as PPC decreased thermal hyperalgesia induced by PPC by 97 % and 79 % (Fig. 1B, $p < 0.001$) compared with [Drug vehicle/PPC]- and [MS Src siRNA/PPC]-treated rats, respectively. In the contralateral paws treated with normal saline, all groups exhibited similar heat sensitivity as in the i.e. [-Drug/-PPC vehicle]- [minus (i.e., without) Drug/minus PPC]- treated control. Src siRNA had no measurable effect on TWL in the injected paw in the absence of inflammation (i.e., in [+ c-Src siRNA/-PPC] group, which suggests that the dose of c-Src siRNA used did not affect basal functions in the paw.

3.2 Inhibition of c-Src decreased levels of (a) IL-1 β , (b) c-Src kinase activity, (c) c-Src/IKK β complex formation and (d) PGE₂ in the DRG

Because thermal hyperalgesia was more robust at 72 h compared to h24 after PPC, we measured all biochemical parameters in the DRG at 72 h only.

(a) IL-1 β levels—In the naïve control rats [-Vehicle/-PPC], no significant changes in IL-1 β contents occurred in either the ipsi- or contra-lateral lumbar DRG (Fig. 2A). PPC increased the levels of IL-1 β (~ 210 %) in the DRG ipsilateral to the inflamed paw compared to the DRG contralateral to the inflamed paw, which would suggest that the increase was not mediated by cytokines in the general circulation. PP2 reduced the magnitude of PPC-induced IL-1 β increase compared to the contralateral DRG in the same group and ipsilateral DRG in the [Drug vehicle/PPC] group suggesting that inhibition of c-Src directly affected IL-1 β synthesis in the DRG. In contrast, AG1478 or SSG had no effect on PPC-induced increase in IL-1 β in the DRG ipsilateral to the inflamed paw, supporting the specificity of c-Src in regulating IL-1 β levels.

(b) c-Src kinase activity—The activity of c-Src kinase was determined by immunocomplex kinase assay as previously described (Igwe, 2003). Src kinase activity did not differ in both the ipsi- and contra-lateral DRG of naïve rats (Fig. 2B). PPC increased c-Src kinase activity in the DRG ipsilateral to the inflamed paw by 77% compared to the corresponding contralateral DRG and the naïve control group. AG1478 had no effect on PPC-induced increase in c-Src kinase activity suggesting that AG 1478 at the dose used did

not inhibit c-Src activity. On the other hand, PP2 significantly decreased PPC-induced increase in c-Src kinase activity in the DRG ipsilateral to the inflamed paws by 62 % compared to the corresponding contralateral DRG side after [Drug vehicle/PPC]. Conversely, SSG increased PPC-induced c-Src kinase activity in both ipsi- and contralateral DRG by equal amounts (27 %) compared to the [Vehicle/PPC] group.

These results again show that nPTPase (e.g., Src homolog phosphatase-1 [SHP-1]) inhibition increases c-Src kinase activity in the DRG ipsilateral to the inflamed paw. In all cases, PPC had no effect on the biochemical parameters in the cervical (C4 – C8) DRG that receive projections from the forepaws (Arvidsson and Pfaller, 1990) and are thus far removed from the inflammatory stimulus.

(c) PGE₂ level as PGE₂ metabolites [PGEM]—[Drug vehicle/PPC] increased PGEM levels in the DRG ipsilateral to the inflamed paw by 239 % compared to the contralateral side (Fig. 2C). Whereas PP2 attenuated a robust PPC-induced increase in PGEM in the DRG ipsilateral to the inflamed paw, PP2 did not abolish it altogether. Contribution by COX-1 activity may also account for the residual PGE₂ increase since we did not correct for it in the data. SSG increased PPC-induced PGEM levels in the DRG ipsilateral to the inflamed paw in [Drug vehicle/PPC] group by ~25 % compared to the corresponding contralateral DRG. PGEM and IL-1 β levels were always significantly higher in the DRG ipsilateral to the inflamed paw following [SSG/PPC] compared to ipsilateral DRG after [Drug vehicle/PPC]. This observation may be an important contributing factor in enhanced sensitivity to heat in [SSG/PPC]-treated rats (Fig. 1).

(d) c-Src-IKK α / β complex—In the absence of an inflammatory stimulus, the level of c-Src-IKK α / β formed in ipsi- and contra-lateral DRG remained unchanged. In all groups, the levels of c-Src-IKK α / β complex formed were unaffected in the DRG contralateral to the inflamed paw, which suggests that afferent drive in the injected paw might be responsible for increased formation of the complex. PPC alone increased the level of c-Src-IKK α / β complex formed in the DRG ipsilateral to the inflamed paw by ~6.2-fold compared to the corresponding contralateral paw (Fig. 3; Table 1).

PP2 reduced PPC-mediated c-Src-associated IKK α / β complex in the DRG ipsilateral to the inflamed paw by more than 50 % [i.e., from ~6.2- to 2.7-fold (Table 1) compared to the DRG in [Drug vehicle/PPC] group (Fig. 3A & Table 1). This reduction suggests that inhibition of c-Src activity (Fig. 2B) also decreased c-Src-IKK α / β complex formation, which may account for a reduction in thermal hyperalgesia as indicated in the behavioral data (Fig. 1A). Intriguingly, [SSG/PPC] treatment increased the level of c-Src-IKK α / β complex formed in the DRG ipsilateral to the inflamed paw by ~7.5-fold compared to the corresponding contralateral DRG (Fig. 3, Table 1). This finding is of interest because SSG was given by intraperitoneal injection. This again suggests that the level of c-Src-IKK α / β formed in DRG ipsilateral to the inflamed paw is related to the degree of thermal hyperalgesia.

3.3 Src siRNA decreased c-Src expression and kinase activity

Because protein levels do not always translate into enzymatic activity, we examined both Src expression and its kinase activity. [Drug vehicle/PPC] or [MS Src siRNA/PPC] increased Src expression quantified as ratios (c-Src: GAPDH) of optical density (OD) signal by 142% and 136% (Fig. 4, $p < 0.001$), respectively, in the DRG ipsilateral to the inflamed paw, compared to the signal in the corresponding contralateral DRG. [Src siRNA/PPC] also increased Src expression but to a much lesser degree (55 %) compared to the corresponding contralateral DRG. This indicates c-Src siRNA reduced Src expression by 92 and 106 %, respectively.

(Fig. 4, $p < 0.01$) respectively, compared with ipsilateral DRG in [Drug vehicle/PPC]- and [MS siRNA/PPC] rats.

c-Src siRNA also reduced PPC-mediated increase in c-Src kinase activity in the ipsilateral DRG by 51% and 56%, respectively, compared to the activities in the corresponding ipsilateral DRG in [Drug vehicle/PPC]- and [MS Src siRNA/PPC] groups. c-Src siRNA treatment did not affect the expression of GAPDH protein, a housekeeping gene product, suggesting that nonspecific or non-target suppression of protein expression did not occur in the injected paw.

4. Discussion and conclusion

We have presented evidence that a prooxidant-induced inflammation can activate c-Src kinase to mediate hyperalgesia quantified as TWL. We have compared the effects of PP2 [a non-receptor Src family kinase inhibitor] (Hanke et al., 1996) and c-Src siRNA on behavioral hyperalgesia with sodium stibogluconate (SSG) [a robust non-receptor protein tyrosine phosphatase inhibitor] (Pathak and Yi, 2001) and AG 1478 [a receptor tyrosine kinase inhibitor] (Ward et al., 1994). PP2 and c-Src siRNA attenuated PPC-induced thermal hyperalgesia, while SSG enhanced it, but AG 1478 had no effect. PP2 also decreased the levels of IL-1 β , c-Src-IKK α/β complex formed and prostaglandin E₂ produced in the DRG ipsilateral to the inflamed paw, while SSG increased the levels of these biochemical parameters. AG 1478 had no effect. c-Src siRNA also decreased Src expression and kinase activity in the DRG ipsilateral to the inflamed paw. Enhanced exogenous oxidant-induced coupling of IKK α/β to c-Src may amplify and perpetuate the expression of inflammatory mediators in the pain pathway (Woolf and Costigan, 1999; Basbaum et al., 2009) to initiate and maintain hyperalgesia (Huang et al., 2003).

Decomposing PPC has been used as a source of reactive oxygen species to examine their effects on biochemical functions (Edwards and Quinn, 1982). PPC can induce chronic inflammation and pain in both mice and rats (Miesel et al., 1995b). PPC-induced inflammation has a clearly defined cause-effect links unlike the adjuvant- and collagen-induced models in which immunogenic interferences quite frequently obscure their modes of action and make data interpretation difficult (Schalkwijk et al., 1987; Miesel and Zuber, 1993). In addition, PPC decays slowly to the same reactants (i.e., ROS) produced by activated phagocytes *in vivo*, thus serving as a continuous and stable source of ROS for a long time with no known off-target effects (Baird et al., 1977; Miesel et al., 1995). We chose to use intraplantar (itp) PPC as a more reliable prooxidant pain model compared to other oxidant models (Meotti et al., 2006).

Our present results further show that PPC and nRTPase (e.g., Src homolog phosphatase-1 (SPH-1) inhibition increased c-Src activity in the DRG ipsilateral to the inflamed paw. Changes in Src kinase activity may directly influence synaptic transmission by phosphorylation of postsynaptic proteins (Yu and Salter, 1999; Zhao et al., 2000), or components of the pre-synaptic releasing system (Smart, 1997). This effect on pain signaling may reflect cooperation between initiation of signal by Src activation and the loss of control of Src-mediated pain/channel gating by nRTPase inhibition. Our present data support the *in vitro* findings that inhibition of nRTPases enhances Src activation (Zhao et al., 1996; Frank et al., 2004). The data also support the hypothesis that prooxidant-induced increases in IL-1 β and c-Src kinase activity in the DRG directly affects sensitivity to thermal hyperalgesia thereby involving the spinal cord in the pain pathway. Therefore, thermal hyperalgesia appears to be enhanced/maintained with increases in the levels of IL-1 β and PGEM in the DRG ipsilateral to the inflamed paw. Furthermore, increased level of PGEM in the DRG ipsilateral to the inflamed paw would suggest that inducible cyclooxygenase 2

(COX-2) and, possibly microsomal prostaglandin E synthase (mPGES)-1 (Jakobsson et al., 1999; Kojima et al., 2011), are functionally coupled and active. In fact, tyrosine phosphorylation (pTyr) of IKK α / β by protein kinase C-dependent c-Src activation has been shown to be involved in enhanced COX-2 expression (Huang et al., 2003). Conversely, decreased PGEM in the DRG ipsilateral to the inflamed paw after [PP2/PPC] may reflect decreased activities of prostanoid biosynthetic enzymes. To provide a reliable estimate of the actual PGE₂ produced following treatments, we quantified PGE₂ as stable PGEM unlike PGE₂, which has a very short half-life *in vivo*.

Activation of c-Src has been implicated in both thermal (Igwe, 2005; Zhang and McNaugy, 2006; Huang et al., 2006) and mechanical (Katsura et al., 2006) hyperalgesia. Furthermore, Src is proposed to be involved in a signaling cascade that inserts a vesicular pool of heat-gated ion channel TRPV1 into the peripheral primary afferent terminals (Zhang et al., 2005). The capsaicin receptor TRPV1 also co-immunoprecipitates with Src kinase and is phosphorylated at the tyrosine (Tyr) residue to maintain channel function (Jin et al., 2004). There is evidence that c-Src can modulate the activation pathway of NF κ B (Schoonbroodt and Piette, 2000; Khadaroo et al., 2003; Ying et al., 2011; Seok et al., 2012). Therefore, co-precipitation of c-Src with IKK α / β suggests that c-Src kinase may phosphorylate a Tyr residue in the IKK α / β complex, which could modify the NF κ B activation pathway possibly through increased formation of (pTyr42)-I κ B α (Huang et al., 2003). Because of the importance of NF κ B activation in response to injury, regulation of (pTyr42)-I κ B α formation as a potential “hyperalgesic primer” produced by environmental stressors, may provide some insight in our understanding of the transition from acute to a chronic pain state (D'Mello and Dickenson, 2008; Voscopoulos and Lema, 2010; Bogen et al., 2012). We define “hyperalgesic primer” as a substance(s) formed *in vivo* in primary afferent nociceptors by oxidants/stressors that may initiate, promote and maintain long-lasting neuroplastic changes in the pain signaling pathways. An important impact of pro-oxidant mediated increase in the formation of (pTyr42)-I κ B α *in vivo* is its potential to also serve as a biomarker for chronic pain of different origins, with crucial import in new drug development.

In the CNS, there is compelling evidence that Src family kinases including c-Src act as a point of convergence for different signaling pathways, which might be crucial for processes that underlie physiological and pathological plasticity (Purcell and Carew, 2003) including pain (Salter and Kalia, 2004). Furthermore, by up-regulating the function of N-methyl-D-aspartate receptors (NMDARs), c-Src may play a role in gating the production of NMDAR-dependent synaptic potentiation and plasticity (Kalia et al., 2004; Salter and Kalia, 2004). We have previously shown that c-Src is expressed not only in the DRG but also in the spinal cord, and contributes to the development and maintenance of inflammatory pain hypersensitivity (Igwe, 2003; Guo et al., 2004). It is likely that the biochemical changes as measured here in the DRG also occur in the spinal microglia and nociceptors to initiate and maintain prooxidant-induced thermal hypersensitivity (Lee et al., 2010). Furthermore, it has shown that injection of a peripheral irritant (capsaicin) can trigger oxidative stress responses in the spinal cord (Lee et al., 2007; Schwartz et al., 2008). Understandably, the spinal cord is the first relay site in the transmission of nociceptive information from the periphery to the brain.

We used *in vivo* RNA interference (RNAi) strategy (Hammond et al., 2001; Dorn et al., 2004; Bumcrot et al., 2006) (Fig. 1B and Fig. 4) to further examine the role of Src in hyperalgesia and Src expression in the DRG ipsilateral to c-Src silencing in conjunction with a prooxidant-induced inflammation. We determined whether *in vivo* Src siRNA would be effective in attenuating PPC-induced Src protein expression, and hence behavioral hyperalgesia quantified as TWL. The approach of administering c-Src siRNA and PPC in

the same paw confirmed that c-Src siRNA duplexes could decrease prooxidant-mediated Src expression and activity in the ipsilateral DRG, at least within the time frame we had examined.

Incomplete knockdown of target c-Src protein (Fig. 4A) may be a result of residual c-Src protein in the tissue, or the short time interval between c-siRNA injection and DRG harvest. In the RNAi segment of this study, we harvested DRG for use in biochemical assays after only 48-h following PPC because the pharmacokinetics of Src-siRNA duplexes was unknown. Interestingly, c-Src siRNA reduced PPC-induced Src kinase activity to a greater degree than c-Src expression. c-Src siRNA treatment would be more efficient in knocking down Src mRNA transcripts than c-Src protein, since the turnover rates of the mRNA and the protein conceivably differ. Furthermore, c-Src-siRNA would not be effective in inhibiting preformed c-Src kinase. This study again confirms Src as a potential target through which inflammatory hyperalgesia may be attenuated. The identity of the cell types in which c-Src mRNA was knocked-down in the DRG after itp c-Src siRNA treatment was not determined in this study. Nonetheless, c-Src mRNA knockdown might have occurred primarily in macrophages, and possibly in neurons in the DRG, as c-Src is expressed in these cells (Kalia et al., 2004; Leu et al., 2006).

In summary, we have shown in this novel prooxidant model of inflammatory pain, that itp c-Src siRNA can produce a significant behavioral reduction of thermal hyperalgesia. This is in correspondence with a significant knockdown of Src protein expression and reduction in c-Src kinase activity in the DRG ipsilateral to the inflamed paw. Conceivably, neither c-Src siRNA reversal of TWL induced by the prooxidant nor suppression of Src expression would be completed at 48-h after its itp administration.

We used DRG derived from cervical (C8) as absolute control in the co-IP/IB experiments because they are anatomically far-removed from the PPC-treated paw, and are least likely to be influenced by treatment. In addition to increased formation of c-Src-IKK α / β complex in the L4 – L6 DRG ipsilateral to the PPC-treated paw and lack thereof from corresponding contra-lateral DRG, our data would suggest that the formation of the complex was due to enhanced prooxidant stress generated by PPC. Thus the C8 DRG data adds more credence to this conclusion.

In conclusion, our present findings demonstrate a potential therapeutic utility of the inhibition of c-Src expression and activity in the treatment of inflammatory pain. A major impact in understanding the role of Src-NF κ B-coupled activation is the potential discovery of new therapeutic target for oxidative/nitrosative inflammatory pain, which is associated with many human diseases. Therefore, prooxidant-induced c-Src/NF κ B coupling represents a novel pathway for dissecting the molecular mechanism of initiation, progression and maintenance of hyperalgesia associated with many pathophysiological processes.

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References

- Arvidsson J, Pfaller K. Central projections of C4–C8 dorsal root ganglia in the rat studied by anterograde transport of WGA-HRP. *J Comp Neurol.* 1990; 292(3):349–362. [PubMed: 1692849]
- Baird MB, Massie HR, Piekelniaik MJ. Formation of lipid peroxides in isolated rat liver microsomes by singlet molecular oxygen. *Chem Biol Interact.* 1977; 16(2):145–153. [PubMed: 403019]

- Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol.* 1996; 14:649–683. [PubMed: 8717528]
- Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. *Cell.* 2009; 139(2):267–284. [PubMed: 19837031]
- Bogen O, Alessandri-Haber N, Chu C, Gear RW, Levine JD. Generation of a pain memory in the primary afferent nociceptor triggered by PKC ϵ activation of CPEB. *J Neurosci.* 2012; 32(6):2018–2026. [PubMed: 22323716]
- Bumcrot D, Manoharan M, Koteliansky V, Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol.* 2006; 2(12):711–719. [PubMed: 17108989]
- Chung JM. The role of reactive oxygen species (ROS) in persistent pain. *Mol Interv.* 2004; 4(5):248–250. [PubMed: 15471906]
- D'Mello R, Dickenson AH. Spinal cord mechanisms of pain. *Br J Anaesth.* 2008; 101(1):8–16. [PubMed: 18417503]
- Dorn G, Patel S, Wotherspoon G, Hemmings-Mieszczak M, Barclay J, Natt FJ, Martin P, Bevan S, Fox A, Ganju P, Wishart W, Hall J. siRNA relieves chronic neuropathic pain. *Nucleic Acids Res.* 2004; 32(5):e49. [PubMed: 15026538]
- Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. siRNA directed against c-Src enhances pancreatic adenocarcinoma cell gemcitabine chemosensitivity. *J Am Coll Surg.* 2004; 198(6):953–959. [PubMed: 15194078]
- Edwards JC, Quinn PJ. Decomposing potassium peroxychromate produces hydroxyl radical (.OH) that can peroxidize the unsaturated fatty acids of phospholipid dispersions. *J Lipid Res.* 1982; 23(7):994–1000. [PubMed: 6292318]
- Fan C, Li Q, Ross D, Engelhardt JF. Tyrosine phosphorylation of I kappa B alpha activates NF kappa B through a redox-regulated and c-Src-dependent mechanism following hypoxia/reoxygenation. *J Biol Chem.* 2003; 278(3):2072–2080. [PubMed: 12429743]
- Frank C, Burkhardt C, Imhof D, Ringel J, Zschornig O, Wieligmann K, Zacharias M, Bohmer FD. Effective dephosphorylation of Src substrates by SHP-1. *J Biol Chem.* 2004; 279(12):11375–11383. [PubMed: 14699166]
- Gao YJ, Ji RR. c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? *Open Pain J.* 2009; 2:11–17. [PubMed: 19898681]
- Gianni D, Bohl B, Courtneidge SA, Bokoch GM. The involvement of the tyrosine kinase c-Src in the regulation of reactive oxygen species generation mediated by NADPH oxidase-1. *Mol Biol Cell.* 2008; 19(7):2984–2994. [PubMed: 18463161]
- Häcker H, Karin M. Regulation and function of IKK and IKK-related kinases. *Sci STKE.* 2006; 2006(357):re13. [PubMed: 17047224]
- Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet.* 1994; 344(8924):721–724. [PubMed: 7915779]
- Halliwell B, Cross CE. Oxygen-derived species: their relation to human disease and environmental stress. *Environ Health Perspect.* 1994; 102(Suppl 10):5–12. [PubMed: 7705305]
- Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet.* 2001; 2(2):110–119. [PubMed: 11253050]
- Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem.* 1996; 271(2):695–701. [PubMed: 8557675]
- Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain.* 1988; 32(1):77–88. [PubMed: 3340425]
- Hinz M, Arslan SÇ, Scheidereit C. It takes two to tango: I κ Bs, the multifunctional partners of NF- κ B. *Immunol Rev.* 2012; 246(1):59–76. [PubMed: 22435547]
- Huang J, Zhang X, McNaughton PA. Inflammatory pain: the cellular basis of heat hyperalgesia. *Curr Neuropharmacol.* 2006; 4(3):197–206. [PubMed: 18615146]
- Huang WC, Chen JJ, Inoue H, Chen CC. Tyrosine phosphorylation of I-kappa B kinase alpha/beta by protein kinase C-dependent c-Src activation is involved in TNF-alpha-induced cyclooxygenase-2 expression. *J Immunol.* 2003; 170(9):4767–4775. [PubMed: 12707358]

- Igwe OJ. c-Src kinase activation regulates preprotachykinin gene expression and substance P secretion in rat sensory ganglia. *Eur J Neurosci.* 2003; 18(7):1719–1730. [PubMed: 14622206]
- Igwe OJ. Modulation of peripheral inflammation in sensory ganglia by nuclear factor (kappa) B decoy oligodeoxynucleotide: involvement of SRC kinase pathway. *Neurosci Lett.* 2005; 381(1–2):114–119. [PubMed: 15882800]
- Igwe OJ. Agents that act by different mechanisms modulate the activity of protein kinase C betaII isozyme in the rat spinal cord during peripheral inflammation. *Neuroscience.* 2006; 138(1):313–328. [PubMed: 16360284]
- Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Dieckmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, Peyron JF. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. *Cell.* 1996; 86(5):787–798. [PubMed: 8797825]
- Jakobsson PJ, Thorén S, Morgenstern R, Samuelsson B. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci U S A.* 1999; 96(13):7220–7225. [PubMed: 10377395]
- Jin X, Morsy N, Winston J, Pasricha PJ, Garrett K, Akbarali HI. Modulation of TRPV1 by nonreceptor tyrosine kinase, c-Src kinase. *Am J Physiol Cell Physiol.* 2004; 287(2):C558–C563. [PubMed: 15084474]
- Kalia LV, Gingrich JR, Salter MW. Src in synaptic transmission and plasticity. *Oncogene.* 2004; 23(48):8007–8016. [PubMed: 15489918]
- Katsura H, Obata K, Mizushima T, Sakurai J, Kobayashi K, Yamanaka H, Dai Y, Fukuoka T, Sakagami M, Noguchi K. Activation of Src-family kinases in spinal microglia contributes to mechanical hypersensitivity after nerve injury. *J Neurosci.* 2006; 26(34):8680–8690. [PubMed: 16928856]
- Khadaroo RG, Kapus A, Powers KA, Cybulsky MI, Marshall JC, Rotstein OD. Oxidative stress reprograms lipopolysaccharide signaling via Src kinase-dependent pathway in RAW 264.7 macrophage cell line. *J Biol Chem.* 2003; 278(48):47834–47841. [PubMed: 12896983]
- Kim HK, Park SK, Zhou JL, Taglialatela G, Chung K, Coggeshall RE, Chung JM. Reactive oxygen species (ROS) play an important role in a rat model of neuropathic pain. *Pain.* 2004; 111:116–124. [PubMed: 15327815]
- Kojima F, Matnani RG, Kawai S, Ushikubi F, Crofford LJ. Potential roles of microsomal prostaglandin E synthase-1 in rheumatoid arthritis. *Inflamm Regen.* 2011; 31(2):157–166. [PubMed: 22308189]
- Lee I, Kim HK, Kim JH, Chung K, Chung JM. The role of reactive oxygen species in capsaicin-induced mechanical hyperalgesia and in the activities of dorsal horn neurons. *Pain.* 2007; 133:9–17. [PubMed: 17379413]
- Lee KY, Chung K, Chung JM. Involvement of reactive oxygen species in long-term potentiation in the spinal cord dorsal horn. *J Neurophysiol.* 2010; 103(1):382–391. [PubMed: 19906875]
- Leu TH, Maa MC. Functional implication of the interaction between EGF receptor and c-Src. *Front. Biosci.* 2003; 8:28–38.
- Leu TH, Charoenfuprasert S, Yen CK, Fan CW, Maa MC. Lipopolysaccharide-induced c-Src expression plays a role in nitric oxide and TNFalpha secretion in macrophages. *Mol Immunol.* 2006; 43(4):308–316. [PubMed: 15869794]
- Meotti FC, Missau FC, Ferreira J, Pizzolatti MG, Mizuzaki C, Nogueira CW, Santos AR. Anti-allodynic property of flavonoid myricitrin in models of persistent inflammatory and neuropathic pain in mice. *Biochem Pharmacol.* 2006; 72(12):1707–1713. [PubMed: 17070780]
- Mercurio F, Manning AM. NF-kappaB as a primary regulator of the stress response. *Oncogene.* 1999; 18(45):6163–6171. [PubMed: 10557108]
- Miesel R, Ehrlich W, Wohler H, Kurpisz M, Kröger H. The effects of interleukin-1 receptor antagonist on oxidant-induced arthritis in mice. *Clin Exp Rheumatol.* 1995a; 13(5):595–601. [PubMed: 8575137]
- Miesel R, Kröger H, Kurpisz M, Weser U. Induction of arthritis in mice and rats by potassium peroxochromate and assessment of disease activity by whole blood chemiluminescence and 99m pertechnetate-imaging. *Free Radic Res.* 1995b; 23(3):213–227. [PubMed: 7581817]

- Miesel R, Zuber M. Elevated levels of xanthine oxidase in serum of patients with inflammatory and autoimmune rheumatic diseases. *Inflammation*. 1993; 17(5):551–561. [PubMed: 8225562]
- Okutani D, Lodyga M, Han B, Liu M. Src protein tyrosine kinase family and acute inflammatory responses. *Am J Physiol Lung Cell Mol Physiol*. 2006; 291(2):L129–L141. [PubMed: 16581827]
- Peng HY, Chen GD, Lai CY, Hsieh MC, Lin TB. Spinal SIRPa1-SHP2 interaction regulates spinal nerve ligation-induced neuropathic pain via PSD-95-dependent NR2B activation in rats. *Pain*. 2012; 153(5):1042–1053. [PubMed: 22425446]
- Purcell AL, Carew TJ. Tyrosine kinases, synaptic plasticity and memory: insights from vertebrates and invertebrates. *Trends Neurosci*. 2003; 26(11):625–630. [PubMed: 14585603]
- Salter MW, Kalia LV. Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci*. 2004; 5(4):317–328. [PubMed: 15034556]
- Salazar EP, Rozengurt E. Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells. *J Biol Chem*. 1999; 274(40):28371–28378. [PubMed: 10497197]
- Salvemini D, Doyle TM, Cuzzocrea S. Superoxide, peroxynitrite and oxidative/nitrative stress in inflammation. *Biochem Soc Trans*. 2006; 34:965–970. [PubMed: 17052238]
- Schalkwijk J, van den Berg WB, van de Putte LB, Joosten LA. An experimental model for hydrogen peroxide induced tissue damage: effect on cartilage and other articular tissues. *Int J Tissue React*. 1987; 9(1):39–43. [PubMed: 3596956]
- Schoonbroodt S, Piette J. Oxidative stress interference with the nuclear factor-kappa B activation pathways. *Biochem Pharmacol*. 2000; 60(8):1075–1083. [PubMed: 11007944]
- Schwartz ES, Lee I, Chung K, Chung JM. Oxidative stress in the spinal cord is an important contributor in capsaicin-induced mechanical secondary hyperalgesia in mice. *Pain*. 2008; 138(3):514–524. [PubMed: 18375065]
- Schwartz ES, Kim HY, Wang J, Lee I, Klann E, Chung JM, Chung K. Persistent pain is dependent on spinal mitochondrial antioxidant levels. *J Neurosci*. 2009; 29:159–168. [PubMed: 19129394]
- SeokYang W, Lee J, Woong Kim T, Hye Kim J, Lee S, Hee Rhee M, Hong S, Youl Cho J. Src/NF- κ B-targeted inhibition of LPS-induced macrophage activation and dextran sodium sulphate-induced colitis by *Archidendron clypearia* methanol extract. *J Ethnopharmacol*. 2012; 142(1):287–293. [PubMed: 22537838]
- Severgnini M, Takahashi S, Tu P, Perides G, Homer RJ, Jung JW, Bhavsar D, Cochran BH, Simon AR. Inhibition of the Src and Jak kinases protects against lipopolysaccharide-induced acute lung injury. *Am J Respir Crit Care Med*. 2005; 171(8):858–867. [PubMed: 15665321]
- Singh S, Darnay BG, Aggarwal BB. Site-specific tyrosine phosphorylation of Ikappa B alpha negatively regulates its inducible phosphorylation and degradation. *J Biol Chem*. 1996; 271(49):31049–31054. [PubMed: 8940099]
- Smart TG. Regulation of excitatory and inhibitory neurotransmitter-gated ion channels by protein phosphorylation. *Curr Opin Neurobiol*. 1997; 7:358–367. [PubMed: 9232798]
- Sun SC. The noncanonical NF- κ B pathway. *Immunol Rev*. 2012; 246(1):125–140. [PubMed: 22435551]
- Voscopoulos C, Lema M. When does acute pain become chronic? *Br J Anaesth*. 2010; 105(Suppl 1):169–185.
- Woolf CJ, Costigan M. Transcriptional and posttranslational plasticity and generation of inflammatory pain. *Proc. Natl. Acad. Sci. USA*. 1999; 96:7723–7730. [PubMed: 10393888]
- Xiao W. Advances in NF-kappaB signaling transduction and transcription. *Cell Mol Immunol*. 2004; (6):425–435. [PubMed: 16293211]
- Ying WZ, Wang PX, Aaron KJ, Basnayake K, Sanders PW. Immunoglobulin light chains activate nuclear factor- κ B in renal epithelial cells through a Src-dependent mechanism. *Blood*. 2011; 117(4):1301–1307. [PubMed: 21098396]
- Yu X-M, Salter MW. Src, a molecular switch governing gain control of synaptic transmission mediated by N-methyl-D-aspartate receptors. *Proc. Natl. Acad. Sci. USA*. 1999; 96:7697–7704. [PubMed: 10393883]
- Zhang J, Somani AK, Siminovich KA. Roles of the SHP-1 tyrosine phosphatase in the negative regulation of cell signalling. *Semin Immunol*. 2000; 12(4):361–378. [PubMed: 10995583]

- Zhang X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO Journal*. 2005; 24:4211–4223. [PubMed: 16319926]
- Zhang X, McNaughton PA. Why pain gets worse: the mechanism of heat hyperalgesia. *J Gen Physiol*. 2006; 128(5):491–493. [PubMed: 17074974]
- Zhao W, Cavallaro S, Gusev P, Alkon DL. Non-receptor Tyr protein kinase pp60c-src in spatial learning: Synapse-specific changes in its gene expression, tyrosine phosphorylation, and protein-protein interactions. *Proc. Natl. Acad. Sci. USA*. 2000; 97:8098–8103. [PubMed: 10884433]
- Zhao Z, Tan Z, Diltz CD, You M, Fischer EH. Activation of mitogen-activated protein (MAP) kinase pathway by pervanadate, a potent inhibitor of tyrosine phosphatases. *J Biol Chem*. 1996; 271(36): 22251–22255. [PubMed: 8703041]

Bulleted statements**What's already known about this topic?**

- There appears to be a relationship between c-Src kinase in the pain pathway and oxidative stress.
- c-Src activates nuclear factor kappa B and this activation may initiate and maintain pain induced by oxidant stress associated with many disease states

What does this study add?

- This study for the first time shows increased formation of c-Src-IKK α/β complex in the L4 – L6 dorsal root ganglia (DRG) ipsilateral to the inflammation.
- Both c-Src kinase inhibitor and c-Src siRNA (in the same paw as the prooxidant) were effective in attenuating pain sensitivity mediated by the prooxidant.

Fig. 1.

Changes in thermal withdrawal latencies (TWLs) to a radiant heat source after PPC at (A) 0-, 24-, and 72-h, and (B) 0- and 48-h. Before Drug and/or PPC treatments, TWL were measured in both hind paws of each rat, which represented baseline TWLs for each test group at $t = h0$. Rats in the naïve control [-Drug vehicle/-PPC] group were handled as in the other groups but were not treated with PPC or drug agent(s). Animals were tested for hyperalgesia at the indicated times after Drug/PPC treatments. Values are expressed as mean $\pm$ SEM with $n = 3 - 7$ rats/ group. For Fig. 1A: $F_{4,7} = 75$ ($P = 0.001$), $*p = 0.01$ compared to [-Drug vehicle/-PPC] i.e., Naïve control; $+p < 0.01$ compared to [Drug vehicle/PPC],

[AG1478/PPC], [PP3/PPC] and [SSG/PPC]-treated groups at corresponding times. For Fig. 1B: $F_{3,5} = 38$ ($P = 0.001$), $*p < 0.01$ compared to [c-Src siRNA/PPC], naïve control (–Vehicle/–PPC) and (– c-Src siRNA/–PPC) groups; $F_{3,5} = 17$ ($P = 0.01$), $+p = 0.05$ compared to [c-Src siRNA/PPC] and [MS Src siRNA/–PPC] groups. [2-way ANOVA/Fisher's PLSD post hoc tests).

Fig. 2.

Levels of (A) IL-1 β , (B) immunoprecipitated c-Src kinase activity, and (C) PGE₂ as PGEM in extracts of the DRG at 72-h after ip Drug and/or itp PPC. Values are expressed as mean $\pm$ SEM (n = pooled DRG from 4 – 6 rats/group); $F_{3,5} = 31.7$ (P = 0.001), *p < 0.05 (P = 0.001) compared to the corresponding ipsilateral values in naïve control (-Vehicle/-PPC) and contralateral DRG in all groups; +p < 0.05 compared to the corresponding ipsilateral values in [Drug vehicle/PPC] and [AG 1478/PPC] groups; **p < 0.01 compared to the ipsilateral sides in the naïve control and [PP2/PPC] groups. [Multiple ANOVA/Fisher's PLSD post hoc tests].

A. **B.** **Fig. 3.**

Western blots of c-Src-IKK α/β complex formed in the DRG after coimmunoprecipitation (co-IP) with anti-c-Src mAb (**A**). Histogram (**B**) showing fold differences in optical density (OD) signal ratios normalized to total β -actin signals (as loading control) after different treatments. The numbers in parenthesis in (**Fig. B**) correspond to the numbers in (**Fig. A**) and denote treatment groups.

Fig. 4.

Levels of (A) Western (immuno) blot of c-Src expression, (B) relative semi-quantitative representation of the optical density (OD) ratios [c-Src: GAPDH] of signal intensities of loaded proteins in the same blots in (A) and (C) immunoprecipitated c-Src activity in the DRG 48-h following itp injections of PPC, c-Src siRNA, or MS c-Src siRNA, or siRNA vehicle in the rat left hind paw. Data are mean $\pm$ SEM (n = pooled 6–8 rats/group with 4–5 experiments performed per parameter. $F_{3,5} = 25.8$ (P = 0.001), *p = 0.01 (P = 0.001) compared to corresponding ipsilateral and contralateral values in naïve control and [c-Src siRNA/PPC] groups; +p = 0.01 compared to the corresponding ipsilateral values in [c-Src siRNA/PPC] group. [Multiple ANOVA/ Fisher's PLSD post hoc tests].

Table 1

Mean fold changes in the OD of c-Src-IKK α / β complex formed [Ipsi-/Contra-lateral DRG] uncorrected for the β -actin signal (Fig. 3A) in DRG homogenate.

Treatments	Mean Fold Changes in Src-IKK α / β complex OD [Ipsi-/Contra-lateral DRG]
1. [-Drug vehicle/-PPC]	1.0
2. [Drug vehicle/PPC]	6.2
3. [PP2/PPC]	2.7
4. [SSG/PPC]	7.5
5. [Drug vehicle/PPC (C8)]	1.1