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p38 MAPK Activation by NGF in Primary Sensory Neurons after Inflammation Increases TRPV1 Levels and Maintains Heat Hyperalgesia

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Summary

Peripheral inflammation induces p38 MAPK activation in the soma of C fiber nociceptors in the dorsal root ganglion (DRG) after 24 hr. Inflammation also increases protein, but not mRNA levels, of the heat-gated ion channel TRPV1 (VR1) in these cells, which is then transported to peripheral but not central C fiber terminals. Inhibiting p38 activation in the DRG reduces the increase in TRPV1 in the DRG and inflamed skin and diminishes inflammation-induced heat hypersensitivity without affecting inflammatory swelling or basal pain sensitivity. p38 activation in the DRG is secondary to peripheral production of NGF during inflammation and is required for NGF-induced increases in TRPV1. The activation of p38 in the DRG following retrograde NGF transport, by increasing TRPV1 levels in nociceptor peripheral terminals in a transcription-independent fashion, contributes to the maintenance of inflammatory heat hypersensitivity.

Introduction

Mitogen-activated protein kinases (MAPK) transduce a broad range of extracellular stimuli into diverse intracellular responses by producing changes in transcription as well as by posttranslational modifications of target proteins (Widmann et al., 1999; Sweatt, 2001). The ERK (extracellular signal-regulated kinase) member of the MAPK family is activated by neuronal activity and is involved in neuronal plasticity, including long-term potentiation, learning and memory, and pain hypersensitivity (Impey et al., 1999; Ji and Woolf, 2001; Sweatt, 2001). p38, a MAPK which operates through a separate intracellular cascade, is activated in many cells by cellular stress and cytokines (Widmann et al., 1999). Although an activity-dependent p38 activation occurs in neurons (Mao et al., 1999), and p38 exerts effects in the hippocampus that oppose that of ERK (Bolshakov et al., 2000), the contribution of p38 MAPK to neuronal function remains largely unknown.

Intense noxious stimuli and tissue inflammation produce a pain hypersensitivity that results both from peripheral sensitization (an increased responsiveness of the peripheral terminals of nociceptor primary sensory neurons) and central sensitization (an increased excitability of dorsal horn neurons) (Woolf, 1983; Woolf and Salter, 2000). ERK activation in the peripheral terminals of primary sensory neurons contributes to epinephrine-induced peripheral sensitization (Aley et al., 2001), and in dorsal horn neurons, to the central sensitization produced by intraplantar injection of formalin (Ji et al., 1999; Karim et al., 2001). These actions occur with such a short latency (<20 min) that they must be mediated by nontranscriptional processing (Ji et al., 1999). ERK activation in dorsal horn neurons operating over a longer time course (>24 hr) also participates in inflammatory pain hypersensitivity by increasing gene transcription in these cells (Ji et al., 2002).

Peripheral inflammation results in changes in neuropeptide, ion channel, and receptor levels in the cell bodies of DRG neurons (Noguchi et al., 1988; Woolf et al., 1994; Ji et al., 1995; Voilley et al., 2001), but the signal transduction pathways involved have not been identified, nor is it clear what role these changes play in generating pain hypersensitivity. NGF is upregulated in inflamed tissues (Woolf et al., 1994) and plays an important role in inflammatory pain by driving peripheral sensitization, acting directly on the peripheral terminal to produce, within a short period, heat hyperalgesia (Chuang et al., 2001). However, NGF is also retrogradely transported to the cell bodies of sensory neurons in the DRG, where it increases gene expression (Lindsay and Harmar, 1989; Woolf et al., 1994). The contribution of these slower onset NGF-mediated changes in sensory neurons to pain is unknown.

The transient receptor potential ion channel TRPV1, formerly known as vanilloid receptor-1 (VR1), which is expressed in nociceptors, is a capsaicin- and proton-sensitive cation-selective channel that acts as a transducer for peripheral noxious heat stimuli (Caterina et al., 1997). The threshold of heat activation of the receptor can be reduced by protons, repeated heat stimuli, and by PKC activation from noxious to innocuous levels (Tominaga et al., 1998; Premkumar and Ahern, 2000). Although a null mutation of TRPV1 has minimal effects on basal heat threshold, presumably reflecting a contribution of the TRP heat sensor TRPV3 (Smith et al., 2002; Xu et al., 2002), it significantly reduces inflammatory heat hyperalgesia (Caterina et al., 2000; Davis et al., 2000), indicating a particular role for TRPV1 in inflammatory pain sensitivity. We now show a slow-onset, transcription-independent regulation of inflammatory heat hypersensitivity through an increase in TRPV1 levels in peripheral nociceptor terminals that is mediated by a NGF-induced activation of p38 in sensory neuron cell bodies in the DRG.

Results

p38 Is Activated in DRG Neurons Following Peripheral Inflammation

Using a phospho-specific p38 antibody, we find that activated p38 (phospho-p38 [p-p38]) is present in only 15% of DRG neurons in naive rats (Figure 1A). The p-p38 is located in the nucleus and cytoplasm of the neurons, as well as in some surrounding nonneuronal cells. To

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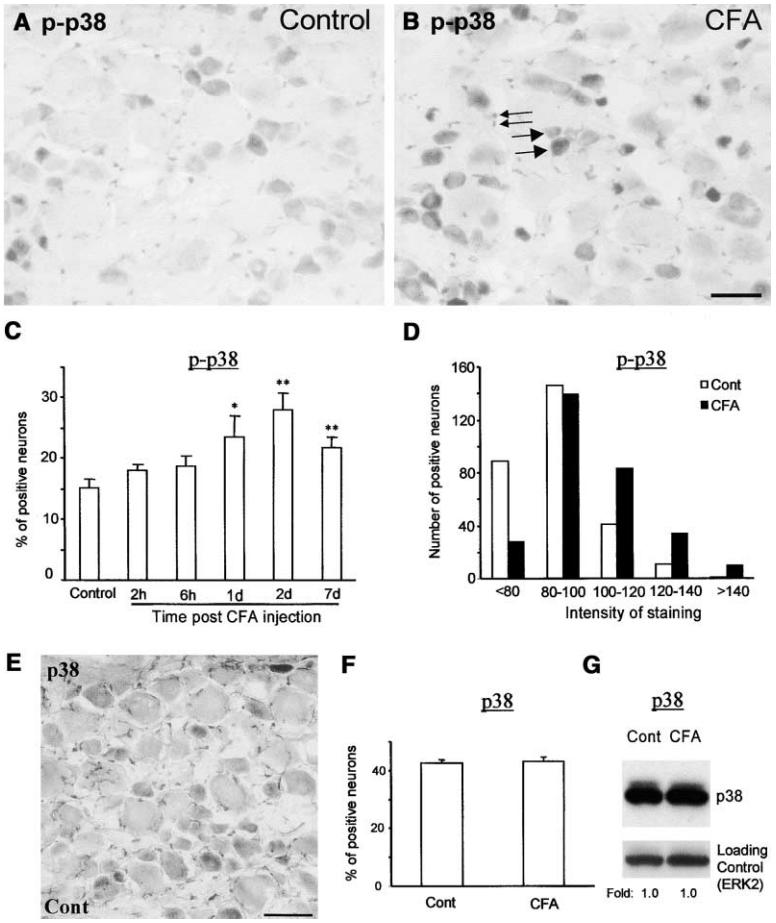

Figure 1. Inflammation Induces Persistent p38 Activation in DRG Neurons

(A and B) p38 phosphorylation (p-p38) is increased in DRG neurons 2 days after CFA injection into a hindpaw. Scale bar, 50 μm. Small and large arrows indicate nonneuronal and neuronal cells, respectively.

(C) Time course of p38 phosphorylation after CFA administration measured by the percentage of p-p38 positive neurons in the DRG ($n = 5$). *$p < 0.05$; **$p < 0.01$, compared to naive control.

(D) Intensity frequency indicates an increase in the intensity of p-p38 immunostained neurons 2 days after inflammation. Three hundred neurons from three animals were measured for each condition.

(E) Total p38 is expressed in many small sized DRG neurons of naive rats. Scale bar, 50 μm.

(F and G) Total p38 level is not changed 2 days after inflammation, as indicated by the percentage of p38 positive neurons (F) and the p38 Western band (G). ERK2, a constitutively expressed protein, was used as a loading control.

test if a localized peripheral inflammation activates p38 in the DRG, 100 μl of CFA was injected into the plantar surface of the left hindpaw. This injection induces a localized inflammation that develops over minutes, lasts for more than a week, and is associated with swelling and erythema, as well as thermal and mechanical pain hypersensitivity (Stein et al., 1988). The inflammation slowly increases the percentage of p-p38 immunoreactive DRG neurons (Figure 1B), reaching significance at 24 hr, with a peak increase at 48 hr, and high levels are maintained at 7 days (Figure 1C). The relative intensity of p-p38-immunoreactive neurons also increases after the inflammation (Figure 1D). Total p38 (nonphosphorylated and phosphorylated) is heavily expressed in nonneuronal and small neuronal cells in the naive DRG (Figure 1E), and its levels do not increase after inflammation (Figures 1F and 1G), indicating that the increase in p-p38 after inflammation is caused by increased phosphorylation rather than elevated substrate.

p-p38 Is Expressed in C Fiber Sensory Neurons

A size frequency analysis indicates that p-p38 is present mainly in small DRG neurons (cross-sectional area $< 600\ \mu m^2$) in both control and inflamed animals (Figure 2A). Double staining of p-p38 with neurofilament-200, a marker for myelinated A fibers, reveals almost no colocalization in either control or inflamed conditions, indicating that p-p38 is predominantly expressed in neurons with unmyelinated axons, the C fibers (Figures 2B and 2C).

C fiber sensory neurons can be divided into two broad groups (Snider and McMahon, 1998): NGF-responsive/TrkA-expressing neurons and GDNF-responsive/c-ret-expressing ones. The former also express the neuropeptides substance P and CGRP; the latter bind to lectin IB4 and express the ATP receptor $P2X_3$ (Averill et al., 1995). We find that p-p38 heavily colocalizes with TrkA 2 days after inflammation (Figures 2D–2F). p-p38 is also expressed in $P2X_3$-positive neurons (data not shown). Moreover, p-p38 heavily colocalizes with TRPV1 (Figure 2G–2I), which is expressed in both types of C fibers (Tominaga et al., 1998).

p38 Is Not Activated in Dorsal Horn Neurons after Peripheral Inflammation

Inflammation induces a sustained increase in ERK phosphorylation in second order dorsal horn neurons. This activation contributes to gene expression and maintenance of inflammatory pain (Ji et al., 2002). However, p38 phosphorylation is not increased in the dorsal horn by peripheral inflammation (measured from 6 hr to 7 days) (Figures 3A and 3B). p-p38 is, moreover, only expressed in nonneuronal cells in the dorsal horn in control and inflamed animals, as shown by absence of colocalization with NeuN, a neuronal marker (Figures 3C and 3D).

p38 Activation Contributes to Inflammatory Pain

To examine if p38 activation in the DRG is involved in the production of inflammatory pain hypersensitivity, we

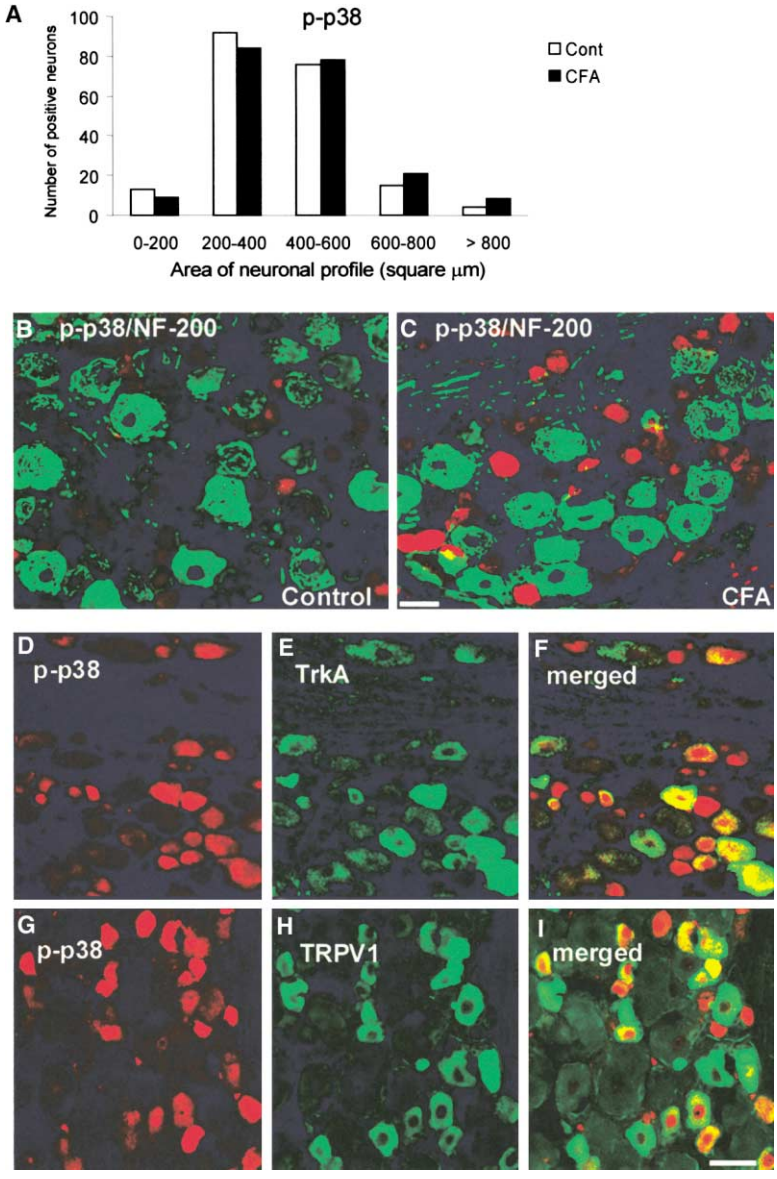

Figure 2. p38 Is Activated in C Fiber Nociceptors in the DRG after Inflammation

(A) A size frequency histogram indicates that the cross-sectional area of the p-38 positive neuronal profile remains similar 2 days after inflammation even though the numbers of such cells increase substantially. Two hundred DRG neurons from each group were measured.

(B and C) Double staining of p-p38 (red) with neurofilament-200 (green), a marker for myelinated A fibers, in DRG neurons of both naive (B) and 2 days inflamed (C) animals indicates that p38 is predominantly activated in unmyelinated C fiber nociceptors. Scale bar, 30 μm.

(D–I) Double staining demonstrates a heavy colocalization of p-p38 (red) with TrkA (D–F) and TRPV1 (G–I) in DRG neurons 2 days after inflammation. Scale bar, 30 μm.

administered a specific p38 inhibitor, SB203580, into the intrathecal space via a catheter whose tip was positioned close to the L4 DRG in order to target p38 activity in the DRG. To obtain a sustained drug infusion, the drug was delivered by an osmotic pump (0.5 μg/μl/hr) connected to a catheter. The catheter was implanted at least 6 hr prior to the CFA injection.

Neither heat nor mechanical basal sensitivity is affected by SB203580 administration in noninflamed rats (Figures 4D and 4E). The SB203580 administration also does not change the earliest phase of the inflammatory pain (6 hr) (Figures 4D and 4E). The inhibitor does, however, reduce inflammation-induced heat hyperalgesia 24 and 48 hr post CFA injection, without an effect on mechanical allodynia (Figures 4D and 4E). Similar results are obtained with bolus intrathecal injections (data not shown). Intrathecal SB203085 infusion does not reduce CFA-induced inflammatory swelling, measured by paw thickness (Figure 4A), and the number of ED-1-positive inflammatory cells (macrophages) in the inflamed skin is not decreased by the p38 inhibitor (Figures 4B and 4C). This indicates that intrathecal administration of SB203580 does not alter peripheral inflammation.

To test if the p38 inhibitor has any effect on established inflammatory pain, a bolus of SB203580 (1 μg) was intrathecally administered 48 hr after the CFA injection, at a time when the inflammatory pain is fully developed, and pain behavior was tested 0.5, 3, and 24 hr later. Posttreatment of SB203580 does not alter established inflammatory pain at 0.5 hr after the injection but starts to decrease heat hyperalgesia at 3 hr and reverses it 24 hr postinjection (Figures 4F and 4G). Mechanical allodynia is not altered by posttreatment with the inhibitor at any time tested (Figures 4F and 4G).

TRPV1 Expression Increases after Peripheral Inflammation

To test if p38 mediates heat hyperglesia by regulating TRPV1 expression, we examined TRPV1 expression in the DRG after inflammation. Previous RT-PCR studies

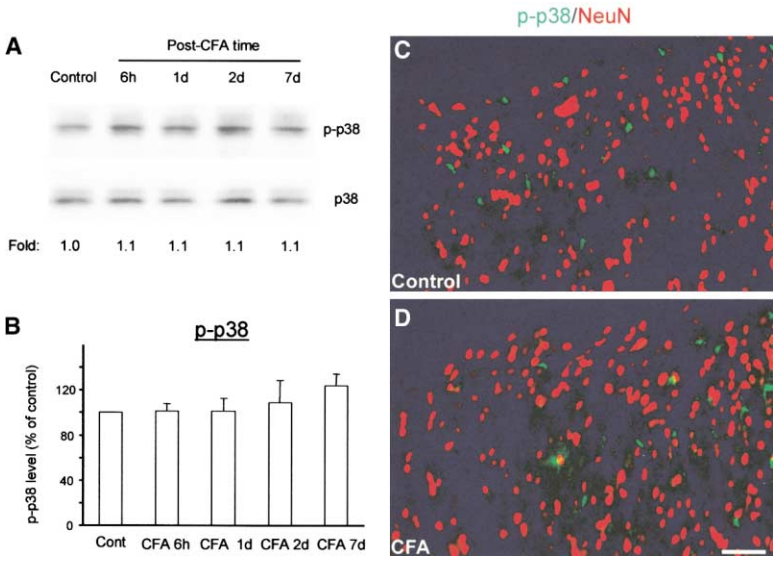

Figure 3. p38 Phosphorylation in the Dorsal Horn Is Not Increased by Inflammation

(A) Western blot analysis demonstrates that p-p38 levels do not change in the dorsal horn after CFA induced peripheral inflammation. Fold represents comparative levels over control after normalizing with total p38 levels.

(B) Quantification of p-p38 levels (density of p-p38 band) in the dorsal horn illustrates no significant change in p-p38 levels after inflammation compared to control ($p > 0.05$, $n = 3$).

(C and D) Double immunostaining of p-p38 and NeuN, a neuronal marker, demonstrates a nonneuronal localization of p-p38 in the medial superficial dorsal horn. p-p38 expression remains unchanged 2 days after inflammation. Scale bar, 50 μm.

do not show an increase in TRPV1 mRNA levels in the DRG after carrageenan or CFA-induced inflammation (Tohda et al., 2001; Voilley et al., 2001). Using an RNase protection assay (RPA), we also find no increase in TRPV1 mRNA levels in the DRG over the entire time course studied (Figures 5A and 5B).

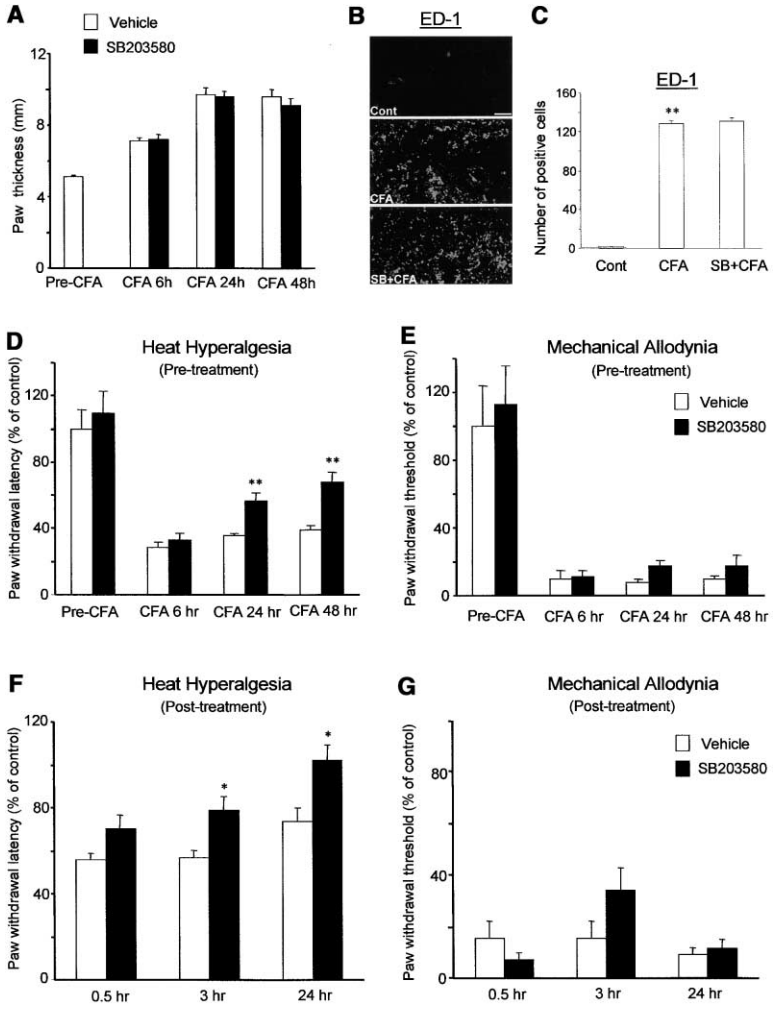

Figure 4. p38 Inhibition Reduces the Late Phase of CFA-Induced Inflammatory Heat Hyperalgesia

(A–C) Intrathecal infusion of the p38 inhibitor SB203580 does not change the development of inflammation of CFA-injected paws, as indicated by measurement of paw thickness (A) and number of ED-1-positive inflammatory cells (cell number per 20× optic field) 2 days after inflammation (C). Inflammation induces a dramatic increase in ED-1-positive cells, which is not affected by the p38 inhibitor (B and C). $^{**}p < 0.01$, compared to control ($n = 3$). SB203580 (1 μg/μl) and control vehicle (saline) were infused by an osmotic pump (0.5 μl/hr) connected to a catheter intrathecally implanted before CFA injection.

(D and E) Pretreatment: infusion of SB203580 as in (A) reduces the late phase of inflammatory heat hyperalgesia (24 and 48 hr after CFA injection). Mechanical allodynia is not decreased. Heat and mechanical sensitivity were measured by paw withdrawal latency and paw withdrawal threshold, respectively, and expressed as the percentage of pre-CFA baseline measurements of vehicle control. $^{**}p < 0.01$, compared to vehicle control ($n = 8$).

(F and G) Posttreatment: intrathecal injection of SB203580 (1 μg) 48 hr post CFA produces a delayed inhibition on inflammatory heat hyperalgesia. Heat and mechanical sensitivity were tested at 0.5, 3, and 24 hr after administration of the inhibitor. $^{*}p < 0.05$, compared to vehicle (saline) control ($n = 8$).

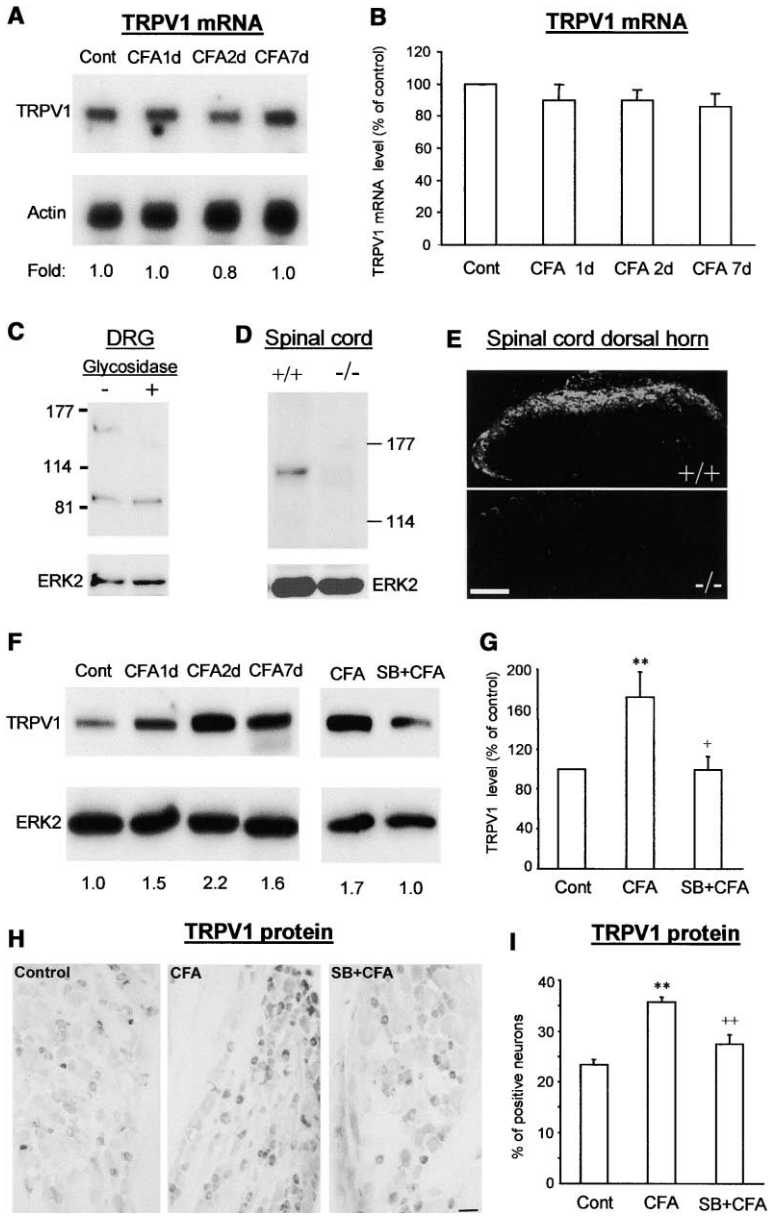

Figure 5. p38 Activation Mediates the Inflammation-Induced Upregulation of TRPV1 in the DRG

(A) RNase protection assay (RPA) reveals no increase in TRPV1 mRNA levels after CFA-induced inflammation (from 1 to 7 days). Fold represents comparative levels over control after normalizing with an actin control.

(B) Quantification of RPA results shows no significant change in TRPV1 mRNA levels after inflammation compared to control ($n = 3$, $p > 0.05$).

(C) The high TRPV1-immunoreactive band around 140 kDa detected by Western blot shifts to a low band of ~90 kDa after treatment of DRG lysate with N-glycosidase F (500 unit for 50 μg) at 37°C for 1 hr, indicating that the high band is a glycosylated form of TRPV1.

(D) A similar high band is also detected in the spinal cord of wild-type mice, but not of TRPV1-deficient mice.

(E) The GSK TRPV1 antibody shows strong labeling in the superficial dorsal horn of wild-type mice, but no specific staining is found in the spinal cord of TRPV1-deficient mice. Scale 50 μm.

(F) Western blot analysis indicates a persistent increase in TRPV1 protein levels after inflammation. This upregulation at 2 days is blocked by intrathecal injection of SB203580 (1 μg, twice a day for 2 days). Fold represents comparative levels over control after normalizing with a loading control.

(G) Quantification of TRPV1 levels, as shown in (F). $^{**}p < 0.01$, compared to control; $^{+}p < 0.05$, compared to CFA, $n = 5$.

(H) Immunohistochemistry confirms an increase in TRPV1 levels 2 days after inflammation, which is decreased by SB203580 delivered via osmotic pump. Scale bar, 50 μm.

(I) Quantification of TRPV1 levels by the percentage of TRPV1-positive neurons as demonstrated in (G). $^{**}p < 0.01$, compared to control; $^{++}p < 0.01$, compared to CFA, $n = 5$.

Using an antibody raised against the C-terminal of the TRPV1 receptor (obtained from C. Parsons, Glaxo Smith Kline), Western blot analysis reveals a band around 140 kDa in samples obtained from DRG, spinal cord, sciatic nerve, and hindpaw skin. This antibody also recognizes a low band around 90 kDa in DRG samples. After incubation of DRG lysate with N-glycosidase F, the high band shifts to the low band (Figure 5C), indicating that the high band is a glycosylated form of TRPV1, as reported before (Kedei et al., 2001; Yiangou et al., 2001). The high band is abolished after preabsorption of the antibody with blocking peptide at 1 μM. Western blot analysis with this antibody shows a similar high band (~140 kDa) in wild-type but not in TRPV1 knockout mice (Figure 5D). Immunohistochemistry using this TRPV1 antibody reveals dense staining in the superficial dorsal horn of wild-type mice, but no staining in the spinal cord of TRPV1 knockout mice (Figure 5E).

Peripheral inflammation induces a sustained increase in TRPV1 protein levels in the DRG, as detected by Western blots using the GSK anti-TRPV1 antibody (Figure 5F). Consistent with these results, immunohistochemistry also demonstrates an increase in the number of TRPV1 immunoreactive neurons in the DRG 2 days after inflammation (Figure 5H). Identical immunostaining results were obtained using a different TRPV1 antibody (produced by D. Julius and M. Caterina). In the contralateral L4, L5 DRGs, we did not find changes of levels of TRPV1 (97% of naive control, $n = 2$) or p-p38 (98% of naive control, $n = 2$) 2 days after inflammation.

p38 Activation and TRPV1 Expression

SB203580 (1 μg) administered intrathecally twice a day for 2 days, with the first injection given 30 min prior to CFA injection, decreases inflammatory heat hyperalgesia as effectively as osmotic pump administration of the

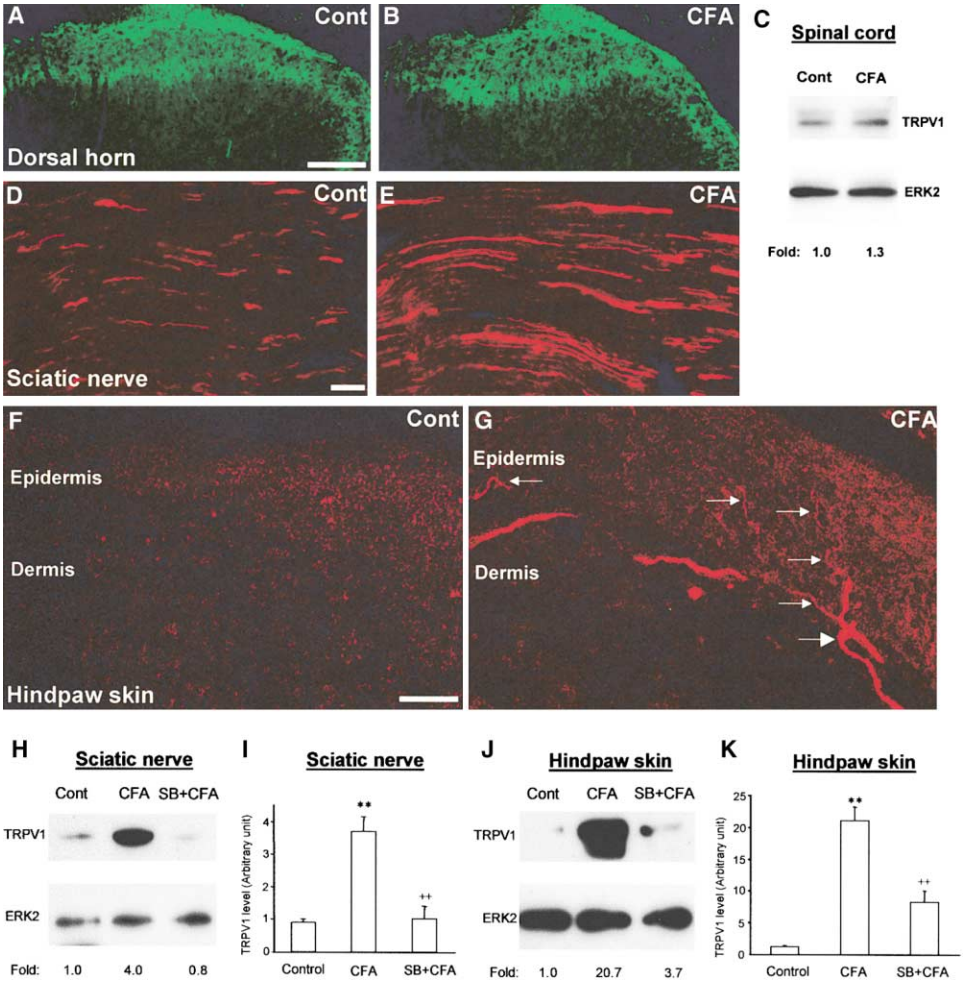

Figure 6. p38 Activation Mediates the Inflammation-Induced Upregulation of TRPV1 in the Sciatic Nerve and Hindpaw Skin

(A–C) Inflammation does not induce a distinct change in TRPV1 levels in the spinal dorsal horn, as shown by TRPV1 immunostaining (A and B) and Western blot (C).

(D–G) Inflammation induces an increase in TRPV1-labeled nerve fibers in the sciatic nerve (D and E) and hindpaw skin (F and G). TRPV1 labeling is found only in nerve fibers in the epidermis and dermis of inflamed hindpaw skin. Large and small arrows indicate nerve bundle and thin fibers, respectively. Scales bars, 50 μm.

(H and J) Western blot analysis indicates an increase in TRPV1 levels in the sciatic nerve (H) and hindpaw skin (J). Both increases are blocked by intrathecal injection of SB203580 (1 μg, twice a day for 2 days). Fold represents comparative levels over control after normalizing with a loading control.

(I and K) Quantification of TRPV1 levels in the sciatic nerve (I) and hindpaw skin (K). $^{**}p < 0.01$, compared to control; $^{++}p < 0.01$, compared to CFA, $n = 3$ for control and CFA; $n = 4$ for SB (SB203580). In all the cases, animals were inflamed with CFA for 2 days.

drug (data not shown). This treatment also blocks the CFA-induced upregulation of TRPV1 protein at 2 days (Figures 5F and 5G). SB203580 also decreases the increase in the number of TRPV1-immunoreactive neurons after inflammation (Figures 5H and 5I).

TRPV1 is anterogradely transported from the DRG, along the sciatic nerve, to peripheral nerve terminals in the skin, and via the dorsal root to central terminals in the spinal cord dorsal horn (Guo et al., 1999). Two days after inflammation, TRPV1 protein levels in the dorsal horn do not change significantly (115% ± 30% of the control, $p > 0.05$, $n = 4$, Western blot analysis) (Figures 6A–6C). In contrast, inflammation produces an increase in TRPV1 protein levels in the sciatic nerve (2.8- to 4.3-fold increase) and an even greater increase in the skin (17.7- to 25-fold increase) (Figures 6D–6K). TRPV1-labeled fibers are barely detectable in control hindpaw skin, but many nerve bundles and single nerve fibers are found in the inflamed dermis and epidermis (Figures 6F and 6G). No nonneuronal TRPV1-labeled cells were identified. The inflammation-induced TRPV1 increase in the sciatic nerve and hindpaw skin is prevented by intrathecal injection of SB203580 (1 μg, twice a day for 2 days, with the first injection given 30 min prior to CFA injection) (Figures 6H–6K).

NGF, p38 Activation, and TRPV1 Expression

p38 is activated in TrkA-expressing DRG neurons (Figure 2). NGF levels increase in inflamed tissues, and this neurotrophin plays an important role in generating inflammatory hyperalgesia (Woolf et al., 1994). To test if NGF has a role in p38 activation, NGF antiserum (5 μl/g

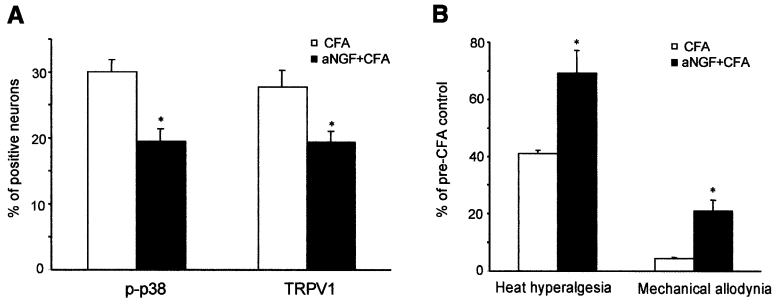

Figure 7. NGF Is Required for p38 Activation and VR1 Upregulation after Inflammation

(A) NGF antiserum treatment (5 μl/g body weight, 84 mg/ml i.p., once a day for 2 days) suppresses the inflammation-induced increase in p-p38 and TRPV1 levels in the DRG 2 days after CFA injection.

(B) This treatment also alleviates inflammatory pain, especially heat hyperalgesia. Heat and mechanical sensitivity were measured by paw withdrawal latency and paw withdrawal threshold, respectively, and expressed as a percentage of pre-CFA baseline measurements of control serum. *$p < 0.05$, compared to CFA, $n = 3$.

body weight, 84 mg/ml, i.p.) was injected once a day for 2 days, with the first injection 1 hr prior to CFA injection, to neutralize NGF (Woolf et al., 1994). This treatment decreases the activation of p38 by CFA, the inflammation-induced upregulation of TRPV1 (Figure 7A), and inflammatory heat hyperalgesia (Figure 7B). In agreement with previous studies (Woolf et al., 1994; Sammons et al., 2000), we did not observe any detectable effect of the neutralizing NGF antibody on the CFA-induced edema or erythema of the inflamed hind paw. We cannot exclude that NGF may have some subtle antiinflammatory role, since NGF appears to regulate the immune response (Stanisz and Stanisz, 2000).

Intrathecal administration of NGF at two doses, 1 or 2 μg, twice a day for 3 days increases TRPV1 protein levels in a dose-dependent way (Figure 8B) without affecting TRPV1 mRNA levels (Figure 8A). Immunohistochemical analysis also demonstrates an increase in p-p38 and TRPV1 levels in DRG neurons following the intrathecal NGF administration (Figures 8C and 8D). SB203580 (1 μg) coadministrated with the NGF (twice a day for 3 days) significantly suppresses the NGF-

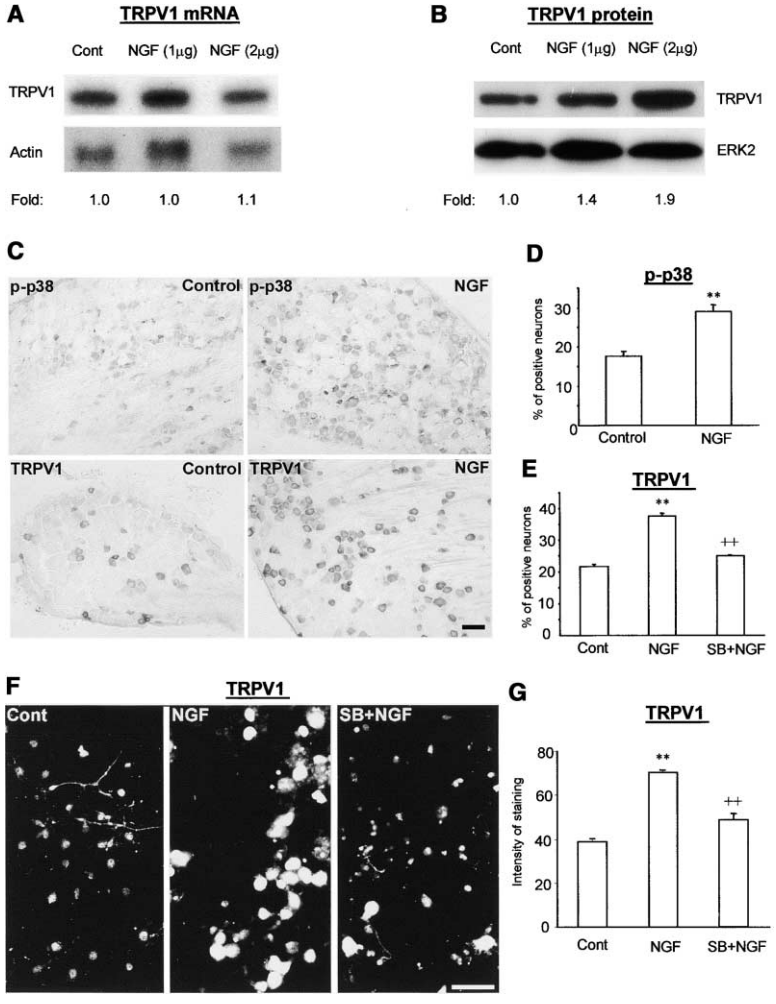

Figure 8. NGF Induces TRPV1 Upregulation in the DRG via p38 Activation Both In Vivo and in DRG Cultures

(A) RNase protection assay reveals no increase in TRPV1 mRNA levels after intrathecal injection of NGF at 1 and 2 μg, twice a day for 3 days. Fold represents comparative levels over control after normalizing with an actin control.

(B) Western blot analysis indicates an increased dose-dependent expression of TRPV1 protein levels after intrathecal NGF. Fold represents comparative levels over control after normalizing with a loading control.

(C and D) Immunohistochemistry indicates increased p-p38 and TRPV1 expression after intrathecal injection of NGF (2 μg, twice a day for 3 days). **$p < 0.01$, compared to control (saline), $n = 3$. Scale bar, 50 μm.

(E) The NGF-induced increase in TRPV1 levels is blocked by the p38 inhibitor SB203580 (1 μg) coadministered with NGF for 3 days. **$p < 0.01$, compared to vehicle (saline); $^{++}p < 0.01$, compared to NGF, $n = 3$.

(F) Immunocytochemistry illustrates an increase in TRPV1 levels 2 days after NGF (100 ng/ml) treatment of adult DRG neuronal cultures. The increase is reversed by cotreatment of SB203580 (20 μM). Scale bar, 50 μm.

(G) Quantification of intensity of TRPV1-labled neurons, as shown in (F). **$p < 0.01$, compared to control; $^{++}p < 0.01$, compared to CFA, $n = 3$.

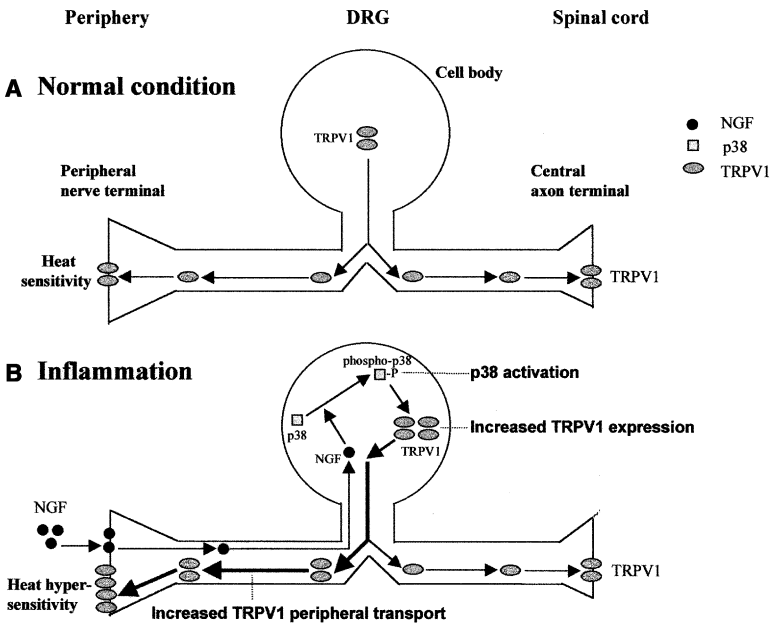

Figure 9. Schematic Representation of the Involvement of p38 in Heat Hyperalgesia after Peripheral Inflammation

In the absence of peripheral inflammation, TRPV1 is synthesized in the cell bodies of primary sensory neurons in the DRG and transported both to peripheral and central terminals, contributing to heat sensitivity in the periphery. After inflammation, NGF is produced in inflamed tissue, taken up by peripheral nerve terminals, and retrogradely transported to the cell body in the DRG. NGF in the cell body activates p38, which in turn increases TRPV1 translation. Increased TRPV1 is anterogradely transported to peripheral terminals, increasing heat sensitivity.

induced TRPV1 increase, as indicated both by the percentage of TRPV1-labeled neurons (Figure 8E) as well as the intensity of TRPV1-positive neurons (data not shown).

NGF regulates capsaicin sensitivity and TRPV1 expression in cultured DRG neurons (Nicholas et al., 1999; Winter et al., 1988; Winston et al., 2001). We used adult primary DRG neuron culture, therefore, as an independent system to study p38-mediated TRPV1 regulation. NGF administration (100 ng/ml) for 2 days increases TRPV1 levels in cultured DRG neurons, and these levels are decreased by SB203580 (20 μM) (Figures 8F and 8G).

Discussion

ERK members of the MAPK family act both in the nociceptor peripheral terminal and the dorsal horn to produce pain hypersensitivity within 20 min of their activation by epinephrine and nociceptive synaptic input, respectively, an effect that is likely due to posttranslational processing (Ji et al., 1999; Aley et al., 2001; Karim et al., 2001). Persistent ERK activation in the dorsal horn induced by peripheral inflammation also contributes to transcriptional regulation of dynorphin in superficial dorsal horn neurons (Ji et al., 2002). We now describe yet another influence of MAP kinases on pain, one mediated by p38 and not by ERK, with a slow onset, and which acts not in the nociceptor terminal or dorsal horn, but in the cell bodies of sensory neurons in the DRG. The p38 activation in the DRG is initiated by retrograde transport of NGF released from inflamed tissue and acts to increase translation and transport of TRPV1 to the peripheral nociceptor terminal, where it contributes to the maintenance of inflammatory heat pain hypersensitivity (Figure 9).

Peripheral inflammation does not increase p38 activation in large, low threshold A fibers or in dorsal horn neurons; the effect is restricted to small DRG neurons, most of which are nociceptors (Snider and McMahon, 1998). This indicates that p38 activation in DRG neurons is not a universal response of neurons to stress, activity, or inflammation but is a restricted change induced by a specific signal in a particular subset of neurons. The effects of p38 activation are limited, moreover, to heat hyperalgesia, with no effect on mechanical hypersensitivity, a finding in keeping with a pivotal role for TRPV1 as a downstream target for activation of p38. The elevation in TRPV1 expression in the cell body is accompanied by an increase in TRPV1 in the peripheral but not the central terminals of the C fibers, implying a directed trafficking of the receptor to the peripheral axon after inflammation.

p38 has several proinflammatory roles (Badger et al., 1996), and systemically administered p38 inhibitors produce antiinflammatory effects by reducing the synthesis of the cytokines TNF-α and IL-1β, as well as Cox-2 induction in inflammatory cells (Lee et al., 1994; Paul et al., 1999; Widmann et al., 1999). Reducing Cox-2 will decrease prostaglandin production, a major inflammatory mediator that generates peripheral sensitization by potentiating voltage-gated sodium channels in nociceptor peripheral terminals (Julius and Basbaum, 2001; Woolf and Salter, 2000). The present study indicates an additional role for p38 in inflammation, one that acts in DRG neurons to regulate TRPV1 expression and thereby heat hyperalgesia. Intrathecal administration of a p38 inhibitor targeted to reach DRG neurons innervating the inflamed tissue blocks this action without reducing the local inflammation. The intrathecal route can be used to deliver molecules directly to DRG neurons including NGF because the dural membrane extends onto the capsule of the DRG so that the proximal face of the DRG is in direct continuity with the subarachnoid space (Michael et al., 1997; Porreca et al., 1999). Since p38 is not activated in the spinal cord (Figure 3), any effects of a p38 inhibitor when administered intrathecally will be limited to the DRG.

Neuronal activity has been implicated in the activation of p38 in cerebellar neurons (Mao et al., 1999). However, it is unlikely that afferent activity initiated in the periphery by the release of local inflammatory mediators plays a major role in p38 activation in the DRG, because no activation was detected for up to 6 hr after the CFA injection, even though the tissue is inflamed and both thermal and mechanical hypersensitivity are fully expressed at this time. The suppressant effect of NGF antiserum on the inflammation-induced activation of p38 and the opposite effect produced by intrathecal NGF administration clearly suggest a role for NGF in the activation of p38 in the DRG. The delay in p38 activation after inflammation closely matches the time required for the retrograde transport of NGF from the inflamed paw to the DRG (Miller and Kaplan, 2001), and NGF induces a sustained activation of p38 in PC 12 cells (Morooka and Nishida, 1998). The mechanisms by which NGF might trigger p38 activation remain to be established. One possibility is that NGF regulates p38 via the Ras/Rho cascade. The Rho family of guanine nucleotide binding proteins, including Rho, Rac1, and Cdc42, regulate the activation of p38 kinase (Coso et al., 1995; Xing et al., 1998). However, not all neurons that are labeled with p-p38 express TrkA, so NGF is unlikely to be the only peripheral signal responsible for p38 activation in DRG neurons.

Inflammation increases transcription of several genes in DRG neurons, including the neuropeptides substance P and CGRP (Noguchi et al., 1988), the neurotrophin BDNF (Mannion et al., 1998), the growth promoting protein GAP-43 (Leslie et al., 1995), and the ion channels $Na_V1.8$ (SNS) (Tanaka et al., 1998) and ASIC (Voilley et al., 2001). NGF is a major factor in regulating gene transcription after inflammation (Lindsay and Harmar, 1989; Leslie et al., 1995; Mannion et al., 1998), and p38 may underlie the transcriptional regulation of these genes. Interestingly, TRPV1 differs from many other molecules in the DRG, which show parallel changes in their mRNA and protein levels (Hokfelt et al., 1994). In agreement with several previous studies (Sanchez et al., 2001; Tohda et al., 2001; Voilley et al., 2001), we did not detect any increase in TRPV1 mRNA levels in the DRG after induction of peripheral inflammation (Figures 5A and 5B). Neither did we find an increase in TRPV1 mRNA after intrathecal NGF treatment (Figure 8A). However, both inflammation and NGF increased the expression of TRPV1 protein levels in the DRG (Figures 5F and 8B). p38 can potentially regulate protein expression in several different ways, activating transcription factors such as ATF-2, ELK-1, and CREB to increase transcription (Xing et al., 1998; Widmann et al., 1999; Ji and Woolf., 2001), increasing mRNA stability (Faour et al., 2001) or increasing translation. One major target of p38 is the translational factor eIF4E, the phosphorylation of which results in increased affinity of eIF4E for capped RNA (Gingras et al., 1999; Raught and Gingras, 1999). Both ERK and p38 MAPK phosphorylate eIF4E via Mnk1/2 (MAPK-interacting kinase 1 and 2) (Wang et al., 1998; Waskiewicz et al., 1997) and in this way could increase translation.

The sensitivity of TRPV1 in the peripheral terminal is controlled by many factors, including protons, endocannabinoids, as well as NGF and bradykinin, which act either directly or through PKC to sensitize the receptor (Premkumar and Ahern, 2000; Tominaga et al., 1998; Zygmunt et al., 1999). p38 alters heat transduction by changing the number of heat transducers in the terminal, rather than their sensitivity, a novel form of regulation. Previous studies have reported increased TRPV1 immunoreactive nerve terminals after inflammation (Carlton and Coggeshall, 2001; Yiangou et al., 2001). NGF and inflammation also increase the sensitivity of DRG neurons to capsaicin (Nicholas et al., 1999; Winter et al., 1988), an effect that is likely to reflect increased TRPV1 translation. Our data show a 20-fold increase of TRPV1 in inflamed hindpaw skin, with a relatively lower increase in the DRG. The redistribution of TRPV1 out of the DRG and toward the periphery resembles the alterations reported for the sodium channel Nav 1.8 after peripheral nerve injury (Novakovic et al., 1998). The very small increase in TRPV1 in the dorsal horn, where the central terminals of C fibers are located, seems to indicate a directed transport down peripheral axons after inflammation. The BNAC1α sodium channel is only transported down the peripheral axons of DRG neurons (Garcia-Anoveros et al., 2001), so some sorting mechanism must be present at the axon branch point in the DRG to achieve such directed traffic.

Inflammatory heat hyperalgesia involves both acute changes resulting from posttranslational modifications of existing TRPV1 in nociceptor peripheral terminals, as well as more delayed changes resulting from increased transport of TRPV1 from the cell body to the peripheral terminal. The latter is the consequence of a NGF-mediated activation of p38 in the DRG cell body after retrograde transport of NGF from the periphery to the cell body. The role of p38 in increasing translation and transport of the TRPV1 ion channel without changing its transcription represents a novel form of regulation of sensory neuronal function, one that would not be detected by transcription profiling techniques such as high density oligonucleotide microarrays.

Experimental Procedures

Animals

Adult male Sprague-Dawley rats (240–320 g) were used according to Massachusetts General Hospital Animal Care institutional guidelines. All procedures were performed under pentobarbital anesthesia (50 mg/kg, i.p.). Complete Freund's adjuvant (CFA, 100 μl) was injected into the plantar surface of the left hindpaw. For intrathecal drug delivery, a PE10 catheter was implanted into the intrathecal space of the spinal cord at the L4 DRG level, and 10 μl of p38 inhibitor SB203580 (1 μg; Calbiochem) was administered. For sustained drug delivery, an Alzet osmotic pump (7 day pump, 0.5 μl/hr) was filled with the p38 inhibitor SB203580 (1 μg/μl) in saline, and the associated catheter implanted intrathecally at least 6 hr before CFA injection. Saline was used as vehicle control for the osmotic pump. NGF antiserum (Woolf et al., 1994) was injected daily for 2 days (i.p., 84 mg/ml, 5 μl/gram body weight), with the first injection 1 hr before CFA injection. NGF (1 or 2 μg in 10 μl; Boeringer) was intrathecally injected twice a day for 3 days. NGF and SB203580 were coadministered in some animals.

Immunohistochemistry

Rats were perfused through the ascending aorta with saline followed by 4% paraformaldehyde with 1.5% picric acid. L4 and L5 DRGs, L4-L5 spinal cord segments, sciatic nerves, and plantar surface of hindpaw skin were dissected. For mice tissue, fresh DRGs and spinal cords were dissected and fixed in the same fixative for 3 hr. DRG

and transverse spinal cord sections (15 μm) were cut and processed for immunohistochemistry using the ABC method (Ji et al., 1995). The following antibodies were used: polyclonal anti-p38 and phospho-p38 (1:300; New England BioLabs), and polyclonal anti-VR1 (1:5000; provided by GSK and Dr. David Julius). Immunofluorescence was performed for double staining for phospho-p38 (anti-rabbit, 1:300) with VR1 (anti-guinea pig, 1:3000; Neuromics), $P2X_3$ (anti-guinea pig, 1:3000; Neuromics), or NeuN (anti-mouse, 1:5000; Chemicon), and for single staining for ED-1 (anti-mouse, 1:5000; Serotec). In brief, DRG and spinal sections were incubated with a mixture of two primary antibodies overnight at 4°C and followed by a mixture of FITC- and CY3-congugated secondary antibodies (1:300; Jackson Immunolabs) for 2 hr at room temperature (RT) (Ji et al., 2002). The sciatic nerve sections (10 μm) and skin sections (40 μm) were processed for immunofluorescence using the GSK VR1 antibody. The Tyramide Signal Amplification (TSA; NEN) kit was used to perform double staining with two polyclonal rabbit antibodies (phospho p38 and TrkA) (Amaya et al., 2000).

Western Blot

The L4 and L5 DRGs and dorsal horns (lumbar enlargement) were homogenized in a buffer containing a cocktail of proteinase and phosphatase inhibitors. The sciatic nerves and hindpaw skins were homogenized by polytron in the same buffer. Protein samples were separated on a SDS-PAGE gradient gel (4%–15%; Bio-Rad) and transferred to PVDF filters. The blots were blocked with 5% milk for 1 hr and incubated with phospho-p38 antibody (1:1000) or VR1 antibody (1:1000) overnight at 4°C. The blots were then incubated in HRP-conjugated secondary antibody (1:5000) for 1 hr at RT, developed in ECL solution (NEN) for 1 min, and exposed onto X-films (superfilm; Amersham) for 2–30 min. The blots were then incubated in stripping buffer (100 μM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris [pH 6.7]) at 50°C for 30 min and reprobed with p38 and ERK2 antibody (1:3000; New England Biolabs) as loading control. For the deglycosylaition experiment, 10 μl DRG lysate (50 μg protein) was treated with 1 μl N-glycosidase F (500 unit per μl; New England Biolabs) at 37°C for 1 hr, followed by VR1 Western blot.

RNase Protection

The L4, L5 DRGs were rapidly removed. VR1 cDNA was generated by RT-PCR from rat DRG total RNA using primers 5′-AAACTCCACC CCACGCTGAA-3′ and 5′-GTCGGTTCAAGGGTTCCACG-3′. The PCR product was cloned into pCRII (Invitrogen). The plasmid was linearized with BamH1, and an antisense probe was synthesized using T7 RNA polymerase and labeled with ^{32}P-UTP (800 Ci/mmol; NEN). RNase protection assays were performed using the RPA III (Ambion) protocol (Samad et al., 2001). Briefly, 5 μg of RNA samples were hybridized with labeled probe overnight at 42°C and then digested with RNase A/RNase T1 mix in RNase digestion buffer for 30 min at 37°C. Finally, samples were separated on denaturing acrylamide gel and exposed to X-films. β-actin probe was used for each sample as loading controls.

DRG Culture

DRGs were aseptically removed from adult rats, incubated with collagenase (5 mg/ml; GIBCO) at 37°C for 60 min, and digested with 2.5% trypsin-0.05% DNase (GIBCO) for 5 min at 37°C, followed by 0.25% trypsin inhibitor. Cells were mechanically dissociated with a flame-polished Pasteur pipette in the presence of 0.05% DNase. The cell suspensions were layered on a cushion of 15% fatty acid-free BSA and Percoll gradient to remove connective tissue and debris. Cells were plated onto polylysine- and laminin-coated plates and grown in a neurobasal defined medium (with 2% B27 supplement; GIBCO) in the presence of 5 μm AraC. NGF (100 ng/ml) and SB203580 (20 μM) were added to the medium 4 hr after cells were plated. Two days after the treatment, the cultures were fixed with 4% paraformaldehyde for 20 min and processed for VR1 immunofluorescence.

Behavioral Analysis

Animals were habituated and basal pain sensitivity was tested before drug administration or surgery (Ji et al., 2002). Mechanical withdrawal threshold on the plantar surface of the hindpaw was measured with a set of von Frey hairs. The threshold was taken as the lowest force that repetitively evoked a brisk withdrawal response. Thermal paw withdrawal latency was measured using the Hargreaves radiant heat apparatus and averaged over three trials.

Quantification and Statistics

The images of immunostained DRG sections were captured with a CCD camera, and the intensity was measured (IP lab software) and number of immunoreactive neuronal profiles was counted in a blinded fashion. Control and treated DRG sections were mounted on the same slides and processed under the same conditions. Every fifth section was picked from a series of consecutive DRG sections (20 μm), and four sections were counted for each DRG and expressed as the percentage of total neuronal profiles (Ji et al., 1996). For cell counting in the skin, four sections were randomly selected from each rat, and labeled cells in a 20× optic field on each section were counted. For Western blot and RNase protection assay, the films were scanned, and the density of specific bands was measured and normalized with an internal loading control band. Data are represented as mean ± SEM. Differences between groups were compared using Student's t test or ANOVA, followed by Fisher's PLSD. For nonparametric data, the Mann-Whitney U test was applied. The criterion for statistical significance was $p < 0.05$.

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