

Diabetes-Induced Mechanical Hyperalgesia Involves Spinal Mitogen-Activated Protein Kinase Activation in Neurons and Microglia via *N*-Methyl-D-aspartate-Dependent Mechanisms

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ABSTRACT

Molecular mechanisms underlying diabetes-induced painful neuropathy are poorly understood. We have demonstrated, in rats with streptozotocin-induced diabetes, that mechanical hyperalgesia, a common symptom of diabetic neuropathy, was correlated with an early increase in extracellular signal-regulated protein kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) phosphorylation in the spinal cord and dorsal root ganglion at 3 weeks after induction of diabetes. This change was specific to hyperalgesia because nonhyperalgesic rats failed to have such an increase. Immunoblot analysis showed no variation of protein levels, suggesting a post-translational regulation of the corresponding kinases. In diabetic hyperalgesic rats, immunocytochemistry revealed that all phosphorylated mitogen-activated protein kinases (MAPKs) colocalized with both the neuronal (NeuN) and microglial (OX42) cell-specific markers but not with the astrocyte marker [glial fibrillary acidic protein (GFAP)] in the superficial dorsal horn-laminae of the spinal cord. In these same rats, a 7-day administration [5 μ g/rat/day, intrathecal (i.t.)] of 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenyl-

thio)butadiene (U0126), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), and anthra(1,9-*cd*)pyrazol-6(2*H*)-one (SP600125), which inhibited MAPK kinase, p38, and JNK, respectively, suppressed mechanical hyperalgesia, and decreased phosphorylation of the kinases. To characterize the cellular events upstream of MAPKs, we have examined the role of the NMDA receptor known to be implicated in pain hypersensitivity. The prolonged blockade of this receptor during 7 days by (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]-cyclohepten-5-10-imine hydrogen maleate (MK801; 5 μ g/rat/day, i.t.), a noncompetitive NMDA receptor antagonist, reversed hyperalgesia developed by diabetic rats and blocked phosphorylation of all MAPKs. These results demonstrate for the first time that NMDA receptor-dependent phosphorylation of MAPKs in spinal cord neurons and microglia contribute to the establishment and long-term maintenance of painful diabetic hyperalgesia and that these kinases represent potential targets for pain therapy.

Sensitive peripheral neuropathies represent a common and debilitating complication of diabetes (types 1 and 2) and affect an increasing proportion of diabetic patients as the disease progresses. Even though antidepressant and antiepileptic agents have been shown to be partially effective, clinical studies have reported the difficulty of managing pain caused by these neuropathies (Sindrup and Jensen, 1999),

which may be due to changes in peripheral nerve or to neuronal processing (e.g., central sensitization in the spinal cord) (Woolf and Salter, 2000). Whereas the pathophysiological and neuroanatomical changes associated with the development of neuropathic pain are well documented, the molecular and cellular mechanisms underlying its initiation and maintenance remain poorly understood.

The mitogen-activated protein kinase (MAPK) cascade is a family of serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues. The

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase kinase; DRG, dorsal root ganglion; STZ, streptozotocin; NMDA, *N*-methyl-D-aspartate; i.t., intrathecal; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; SB503580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SP600125, anthra(1,9-*cd*)pyrazol-6(2*H*)-one; MK801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]-cyclohepten-5-10-imine hydrogen maleate; RT, room temperature; GFAP, glial fibrillary acidic protein; NeuN, neuronal specific nuclear protein; DMSO, dimethyl sulfoxide.

MAPKs play a pivotal role in transducing extracellular stimuli into intracellular post-translational and transcriptional responses (Widmann et al., 1999) and include extracellular signal-regulated protein kinase (ERK), p38-MAPK (p38), and c-Jun N-terminal kinase (JNK). The MAPKs have characteristically been involved as regulators of cell proliferation, differentiation, and survival but are now recognized to have a major role in the generation of pain hypersensitivity.

In several models associated with peripheral tissue inflammation, persistent inflammatory hyperalgesia can be prevented by MEK inhibitors (Galan et al., 2002; Ji et al., 2002a) or p38 inhibitors (Ji et al., 2002b; Svensson et al., 2003a,b). Recent studies have reported that MAPKs are activated in experimental neuropathic pain models. After peripheral nerve damage, phosphorylation at the spinal level of ERK (Ciruela et al., 2003; Obata et al., 2004a,b; Zhuang et al., 2005) or p38 (Jin et al., 2003; Schafers et al., 2003; Tsuda et al., 2004) contributes to the painful behavior generated in these models. Moreover, peripheral axotomy has been shown to induce long-term JNK activation in dorsal root ganglion (DRG) neurons (Kenney and Kocsis, 1998). In the lumbar DRG of streptozotocin (STZ)-induced diabetic rats, a model of type 1 diabetes, ERK and p38 are activated 8 weeks after the induction of the disorder, whereas JNK is activated later, at 12 weeks (Fernyhough et al., 1999; Purves et al., 2001). At 12 weeks' duration, JNK is also activated in the rat (Fernyhough et al., 1999) and human (Purves et al., 2001) sural nerve. Long-term treatment of STZ-induced diabetic rats with a p38 inhibitor prevents neuronal dysfunction such as the archetypal defect of slowed nerve conduction (Agthong and Tomlinson, 2002). All the animal studies have been carried out at an advanced stage of the diabetes, whereas the painful symptoms, such as hyperalgesia, appeared more precociously, from 3 weeks (Courteix et al., 1993). Moreover, most reports did not correlate the pain behavior of diabetic rats with the MAPK activation. In addition, the status of the three MAPK isoforms has never been studied simultaneously in the model of diabetic neuropathy. In view of that, the present study performed in an STZ-induced model of diabetes investigated whether 1) early activation of ERK, JNK, and p38, assessed by their phosphorylation status, occurred in the spinal cord and in DRG, in which cells it happened at the spinal level and 2) this activation contributes to the mechanical hyperalgesia developed by diabetic rats.

In the context of chronic diabetes-induced hyperalgesia, the upstream effectors of spinal MAPK activation have never been investigated. The NMDA receptor has been shown to be involved in diabetic hyperalgesia (Malcangio and Tomlinson, 1998; Begon et al., 2000). In addition, in acute and inflammatory pain models, activation of the p38 (Svensson et al., 2003a) and ERK pathways (Ji et al., 1999; Kawasaki et al., 2004) are NMDA receptor-dependent. Another aim of our study, therefore, was to determine the link between the NMDA receptor, MAPK activation, and hyperalgesia in diabetic rats.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Les Oncins, France), initially weighting 201 to 225 g, were housed four per cage under standard laboratory conditions and allowed food and

water ad libitum. Animal care and experiments were carried out in accordance with the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983).

Induction of Diabetes

Rats were rendered diabetic with an i.p. injection of 75 mg/kg STZ (Zanosar; Pfizer, Paris, France) dissolved in distilled water. Diabetes was confirmed 2 and 3 weeks later by measurement of tail vein blood glucose levels with Glucotite test strips and a reflectance colorimeter (Glucometer 4; Bayer Diagnostics, Puteaux, France). Only rats with a final blood glucose level >14 mM were included in the study. Control rats were administered only with distilled water (1 ml/kg, i.p.). This model has been previously described in detail by Courteix et al. (1993).

Behavioral Studies

Nociceptive Test Procedure. Animals were habituated to the testing environment daily for 3 days before baseline testing. The rats were submitted to the paw-pressure test described previously (Randall and Selitto, 1957). Nociceptive thresholds, expressed in grams, were measured using an Ugo Basil analgesimeter (Bioseb, Chaville, France) by applying increasing pressures to the left hind paw until a squeak (vocalization threshold) was elicited. The test was repeated until three stable threshold values were obtained. The maximal pressure was set at 450 g.

Experimental Design. To correlate the painful status of animals and the expression of phosphorylated forms of MAPKs, rats were subjected to the paw-pressure test 3 weeks after the induction of diabetes. Rats were considered hyperalgesic when reduction in nociceptive pain thresholds was more than 15% of the value obtained before the STZ injection.

The study of both MAPK pathways and NMDA receptor involvement in diabetes-induced hyperalgesia was performed by inhibiting MEK (which is an upstream kinase of ERK), JNK and p38 by U0126, SP600125, SB503580, respectively, and by blockade of the NMDA receptor with MK801. Fourteen days after induction of diabetes, only diabetic hyperalgesic rats were included. A long-term treatment by intrathecal (i.t.) route with MAPKs inhibitors (5 μ g/rat/day) was performed in these rats as described in previous studies (Ji et al., 2002a; Jin et al., 2003; Obata et al., 2004a; Sweitzer et al., 2004) between the 2nd and the 3rd week of the disease. The dose of MK801 (5 μ g/rat/day, i.t.) has been determined after preliminary data and according to previous studies performed in diabetic rats showing the need of long-term treatment to reverse hyperalgesia (Malcangio and Tomlinson, 1998; Begon et al., 2000). Therefore, the diabetic hyperalgesic rats were intrathecally treated with the different drugs or the vehicle from days 15 to 21 after the induction of the trouble. Each experiment was performed blind using different animals in randomized blocks to assess the effects of the different treatments under the same lapse of time and environmental conditions. At day 22, rats whose vocalization thresholds were increased by 25% compared with nociceptive thresholds measured before treatment (at day 14) were considered to have an improved pain status. Rats were then sacrificed by decapitation. Lumbar enlargements of the spinal cord and L4–L5 DRG were rapidly removed, frozen in liquid nitrogen, and kept at -80°C .

Drugs and Intrathecal Administration. U0126, SB503580, and SP600125 were purchased from BIOMOL Research Laboratories (Le Perray en Yvelines, France). The NMDA receptor antagonist MK801 was obtained from Sigma (St. Quentin Fallavier, France). Intrathecal injections of drugs or vehicle (saline containing 10% dimethyl sulfoxide) were performed under isoflurane anesthesia (4% induction, 2% maintenance), as described previously (Mestre et al., 1994). In brief, the anesthetized rat was held in one hand by the pelvic girdle, and a 25-gauge, 1-inch needle connected to a 25- μ l Hamilton syringe was inserted into the subarachnoid space between

lumbar vertebrae L5 and L6 until a tail-flick was elicited. The syringe was held in position for few seconds after the injection of a volume of 10 μ l/rat. Intrathecal injection of vehicle had no effect on nociceptive thresholds/behavior, protein expression and protein phosphorylation.

Immunoblotting Analysis

Spinal cord enlargements and DRG, homogenized in 400 μ l of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $\text{Na}_2\text{P}_2\text{O}_7$, 2 mM orthovanadate, 100 mM NaF, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 20 μ M leupeptin, and 100 IU/ml aprotinin; Sigma), were incubated for 20 min at 4°C and then centrifuged at 16,000g for 15 min. The protein concentration of tissue lysates was determined with a BCA protein assay kit (Interchim, Paris, France). Proteins were then electrophoresed in 10% SDS polyacrylamide gels and transferred to nitrocellulose membrane (BioTrace NT; BioTrace S.A., Villeneuve la Garenne, France). Membranes were blocked 1 h at room temperature (RT) with Tris-buffered saline/Tween 20 (10 mM Tris-HCl, pH 7.6, 140 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4°C with the indicated antibodies (anti-total ERK1/ERK2, JNK1, p38 antibodies, polyclonal, anti-rabbit, 1:200; Tebu, Le Perray en Yvelines, France; anti-phospho-ERK, phospho-p38, and phospho-JNK antibodies that recognize ERK, p38, and JNK only when activated by dual phosphorylation at Thr202/Tyr204, Thr180/Tyr182, and Thr183/Tyr185, respectively, and do not detect nonphosphorylated MAPKs, monoclonal, anti-mouse, 1:2000; Cell Signaling Technology, Beverly, MA). The blots were washed three times in Tris-buffered saline/Tween 20 and incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL), developed in ECL solution (SuperSignal West Pico Chemiluminescent Substrate) for 5 min and exposed onto Kodak BioMax hyperfilms (Amersham Biosciences, Saclay, France) for 1 to 30 min. The intensity of immunoreactive bands was quantified using Phoretix Advanced Software and normalized against a loading control. Results were expressed as the percentage change from control, where control is the mean result from at least three spinal cords of animals that received saline or vehicle.

Immunohistochemistry

Three-week-old diabetic, hyperalgesic rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4. The lumbar spinal cords were removed, postfixed in the same fixative for 1 h at 4°C, dehydrated, and included in paraffin. Antigen retrieval was performed by bringing slides to sub-boiling 10 min in 10 mM sodium citrate, pH 6, and cooling at RT for 30 min. Then, spinal sections were incubated for 10 min in a solution containing 3% H_2O_2 and blocked with 5% donkey serum for 1 h at RT. For double immunofluorescence staining, spinal cords were incubated overnight at 4°C with a mixture of monoclonal anti-phospho-MAPK antibody (anti-phospho-p38, phospho-ERK, phospho-JNK, anti-rabbit, 1:100; Cell Signaling Technology) and monoclonal neuronal specific nuclear protein (NeuN; neuronal marker, anti-mouse, 1:200; Chemicon, Temecula, CA), glial fibrillary acidic protein (GFAP) (astrocyte marker, anti-mouse, 1:200; Chemicon), or anti-OX-42 (CD11b, microglial marker, anti-mouse, 1:200; Chemicon) antibodies followed by a mixture of fluorescein isothiocyanate- and rhodamine-conjugated secondary antibody (anti-rabbit or anti-mouse, 1:100; FluoProbes, France) for 2 h at RT. Nonspecific staining was determined by excluding the primary antibodies. Images were captured using a microscope (Nikon, Tokyo, Japan) equipped for epifluorescence operated by Lucia software.

Statistical Analysis

Three to eight rats were included for each group for quantification of immunoblots and behavioral studies. Data are expressed as mean \pm S.E.M. Differences between groups were compared by a

two-way analysis of variance (ANOVA) followed by either a Dunnett's test to analyze the time course of the effect of different treatments on vocalization thresholds, or by Student's *t* test in unpaired series to compare kinase activity obtained after different treatments or for relationships between pain thresholds and phosphorylation of MAPKs. Correlations were assessed by Pearson's simple correlation coefficient. The criterion for statistical significance was $p < 0.05$.

Results

Biological and Clinical Parameters

Two weeks after the STZ injection, 70.7% ($n = 81$) of the rats presented a significant 3.58 ± 0.3 -fold increase ($p < 0.001$) in glycemia compared with that before injury (before STZ, 7.76 ± 0.14 mM; week 2, 27.79 ± 1.05 mM). Only diabetic rats were included in the studies. No significant difference was observed for glycemia after long-term treatment with the vehicle, selective MAPK inhibitors, or the NMDA receptor antagonist, demonstrating that these treatments had no effect on this parameter.

The analysis of the pain behavior showed a significant reduction ($41.4 \pm 1.5\%$, $p < 0.01$) in the vocalization thresholds 2 weeks after the induction of diabetes in 72.8% ($n = 59$) of the diabetic rats (before STZ, 357.1 ± 8.2 g; week 2, 209.1 ± 9 g). In the absence of treatment, this decrease was maintained 3 weeks after the induction of diabetes (198.3 ± 12 g). No variation in the thresholds was observed in 27.2% of the diabetic rats and in control animals 2 and 3 weeks after the injection of STZ or distilled water, respectively.

The mean body weight of all the rats ($n = 122$) used in the experiments was 213 ± 2.5 g before STZ and 249 ± 6 or 284.1 ± 4.2 g at 2 weeks after injection of STZ or distilled water, respectively. General health state of the animals has been considered. Loss of more than 10% of the initial body weight, loss of activity, and piloerection were criteria that justified the removal of diabetic animals. Thus, hyperalgesia observed in STZ-induced diabetic rats was specific to diabetes and was not due to the general health state of the animals.

Spinal MAPK Involvement in Diabetes-Induced Hyperalgesia

Expression of Phosphorylated Forms of MAPKs. We first investigated whether the phosphorylation of MAPK isoforms was modified at 3 weeks of diabetes, an early stage of the disease with hyperalgesia (Courteix et al., 1993).

Immunoblots analysis with specific anti-phospho-ERK, anti-phospho-p38, or anti-phospho-JNK antibodies, which detect ERK, p38, and JNK only when activated by dual phosphorylation, revealed an increased phosphorylation of MAPK in the spinal cord (Fig. 1, A–C; top) and in DRG (Fig. 2, A–C; top) of diabetic hyperalgesic rats compared with control or diabetic nonhyperalgesic rats. Phospho-ERK and phospho-p38 were not detectable in the spinal cord of control and diabetic nonhyperalgesic rats (Fig. 1, A and B; top), whereas the three phosphorylated MAPKs were below detection levels in the DRG (Fig. 2, A–C; top) of the same animals, even if membranes were autoradiographed for a longer period of time. These results suggest that very low levels of phosphorylated forms of MAPK were present in control and diabetic nonhyperalgesic rats. In the spinal cord, densitometric quantification of JNK immunoblots showed significant $6.13 \pm$

0.5-fold and 5.5 ± 0.2 -fold increases ($p < 0.001$) in phosphorylation of the kinase in diabetic hyperalgesic rats compared with control and diabetic nonhyperalgesic animals, respectively (Fig. 1C, top). Regression analysis showed that diabetes-induced phosphorylation of MAPKs conversely correlated significantly with pain thresholds in the spinal cord (P-ERK; $r = 0.81$; P-p38; $r = 0.82$; P-JNK; $r = 0.84$; $p < 0.0001$; Fig. 1, D–F) and in DRG (P-ERK; $r = 0.80$; P-p38; $r = 0.75$; P-JNK; $r = 0.85$; $p < 0.0001$; Fig. 2, D–F). No variation of total ERK1/ERK2, p38, or JNK1 protein expression was observed in the spinal cord (Fig. 1, A–C; bottom) and in DRG (Fig. 2, A–C; bottom), whatever the behavioral or glycemic status of animals. Together, these results suggested that post-translational activation of MAPKs in the spinal cord and in DRG was specific to hyperalgesia and was not due directly to metabolic alterations of diabetes.

Cellular Localization of Spinal Activated MAPKs. Phosphorylated forms of ERK, p38, and JNK were localized mainly to the superficial dorsal horn laminae of the spinal cord (Fig. 3A). To identify the cell types that expressed spinal phospho-MAPKs in 3-week-old diabetic hyperalgesic rats, double immunofluorescence staining of phospho-MAPKs was performed with several cell-specific markers: NeuN for neurons, GFAP for astrocytes, and OX-42 (CD11b) for microglia. The three MAPKs in their activated state were expressed in neuronal (Fig. 3, B–D) and microglial (Fig. 3, E–G) cells because they colocalized with NeuN and OX-42, respectively. However, the three MAPKs did not seem to be expressed in

astrocytes, in that no colocalization could be detected between these isoforms and GFAP (Fig. 3, H–J).

Influence of MAPK Inhibition on Diabetes-Induced Hyperalgesia. To determine the role of spinal MAPK phosphorylation in nociceptive behavior, we examined the ability of U0126, SB503580, and SP600125, which are specific inhibitors of MEK (an upstream kinase in the activating cascade of ERK), p38, and JNK, respectively, to antagonize mechanical hyperalgesia evoked by diabetes. The long-term intrathecal treatment ($5 \mu\text{g}/\text{rat}/\text{day}$ for 7 days) of U0126 (Fig. 4A), SB503580 (Fig. 5A), or SP600125 (Fig. 6A) in diabetic, hyperalgesic rats significantly increased nociceptive thresholds by $57.4 \pm 0.3\%$ (from 235.0 ± 40.9 to 370.0 ± 52.9 g; $p < 0.05$), $78.8 \pm 3.9\%$ (from 185.0 ± 6.5 to 330.8 ± 32.2 g; $p < 0.01$), and $54.7 \pm 0.4\%$ (from 240.0 ± 19.8 to 371.3 ± 28.1 g; $p < 0.01$), respectively. The long-term administration of DMSO, used as vehicle, failed to induce any change in pain scores. Nociceptive thresholds obtained after a 7-day inhibition of MEK, p38, or JNK were not significantly different from prediabetes scores (thresholds after U0126 treatment, 370.0 ± 52.9 g versus 392.5 ± 53.8 g before diabetes; after SB503580 treatment, 330.8 ± 32.2 g versus 355.0 ± 17.3 g before diabetes; after SP600125 treatment, 371.3 ± 28.1 g versus 405.0 ± 20.1 g before diabetes), demonstrating that the three MAPK pathways participate in mechanical hyperalgesia observed in diabetic rats (Figs. 4–6A).

Immunoblot analyses were subsequently carried out on the lumbar spinal cord of the vehicle and MAPK inhibitor-

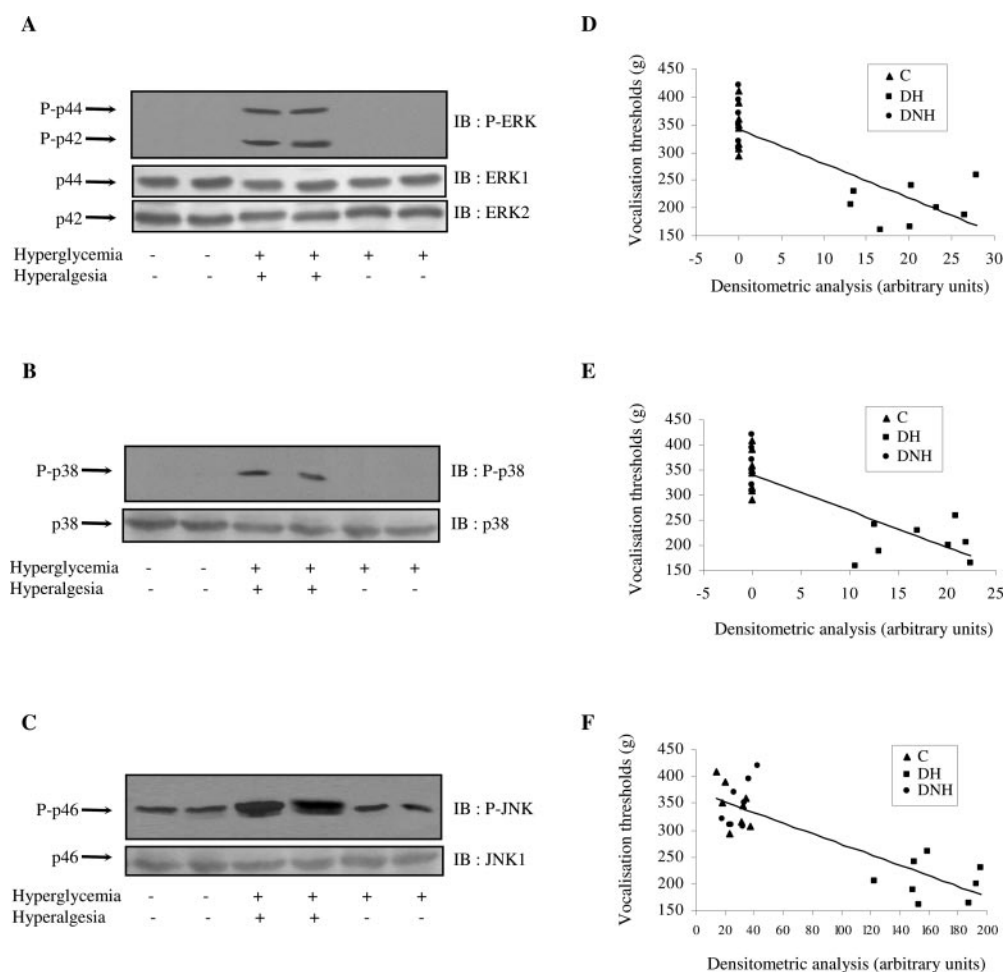


Fig. 1. Spinal MAPK involvement in diabetes-induced hyperalgesia. Immunoblots showed the increased phosphorylation of ERK1 (P-p44), ERK2 (P-p42), p38 (P-p38), and JNK1 (P-p46) in the lumbar spinal cord homogenates from 3-week-old diabetic hyperalgesic rats compared with control and diabetic nonhyperalgesic animals (A–C, top). The bottom in A to C indicates levels of total ERK1/ERK2, p38, and JNK1 proteins. The glycemic and behavioral (hyperalgesic) status of the animals are described under the blots. Representative autoradiograms from eight independent experiments are shown. D–F, correlations between vocalization thresholds and phosphorylation of ERK, p38, and JNK in control (C), diabetic hyperalgesic (DH), and diabetic nonhyperalgesic (DNH) rats.

treated diabetic hyperalgesic animals. When nociceptive thresholds were increased after U0126, SB503580, or SP600125 treatment, phosphorylation of ERK1/ERK2 (Fig. 4B), p38 (Fig. 5B), or JNK1 (Fig. 6B) isoforms at the spinal level significantly decreased ($p < 0.001$) by 90.5 ± 2.2 , 73.6 ± 10.8 , 87.2 ± 2.5 , and $62.8 \pm 4.0\%$, respectively, compared with the phosphorylation measured in the vehicle-treated rats. No significant difference in the expression of total ERK1/ERK2 (ERK1, $91.6 \pm 5.7\%$; ERK2, $108.6 \pm 5.4\%$; Fig. 4C), p38 ($113.2 \pm 6.3\%$; Fig. 5C), or JNK1 ($96.0 \pm 5.1\%$; Fig. 6C) proteins was observed in rats treated with the MEK, p38, or JNK inhibitors, respectively, compared with vehicle-treated animals, suggesting a post-translational regulation of the corresponding proteins. Then, the specificity of the three MAPK inhibitors was tested on the same lumbar enlargements of the spinal cord. The phosphorylation of p38 and JNK1 in U0126-treated rats was not significantly modified compared with that measured in vehicle-treated rats (P-p38, $91.7 \pm 8.3\%$; P-JNK1, $110.5 \pm 13.8\%$; after U0126 treatment versus after vehicle treatment). SB503580 is specific to the p38 pathway because the phosphorylation of ERK1/ERK2 and JNK1 was not different in vehicle- and SB503580-treated rats (P-ERK1, $92.1 \pm 13.6\%$; P-ERK2, $88.1 \pm 14.1\%$; P-JNK1, $127.1 \pm 16.8\%$; after SB503580 treatment versus after vehicle treatment). In addition, the JNK inhibitor was specific to this signaling pathway because it did not affect ERK and p38 phosphorylation (P-ERK1, $121.1 \pm 28.8\%$; P-ERK2, $89.6 \pm 12.0\%$; P-p38, $112.4 \pm 27.5\%$; after SP600125 treatment versus after vehicle treatment).

Involvement of the NMDA Receptor in Diabetic Hyperalgesia-Induced MAPK Activation. Because the NMDA receptor was involved in diabetic hyperalgesia (Malcangio and Tomlinson, 1998; Begon et al., 2000), we examined the effect of MK801, a noncompetitive NMDA receptor antagonist, on both the vocalization thresholds and the phosphorylation of MAPK in diabetic hyperalgesic rats. Intrathecal administration of MK801 ($5 \mu\text{g}/\text{rat}/\text{day}$ for 7 days) significantly increased ($p < 0.001$) nociceptive thresholds by $55.8 \pm 0.7\%$, from 192.0 ± 17.4 to 299.2 ± 29.6 g (Fig. 7A) and led to pain scores not significantly different from those before induction of diabetes (299.2 ± 29.6 versus 342.0 ± 8.8 g). No change in hyperalgesia was observed after the injection of the vehicle, and no side effects, such as stereotypies and/or motor dysfunction, were observed after the administration of the intrathecal dose of MK801.

In MK801-treated rats, the phosphorylation of ERK1/ERK2, p38, and JNK1 was significantly decreased ($p < 0.001$) by 65.9 ± 7.44 , 68.8 ± 10.6 , 68.0 ± 8.6 , and $76.1 \pm 3.9\%$, respectively, compared with the phosphorylation measured in vehicle-treated rats (Fig. 7, B–D).

Discussion

MAPK Activation in Diabetes-Induced Mechanical Hyperalgesia. The present study reports, for the first time, that an increase of ERK, JNK, and p38 phosphorylation in the spinal cord and in DRG, which reflects an increase of their activation, is correlated with a diabetic hyperalgesic

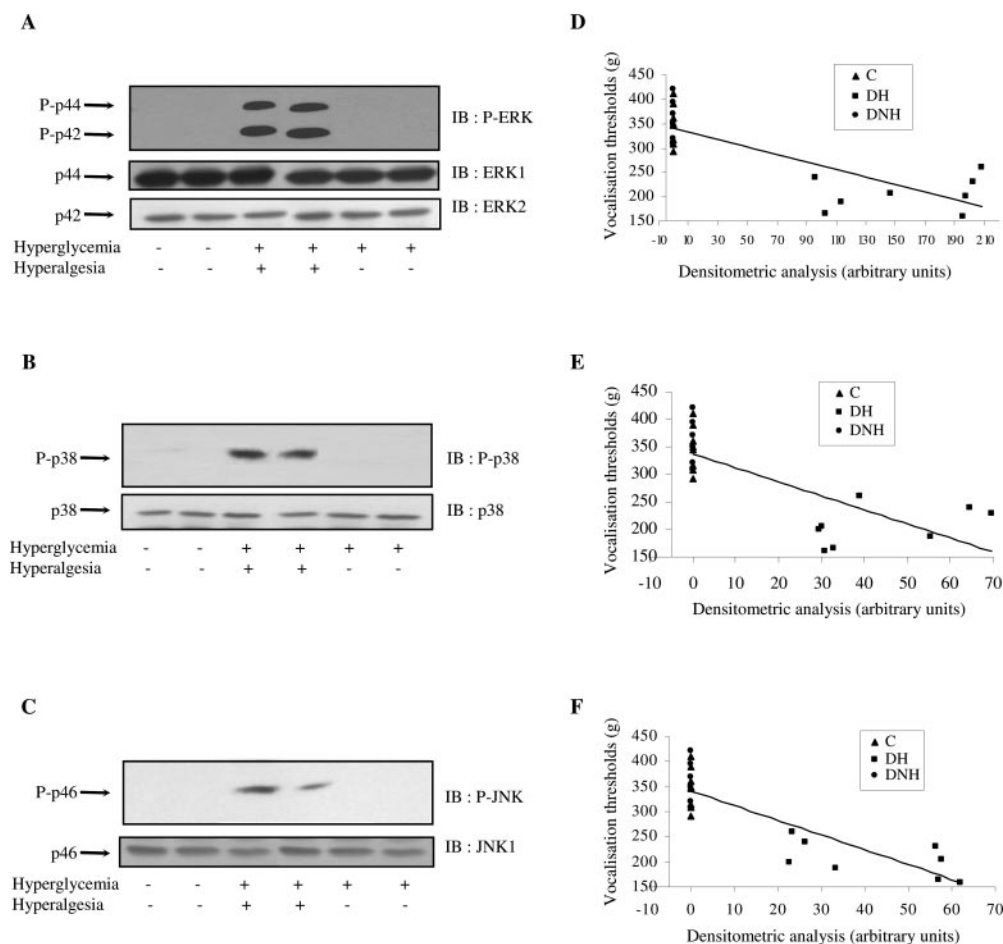


Fig. 2. MAPK involvement in DRG in diabetes-induced hyperalgesia. Immunoblots showed the increased phosphorylation of ERK1 (P-p44), ERK2 (P-p42), p38 (P-p38), and JNK1 (P-p46) in the lumbar DRG homogenates from 3-week-old diabetic hyperalgesic rats compared with control and diabetic nonhyperalgesic animals (A–C, top). The bottom in A to C indicates levels of total ERK1/ERK2, p38, and JNK1 proteins. The glycemic and behavioral (hyperalgesic) status of the animals are described under the blots. Representative autoradiograms from eight independent experiments are shown. D to F, correlations between vocalization thresholds and phosphorylation of ERK, p38, and JNK in control (C), diabetic hyperalgesic (DH), and diabetic nonhyperalgesic (DNH) rats.

state in rats at an early stage of an experimental painful diabetic neuropathy. These cellular changes were not linked only to hyperglycemia, because diabetic, nonhyperalgesic rats failed to have such an increase. These data suggest that hyperglycemia is a necessary, but not sufficient, condition to obtain hyperalgesia, which is in accordance with the ability of insulin to relieve this symptom in both humans and animals with diabetes (Courteix et al., 1996). Thus, hyperglycemia is not the sole trigger for mechanical hyperalgesia, and

other downstream factors, such as MAPK activation, are needed to determine development of altered pain reactions during diabetes. However, the mechanisms by which hyperglycemia induced MAPK activation remain to be elucidated, and additional work will be required to verify the ability of insulin to reverse this cellular transduction pathway. Regarding clinical data, it would also be of great interest to understand these signaling cascades and their contribution to determine the factors of neuropathic pain sensitivity in diabetes disease. The involvement of MAPK phosphorylation in diabetes-induced mechanical hyperalgesia was confirmed by the suppression of this painful symptom in diabetic rats after 7-day treatment with well tolerated MAPK inhibitors. On the contrary, the acute administration of these inhibitors was without effect on pain thresholds (data not shown). However, in both the STZ and the chronic constriction injury models, Ciruela et al. (2003) reported that a single intrathecal administration of a MEK inhibitor 2 weeks after the induction of the neuropathy suppressed tactile allodynia and activation of ERK1/ERK2 30 min after treatment. This discrepancy may result from a different spatiotemporal kinetic of ERK activation according to the etiology or the symptomatology of the neuropathic pain models studied. The fact that MAPK inhibition had no immediate effect on hyperalgesia in our model of neuropathy suggests that long-lasting phenomena are involved after activation of ERK, p38, and JNK and contribute to the generation but also the maintenance of this painful symptom. The observed antihyperalgesic effects of the three MAPK inhibitors are compatible with a role for MAPK activation via a transcriptional regulation. However, the specific targets regulated by MAPK in the spinal cord after painful diabetic neuropathy remain largely unknown. A cascade involving ERK activation, CREB phosphorylation, induction of immediately early genes (e.g., *c-fos*, *Zif 268*, *Cox-2*), and induction of late response genes encoding neuropeptides (e.g., prodynorphin or neurokinin-1), and/or their receptors has been proposed (Ji et al., 2002a,b; Kawasaki et al., 2004). Activation of each element in this cascade could have a different time course. ERK activation seems to be an initial trigger, and persistent activation of ERK in dorsal horn neurons for several weeks may play a role in maintaining gene expression (Zhuang et al., 2005). p38 is also known to regulate long-term adaptive changes (hours to days) in expression of proteins or enzymes (e.g., cyclooxygenase 2) that are important for spinal sensitization (Lasa et al., 2000). Our findings demonstrate a similar implication of the three MAPKs in the suppression of diabetes-induced hyperalgesia, suggesting that they might have common downstream pathways. It is possible that the transcriptional regulation by the three isoforms leads to the altered expression of one or several similar genes involved in diabetes-induced hyperalgesia. Moreover, it cannot be excluded that MAPK activation may also contribute to changes by nontranscriptional means, probably via the phosphorylation of kinases, key receptors, and ion channels, leading to an increase of the neuronal excitability. The three major MAPKs phosphorylate their substrates on the consensus (T/S)P sequence, and it is possible that they could target some common substrates. Many potential substrates contain this motif and could thus be good candidates for the maintenance of pain hypersensitivity. Our results also report that the three MAPK cascades function in an independent-manner to maintain hyperalgesia

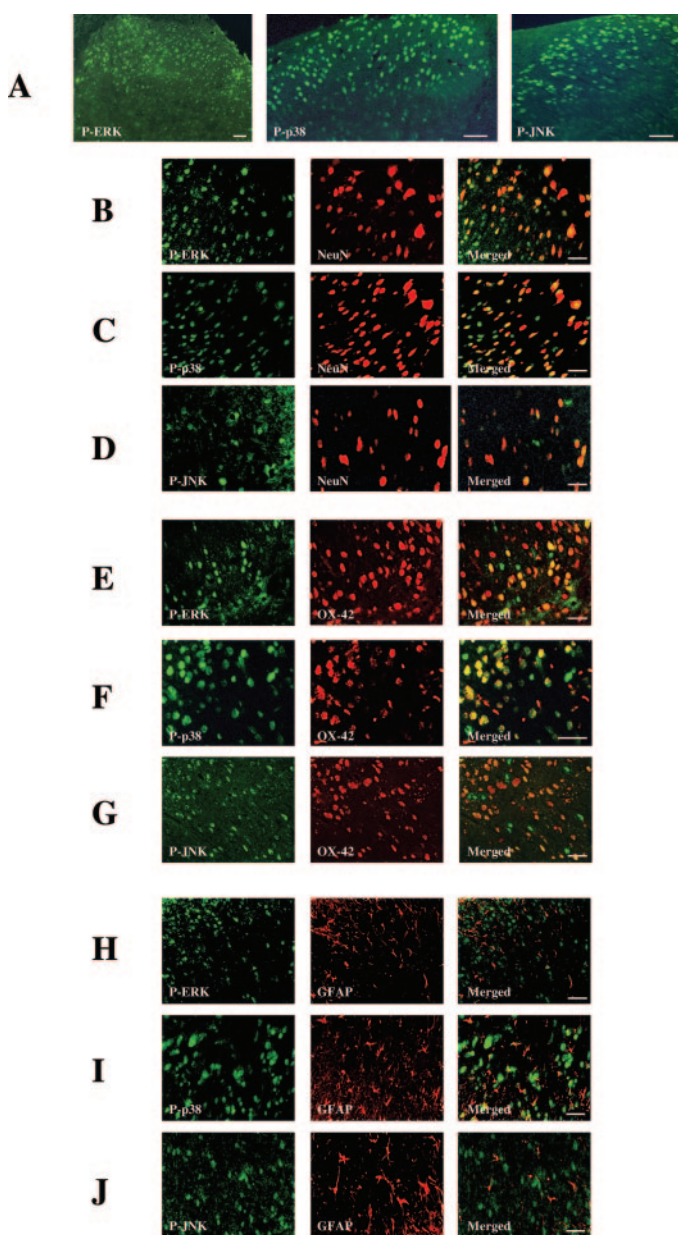


Fig. 3. Activated MAPKs were localized in neuronal and microglial cells in diabetic hyperalgesic rat spinal cord. A, immunocytochemistry with P-ERK, P-p38, and P-JNK antibodies indicates that increased P-MAPK immunoreactivity levels were observed predominantly in the superficial dorsal horn laminae of the spinal cord. Double immunofluorescence staining showed that spinal P-ERK (green), P-p38 (green), and P-JNK (green) are expressed in neurons and microglia because they were colocalized with NeuN (red, B–D) and OX-42 (red, E–G) in the spinal cord of 3-week-old diabetic hyperalgesic rats (merged overlap appears as yellow; $n = 4$ /group). Immunohistochemistry showed no colocalization between P-MAPKs (green) and GFAP (red, H–J), indicating that P-MAPKs were not expressed by astrocytes ($n = 4$ /group). Scale bars, 100 μ m.

at 3 weeks of diabetes, with no cross-talk between these pathways, because the inhibition of one MAPK pathway did not affect the other two. Two mechanisms prevent inappropriate cross-talk between the different MAPK modules

(Pouyssegur et al., 2002). First, scaffold proteins create multi-enzyme complexes that bring together components of a single kinase cascade. Second, specific docking sites on MAPKs that aid the binding of substrates, activators, and

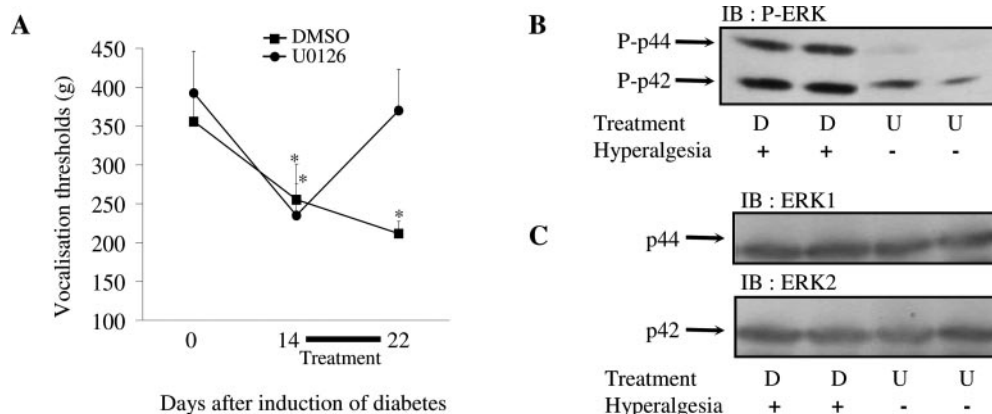


Fig. 4. Involvement of the ERK pathway in diabetes-induced mechanical hyperalgesia. A, vocalization thresholds measured before, 14 and 22 days after the induction of diabetes in rats treated from days 15 to 21 with intrathecal vehicle (DMSO; 10 μ l/rat) or MEK inhibitor (U0126; 5 μ g/rat). Values are mean \pm S.E.M. ($n = 4$ /group). *, $p < 0.05$ versus thresholds on day 0. B–C, representative immunoblots showed an increase in P-ERK1/P-ERK2 (B), but no change in total ERK1 and ERK2 proteins (C) in lumbar spinal cord homogenates from diabetic rats treated with DMSO (D) or U0126 (U). The treatments and hyperalgesic state of rats are indicated under the blots.

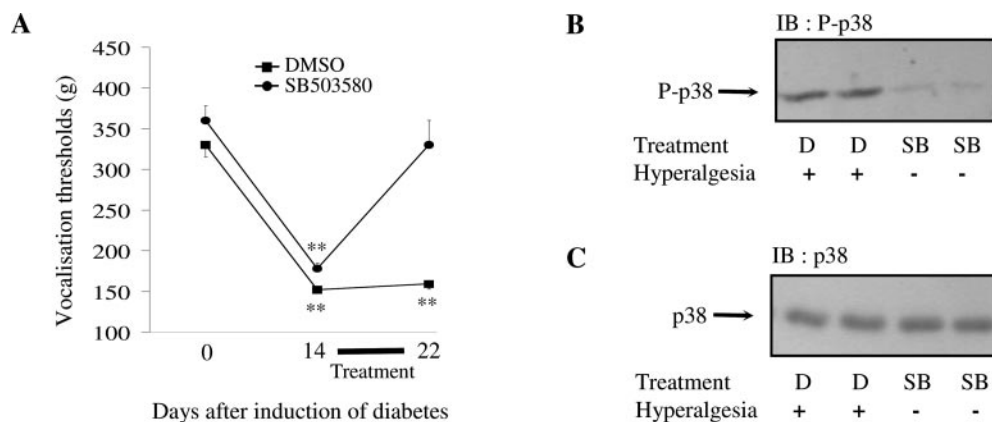


Fig. 5. Involvement of the p38 pathway in diabetes-induced mechanical hyperalgesia. A, vocalization thresholds measured before, 14 days after, and 22 days after the induction of diabetes in rats treated from days 15 to 21 with intrathecal vehicle (DMSO; 10 μ l/rat) or p38 inhibitor (SB503580; 5 μ g/rat). Values are mean \pm S.E.M. ($n = 6$ /group). **, $p < 0.01$ versus thresholds on day 0. B and C, representative immunoblots showed an increase in P-p38 (B) but no change in total p38 protein (C) in lumbar spinal cord homogenates from diabetic rats treated with DMSO (D) or SB503580 (SB). The treatments and hyperalgesic state of rats are indicated under the blots.

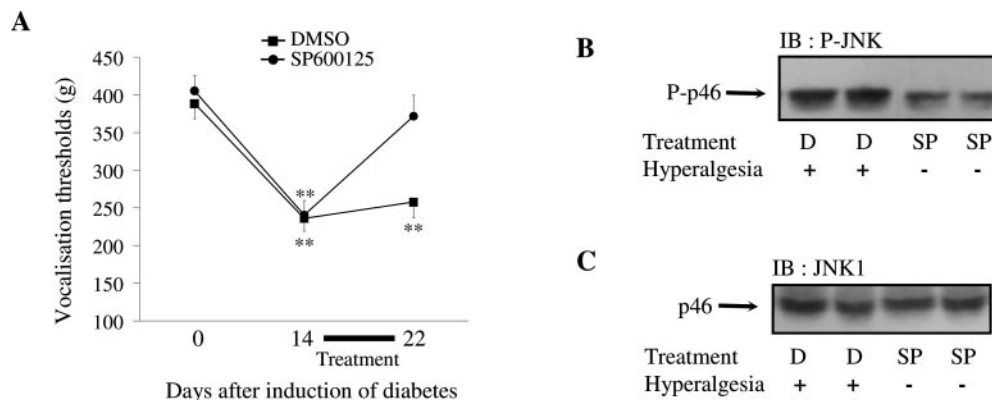


Fig. 6. Involvement of the JNK pathway in diabetes-induced mechanical hyperalgesia. A, vocalization thresholds measured before, 14 days after, and 22 days after the induction of diabetes in rats treated from days 15 to 21 with intrathecal vehicle (DMSO; 10 μ l/rat) or JNK inhibitor (SP600125; 5 μ g/rat). Values are mean \pm S.E.M. ($n = 6$ /group). **, $p < 0.01$ versus thresholds on day 0. B and C, representative immunoblots showed an increase in P-JNK1 (B), but no change in total JNK1 protein (C) in lumbar spinal cord homogenates from diabetic rats treated with DMSO (D) or SP600125 (SP). The treatments and hyperalgesic state of rats are indicated under the blots.

regulators increase the fidelity and the efficiency of the enzymatic reactions.

Spinal MAPK Activation in Neurons and Microglia. Immunohistochemistry studies performed at 3 weeks after diabetes induction revealed that spinal enhanced MAPK immunoreactivities were observed mainly in the superficial dorsal horn-laminae and were present in NeuN- and OX-42-positive cells, which are specific markers of neuronal and microglial cells, respectively. Although hyperalgesia was originally thought to result exclusively from altered neuronal activity in the primary sensory and spinal cord neurons, evidence indicates that glial cells may also play a role in the pathogenesis of pain, probably via the release of neuroactive factors including prostanooids and cytokines (Watkins et al., 1997, 2001). Microglia is activated in the spinal cord in cancer pain models (Mantyh et al., 2002) or after nerve injury (Winkelstein et al., 2001), and neuropathic pain is reduced by minocycline, a microglial inhibitor (Raghavendra et al., 2003). p38 in microglia have been implicated in the generation of mechanical allodynia and thermal hyperalgesia observed in inflammatory (Svensson et al., 2003a,b) and nerve injury pain models (Jin et al., 2003). On the other hand, spinal nerve ligation induces ERK activation in microglia (Zhuang et al., 2005). A crucial question is the nature of communication between sensory neurons and microglia. It is difficult to determine whether microglial and/or neuronal MAPKs are responsible for the effects seen in this study. The activation of microglial MAPKs might represent a direct effect of glutamate, released from the afferent terminals and binding to NMDA receptors present on microglia. But it is also possible that the activation of MAPKs in microglia is an indirect effect of neuronal NMDA receptor activation, mediated by factors released from neurons upon stimulation.

NMDA Receptors Involved Upstream of MAPK Activation in Painful Diabetic Hyperalgesia. Multiple transmitter receptors, such as ionotropic NMDA receptors, non-NMDA glutamate receptors, and G-protein-coupled receptors, are coupled to MAPK activation in the superficial laminae of the spinal cord. The NMDA receptor functions as a Ca^{2+} channel, and glutamate signaling through this receptor induces phosphorylation of p38 in primary neuronal cultures (Chen et al., 2003) and in dorsal horn microglia (Svens-

son et al., 2003a,b). Moreover, inhibition of p38 attenuates NMDA-evoked release of prostaglandin E2 and the resulting hyperalgesia (Svensson et al., 2003a). Because hyperalgesia caused by diabetic neuropathy is supported by pharmacological studies implicating glutamatergic pathways (Malcangio and Tomlinson, 1998; Begon et al., 2000), we investigated whether the NMDA receptor might be involved upstream of spinal MAPK activation in diabetic hyperalgesia. Our results indicate that blockade of the NMDA receptor with the MK801 antagonist administered by intrathecal route suppressed hyperalgesia and decreased ERK, JNK, and p38 phosphorylation in the same diabetic rats. This suggests that NMDA receptor activation is needed to induce spinal MAPK phosphorylation and consequently hyperalgesia. This conclusion is reinforced by the observation of a similar degree of antihyperalgesia and enzyme inhibition by both NMDA antagonists and MAPK inhibitors (55.8 and 54.7% vocalization threshold increase and 76.1 and 62.8% enzyme inhibition for MK801 and JNK inhibitor, respectively). Previous studies found that phosphorylation of ERK is partially inhibited by NMDA antagonists after C-fiber stimulation induced by electrical stimulation or application of capsaicin whereas blockade of both NMDA and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors totally suppressed activation of ERK (Kawasaki et al., 2004). Calcium entry through the NMDA receptor ion channel or other receptors may be responsible for initiating activation of the three MAPK pathways in our painful diabetic neuropathy. Several intermediates depending on calcium, such as nitric oxide synthesis (Yun et al., 1999), protein kinase C, calmodulin kinase (Fukunaga and Miyamoto, 1998), or the proline-rich tyrosine kinase 2 (Lev et al., 1995), a member of the Src-family protein tyrosine kinases, may be good candidates to participate in the activation of MAPK in our model of diabetic neuropathy.

In conclusion, we provide for the first time substantive evidence that a cascade initiated by NMDA ionophore activation, leading to diabetic hyperalgesia, is dependent on the phosphorylation of the three MAPK isoforms in both spinal neurons and microglial cells. Our results add further support to the hypothesis that glial cells play an important role in the genesis of diabetic neuropathic pain and cooperate with neurons to regulate its development. It is likely that MAPKs

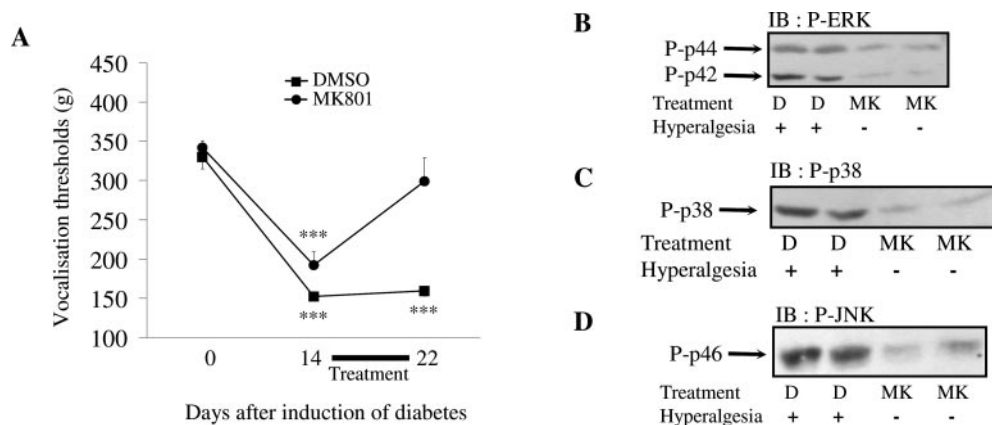


Fig. 7. Involvement of NMDA receptor in diabetic hyperalgesia-induced MAPKs activation. A, vocalization thresholds measured before, 14 days after, and 22 days after the induction of diabetes in rats treated from days 15 to 21 with intrathecal vehicle (DMSO; 10 $\mu\text{l}/\text{rat}$) or NMDA receptor antagonist (MK801; 5 $\mu\text{g}/\text{rat}$). Values are mean \pm S.E.M. ($n = 7/\text{group}$). ***, $p < 0.001$ versus thresholds on day 0. B and C, representative immunoblots showed an increase in P-ERK1/P-ERK2 (B), P-p38 (C), and P-JNK1 (D) in lumbar spinal cord homogenates from diabetic rats treated with DMSO (D) or MK801 (MK). The treatments and hyperalgesic state of rats are indicated under the blots.

mediate alterations in gene expression and then contribute to the establishment and maintenance of this neuropathic pain. Thus, MAPK pathways play a significant role in spinal sensitization processing induced by diabetes and represent potential targets for pain therapy of these painful metabolic neuropathies.

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