Diabetes-induced mechanical hyperalgesia involves spinal MAPKs activation in neurons and microglia via NMDA-dependent mechanisms

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List of Abbreviations:
CCI : Chronic constriction injury ; COX-2 : Cyclooxygenase-2 ; DRG : Dorsal Root Ganglion ; ERK : Extracellular signal-regulated protein kinase ; GFAP : Glial fibrillary acidic protein ; i.t. : Intrathecal ; JNK : c-Jun N-terminal kinase ; MAPKs : Mitogen-activated protein kinases ; NeuN : Neuronal specific nuclear protein ; NMDA : N-Methyl D-Aspartate ; NK-1 : Neurokinin-1 ; NO : Nitric oxyde ; PKC : Protein kinase C ; PYK2 : Proline-rich tyrosine kinase 2 ; SNL : Spinal nerve ligation ; STZ : Streptozotocin.
Molecular mechanisms underlying diabetes-induced painful neuropathy are poorly understood. In the streptozotocin-induced diabetic rats, we have demonstrated that mechanical hyperalgesia, a common symptom of diabetic neuropathy, was correlated with an early increase of ERK, p38 and JNK phosphorylation in the spinal cord and dorsal root ganglion at three weeks of the diabetes. This change was specific of hyperalgesia because non hyperalgesic rats failed to have such an increase. Immunoblots 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 MAPKs colocalized both with the neuronal (NeuN) and microglial (OX42) cell-specific markers but not with the astrocyte marker (GFAP) in the superficial dorsal horn-laminae of the spinal cord. In these same rats, a seven-day administration (5 µg/rat/day, i.t.) of U0126, SB203580 and SP600125, which inhibit MEK, 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 seven days by MK801 (5 µg/rat/day, i.t.), a non-competitive 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 chronic maintenance of painful diabetic hyperalgesia and that these kinases represent potential targets for pain therapy.
INTRODUCTION

Peripheral sensitive neuropathies represent a common and debilitating complication of diabetes (type 1 and 2) and affect an increasing proportion of diabetic patients as their disease progress. Even though antidepressants and antiepileptics have been shown to be partially effective, clinical studies have reported the difficulty of managing pain due to 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). While 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 still 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 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 been classically 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. Chronic treatment of STZ-induced diabetic rats with a p38 inhibitor prevents neuronal dysfunction such as the classical 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, 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 MAPKs activation. In addition, the status of the three MAPKs isoforms has never been studied simultaneously in the model of diabetic neuropathy. In view of that, the present study performed in a STZ-induced model of diabetes firstly investigated whether 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 if this activation contributes to the mechanical hyperalgesia developed by diabetic rats.

In the chronic context of diabetes-induced hyperalgesia, the upstream effectors of spinal MAPKs 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. An other aim of our study was therefore to determine the link between the NMDA receptor, MAPKs activation and hyperalgesia in diabetic rats.
MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley rats (Charles River, France), initially weighting 201-225 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 IASP (Zimmermann, 1983).

2. Induction of diabetes

Rats were rendered diabetic with an intraperitoneal (i.p.) injection of STZ (75 mg/kg) (Zanosar®, Pharmacia, France) dissolved in distilled water. Diabetes was confirmed 2 and 3 weeks later by measurement of tail vein blood glucose levels with Glucotide® and a reflectance colorimeter (Glucometer® 4, Bayer Diagnostics, 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).

3. Behavioral studies

3.1. 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 previously described (Randall and Selitto, 1957). Nociceptive thresholds, expressed in grams, were measured using an Ugo Basil analgesimeter (Bioseb, France) by applying increasing pressures to the left hind paw until a
squeak (vocalisation threshold) was elicited. The test was repeated until three stable thresholds values were obtained. The maximal pressure was set at 450 g.

### 3.2. Experimental design

To correlate the painful status of animals and the expression of phosphorylated forms of MAPKs, rats were submitted 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 MAPKs 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 chronic treatment by intrathecal (i.t.) route with MAPKs inhibitors (5 µg/rat/day) was performed in these rats according to 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 µg/rat/day, i.t.) has been determined after preliminary data and according to previous studies performed in diabetic rats showing the need of chronic treatment to reverse hyperalgesia (Malcangio et al., 1998; Begon et al., 2000). Therefore, the diabetic hyperalgesic rats were intrathecally treated with the different drugs or the vehicle from day 15 to day 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 vocalisation thresholds were increased by 25 % compared to nociceptive thresholds measured before treatment (at day 14) were considered as having 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.

### 3.3. Drugs and intrathecal administration

U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene), SB503580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) and SP600125 (Anthra[1,9-cd]pyrazol-6(2H)-one) were purchased from Biomol (France). The NMDA receptor antagonist MK801 ((5R,10S)-(+)5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5-10-imine hydrogen maleate) was obtained from Sigma (France). Intrathecal injections of drugs or vehicle (saline containing 10 % dimethylsulfoxide) were performed under isoflurane anaesthesia (4 % induction, 2 % maintenance), as previously described (Mestre et al., 1994). Briefly, the anaesthetised rat was held in one hand by the pelvic girdle and a 25-gaugeX1-inch needle connected to a 25 µl Hamilton syringe was inserted into the subarachnoidal 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 µl/rat. Intrathecal injection of vehicle had no effect on nociceptive thresholds/behavior, protein expression and protein phosphorylation.

### 4. Immunoblotting analysis

Spinal cord enlargements and DRG, homogenized in 400 µl of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM Na₄P₂O₇, 2 mM orthovanadate, 100 mM NaF, 1 % Triton X-100, 0.5 mM phenylmethylsulfonylfluoride, 20 µM leupeptin, 100 IU/ml aprotinin, Sigma, France), were incubated for 20 min at 4°C and then centrifuged at 16,000 g for 15 min. The protein concentration of tissue lysates was determined with a BCA Protein Assay Kit (Interchim, France). Proteins were then electrophoresed in 10 % SDS
polyacrylamide gels and transferred to nitrocellulose membrane (BioTrace NT, France). Membranes were blocked 1 h at room temperature (RT) with TBS/T (10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 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, France ; anti-phospho-ERK, phospho-p38, 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 non phosphorylated MAPKs, monoclonal, anti-mouse, 1:2000, Cell Signaling Technology, Beverly, MA). The blots were washed three times in TBS/T and incubated for 1 h at RT with HRP-conjugated secondary antibody (Pierce, Rockford, IL), developed in ECL solution (SuperSignal West Pico Chemiluminescent Substrate, Pierce) for 5 min and exposed onto BioMax hyperfilms (Kodak, France) for 1-30 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.

5. Immunohistochemistry

Three weeks-aged diabetic hyperalgesic rats were perfused transcardially with 4 % paraformaldehyde in 0.1 M PBS, pH 7.4. The lumbar spinal cords were removed, postfixed in the same fixative for 1 h at 4°C, deshydrated 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 10 min in a solution containing 3 % H2O2 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, Beverly, MA) 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 FITC- and rhodamine-conjugated secondary antibody (anti-rabbit or anti-mouse, 1:100, FluoProbes, France) for 2 h at RT. Non-specific staining was determined by excluding the primary antibodies. Images were captured using a Nikon microscope equipped for epifluorescence (Tokyo, Japan) operated by a Lucia® Software.

6. Statistical analysis

Three to eight rats were included for each group for quantification of immunoblots and behavioral studies. Data are expressed as mean ± S.E.M. Differences between groups were compared by a two-way analysis of variance (ANOVA) followed by either a Dunnett's test to analyse the time course of the effect of different treatments on vocalisation 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

1. 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 to 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 chronic treatment with the vehicle, selective MAPKs inhibitors or the NMDA receptor antagonist, demonstrating that these treatments had no effect on this parameter.

The analysis of the pain behavior showed a significative reduction (41.4 ± 1.5 %, p < 0.01) in the vocalisation thresholds two 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 three weeks after the induction of diabetes (week 3 : 198.3 ± 12 g). No variation in the thresholds were observed in 27.2 % of the diabetic rats and in control animals two and three 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, 249 ± 6 g and 284.1 ± 4.2 g two weeks after injection of STZ or distilled water, respectively. General health state of the animals have been considered. Loss of more than 10 % of the initial body weight, loss of activity and piloerection were criteria that justify the removal of diabetic animals. Thus, hyperalgesia observed in STZ-induced diabetic rats was specific to diabetes and not due to the general health state of the animals.
2. Spinal MAPKs involvement in diabetes-induced hyperalgesia

2.1. Expression of phosphorylated forms of MAPKs

We first investigated whether the phosphorylation of MAPKs isoforms was modified at three 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, that detect ERK, p38 and JNK only when activated by dual phosphorylation, revealed an increased phosphorylation of MAPKs in the spinal cord (Fig. 1A-C; upper panels) and in DRG (Fig. 2A-C; upper panels) of diabetic hyperalgesic rats compared to control or diabetic non hyperalgesic rats. Phospho-ERK and phospho-p38 were not detectable in the spinal cord of control and diabetic non hyperalgesic rats (Fig. 1A-B; upper panels) while the three phosphorylated MAPKs were below detection levels in the DRG (Fig. 2A-C; upper panels) of the same animals, even if membranes are autoradiographed for a longer period of time. These results suggest that very low levels of phosphorylated forms of MAPKs were present in control and diabetic non hyperalgesic rats. In the spinal cord, densitometric quantification of JNK immunoblots showed a significant 6.13 fold ± 0.5 and 5.5 ± 0.2 increase (p < 0.001) in phosphorylation of the kinase in diabetic hyperalgesic rats compared to control and diabetic non hyperalgesic animals, respectively (Fig. 1C; upper panel). 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. 1D-F) and in DRG (P-ERK : r= 0.80 ; P-p38 : r=0.75 ; P-JNK : r=0.85 ; p < 0.0001 ; Fig. 2D-F). No variation of total ERK1/ERK2, p38 or JNK1 protein expression was observed in the spinal cord (Fig. 1A-C; lower panels) and in DRG (Fig. 2A-C; lower panels), 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 of hyperalgesia and not directly due to metabolic alterations of diabetes.

2.2. 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 three weeks-aged diabetic hyperalgesic rats, double immunofluorescence staining of phospho-MAPKs was performed with several cell-specific markers: neuronal specific nuclear protein (NeuN) for neurons, glial fibrillary acidic protein (GFAP) for astrocytes and OX-42 (CD11b) for microglia. The three MAPKs in their activated sated were expressed in neuronal (Fig. 3B-D) and microglial (Fig. 3E-G) cells since they colocalized with NeuN and OX-42, respectively. However, the three MAPKs do not appear to be expressed in astrocytes, as no colocalization could be detected between theses isoforms and GFAP (Fig. 3H-J).

2.3. Influence of MAPKs inhibition on diabetes-induced hyperalgesia

To determine the role of spinal MAPKs 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 chronic intrathecal treatment (5 µg/rat/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 ± 0.3 % (from 235.0 ± 40.9 g to 370.0 ± 52.9 g; p < 0.05), 78.8 ± 3.9 % (from 185.0 ± 6.5 g to 330.8 ± 32.2 g; p < 0.01) and 54.7 ± 0.4 % (from 240.0 ± 19.8 g to 371.3 ± 28.1 g; p < 0.01), respectively. The chronic administration of DMSO, used as vehicle, failed to induce any
change in pain scores. Nociceptive thresholds obtained after a seven-day inhibition of MEK, p38 or JNK were not significantly different from pre-diabetes 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 MAPKs pathways participate in mechanical hyperalgesia observed in diabetic rats (Fig. 4-6A).

Immunoblots analysis were subsequently carried out on the lumbar spinal cord of the vehicle and MAPK inhibitor-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 ± 4.0 %, respectively, compared to the phosphorylation measured in the vehicle-treated rats. No significant difference in the expression of total ERK1/ERK2 (ERK1: 91.6 ± 5.7 %; ERK2: 108.6 ± 5.4 %; Fig. 4C), p38 (113.2 ± 6.3 %; Fig. 5C) or JNK1 (96.0 ± 5.1 %; Fig. 6C) proteins was observed in rats treated with respectively the MEK, p38 or JNK inhibitor compared to vehicle-treated animals, suggesting a post-translational regulation of the corresponding proteins. Then, the specificity of the three MAPKs 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 to that measured in vehicle-treated rats (after U0126 treatment: P-p38: 91.7 ± 8.3 %; P-JNK1: 110.5 ± 13.8 % versus after vehicle treatment).

SB503580 is specific to the p38 pathway since the phosphorylation of ERK1/ERK2 and JNK1 was not different in vehicle and SB503580-treated rats (after SB503580 treatment: P-ERK1: 92.1 ± 13.6 %; P-ERK2: 88.1 ± 14.1 %; P-JNK1: 127.1 ± 16.8 % versus after vehicle treatment). In addition, the JNK inhibitor was specific of this signaling pathway since
it did not affect ERK and p38 phosphorylation (after SP600125 treatment: P-ERK1: 121.1 ± 28.8 %; P-ERK2: 89.6 ± 12.0 %; P-p38: 112.4 ± 27.5 % versus after vehicle treatment).

3. Involvement of the NMDA receptor in diabetic hyperalgesia-induced MAPKs activation

As the NMDA receptor was involved in diabetic hyperalgesia (Malcangio et al., 1998; Begon et al., 2000), we examined the effect of MK801, a non-competitive NMDA receptor antagonist, on both the vocalisation thresholds and the phosphorylation of MAPKs in diabetic hyperalgesic rats. Intrathecal administration of MK801 (5 µg/rat/day for 7 days) significantly increased (p < 0.001) nociceptive thresholds by 55.8 ± 0.7 % from 192.0 ± 17.4 g to 299.2 ± 29.6 g (Fig. 7A) and led to pain scores not significantly different from pre-diabetes ones (299.2 ± 29.6 g versus 342.0 ± 8.8 g). No change in hyperalgesia was observed after the injection of the vehicle and no side-effects like stereotypies and/or motor dysfunction was 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 ± 3.9 %, respectively, compared to the phosphorylation measured in vehicle-treated rats (Fig. 7B-D).
DISCUSSION

MAPKs 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 state in rats, at an early stage of an experimental painful diabetic neuropathy. These cellular changes were not only linked to hyperglycemia because diabetic non hyperalgesic rats failed to have such an increase. These data suggest that hyperglycemia is a necessary, but not sufficient, condition to obtain hyperalgesia, which agree with the ability of insulin to relieve this symptom both in diabetic humans and animals (Courteix et al., 1996). Thus, hyperglycemia is not the sole trigger for mechanical hyperalgesia and other downstream factors, like MAPKs activation, are needed to determine development of altered pain reactions during diabetes. However, the mechanisms by which hyperglycemia induced MAPKs activation remains 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 be also of a great interest to understand these signaling cascades and their contribution to determine the factors of neuropathic pain sensitivity in diabetes disease. The involvement of MAPKs phosphorylation in diabetes-induced mechanical hyperalgesia was confirmed by the suppression of this painful symptom in diabetic rats after seven days-treatment with well-tolerated MAPKs inhibitors. On the contrary, the acute administration of these inhibitors was without effect on pain thresholds (not shown observation). However, in both the STZ and the chronic constriction injury (CCI) models, Ciruela et al. 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 (Ciruela et al., 2003). This discrepancy may result from a
different spatio-temporal kinetic of ERK activation according to the etiology or the symptomatology of the neuropathic pain models studied. The fact that MAPKs inhibition had no immediate effect on hyperalgesia in our model of neuropathy suggests that long lasting phenomenon 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 MAPKs inhibitors are compatible with a role of MAPKs activation via a transcriptional regulation. However, the specific targets regulated by MAPKs in the spinal cord after painful diabetic neuropathy remain largely unknown. Recently, it has been proposed a cascade that involves ERK activation, CREB phosphorylation, induction of immediately early genes, e.g. c-fos, Zif 268, Cyclooxygenase 2 (Cox-2), and induction of late response genes encoding neuropeptides, e.g. prodynorphin or NK-1, and/or their receptors (Ji et al., 2002a,b; Kawasaki et al., 2004). Activation of each element in this cascade could have different time course. ERK activation appears 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 adaptative changes (hours to days) in expression of proteins or enzymes, e.g. Cox-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 MAPKs activation may also contribute to changes by non transcriptional 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 thus could be good candidates for the maintenance of pain hypersensitivity. Our results also report that the three MAPKs cascades function in an independent-manner to maintain hyperalgesia at three weeks of diabetes, with no cross-talk between these pathways, since the inhibition of one MAPK pathway did not affect the two other ones. Two mechanisms prevent inappropriate cross-talk between the different MAPKs 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 which serve for the binding of substrates, activators and regulators increase the fidelity and the efficiency of the enzymatic reactions.

**Spinal MAPKs activation in neurons and microglia**

Immunohistochemistry studies performed at three weeks of diabetes revealed that spinal enhanced MAPKs immunoreactivities were mainly observed 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, there is evidence indicating that glial cells may also play a role in the pathogenesis of pain, probably via the release of neuroactive factors including prostanoids 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 MAPKs activation in painful diabetic hyperalgesia**

Multiple transmitter receptors, as ionotropic NMDA, non-NMDA glutamate receptors and G-protein coupled receptors, are coupled to MAPKs 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 (Svensson et al., 2003a,b). Moreover, inhibition of p38 attenuates NMDA-evoked release of prostaglandin E2 and the resulting hyperalgesia (Svensson et al., 2003a). As hyperalgesia due to diabetic neuropathy is supported by pharmacological studies implicating glutamatergic pathways (Malcangio and Tomlinson, 1998; Begon et al., 2000), we investigated if the NMDA receptor may be involved upstream of spinal MAPKs 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 MAPKs phosphorylation and consequently hyperalgesia. This conclusion is reinforced by the observation of a similar degree of antihyperalgesic effect and enzyme inhibition by both NMDA antagonist and MAPKs inhibitors (e.g. 55.8% and 54.7% of vocalisation thresholds
increase, 76.1% and 62.8% of 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 AMPA 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 MAPKs pathways in our painful diabetic neuropathy. Several intermediates depending on calcium such as NO synthesis (Yun et al., 1999), PKC or calmodulin kinase (Fukunaga and Miyamoto, 1998) or the protein tyrosine kinase PYK2 (Lev et al., 1995), a member of the Src-family protein tyrosine kinases, may be good candidates to participate in the activation of MAPKs 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 MAPKs 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 mediate alterations in gene expression and then contribute to the establishment and chronic maintenance of this neuropathic pain. Thus, MAPKs 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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LEGENDS

**Fig. 1** : Spinal MAPKs 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 weeks-aged diabetic hyperalgesic rats compared with control and diabetic non hyperalgesic animals (A-C, upper panels). The lower panels (A-C) indicate 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 8 independent experiments are shown. (D-F) Correlations between vocalisation thresholds and phosphorylation of ERK, p38 and JNK in control (C), diabetic hyperalgesic (DH) and diabetic non hyperalgesic (DNH) rats.

**Fig. 2** : MAPKs 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 weeks-aged diabetic hyperalgesic rats compared with control and diabetic non hyperalgesic animals (A-C, upper panels). The lower panels (A-C) indicate 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 8 independent experiments are shown. (D-F) Correlations between vocalisation thresholds and phosphorylation of ERK, p38 and JNK in control (C), diabetic hyperalgesic (DH) and diabetic non hyperalgesic (DNH) rats.
**Fig. 3: Activated MAPKs were localized in neuronal and microglial cells in diabetic hyperalgesic rat spinal cord**

(A) Immunochemistry with P-ERK, P-p38 and P-JNK antibodies indicates that increased P-MAPKs 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 since they were colocalized respectively with NeuN (red, B-D) and OX-42 (red, E-G) in the spinal cord of three weeks-aged 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 µm.

**Fig. 4: Involvement of the ERK pathway in diabetes-induced mechanical hyperalgesia**

(A) Vocalisation thresholds measured before, 14 and 22 days after the induction of diabetes in rats treated from day 15 to day 21 with intrathecal vehicle (DMSO; 10 µl/rat) or MEK inhibitor (U0126; 5 µg/rat). Values are mean ± SEM (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) Vocalisation thresholds measured before, 14 and 22 days after the induction of diabetes in rats treated from day 15 to day 21 with intrathecal vehicle (DMSO; 10 µl/rat) or p38 inhibitor (SB503580; 5 µg/rat). Values are mean ± SEM (n = 6/group). ** p < 0.01 versus thresholds
on day 0. (B-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) Vocalisation thresholds measured before, 14 and 22 days after the induction of diabetes in rats treated from day 15 to day 21 with intrathecal vehicle (DMSO ; 10 µl/rat) or JNK inhibitor (SP600125 ; 5 µg/rat). Values are mean ± SEM (n = 6/group). ** p < 0.01 versus thresholds on day 0. (B-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.

**Fig. 7 : Involvement of NMDA receptor in diabetic hyperalgesia-induced MAPKs activation**

(A) Vocalisation thresholds measured before, 14 and 22 days after the induction of diabetes in rats treated from day 15 to day 21 with intrathecal vehicle (DMSO ; 10 µl/rat) or NMDA receptor antagonist (MK801 ; 5 µg/rat). Values are mean ± SEM (n = 7/group). *** p < 0.001 versus thresholds on day 0. (B-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.
Fig. 2

A

IB : P-p38
IB : p38

Hyperglycemia - - +          +         +         +
Hyperalgesia - - +          +         - -

B

IB : P-p46
IB : JNK1
IB : ERK1
IB : ERK2

Hyperglycemia - - +          +         +           +
Hyperalgesia - - +          +         - -

C

IB : P-ERK

Hyperglycemia - - +          +         +         +
Hyperalgesia - - +          +         - -

D

E

F

Vocalisation thresholds (g)

Densitometric analysis (arbitrary units)
Fig. 3

A

P-ERK | P-p38 | P-JNK

B

P-ERK | NeuN | Merged

C

P-p38 | NeuN | Merged

D

P-JNK | NeuN | Merged

E

P-ERK | OX-42 | Merged

F

P-p38 | OX-42 | Merged

G

P-JNK | OX-42 | Merged

H

P-ERK | GFAP | Merged

I

P-p38 | GFAP | Merged

J

P-JNK | GFAP | Merged
Fig. 4

(A) Vocalisation thresholds (g) against days after induction of diabetes. The graph shows the differences between DMSO (black line) and U0126 (red line) treatments.

(B) Western blot analysis showing P-ERK, P-p44, and P-p42 with treatment D (DMSO) and D (U0126). Hyperalgesia is indicated as + or -.

(C) Western blot analysis showing ERK1 and ERK2 with treatment D (DMSO) and D (U0126). Hyperalgesia is indicated as + or -.
Days after induction of diabetes

A B

IB: p38

<table>
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<th>D</th>
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C

IB: p38

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<tr>
<td>Hyperalgesia</td>
<td>+</td>
<td>+</td>
<td>-</td>
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Fig. 5

Vocalisation thresholds (g)

A

DMSO
SB503580
Days after induction of diabetes

Vocalisation thresholds (g)

DMSO
SB503580

Days after induction of diabetes
Fig. 6

A

Vocalisation thresholds (g)

Days after induction of diabetes

DMSO
SP600125

**

B

P-p46

Treatment
D  D  SP  SP
Hyperlgesia  +  +  -  -

C

IB : JNK1

p46

Treatment
D  D  SP  SP
Hyperlgesia  +  +  -  -

**
Fig. 7

A

B

C

D

Vocalisation thresholds (g)

0 14 22

Days after induction of diabetes

DMSO
MK801

P-p44
P-p42

Treatment

Hyperalgesia

+ + - -

IB : P-ERK

P-p38

IB : P-p38

P-p46

IB : P-JNK

***

***

***

0 14 22

Treatment

Hyperalgesia

+ + - -

+ + - -

+ + - -