A role for mitogen-activated protein kinases in the etiology of diabetic neuropathy

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ABSTRACT **The onset of diabetic neuropathy, a complication of diabetes mellitus, has been linked to poor glycemic control. We tested the hypothesis that the mitogen-activated protein kinases (MAPK) form transducers for the damaging effects of high glucose. In cultures of adult rat sensory neurons, high glucose activated JNK and p38 MAPK but did not result in cell damage. However, oxidative stress activated ERK and p38 MAPKs and resulted in cellular damage. In the dorsal root ganglia of streptozotocin-induced diabetic rats (a model of type I diabetes), ERK and p38 were activated at 8 wk duration, followed by activation of JNK at 12 wk duration. We report activation of JNK and increases in total levels of p38 and JNK in sural nerve of type I and II diabetic patients. These data implicate MAPKs in the etiology of diabetic neuropathy both via direct effects of glucose and via glucose-induced oxidative stress.—Purves, T., Middlemas, A., Agthong, S., Jude, E. B., Boulton, A. J. M., Fernyhough, P., Tomlinson, D. R. A role for mitogen-activated protein kinases in the etiology of diabetic neuropathy.** ***FASEB J.*** **15, 2508–2514 (2001)**

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Symmetrical neuropathies of the sensory and autonomic nervous systems affect an increasing proportion of diabetic patients as their disease progresses (1). At 20 years of diabetes, the incidence of neuropathy may be as high as 50% of the type I diabetic population (2). Incidence and severity of neuropathy are increased by poor control of glycemia (3), indicating that excess glucose may be the biochemical trigger in pathogenesis. The pathology of neuropathies is multifocal with changes in axons, Schwann cells, microvascular elements in the endoneurium, and extracellular matrix (4–6). However, a generic component is a change in the phenotype of the above cells, presumably induced by hyperglycemia. Thus, a critical feature of the etiology of neuropathies is the mechanism by which raised extracellular glucose alters the pattern of gene expression that constitutes cell phenotype.

The effects of glucose may be primary or secondary via the polyol pathway, oxidative stress, protein glycation, or other unidentified consequences of hyperglycemia (5, 7, 8). The potential for a pivotal role for oxidative stress in the etiology of diabetic neuropathies was indicated some time ago based on functional measures associated with antioxidant treatment (9–12). Our proposal extends this to take account of the need to explain the alteration in cellular phenotype that is certainly a feature of neuropathies and may be the critical stage in the other complications.

Activation of the mitogen-activated protein kinases (MAPK) offers a link between these events (13). All three groups of MAPK can be activated by osmotic perturbations derived from glucose itself or from the polyol pathway (14, 15), by oxidative stress (16), and by advanced glycation end products (AGE) via the receptors for AGE (RAGE) (13, 17). There are three distinct subfamilies of MAPKs: extracellular signal-regulated kinases (ERK), c-Jun NH_2-terminal kinases (JNK), and p38 kinases. Through regulation of transcription factor activation, MAPKs play a central role in diverse cellular responses including survival, growth, differentiation, maintenance of phenotype, and death (18). The MAPKs respond to mitogens and growth factors and are implicated in growth and survival; they also respond to stress stimuli (e.g., UV, oxidants, cytokines), may be causal in apoptosis, and can be termed stress-activated protein kinases (SAPKs).

Other work implicates MAPKs in the etiology of diabetic complications. High glucose levels phosphorylate ERKs in rat glomeruli and mesangial cells (19, 20), p38 MAPK in cultured vascular cells, and aorta derived from diabetic rats (21). RAGE activation by AGE binding activates MAPKs in PC12 and rat pulmonary artery smooth muscle cells (17, 22, 23). Oxidative stress is a potent activator of MAPKs in various cell types (24–29). In this study, cultures of adult rat dorsal root ganglion (DRG) neurons were used to examine neuronal MAPK signaling pathways in response to different diabetes-related cellular stressors. We have also examined activation of MAPKs in vivo in DRG from 8 and 12 wk streptozotocin-induced diabetic rats and sural nerve from type I and type II diabetic patients. The data

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presented here implicate MAPK signaling in the etiology of diabetic neuropathy.

MATERIALS AND METHODS

Sensory neuron cultures

DRG from adult male Wistar rats (300 g) were dissociated using a previously described method (30). The cells were plated onto poly ornithine-laminin-coated 3.5 cm dishes or 48-well plates and cultured in serum-free F12 medium with N2 additives (0.1 mg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, and 1 mg/ml BSA), cytosine arabinoside (0.01 mM), and 10 pM insulin at 37°C in a 95% air/5% CO_2 humidified incubator (all additives were from Sigma, Poole, UK; culture medium was from Life Technologies, Paisley, UK). Cells were cultured overnight, starved of insulin (4 h), and treated. Cultures were pretreated with inhibitors (1 h).

Experimental diabetes

Male Wistar rats (300 g) were made diabetic by single intraperitoneal injection of streptozotocin (STZ, 55 mg/kg) (Sigma). Tail blood glucose was assayed 3 days after injection using glucose test strips (BM-Accutest®, Roche Diagnostics, Basel, Switzerland) to confirm diabetes. Rats were maintained for 12 wk with free access to water and chow. Rats were decapitated and exsanguinated, and L4 and L5 DRG were removed and snap frozen in liquid nitrogen. At death, plasma glucose levels were 34.5 ± 3.05 mmol/l for diabetic rats and 5.91 ± 0.54 mmol/l for age-matched controls (D-glucose detection kit, Digene, Gaithersburg, MD).

Measurement of protein levels using Western blotting

Samples (primary DRG cells, L4/L5 ganglia, or human sural nerve) were homogenized in ice-cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EGTA pH 8.0, 100 mM NaF, 1.5 mM $MgCl_2$, 10% v/v glycerol, 1% v/v Triton-X_{100}) containing 1 mM PMSF, 1 mM $NaVO_4$, and protease inhibitor mixture. SDS buffer was added, samples were boiled, and protein concentration determined using the Bramhall protein assay (31). SDS-PAGE (10% acrylamide) was performed on 5 μg protein and proteins were transferred to nitrocellulose (Hybond™ECL™, Amersham Pharmacia Biotech, Little Chalfont, UK) using a semi-dry graphite blotter. Primary antibodies used were antibodies to total (code FL; 1:1000) and phosphospecific (code G-7, 1:200; phosphorylated on Thr183 and Tyr185) JNK1 and 2 (Santa Cruz Biotechnology, Santa Cruz, CA); antibodies to total and phosphospecific ERK1 and 2 (1:5000, New England Biolabs, Beverly, MA); and antibodies to phosphospecific p38 (1:1000, phosphorylated on Thr180 and Tyr182, Biosource Europe, Nivelles, Belgium) and total p38 (1:2000, New England Biolabs). Detection was with an HRP-detection kit (LumiGLO™, New England Biolabs). The immunoblots were scanned with a flatbed scanner (Astra 1220S, UMAX, Willich, Germany) with a transparency adapter (UTA-3A, UMAX) and converted to numerical values using Molecular Analyst software (Version 1.5, Bio-Rad, Hercules, CA). Results are expressed graphically as a ratio of phosphorylated to total protein.

Cytotoxicity assays

Cell viability was examined using lactate dehydrogenase (LDH) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays. LDH assay measures cell death, MTT assay measures viable cells. The LDH assay was performed using the CytoTox 96® nonradioactive cytotoxicity assay (Promega, Madison, WI). MTT reagent was from Sigma and the assay was carried out as described (32).

Human sural nerve samples

Sural nerves were dissected from diabetic patients undergoing lower limb amputation due to end-stage neuropathy. Samples were obtained at the time of surgery and snap frozen immediately in liquid nitrogen. Control tissue was obtained from nondiabetic patients undergoing lower limb amputation.

Statistical analyses

Data is represented as a mean ± SE. Comparisons were made with a Student's *t* test using Excel (Microsoft). $P < 0.05$ was considered statistically significant. Experiments represented by one set of data were repeated a minimum of three times.

RESULTS

MAPK activation in cultured sensory neurons

Cultured adult rat sensory neurons were exposed to diabetes-related stresses, including high glucose and the pro-oxidants hydrogen peroxide and diethyl maleate (DEM). Activation of MAPK pathways was examined using Western blotting: antibodies raised against phosphorylated epitopes of the kinases and antibodies raised against the kinases irrespective of their phosphorylation states were used to detect the proteins. A ratio of phosphorylated to total protein (p/t) was then determined and changes in overall phosphorylation states were analyzed.

Sensory neurons treated with increasing glucose showed a dose-dependent activation of p38 and JNK at 16 h of treatment (**Fig. 1**). Cells were treated with glucose ranging from 10 mM to 200 mM; results for 10, 25, and 50 mM only are represented graphically (Fig. 1*a*). Streptozotocin-induced diabetic rats exhibit plasma glucose concentrations ranging between 25 and 50 mM glucose. High glucose (50 mM) increased p38 activation 2.64 ± 0.18-fold relative to controls (10 mM glucose), p46 JNK 2.00 ± 0.42-fold and p56/54 JNK 2.01 ± 0.38-fold relative to controls (n=4) (Fig. 1*a*). No activation of ERK isoforms was detected. Representative immunoblots of p38 and JNK are shown (Fig. 1*b*).

Glucose has been implicated in the onset of neuropathy by inducing an intracellular oxidative stress caused by depletion of NADPH compromising the antioxidant defense mechanism of the neuronal cells. These stresses were mimicked in vitro using H_2O_2 or DEM. DEM binds to glutathione, compromising the glutathione redox cycle (33), and may prevent GSH from neutralizing lipid peroxidation products (34). DEM activated p38 and ERK but not JNK. DEM (500 μM) at control glucose (10 mM) increased p38 activation 1.9-fold relative to controls (10 mM glucose alone) at

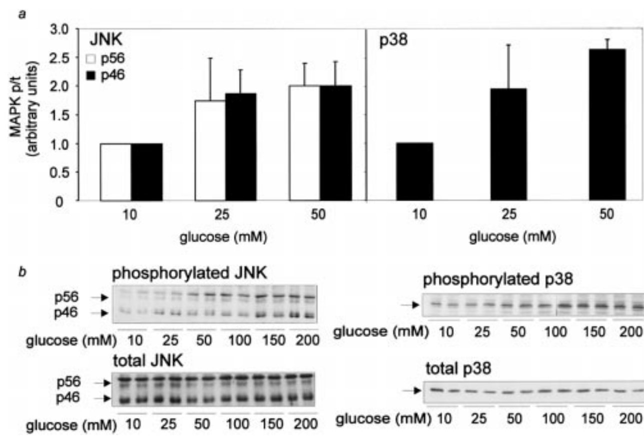

Figure 1. Dose-dependent activation of JNK and p38 MAPKs in response to glucose in cultured DRG neurons. Primary DRG neurons were treated with increasing glucose (concentrations below graphs and blots), and cell lysates were subjected to Western blotting. The immunoblots were scanned and the data represented graphically as a ratio of phosphorylated to total (p/t) protein. *a)* The graphs indicate the dose-dependent increase in p/t p38 and JNK MAPK. The data from 4 separate experiments is represented and all data have been normalized such that control values (10 mM) are equal to 1. The bars represent means ± SE. *b)* The immunoblots represent one experiment. Upper immunoblots were developed with an antibody against phosphorylated JNK MAPK (left) and an antibody against phosphorylated p38 MAPK (right). Lower immunoblots were developed with an antibody against total JNK MAPK (left) and an antibody against total p38 MAPK (right).

16 h treatment (**Fig. 2*a***). At the same time point, DEM in conjunction with 50 mM glucose further increased the activation of p38 seen with high glucose alone (1.5-fold relative to 10 mM glucose) to 3-fold relative to 10 mM glucose (Fig. 2*a*). DEM increased ERK activation between three- and fivefold relative to 10 mM and 50 mM glucose (Fig. 2*a*).

H_2O_2 (500 μM) increased p38 and ERK activation but not JNK at 10 min (Fig. 2*b*). At low H_2O_2 (2 μM) with 50 mM glucose, H_2O_2 increased the activation of p38 above that seen at 50 mM glucose alone compared to controls (10 mM glucose) at 16 h treatment in an apparent additive affect (Fig. 2*c*). This low dose of H_2O_2 in conjunction with high glucose did not have the same effect on ERK (Fig. 2*c*).

Involvement of p38 and ERK MAPK activation in cell damage

The involvement of MAPK activation in cell viability was examined using LDH and MTT cytotoxicity assays. The LDH assay measures LDH released into supernatant on cell rupture i.e., cell death. The MTT assay measures the toxic MTT reagent converted to a nontoxic compound by mitochondrial dehydrogenase i.e., cell survival. Both assays indicated that high glucose did not induce cell death at 16 h treatment (**Fig. 3**). Treatment with DEM (Fig. 3*a*) or H_2O_2 (Fig. 3*b*) resulted in decreased cell viability at 16 h treatment. This decrease was partially reversed by the addition of either an ERK pathway inhibitor, U0126 (10 μM, Promega, WI), or a p38 pathway inhibitor, SB202190 (2 μM, Calbiochem, CN Biosciences Inc, Darmstadt, Germany) (Fig. 3). High glucose caused an apparent increase in cell viability as measured by MTT (Fig. 3*b*), presumably by increasing activity of the mitochondrial enzyme succinate dehydrogenase.

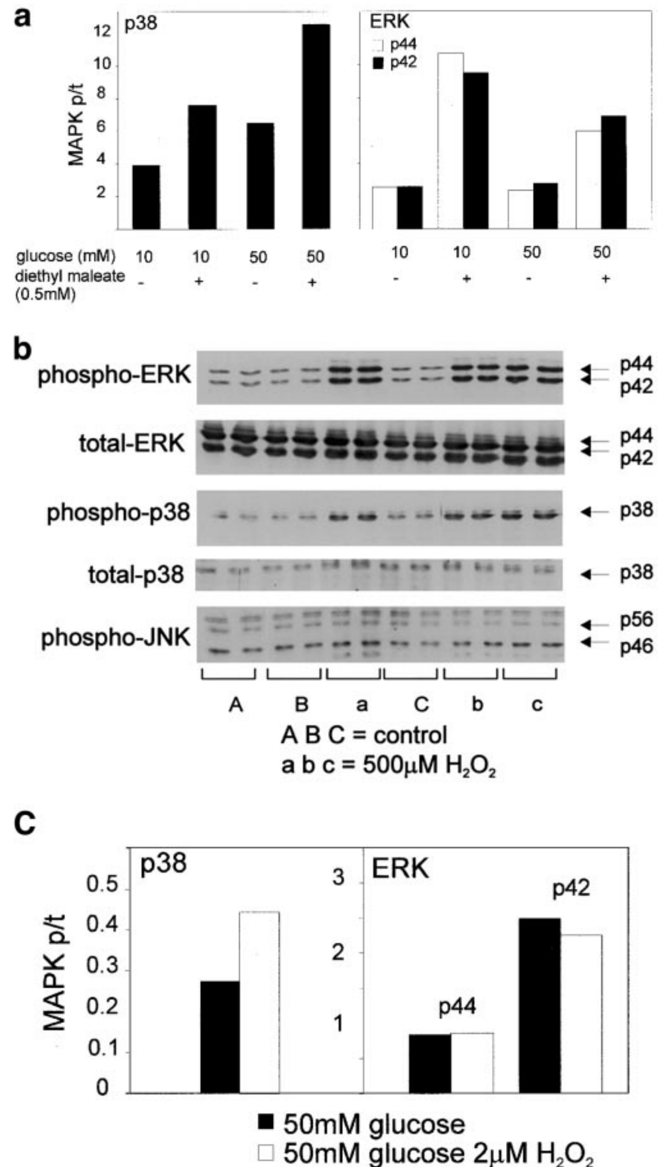

Figure 2. Activation of p38 and ERK MAPKs in response to H_2O_2- and DEM-induced oxidative stress. Cell lysates were subjected to Western blotting; immunoblots were scanned and the data represented graphically as a ratio of phosphorylated to total (p/t) protein. *a)* DEM activated p38 and ERK, but not JNK MAPK. p38 activation was increased by glucose and DEM together compared with the activation of p38 with each separate treatment. *b)* H_2O_2 activated ERK and p38, but not JNK MAPK. No change in expression of total MAPK occurred. *c)* Low H_2O_2 further increased the activation of p38 seen with high glucose alone at 16 h. Activation of ERK MAPK was also observed.

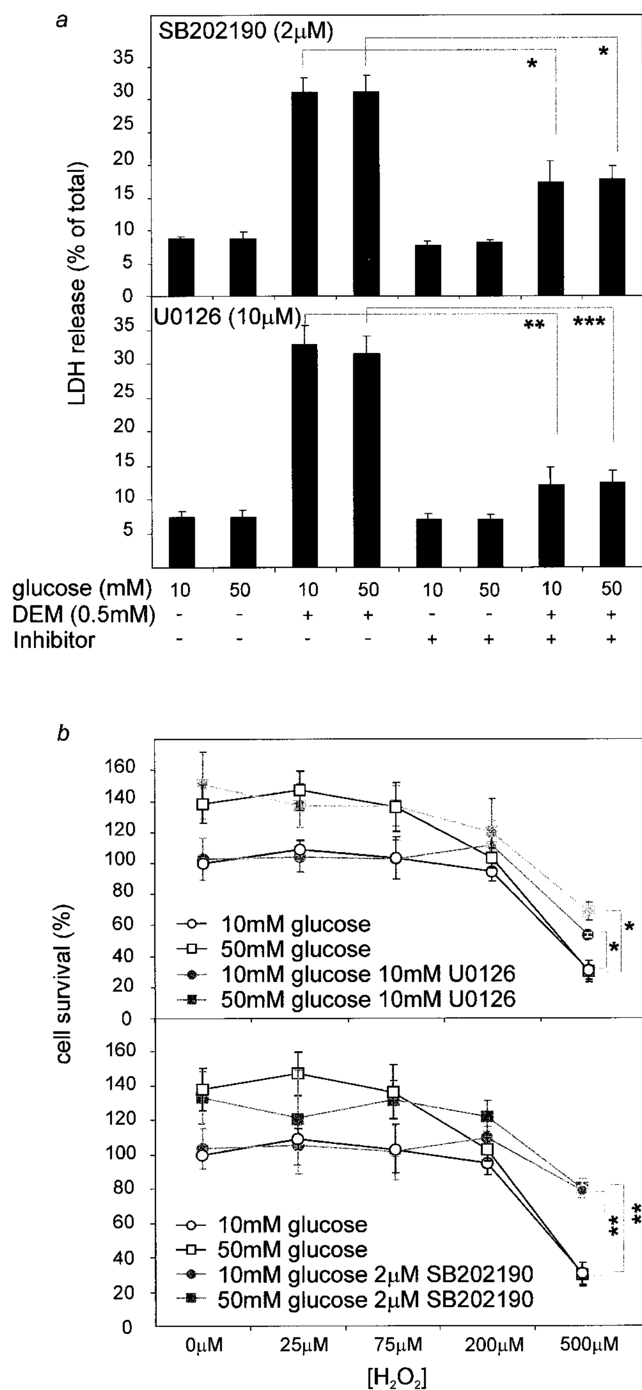

Figure 3. ERK and p38 MAPK activation by oxidative stress are death signals in sensory neuronal cultures. Bars indicate means ± SE, $n = 4$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *a)* DEM treatment increased LDH release (i.e., cell death), which was blocked by either a p38 pathway-specific inhibitor (SB202190) or a ERK pathway-specific inhibitor (U0126). High glucose did not change LDH release. High glucose in conjunction with DEM did not increase LDH release above that seen with DEM alone. *b)* H_2O_2 (concentration range below graph) resulted in a decrease in cell viability at high and low glucose. This was partially prevented by the addition of either a p38 pathway-specific inhibitor or an ERK pathway-specific inhibitor.

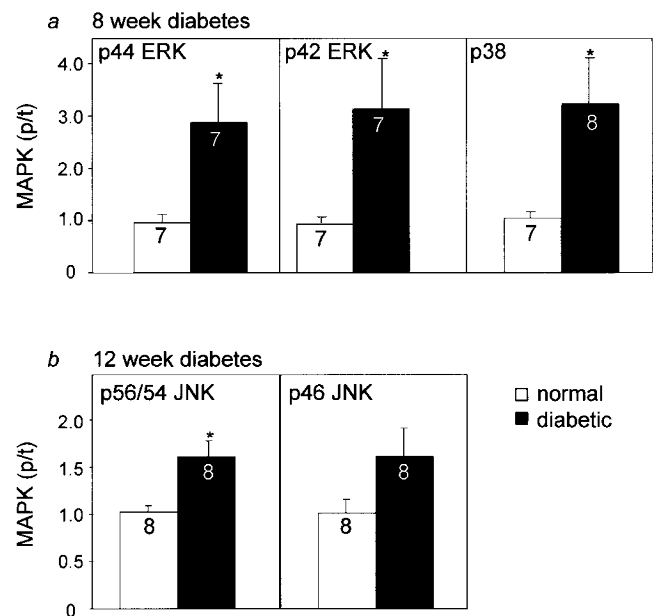

Figure 4. MAPK activation in streptozotocin-induced diabetic rats. Bars denote means ± SE and the numbers in the bars represent sample number. *$P < 0.05$. *A)* ERK and p38 MAPKs are activated in DRG of 8 wk diabetic rats. *B)* p56/54 JNK MAPK is activated in 12 wk diabetic rats.

MAPK activation in DRG of streptozotocin-induced diabetic rats (STZ rats)

DRG from rats with diabetes of 8 and 12 wk duration were subjected to immunoblotting. A significant increase in p38 and ERK activation was seen in 8 wk STZ rats vs. age-matched control rats (**Fig. 4*a***). A significant increase in p56/54 JNK activation was seen in 12 wk STZ rats when compared with age-matched controls (Fig. 4*b*); similar findings have previously been reported (35).

MAPK activation in sural nerve from type I and II diabetic patients

Human sural nerve from type I and type II diabetic patients and control sural nerve from nondiabetic patients with lower limb amputations were subjected to immunoblotting. There was an increase in p38 activation in type I and type II diabetic patients that was not significant; however, total p38 was significantly increased in both type I and type II patients (**Fig. 5*a***). Results indicated a significant increase of total p56/54 JNK in type I and type II diabetic patients (Fig. 5*b*) and a significant increase in phosphorylation of p56/54 JNK in type II and p46 JNK in type I patients. The data for ERK fluctuated between patients and no significant changes were seen (results not shown).

DISCUSSION

We have identified the MAPKs (p38, JNK, and ERK) as targets of the signal transduction pathways that are

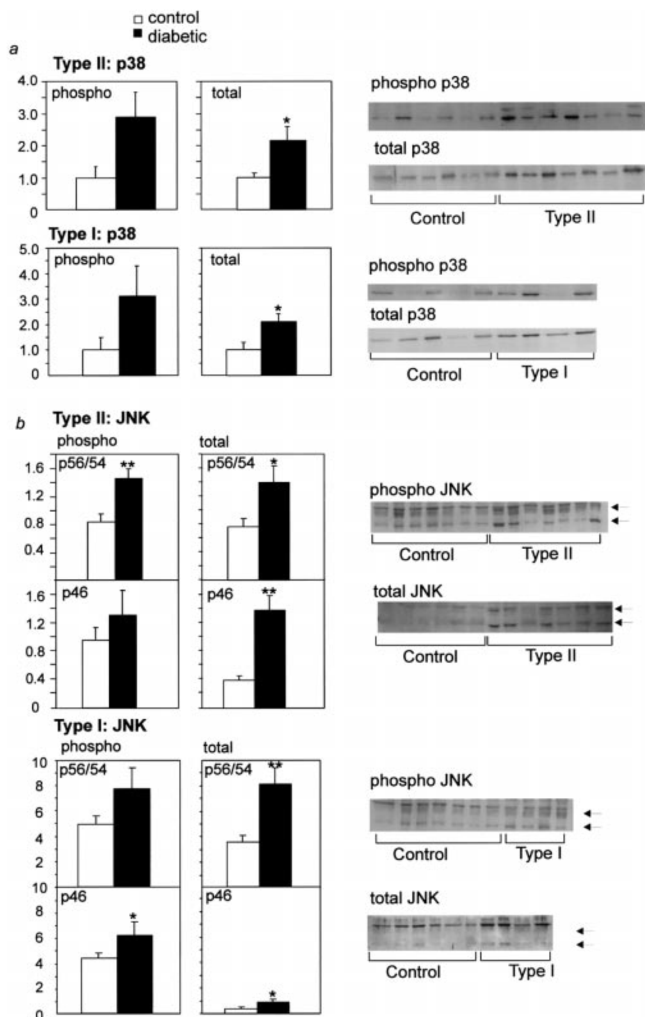

Figure 5. MAPK activation in sural nerve from type I and type II diabetic patients. *a)* Total p38 levels were significantly increased in type I and type II patients. Bars denote mean ± SE. $*P < 0.05$, $**P < 0.001$. *b)* All isoforms of total JNK were significantly increased in both type I and type II patients. Phosphorylated levels of p56/54 were significantly increased in type II patients and phosphorylated levels of p46 were significantly in type I patients.

altered by high glucose and/or oxidative stress in cultured sensory neurons, by diabetes in rats, and in human sural nerve specimens from patients with diabetes. The results suggest that MAPKs may act as transducers linking raised glucose and secondary metabolic stresses to cellular damage and diabetic neuropathy.

There was activation of JNK/p38 by high glucose in cell culture without an effect on ERK (Fig. 1), whereas in response to oxidative stress there was activation of ERK/p38, but no effect on JNK (Fig. 2). Cotreatment with high glucose and oxidative stress resulted in an additive effect upon p38 activation and no additional effect upon ERK activation (Fig. 2*c*). These results suggest alternative mechanisms of high glucose vs. oxidative stress-dependent MAPK activation in this short-term (16 h) cell culture treatment regime. We can only speculate at present, but glucose-dependent activation of JNK/p38 may be the result of a direct effect of the glucose molecule itself, elevated polyol pathway intermediates, or transcription-dependent induction of activators of JNK/p38, i.e., gene 33 (see below). At this early point in culture, it would appear that the metabolism of high glucose concentrations—for example, via the polyol pathway or possibly through increased rates of pyruvate-driven oxidative phosphorylation in the mitochondria—is not linked to overproduction of reactive oxygen species or other factors that may cause oxidative stress.

In culture, high glucose resulted in a sustained activation of p38 and JNK at 16 h and no short-term transient activation of the kinases was observed. The sustained activation of JNK can be explained by the function of an immediate early gene, gene 33, which is transcriptionally induced by a diverse array of extracellular stimuli. Induction of gene 33 requires activation of JNK and, in a feedback loop, transient expression of gene 33 results in the selective activation of JNK (36). Gene 33 expression levels increase soon after onset of diabetes and continue throughout the progression of diabetic nephropathy (36); thus, it is possible that such a mechanism is involved in diabetic neuropathy and is responsible for the sustained activation of JNK in vitro and in vivo.

Studies of 8–12 wk STZ rats showed that all the MAPKs were activated in DRG, although with a delayed activation of JNK compared with p38/ERK (Fig. 4). Clearly, the important observation that glucose can activate MAPKs in vitro has a bearing on these changes in diabetic rats in vivo, but the different situations and time courses of development make direct extrapolation impossible. Accordingly, we suggest that diabetes activates the MAPKs in vivo both directly via hyperglycemia and indirectly, via oxidative stress and other changes. Results from human sural nerve specimens indicate that changes in MAPK activation associated with diabetes are remarkably similar to those seen in diabetic rats.

Downstream targets of the MAPKs include transcription factors such as c-Jun, which has been shown to be activated in DRG of diabetic rats (35) and has been related to degeneration, survival, and neuroprotection (37). Hyperphosphorylation of cytoskeletal elements such as neurofilaments have been proposed as part of the neurodegenerative process, and there is evidence this is mediated by MAPKs including JNK (35, 38) and ERK (39).

Does the activation of MAPKs in cellular stress form part of the chain of damage inflicted on the cell by stress or do they form part of the protective response of the cell? In this study, high glucose activated p38 and JNK in primary DRG neurons (Fig. 1) but did not result in cell death (Fig. 3) at 16 h. In vivo, diabetes results in a loss of axonal caliber and a shrinkage of perikaryal volume, but no significant decrease in cell number (40, 41). Oxidative stress (DEM or H_2O_2) activated p38 and ERK in primary DRG neurons and resulted in decreased cell viability (Fig. 3), indicating a role for these kinases in cell damage. Inhibition of either p38 or ERK

with pathway-specific inhibitors prevented the decrease in cell viability induced by either DEM or H_2O_2 (Fig. 3). The oxidative stress treatments used serve as partial surrogates for the stress caused by glucose over longer periods and in vivo. It is known that the combination of MAPKs activated play an important role in biological outcome (42). As an example, HeLa or HepG2 cells treated with butylated hydroxyanisole, which generates intracellular H_2O_2, show rapid activation of ERK2 and a slower activation of JNK1. Pretreatment with antioxidants such as vitamin E and N-acetylcysteine attenuated the ERK response but not the JNK response (28), implicating ERK activation as an early component of the damaging effects, with the possibility that JNK activation could form part of a protective response.

In diabetic rats, p38 and ERK activation was significantly increased in L4/L5 DRG by diabetes of 8 wk duration (Fig. 4*a*). This was followed by a significant increase in JNK activation in L4/L5 DRG by 12 wk diabetes (Fig. 4*b*). Structural nerve damage in STZ rats has not been reported before 12 wk duration, but a loss of axonal caliber has been detected at 12 wk (43); this structural alteration may be the signal that activates JNK. After sciatic nerve injury, rapid, long-term upregulation of JNK activity occurs as part of the neuronal stress response, but no apoptosis ensues and the upregulation of JNK and its downstream target, c-Jun, may be required for regeneration (44, 45). Previous reports have indicated an increase in JNK but not ERK activation in diabetic rat sural nerve (35). This is remarkably similar to the pattern of activation reported here for sural nerves from diabetic patients. The data from diabetic rats and diabetic patients in combination with the implication of ERK and p38 activation in cell damage suggest a role for the MAPKs in the etiology of diabetic neuropathy.

In summary, our study has identified p38, JNK, and ERK as targets activated by high glucose, oxidative stress, and diabetes. ERK and p38 MAPKs have been implicated in the damaging effects of oxidative stress and diabetes on sensory neurons and we propose that activation of these kinases contribute to the onset of diabetic neuropathy. We propose that the MAPKs provide a focus for the synergy of the different biochemical and metabolic changes brought about by high glucose and that the ability to modulate the activity of these pathways in vivo may form the basis of a preventative treatment for diabetic neuropathy and other diabetic complications. FJ

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