

# Mitogen-Activated Protein Kinase p38 Mediates Reduced Nerve Conduction Velocity in Experimental Diabetic Neuropathy

## Interactions With Aldose Reductase

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**This study examined the role of p38 mitogen-activated protein (MAP) kinase in transducing high glucose into deficits in nerve conduction velocity (NCV) that are characteristic of diabetic neuropathy. p38 activation and NCV were measured in streptozocin-induced diabetic rats treated with a p38 inhibitor, an aldose reductase inhibitor, and insulin. Dorsal root ganglia (DRG) from diabetic animals showed marked activation of p38 at 12 weeks of diabetes. Insulin treatment for the last 4 of 12 weeks of diabetes normalized p38 activation. Furthermore, activation was completely prevented by 12 weeks' treatment with the aldose reductase inhibitor, fidarestat. Immunocytochemistry localized activation of p38 to the nuclei of virtually all sensory neuronal phenotypes in the DRG, and activation was clear in diabetes, as was inhibition by fidarestat and by the p38 inhibitor SB 239063. In the ventral horn of the spinal cord, p38 was present in motoneuron cell bodies; and again, activation in diabetes and fidarestat inhibition was clear. Treatment of diabetic animals with a specific inhibitor of p38 (SB 239063), fidarestat, or insulin also prevented reductions in both motor and sensory NCV. These findings suggest that increased polyol pathway flux in diabetic animals leads to the activation of p38. This activation can mediate changes in gene transcription and cellular phenotype that are likely to underlie the NCV deficits. Insulin and aldose reductase inhibitors can prevent excess polyol pathway flux, and hence these agents may prevent NCV deficits by preventing p38 MAP kinase activation. *Diabetes* 53:1851–1856, 2004**

**R**educed nerve conduction velocity (NCV) is an established hallmark of diabetic neuropathy, occurring in diabetes in both patients (1) and diabetic laboratory animals (2). Since the earliest demonstration (3) that aldose reductase inhibitors prevented slowing of motor NCV (MNCV) in rats with experimental diabetes, it has become a well-defined end point for the evaluation of this class of drugs. Conduction changes have also been the most responsive component of neuropathic diabetic defects in the clinic (4,5). Despite this, we have no proven link between exaggerated flux through the sorbitol pathway and the molecular basis of the conduction velocity deficit.

The Diabetes Control and Complications Trial (6) has focused our attention on elevated glucose as a primary cause of diabetes complications, thus we assume that excessive glucose causes biochemical derangements that ultimately develop into diabetes complications. We have recently implicated the mitogen-activated protein (MAP) kinase p38 as a de facto glucose transducer in promoting some of the changes in neuronal phenotype caused by raised glucose. We showed consistent activation of p38 in neurons cultured with raised glucose in nerve from diabetic rats and in sural nerve specimens from diabetic patients (7). The present study was designed to test the hypothesis that nerve conduction deficits form a functional component of the downstream effects of persistent p38 activation in diabetic rats. To do this, the second-generation p38 inhibitor SB 239063 was used because this specifically inhibits p38 $\alpha$  and - $\beta$  isoforms, the main isoforms expressed in neuronal tissue (8), and has no effect on other MAP, tyrosine, or lipid kinases (9). Since nerve conduction defects also respond to aldose reductase inhibitors, we also tested the possibility that the sorbitol pathway is involved in the activation of p38 by diabetes by testing the effect of aldose reductase inhibition on p38 activation and NCV in peripheral nerve.

### RESEARCH DESIGN AND METHODS

This study comprised several self-contained experiments (i.e., each with its own nondiabetic and diabetic control groups). One study tested the effect of treatment with an aldose reductase inhibitor on the expression and state of activation of p38 MAP kinase. A second study examined the effect of glycemic control with insulin. A third experiment examined the effect of a specific inhibitor of p38 MAP kinase on MNCV and sensory NCV (SNCV).

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Received for publication 4 April 2003 and accepted in revised form 19 April 2004.

ChAT, choline acetyltransferase; DRG, dorsal root ganglia; MAP, mitogen-activated protein; MNCV, motor nerve conduction velocity; NCV, nerve conduction velocity; SNCV, sensory NCV; STZ, streptozocin.

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TABLE 1  
Changes in body weight, plasma glucose, and nerve polyol pathway metabolites (where measured) with all treatments

Animal group	n	Body weight (g)		Plasma glucose (mmol/l)	Sciatic nerve content (nmol/mg)		
		Start	End		Glucose	Fructose	Sorbitol
Control	12	344 ± 6.0	527 ± 18.9	4.99 ± 0.54	7.19 ± 0.51 <sup>a</sup>	4.13 ± 0.23 <sup>a</sup>	0.46 ± 0.04 <sup>a</sup>
Diabetic	13	345 ± 5.6	349 ± 9.7	23.60 ± 1.43	33.71 ± 2.06 <sup>b,c</sup>	15.83 ± 0.92 <sup>b</sup>	8.58 ± 0.90 <sup>b</sup>
Diabetic + fidarestat	11	347 ± 6.0	336 ± 11.2	29.36 ± 1.42	41.48 ± 3.07 <sup>b,d</sup>	4.22 ± 0.41 <sup>a</sup>	0.29 ± 0.05 <sup>a</sup>
Control	12	380 ± 20.2	602 ± 15.7	7.90 ± 0.60	NM	NM	NM
Diabetic	12	390 ± 21.6	358 ± 12.9	40.28 ± 2.45	NM	NM	NM
Diabetic + SB 239063	11	396 ± 26.5	356 ± 11.6	36.44 ± 3.25	NM	NM	NM
Control	6	314 ± 3.0	458 ± 6.3	4.68 ± 0.29	NM	NM	NM
Diabetic	10	306 ± 4.6	291 ± 13.2	23.45 ± 1.40	NM	NM	NM
Diabetic + insulin	10	300 ± 5.7	409 ± 12.1	5.55 ± 1.41	NM	NM	NM

Data are means ± 1SD. Massive differences in body weight and blood glucose were not tested. For polyol pathway metabolites within experiments: a versus b,  $P < 0.01$ ; c versus d,  $P < 0.05$ . NM, not measured.

**Animals and diabetes.** All studies used male Wistar rats with starting weights in the range of 280 to 400 g. Diabetes was induced by an intraperitoneal injection of streptozocin (STZ; Sigma, St. Louis, MO) freshly dissolved in normal saline at a dose of 55 mg/kg, administered the morning after an overnight fast. Three days later, glucose was measured by reflectance photometry on tail vein blood, and any STZ-administered rats with blood glucose <15 mmol/l were rejected from the study. Animals were then group housed with full access to food and water for durations of 12 weeks, and treatments were given as defined below.

**Treatments.** Aldose reductase inhibition was achieved using fidarestat. We measured daily food consumption at between 80 and 100 g/rat; hence, 240–300 g/kg body wt. Rats were housed in groups of three, and sufficient fidarestat to provide a dose of 2 mg/kg was added to 200 g diet, which was made into a stiff slurry with water. Thus, 200 g medicated diet was provided daily, plus an excess of normal diet. Rats always preferred the slurry and consumed the entire amount each day, thereby guaranteeing adequate dosing over the three animals. Because weight loss was evenly spread, we judged the amount consumed to be roughly equal between “cage mates.” The p38 inhibitor SB 239063 was given at a dose of 20 mg · kg<sup>-1</sup> · day<sup>-1</sup> by gavage of an aqueous suspension. Treatment was begun immediately after confirmation of diabetes (day 3 after STZ administration) and was maintained until the end of the protocol. Insulin treatment (given via two subcutaneous Linplants, see [10] for details) was started after 8 weeks’ untreated diabetes and maintained for the final 4 weeks.

**Tissues and measurements.** At the end of the designated protocol, rats were anesthetized (isoflurane in oxygen) and MNCV and SNCV were measured exactly as described elsewhere (11). The animals were then exsanguinated from the right ventricle under anesthetic, and tissues were dissected and placed in tubes on dry ice for subsequent assay.

In experiments involving fidarestat, one sciatic nerve was removed from each rat for measurement of glucose, fructose, and sorbitol by capillary gas chromatography of trimethylsilyl derivatives exactly as described elsewhere (12). In all experiments with treatments, the L4 and L5 dorsal root ganglia (DRG) were removed for SDS-PAGE and Western blotting, again exactly as described elsewhere (7). Blots were exposed to either an antibody directed at a nonphosphorylated epitope of p38 MAP kinase to measure total protein (rabbit polyclonal at a dilution of 1:2,000; New England Biolabs, Beverly, MA) or a phosphospecific antibody to measure activation of p38 (rabbit polyclonal at a dilution of 1:4,000; Biosource, Camarillo, CA). Gels were then scanned and immunoreactivity for p38 bands estimated using proprietary densitometry software (Molecular Analyst). All gels comprised 20 lanes, and in every gel there were 10 lanes from control samples (10 different rats). Thus, within each gel, each diabetic sample (either untreated or treated, depending on the experiment) was normalized to the mean value for all controls in the same gel. That done, phosphospecific p38 immunoreactivity was normalized to total p38 protein for the same tissue sample. This enabled an estimate of activation of p38 independent of any changes in expression; in fact, no systematic changes in p38 total immunoreactivity were seen, but the procedure was maintained as a fail-safe.

In the fidarestat study, 10 rats from each group were fixed by arterial perfusion (4% paraformaldehyde in PBS) under anesthetic and sections prepared for immunocytochemistry as described elsewhere (13). Sections of L4 or L5 DRG and of L1 spinal cord were stained for p38 immunoreactivity (total protein, rabbit polyclonal antibody at 1:200 dilution; Santa Cruz Bio-

technology, Santa Cruz, CA; and phospho-protein, rabbit polyclonal at 1:250 dilution; Biosource).

**Analysis.** All comparisons were made within each self-contained experiment (see Table 1 for an example). Group means were compared using one-way ANOVA, and, where the  $F$  ratio is indicated, individual means were compared using Scheffe’s range tests.

## RESULTS

### Effects of treatments on body weight and diabetes.

All data are in Table 1. Neither the aldose reductase inhibitor nor the p38 inhibitor affected body weight or glycemia; thus any neurological changes seen with these cannot be attributed to attenuation of diabetes severity. However, the insulin implant normalized morning glycemia and allowed the animals to gain weight dramatically, obtaining similar weights to the nondiabetic controls.

### Effects of treatments on biochemical and functional variables.

Fidarestat significantly reduced flux through the sorbitol pathway (Table 1). Indeed, the levels of both sorbitol and fructose were normalized by fidarestat at 2.0 mg/kg. The profound inhibition of flux through the pathway by fidarestat was associated with a significant increase in nerve glucose.

p38 MAP kinase was markedly activated in Western blots made from lumbar DRG from diabetic rats, without any significant change in the amount of protein expression. This was consistent throughout all treatment experiments (all data in Fig. 1). Treatment with fidarestat reduced p38 activation below that of controls, and this effect was also seen by immunocytochemistry (see below). Insulin treatment for the final 4 weeks of the protocol also normalized the activation of p38, as did treatment with SB 239063 (Fig. 1). This was evident in Western blots from DRG, and because the MAP kinase may have had effects on the nerve axons in relation to its effect on nerve conduction, we also found inhibition of the enzyme in blots made from the sciatic nerve (Fig. 2). In all cases, treatment resulted in a level of active p38 similar to the physiological levels of active p38 displayed by control animals. The dose of SB 239063 was slightly lower than the dose that has been demonstrated (14) to display complete inhibition of p38. Alternatively, the phosphospecific p38 antibody recognizes the  $\gamma$ -isoform of p38, which is not inhibited by SB 239063. Ganglia from these SB 239063-treated rats were also subjected to Western blotting for

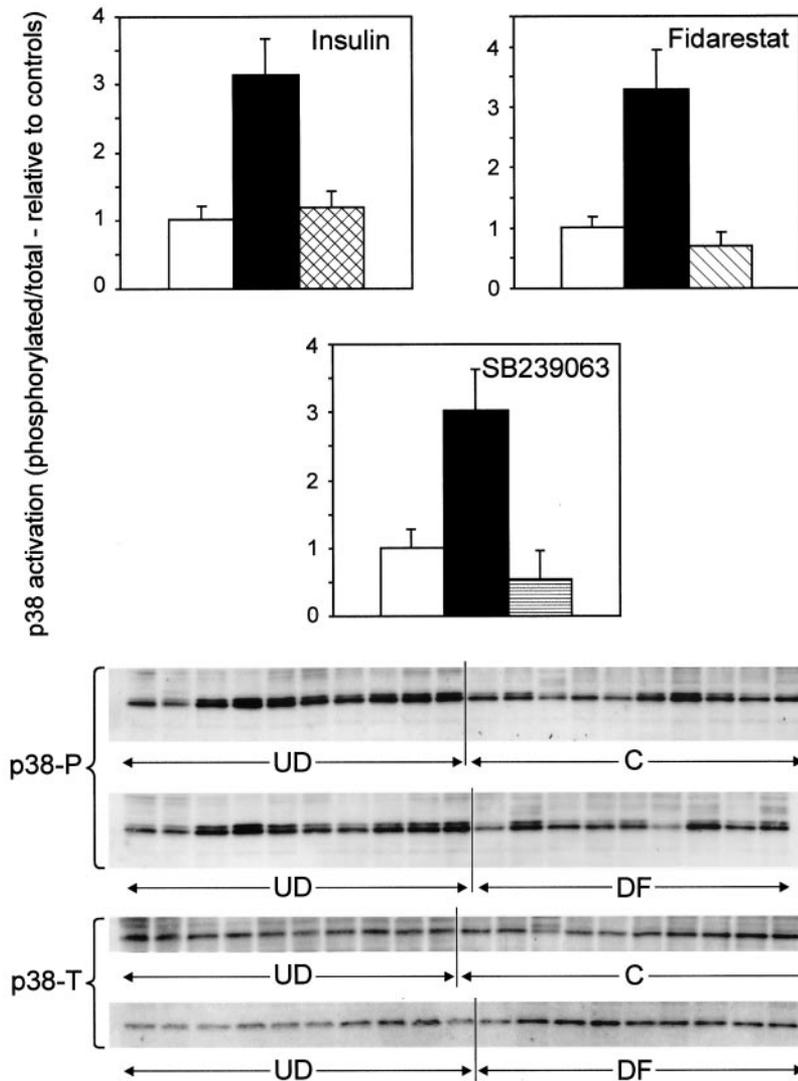


FIG. 1. Bar charts from three experiments showing the effects of treatment of diabetic rats with the aldose reductase inhibitor, fidarestat ( $2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  per os), with insulin (subcutaneous implants), and the specific inhibitor of p38 MAP kinase, SB 239063 ( $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  per os) on diabetes-induced activation of p38. Open columns (□) show control rats, filled columns (■) show untreated diabetic rats, and shaded columns (▨, ▩, or ▪) show animals treated with insulin, fidarestat, and SB239063, respectively. Error bars show  $\pm 1$  SD. In all cases, the effect of diabetes against controls was significant ( $P < 0.01$ ), as were the effects of fidarestat, insulin, and SB 239063 against their untreated diabetic groups ( $P < 0.01$ ). Below are shown sample gels from the fidarestat study. The same samples are shown in the upper pair of gels (immunostained for phosphorylated p38) as in the lower pair (immunostained for total p38). C, control rats; DF, fidarestat-treated diabetic rats; UD, untreated diabetic rats.

extracellular signal-related kinase and c-Jun NH<sub>2</sub>-terminal kinase to monitor specificity of the enzyme against the other MAP kinases, both of which are reproducibly activated by STZ-induced diabetes (7). The inhibitor was without effect.

Immunocytochemistry on DRG and spinal cord sections was carried out in 10 control, 10 untreated diabetic, 10 fidarestat-treated diabetic, and 5 SB 239063-treated diabetic rats using the phosphospecific p38 antibody. These results confirmed those obtained by Western blotting, and typical micrographs from animals from each of these

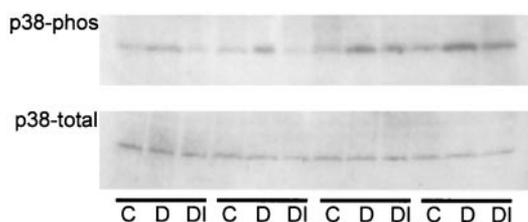
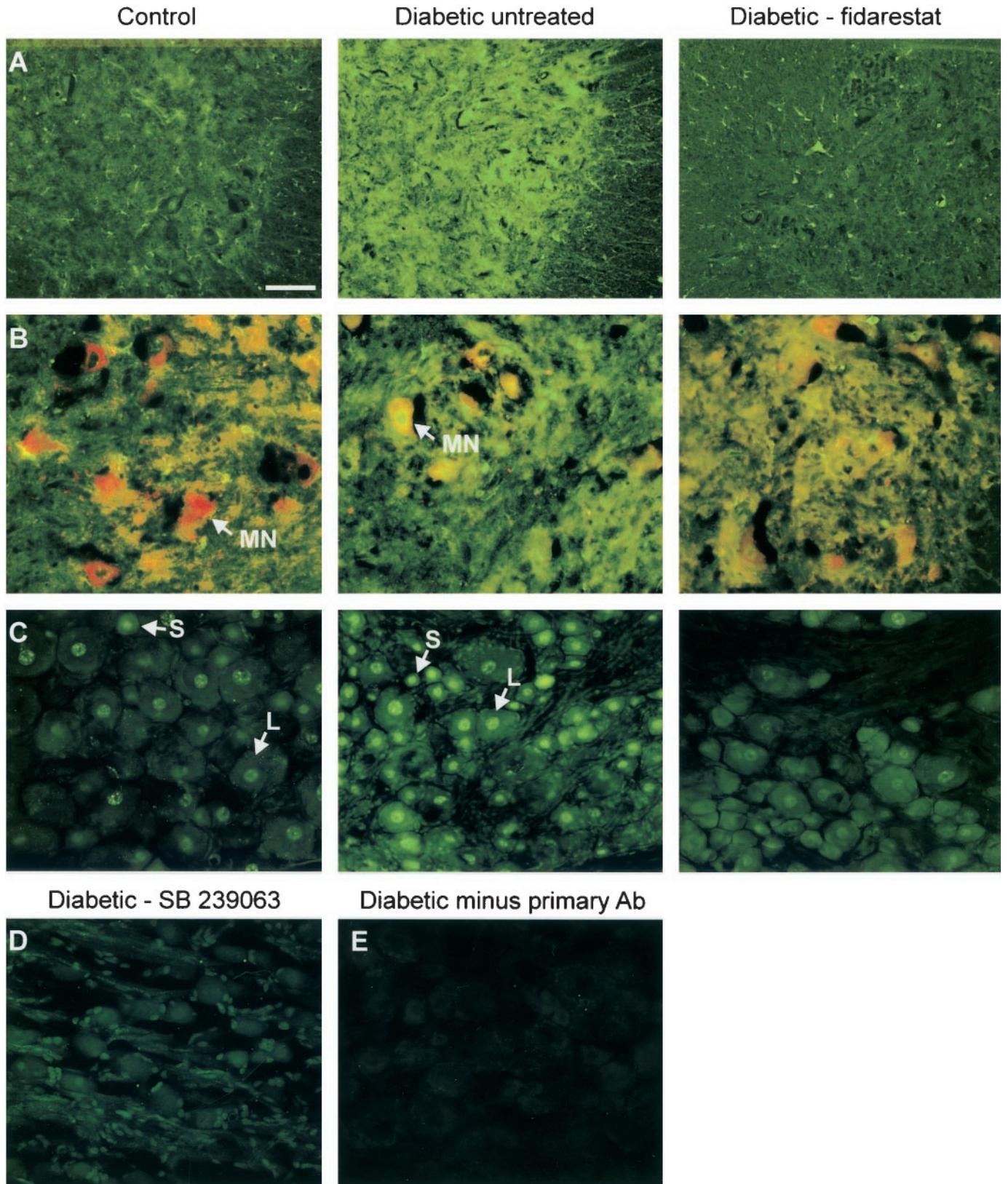


FIG. 2. Western blots showing p38 MAPK activation in the sciatic nerve. In nerve from diabetic rats, the level of activation relative to controls ( $P < 0.01$ ) was similar to that seen in DRG (Fig. 1) and, as with DRG, was blocked completely ( $P < 0.01$ ) by treatment with the inhibitor SB 239063. C, control rats; D, diabetic rats; DI, diabetic rats treated with the p38 inhibitor SB239063.

groups are shown in Fig. 3. Sections exposed to secondary antibody without prior exposure to the phospho-p38 antiserum showed no immunoreactivity (Fig. 3E). Ganglia from control rats showed patchy immunofluorescence from the phospho-p38 antibody. This was not clearly related to any identifiable neuronal phenotype, such that examples of both large and small cell-body nuclei showed punctate fluorescence of varying intensities. In striking contrast, ganglia from diabetic rats displayed intense fluorescence in virtually all neuronal nuclei, indicating a strong activation of p38. There was also a diffuse fluorescence in the cytoplasm of many cells. This predominantly nuclear localization is consistent with the notion that activated p38 is translocated to the nucleus, where it can have profound effects on gene transcription. The diabetes-induced activation was clearly prevented by fidarestat, demonstrated by the distinct reduction in fluorescence so that the general level of fluorescence in neuronal nuclei appeared to be lower than that of controls (Fig. 3). Nuclear fluorescence in DRG cells was also prevented by treatment with SB 239063. In the L1 spinal cord, diabetes promoted p38 MAP kinase activation in motoneuron cell bodies, which was identified by colocalization of choline acetyltransferase (ChAT) (Fig. 3). There was also intense p38 activation in small cells, which



**FIG. 3.** Micrographs from different rats showing immunoreactivity attributed to phospho-p38 (yellow-green) in L1 spinal cord and L4 DRG. Experimental conditions are grouped vertically, as denoted by headings, and the tissue/nature of micrographs horizontally (rows A–C), except for the bottom micrographs, which show the appearance of DRG cells in rats treated with the p38 MAPK inhibitor SB 239063 (D) and a section from DRG of a diabetic rat exposed to the secondary antibody, but not to the primary (anti-p38 phos) antibody (E). A: Low-power micrographs of the gray matter of the cord. B: Higher-power micrographs of the ventral horn. C: DRG are shown. In the cord, there was general activation of p38 MAP kinase by diabetes in all cells (A and B). At higher power, activity was clear in the nuclei of motoneurons (MN), identified by staining for ChAT (orange). Treatment with fidarestat reduced the p38 activation in nerve cells. In diabetes, ChAT staining was clearly reduced, and this was partly reversed by fidarestat. In the DRG of control animals (row C), many nuclei showed modest immunoreactivity; in untreated diabetic rats virtually all nuclei show strong staining, whereas in fidarestat-treated diabetic rats, staining is virtually absent. The marker bar represents 100  $\mu$ m for row A and 50  $\mu$ m for rows B and C and for micrographs D and E.

TABLE 2  
Nerve conduction data in meters per second: effects of insulin or fidarestat or the p38 MAP kinase inhibitor, SB239063

	MNCV	SNCV
Controls	63.8 ± 3.1 <sup>a</sup>	71.5 ± 4.7 <sup>a</sup>
Diabetic	49.1 ± 4.2 <sup>b</sup>	52.8 ± 5.5 <sup>b</sup>
Diabetic + insulin	62.5 ± 4.8 <sup>a</sup>	65.0 ± 4.8 <sup>a</sup>
Controls	59.2 ± 2.2 <sup>a</sup>	76.4 ± 3.7 <sup>a</sup>
Diabetic	50.7 ± 2.4 <sup>b</sup>	61.8 ± 4.2 <sup>b</sup>
Diabetic + fidarestat	62.0 ± 6.0 <sup>a</sup>	71.2 ± 3.4 <sup>a</sup>
Controls	65.8 ± 3.4 <sup>a</sup>	71.7 ± 3.1 <sup>a</sup>
Diabetic	47.6 ± 3.5 <sup>b</sup>	58.4 ± 4.1 <sup>b</sup>
Diabetic + SB 239063	69.9 ± 7.7 <sup>a</sup>	69.6 ± 6.5 <sup>c</sup>

Data are means ± 1SE. Within-individual experiment levels of significance were a versus b,  $P < 0.01$ , and b versus c,  $P < 0.05$ .

might be microglia, and diffuse activation in neuronal and nonneuronal cells of the gray matter. All of this activation was inhibited by fidarestat. It was also noted that ChAT staining was reduced in motoneuron cell bodies of diabetic rats (Fig. 3). Again, this was prevented by fidarestat.

To determine whether this inhibition of p38 signaling had functionally relevant consequences in diabetic rats, the MNCV and SNCV were measured. Diabetic rats displayed a significant reduction in both MNCV and SNCV compared with control animals ( $P < 0.01$ ) (Table 2). This deficit was completely prevented by treatment with SB 239063 and fidarestat, such that the conduction velocities were not significantly different from those of the nondiabetic control groups. Insulin-treated diabetic rats also had similar conduction velocities to control animals, indicating that the 4 weeks' treatment completely reversed the deficits that developed over the previous 8 weeks of untreated diabetes.

## DISCUSSION

This study shows clear activation of MAP kinase p38 in neurons of the lumbar DRG of diabetic rats. Activation of p38 was inhibited by SB 239063, consistent with the inhibitor competing with ATP for active p38 and also binding to inactive p38 (15). Activation of p38 derives from diabetes, as is confirmed by the effect of insulin in normalizing levels of phospho-p38. In none of our immunoblots did we see any systematic alteration of expression of the p38 protein itself, thus the changes in the phospho-epitope can be ascribed to real changes in the activation status (phosphorylation) of the enzyme. Immunocytochemistry showed that the changes occurred predominantly in the nucleus and that all of the different neuronal phenotypes of the DRG were involved in the diabetes-induced activation of p38. We made no attempt to correlate p38 immunostaining with staining to define specific neuronal phenotypes because the p38 immunoreactivity was so uniform (Fig. 2). In the spinal cord of diabetic rats, p38 MAP kinase activation was seen in all motoneurons, as well as in other cell types of the gray matter. The decreased ChAT activity in motoneuron cell bodies of diabetic rats is in accordance with old observations (3,16) of reduced axonal transport of ChAT in sciatic nerves of diabetic rats. Indeed this observation provides an explanation for the older finding, in that there is clearly a reduction in ChAT expression in motoneuron cell bodies, which would translate to a reduced level of enzyme accumulating proximal to ligatures in the axonal transport studies. Thus, there was probably no flaw in

the transport process per se, rather translocation of reduced amounts from the cell body secondary to reduced synthesis of the ChAT enzyme. Further agreement comes from our present observation that the aldose reductase inhibitor normalized expression of ChAT in the cell body, just as it normalized the amount accumulating at the ligature in the earlier studies (3,17).

We have shown that p38 can be activated in vitro by combined oxidative stress and elevated glucose concentration (25–50 mmol/l) (7). This observation, plus the effect of insulin treatment seen here, strongly implicate hyperglycemia in causing activation of neuronal p38 in diabetic rats. Furthermore, the effect of fidarestat on p38 activation strongly implicates the polyol pathway as an intermediate mechanism. There may also be a direct effect of glucose itself, though such a response has not been documented. It may occur as a result of osmotic stress (18–20) or of oxidative stress (21–23). However, the elevation of nerve glucose associated with fidarestat treatment argues against a direct glucose effect on MAP kinase activation. In the longer term (i.e., not mimicked by <24 h exposure to raised glucose in tissue culture) activation of receptor of advanced glycation end products receptors by glycation end products might also activate p38 MAP kinase (24,25). This latter mechanism could certainly have participated in our in vivo study.

As stated, a role for flux through the polyol pathway in neuronal p38 activation in diabetic rats is clear. This might derive from either osmotic or oxidative stress. In a renal cell line, osmotic stress has been shown to both activate p38 and to induce aldose reductase expression, though the cellular signaling pathways for these two phenomena were different (26). This does not preclude synergy, with amplification of a direct osmotic effect on p38 by a secondary effect via the polyol pathway. There is also good evidence for reduction of oxidative stress by inhibition of aldose reductase in peripheral nerve (27,28). Thus, we have the potential for a funneling of different cellular stresses, derived from diabetes, through p38 MAP kinase to influence cellular phenotype and downstream function.

Although we have been able to activate neuronal p38 under tissue culture conditions (7), it was not possible to test the relationship of its activation to neurophysiological (functional) defects because a feasible time course for the development of dysfunction cannot be maintained in tissue culture without introduction of other extraneous effects. Hence, the inhibition of p38 and its effects on nerve conduction reveals important links between hyperglycemia and a classical index of nerve dysfunction in diabetic neuropathy. In a previous pilot study (29), we found that a much shorter duration of treatment (enforced by the amount of compound available to us) with the same inhibitor normalized only SNCV. However, the findings reported here, together with the immunocytochemistry, indicate a clear involvement of p38 activation in the motor deficit. This might also provide an explanation for the protection of nerve function given by aldose reductase inhibitors. Because the activation of p38 seen in the DRG was primarily a nuclear event and the known targets of p38 are transcription factors (30–33), the observations reported here also suggest that the nerve conduction defects seen in diabetes derive from disordered gene expression. However, the activation of p38 MAP kinase in

the sciatic nerve itself, plus the inhibition of that activation by SB 239063, does not preclude local mechanisms operating in the axon or even effects on nerve blood flow or Schwann cell function. Further studies will clarify these possibilities. Whatever the mechanism, this study clearly implicates p38 MAP kinase in the development of nerve conduction deficits. Further studies will also seek the substrate downstream of p38 MAP kinase. So far we have examined activation of both activating transcription factor-2 and c-Jun, but the former was unaffected by diabetes and the latter unaffected by the inhibitor (data not shown). In any case, both of these substrates are activated by other upstream events, such as phosphorylation of c-Jun NH<sub>2</sub>-terminal kinase, which is also increased in diabetes (7).

In conclusion, the present study provides a possible mechanism to explain the causation of nerve conduction deficits by exaggerated flux through the polyol pathway, namely activation of MAP kinase p38. We suggest that this phosphorylates transcription factors, leading to reduced expression of proteins critical to the propagation of the nerve action potential. Given that p38 could be activated by a combination of osmotic and oxidative stresses—both attributable to and independent of the polyol pathway—plus the action of advanced glycation end products via receptor of advanced glycation end products receptors, it may act as a synergy funnel, whereby different biochemical anomalies affect a single function.

#### ACKNOWLEDGMENTS

S.A. was in receipt of a scholarship from the government of Thailand. Financial support from the Medical Research Council of the U.K. (to D.R.T. and supporting S.A.P.) is gratefully acknowledged.

We thank Sanwa (fidarestat) and GlaxoSmithKline (SB 239063) for gifts of drugs.

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