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Interplay of sorbitol pathway of glucose metabolism, 12/15-lipoxygenase, and mitogen-activated protein kinases in the pathogenesis of diabetic peripheral neuropathy

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ABSTRACT

The interactions among multiple pathogenetic mechanisms of diabetic peripheral neuropathy largely remain unexplored. Increased activity of aldose reductase, the first enzyme of the sorbitol pathway, leads to accumulation of cytosolic Ca^{2+}, essentially required for 12/15-lipoxygenase activation. The latter, in turn, causes oxidative–nitrosative stress, an important trigger of mitogen activated protein kinase (MAPK) phosphorylation. This study therefore evaluated the interplay of aldose reductase, 12/15-lipoxygenase, and MAPKs in diabetic peripheral neuropathy. In experiment 1, male control and streptozotocin-diabetic mice were maintained with or without the aldose reductase inhibitor fidarestat, 16 mg kg^{-1} d^{-1}, for 12 weeks. In experiment 2, male control and streptozotocin-diabetic wild-type (C57Bl6/J) and 12/15-lipoxygenase-deficient mice were used. Fidarestat treatment did not affect diabetes-induced increase in glucose concentrations, but normalized sorbitol and fructose concentrations (enzymatic spectrofluorometric assays) as well as 12(S)-hydroxyeicosatetraenoic concentration (ELISA), a measure of 12/15-lipoxygenase activity, in the sciatic nerve and spinal cord. 12/15-lipoxygenase expression in these two tissues (Western blot analysis) as well as dorsal root ganglia (immunohistochemistry) was similarly elevated in untreated and fidarestat-treated diabetic mice. 12/15-Lipoxygenase gene deficiency prevented diabetes-associated p38 MAPK and ERK, but not SAPK/JNK, activation in the sciatic nerve (Western blot analysis) and all three MAPK activation in the dorsal root ganglia (immunohistochemistry). In contrast, spinal cord p38 MAPK, ERK, and SAPK/JNK were similarly activated in diabetic wild-type and 12/15-lipoxygenase$^{-/-}$ mice. These findings identify the nature and tissue specificity of interactions among three major mechanisms of diabetic peripheral neuropathy, and suggest that combination treatments, rather than monotherapies, can sometimes be an optimal choice for its management.

1. Introduction

Diabetic peripheral neuropathy (DPN) affects at least 50% of patients with both Type 1 and Type 2 diabetes, and is a leading cause of foot amputation [1–3]. DPN is manifested by nerve blood flow and motor (MNCV) and sensory (SNCV) nerve conduction velocity deficits as well as by increased vibration and thermal perception thresholds that progress to sensory loss, occurring in conjunction with degeneration of all fiber types in the peripheral nerve [4]. A significant proportion of patients with DPN also describe abnormal sensations such as paresthesias, allodynia, hyperalgesia, and spontaneous pain [3–5].

The pathogenesis of DPN has extensively been studied in animal models of diabetes, and involves complex interactions between vascular and non-vascular mechanisms [6,7]. Multiple biochemical changes including, but not limited to, increased activity of the sorbitol pathway of glucose metabolism [8,9], non-enzymatic glycation/glycoxidation [10,11], activation of protein kinase C (PKC) and mitogen activated protein kinases (MAPKs) [12–15], oxidative–nitrosative stress [16–19], impaired neurotrophism [20], activation of poly(ADP-ribose) polymerase (PARP [21,22]) as well as of the enzymes of arachidonic acid metabolism, cyclooxygenase-2 [23] and 12/15-lipoxygenase (LO [24,25]), participate in the development of nerve conduction velocity deficits and small sensory nerve fiber dysfunction. Increased sorbitol pathway activity [26–28], impaired neurotrophic support [29,30], oxidative–nitrosative stress [31], PARP [32], cyclooxygenase-2 [23], and LO [25] activation have also been implicated in

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axonal atrophy of large myelinated fibers and/or small sensory nerve fiber degeneration. The interactions among some of biochemical mechanisms, *e.g.* (1) increased activity of the sorbitol pathway and oxidative–nitrosative stress [8,27,28,33–35], PKC [12], p38 MAPK [14], and PARP [33] activation; (2) oxidative stress and impaired neurotrophic support [36]; (3) oxidative–nitrosative stress and PARP activation [19,32], in DPN have been identified, but many others remain largely unexplored. Diabetes-induced increase in activity of aldose reductase (AR), the first enzyme of the sorbitol pathway, has been reported to lead to accumulation of cytosolic Ca^{2+} [37], essentially required for LO activation [38,39]. The latter, in turn, causes oxidative–nitrosative stress [24], an important trigger of MAPK phosphorylation [40]. The present study therefore evaluated the interplay of AR, LO, and MAPKs in tissue sites for DPN including peripheral nerve, spinal cord, and dorsal root ganglion (DRG) neurons. The experiments were performed in C57Bl6/J mice, a robust animal model of DPN, that is manifested by MNCV and SNCV deficits, small sensory nerve fiber dysfunction and degeneration, and axonal atrophy of large myelinated fibers [9,19,24,25,27], and is amenable to treatment with AR [9,27], LO [24,41], and p38 MAPK [15] inhibitors.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all chemicals were of reagent-grade quality, and were purchased from Sigma Chemical Co., St. Louis, MO, USA. For Western blot analysis, rabbit polyclonal (clone H-100) anti-12-lipoxygenase (LO) antibody, rabbit polyclonal (clone H-147) anti-p38 MAPK antibody, mouse monoclonal anti-ERK antibody (clone MK1), rabbit polyclonal (clone C17) anti-JNK1 antibody were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Rabbit polyclonal anti-phospho-p38 MAPK antibody, rabbit monoclonal (clone D13.14.4E) anti-phospho-ERK antibody, and rabbit polyclonal anti-phospho-SAPK/JNK antibody were purchased from Cell Signaling Technology, Boston, MA, USA. For immunohistochemistry, 12-lipoxygenase (murine leukocyte) polyclonal antiserum was purchased from Cayman Chemical, Ann Arbor, MI, USA. Rabbit polyclonal antibodies against p38 MAPK (clone H147), SAPK/JNK (clone FL), phospho-ERK, and phospho-SAPK/JNK were obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-ERK antibody was purchased from Abcam, Cambridge, MA, USA, and rabbit monoclonal (clone D3F9) anti-phospho-p38 MAPK antibody from Cell Signaling Technology, Boston, MA, USA. Secondary Alexa Fluor 594 goat anti-rabbit antibody, Prolong Gold Antifade Reagent, and Image-iT FX Signal Enhancer were purchased from Invitrogen, Eugene, OR, USA. VECTASHIELD Mounting Medium was obtained from Vector Laboratories, Burlingame, CA, USA.

2.2. Animals

The experiments were performed in accordance with regulations specified by the National Institutes of Health "Principles of Laboratory Animal Care, 1985 Revised Version" and Pennington Biomedical Research Center Protocol for Animal Studies. Mature C57Bl6/J mice were purchased from Jackson Laboratories. All the mice were fed standard mouse chow (PMI Nutrition International, Brentwood, MO, USA) and had *ad libitum* access to water.

In experiment 1, the mice were randomly divided into two groups. In one group, diabetes was induced by streptozotocin (STZ) as we described previously [42]. Blood samples for glucose measurements were taken from the tail vein three days after STZ injection and the day before the animals were killed. The mice with blood glucose $\geq$13.8 mM were considered diabetic. Then control and diabetic mice were maintained with or without treatment with the aldose reductase inhibitor fidarestat (SNK-860, Sanwa Kagaku Kenkyusho, Nagoya, Japan), at 16 mg kg^{-1} d^{-1} for 12 weeks.

The "leukocyte-type" 12/15-lipoxygenase-null ($LO^{-/-}$) mice were originally generated by Dr. Colin Funk, and the procedure was described in detail [43]. In Dr. Jerry Nadler's laboratory, $LO^{-/-}$ mice have been backcrossed to the B6 background for at least six generations before inbreeding for homozygosity in the experimental mice. Microsatellite testing has confirmed >96% homology between the $LO^{-/-}$ and the C57Bl6/J mice [44]. In experiment 2, a colony of $LO^{-/-}$ mice was established from several breeding pairs provided by Dr. Jerry Nadler's laboratory. Part of wild-type and $LO^{-/-}$ mice was used for induction of STZ diabetes [42]. Then non-diabetic and STZ-diabetic wild-type and $LO^{-/-}$ mice were maintained for 12 weeks.

2.3. Anesthesia, euthanasia and tissue sampling

The animals were sedated by CO_2, and immediately sacrificed by cervical dislocation. Sciatic nerves and spinal cords were rapidly dissected and frozen in liquid nitrogen for further assessment of glucose, sorbitol, fructose, LO expression, and 12(S)HETE concentrations in experiment 1, and total and phosphorylated p38 MAPK, ERK, and SAPK/JNK expression in experiment 2. Dorsal root ganglia were dissected and fixed in normal buffered 4% formalin, for subsequent evaluation of LO expression (experiment 1), and total and phosphorylated p38 MAPK, ERK, and SAPK/JNK expression in experiment 2.

2.4. Specific methods

2.4.1. Glucose and sorbitol pathway intermediates in sciatic nerve and spinal cord

Sciatic nerve and spinal cord glucose, sorbitol, and fructose concentrations were assessed by enzymatic spectrofluorometric methods with hexokinase/glucose 6-phosphate dehydrogenase, sorbitol dehydrogenase, and fructose dehydrogenase as we described in detail [45]. Measurements were taken at LS 55 Luminescence Spectrometer (PerkinElmer, MA, USA).

2.4.2. Western blot analysis of LO and total and phosphorylated p38 MAPK, ERK, and SAPK/JNK in sciatic nerve and spinal cord

To assess LO and total and phosphorylated p38 MAPK, ERK, and SAPK/JNK expression by Western blot analysis, sciatic nerve and spinal cord materials (~3–10 mg) were placed on ice in 100 μl of RIPA buffer containing 50 mmol/l Tris–HCl, pH 7.2; 150 mmol/l NaCl; 0.1% sodium dodecyl sulfate; 1% NP-40; 5 mmol/l EDTA; 1 mmol/l EGTA; 1% sodium deoxycholate and the protease/phosphatase inhibitors leupeptin (10 μg/ml), pepstatin (1 μg/ml), aprotinin (20 μg/ml), benzamidine (10 mM), phenylmethylsulfonyl fluoride (1 mM), sodium orthovanadate (1 mmol/l), and homogenized on ice. The homogenates were sonicated (4 × 10 s) and centrifuged at 14,000 × *g* for 20 min. All the aforementioned steps were performed at 4 °C. The lysates (20 and 40 μg protein for sciatic nerve and spinal cord, respectively) were mixed with equal volumes of 2× sample-loading buffer containing 62.5 mmol/l Tris–HCl, pH 6.8; 2% sodium dodecyl sulfate; 5% β-mercaptoethanol; 10% glycerol and 0.025% bromophenol blue, and fractionated in 10% (total and phosphorylated MAPKs) or 7.5% (LO) SDS-PAGE in an electrophoresis cell (Mini-Protean III; Bio-Rad Laboratories, Richmond, CA, USA). Electrophoresis was conducted at 15 mA constant current for stacking, and at 25 mA for protein separation. Gel contents were electrotransferred (80 V, 2 h) to nitrocellulose membranes using Mini Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA, USA) and Western transfer buffer (10× Tris/glycine buffer, Bio-Rad Laboratories, Richmond, CA, USA) diluted with 20% (v/v) methanol. Free binding sites were blocked in 5% (w/v) BSA in