12/15-Lipoxygenase inhibition counteracts MAPK phosphorylation in mouse and cell culture models of diabetic peripheral neuropathy

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ABSTRACT

Background: Increased mitogen-activated protein kinase (MAPK) phosphorylation has been detected in peripheral nerve of human subjects and animal models with diabetes as well as high-glucose exposed human Schwann cells, and have been implicated in diabetic peripheral neuropathy. In our recent studies, leukocyte-type 12/15-lipoxygenase inhibition or gene deficiency alleviated large and small nerve fiber dysfunction, but not intraepidermal nerve fiber loss in streptozotocin-diabetic mice. Methods: To address a mechanism we evaluated the potential for pharmacological 12/15-lipoxygenase inhibition to counteract excessive MAPK phosphorylation in mouse and cell culture models of diabetic neuropathy. C57Bl6/J mice were made diabetic with streptozotocin and maintained with or without the 12/15-lipoxygenase inhibitor cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC). Human Schwann cells were cultured in 5.5 mM or 30 mM glucose with or without CDC. Results: 12(S) HETE concentrations (ELISA), as well as 12/15-lipoxygenase expression and p38 MAPK, ERK, and SAPK/JNK phosphorylation (all by Western blot analysis) were increased in the peripheral nerve and spinal cord of diabetic mice as well as high glucose-exposed human Schwann cells. CDC counteracted diabetes-induced increase in 12(S)HETE concentrations (a measure of 12/15-lipoxygenase activity), but not 12/15-lipoxygenase overexpression, in sciatic nerve and spinal cord. The inhibitor blunted excessive p38 MAPK and ERK, but not SAPK/JNK, phosphorylation in sciatic nerve and high glucose exposed human Schwann cells, but did not affect MAPK, ERK, and SAPK/JNK phosphorylation in spinal cord. Conclusion: 12/15-lipoxygenase inhibition counteracts diabetes related MAPK phosphorylation in mouse and cell culture models of diabetic neuropathy and implies that 12/15-lipoxygenase inhibitors may be an effective treatment for diabetic peripheral neuropathy.

Keywords: Diabetes; Lipoxygenase; Neuropathy; Schwann Cells; Mitogen-Activated Protein Kinase

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 motor (MNCV) and sensory (SNCV) nerve conduction velocity deficits as well as microvascular dysfunction and 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 [1-4]. A significant proportion of patients with DPN also describe abnormal sensations such as paresthesias, allodynia, hyperalgesia, and spontaneous pain [3]. The pathogenesis of DPN has extensively been studied in animal and cell culture models. Multiple biochemical changes have been attributed to the etiology of diabetic neuropathy including, but not limited to, increased activity of the sorbitol pathway [5-7], gen-
eration of methylglyoxal [8,9] and non-enzymatic glyca-
tion/glycoxidation [10,11], oxidative-nitrosative stress [12-16], impaired neurotrophic support [17,18], and ac-
tivation of protein kinase C [19,20], poly(ADP-ribose) poly-
erase [21,22], and of the enzymes of arachidonic acid metabolism, cyclooxygenase-2 [23] and leukocyte-
type 12/15-lipoxygenase [24,25]. Increased mitogen-
activated protein kinase (MAPK) phosphorylation was
detected in peripheral nerve of human subjects with dia-
betes [26], several animal models of diabetes [26-31], and
high-glucose exposed cultured human Schwann cells [32], and has been implicated in the pathophysiology of
diabetic peripheral neuropathy.

Multiple specific small molecule MAPK inhibitors are
now in clinical trials for chronic diseases including sev-
eral types of cancer, inflammatory and autoimmune dis-
eses, neuropathic pain following nerve trauma, as well
as Parkinson’s and Alzheimer’s diseases [33]. Regardless
of these efforts and outcomes, the search for alternative
approaches to inhibit excessive MAPK phosphorylation
in specific pathological conditions including diabetic
neuropathy is highly warranted. In our previous exper-
iments [24,25,31], 12/15-lipoxygenase inhibition and
gene deficiency improved several diabetic neuropathy
associated endpoints in streptozotocin-diabetic mice in-
cluding nerve conduction deficits and behavioral changes
suggesting that preventing 12/15-lipoxygenase activation
may be an effective treatment for diabetic neuropathy.

2. MATERIALS AND METHODS

2.1. Reagents

Unless otherwise stated, all chemicals were of re-
agent-grade quality, and were purchased from Sigma
Chemical Co., St. Louis, MO, USA. Cinnamyl-3,4-
dihydroxy-alpha-cyanocinnamate (CDC) was obtained
from Enzo Life Sciences International, Plymouth Meet-
ing, PA, USA. For Western blot analyses in mouse tis-
sues, rabbit polyclonal (clone H-100) anti-12-lipoxy-
genase (LO) antibody, rabbit polyclonal (clone H-147)
anti-p38 MAPK antibody, mouse monoclonal (clone
MK1) anti-ERK antibody, and 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 rab-
bit polyclonal anti-phospho-SAPK/JNK antibody were
purchased from Cell Signaling Technology, Boston, MA,
USA. For Western blot analyses in human Schwann cells,
rabbit polyclonal (clone C16) antibody against total ERK
and mouse monoclonal (clone E4) antibody against
phosphorylated ERK were obtained from Santa Cruz
Biotechnology, Santa Cruz, CA. For other MAPKs, the
antibodies listed above were used.

2.2. Animals

The experiments were performed in accordance with
The Guide for the Care and Handling of Laboratory
Animals (NIH Publication No. 85-23) and Pennington
Biomedical Research Center Protocol for Animal Studies.
Mature male C57Bl6/J mice were purchased from Jack-
son Laboratories. All the mice were fed standard mouse
chow (PMI Nutrition International, Brentwood, MO,
USA) and had ad libitum access to water. After a 7-day
acclimation in a new environment, the mice were ran-
domly divided into two groups. In one group, diabetes
was induced by streptozotocin (STZ) as we described
previously [24,25]. The mice with blood glucose ≥13.8
mM, three days post streptozotocin were considered di-
abetic. The control and diabetic mice were kept for 12
weeks without treatment, and then divided into two sub-
groups that were maintained with or without treatment
with cinnamyl-3,4-dihydroxy-alpha-cyanocinnamate (CDC),
8 mg kg/d, s.c., for another 4 weeks. CDC, at the afore-
mentioned dose, counteracted multiple manifestations of
diabetic neuropathy and oxidative-nitrosative stress in
peripheral nerve and spinal cord in our previous study
[24]. Non-fasting blood glucose measurements were
performed after induction of diabetes and at the end of
the study period.

2.3. Anesthesia, Euthanasia and Tissue
Sampling

The animals were sedated by CO2, and immediately
sacrificed by cervical dislocation. Sciatic nerves and
spinal cords were rapidly dissected and frozen in liquid
nitrogen for subsequent assessment of LO as well as total
and phosphorylated p38 MAPK, ERK, and SAPK/JNK
levels, and 12(S)-HETE concentrations.

2.4. Human Schwann Cell Culture

Schwann cells play a key role in the pathology of
various inflammatory, metabolic, and hereditary poly-
neuropathies, including diabetic neuropathy [34,35]. Pre-
vious studies demonstrated that cultured human Schwann
cells (cell line cat. #1700, ScienCell, Carlsbad, CA) mani-
fest increased superoxide production, accumulation of
nitrated and poly(ADP-ribosyl)ated proteins and 4-
hydroxynonenal adducts, inducible nitric oxide synthase
overexpression, 12/15-Lipoxygenase overexpression and
activation, increased p38 MAPK phosphorylation, down-
regulation of taurine transporter, as well as impaired in-
sulin signaling early (1 - 7 d) after exposure to high glu-
cose [24,36-38]. They therefore represent a good model
for studying interactions among individual pathobi-
chemical mechanisms in the peripheral nerve. In the
present study, human Schwann cells (passages 7 - 10)
were cultured in 6-well plates in media containing 5.5
mM D-glucose. At ~70% confluence, the media were replaced with those containing either 5.5 mM D-glucose or 30 mM D-glucose with or without CDC, 10 μM (6 - 8 plates per condition). After 24 hr, the cells were used for assessment of total and phosphorylated p38 MAPK, ERK, and SAPK/JNK.

2.5. Specific Methods

2.5.1. Western Blot Analyses of LO and Total and Phosphorylated p38 MAPK, ERK, and SAPK/JNK

Sciatic nerve and spinal cord materials (~3 - 10 mg) or scraped human Schwann cells were placed on ice in 100 μL of 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 and centrifuged at 14,000 g for 20 min. All the afore-mentioned steps were performed at 4°C. The lysates (20 μg protein for sciatic nerve and 40 μg for spinal cord and human Schwann cells) were mixed with equal volumes of 2x 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% (lipoxygenase) SDS-PAGE in an electrophoresis cell (Mini-Protean III; Bio-Rad Laboratories, Richmond, CA). Electrophoresis was conducted at 15 mA constant current for stacking, and at 25 mA for protein separation. Gel contents were electrotransferred (80 V, 2 hr) to nitrocellulose membranes using Mini Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA) and Western transfer buffer (10X Tris/Glycine buffer, Bio-Rad Laboratories, Richmond, CA) diluted with 20% (v/v) methanol. Free binding sites were blocked in 5% (w/v) BSA in 20 mmol/l Tris-HCl buffer, pH 7.5, containing 150 mmol/l NaCl and 0.05% Tween 20, for 1 h. Primary antibodies against 12/15-lipoxygenase, or phosphorylated p38 MAPK, or SAPK/JNK were applied at 4°C overnight, after which secondary antibodies were applied at room temperature for 1 hr. After extensive washing, protein bands detected by the antibodies were visualized with the Amersham ECL TM Western Blotting Detection Reagent (Little Chalfont, Buckinghamshire, UK). Membranes previously probed for phosphorylated MAPKs were then stripped in the 25 mmol/l glycine-HCl buffer, pH 2.5, containing 2% SDS, and reprobed with antibodies against total p38 MAPK, ERK, and SAPK/ JNK, respectively. Membranes previously probed for 12/ 15-lipoxygenase were stripped again and reprobed with β-actin antibody to confirm equal protein loading.

2.5.2. ELISA 12(S)HETE Measurements

For assessment of 12(S)HETE, sciatic nerve and spinal cord samples were homogenized on ice in 15 mM Tris-HCl buffer (1:100 w/v) containing 140 mM NaCl, pH 7.6, and centrifuged. 12(S)HETE was measured in supernatants with the 12(S)-hydroxyicosatetraenoic acid [12(S)HETE] Enzyme Immuno Assay kit (Assay Designs, Ann Arbor, MI) according to manufacturer’s instructions.

2.6. Statistical Analysis

The results are expressed as Mean ± SEM. Data were subjected to equality of variance F test, and then to log transformation, if necessary, before one-way analysis of variance. Where overall significance (p < 0.05) was attained, individual between group comparisons for multiple groups were made using the Student-Newman-Keuls multiple range test. When between-group variance differences could not be normalized by log transformation (datasets for body weights and plasma glucose), the data were analyzed by the nonparametric Kruskal-Wallis one-way analysis of variance, followed by the Bonferroni/ Dunn test for multiple comparisons. Individual pair-wise comparisons in experiments 3 and 4 were made using the unpaired two-tailed Student’s t-test or Mann-Whitney rank sum test where appropriate. Significance was defined at p < 0.05.

3. RESULTS

3.1. Animal Experiments

3.1.1. Body Weights and Blood Glucose Concentrations

The initial (prior to streptozotocin administration) body weights were similar in all experimental groups (Table 1). Weight gain during the 16-wk study was lower in both untreated and CDC-treated diabetic mice than in the non-diabetic control group. CDC treatment did not affect weight gain in either control or diabetic mice. Initial (after streptozotocin administration) non-fasting blood glucose concentrations were 2.0-fold and 1.9-fold higher in untreated and CDC-treated diabetic mice than in the control group. Hyperglycemia progressed with the prolongation of diabetes, and the differences between final blood glucose concentrations in both diabetic groups and non-diabetic controls exceeded 3-fold. CDC treatment did not affect non-fasting blood glucose concentrations in either non-diabetic or diabetic mice.

3.1.2. 12/15-Lipoxygenase Expression and 12(S)HETE Concentrations

Sciatic nerve (Figures 1(a) and (b)) and spinal cord
(Figures 1(d) and (e)) 12/15-lipoxygenase expression was increased by 40% and 57%, respectively, in diabetic mice compared with controls. CDC treatment did not affect sciatic nerve and spinal cord 12/15-lipoxygenase expression in either control or diabetic mice. Sciatic nerve (Figure 1(c)) and spinal cord (Figure 1(f)) 12(S)HETE concentrations, a measure of 12/15-lipoxygenase activity, were increased by 223% and 48%, respectively, in diabetic mice compared with controls. CDC treatment blunted diabetes-associated sciatic nerve and spinal cord 12(S)HETE accumulation.

3.1.3. MAPK Expression in Sciatic Nerve and Spinal Cord

Diabetic wild-type mice displayed 108%, 35%, and 56% increases in sciatic nerve p38 MAPK (Figures 2(a)).

Table 1. Initial and final body weights and blood glucose concentrations in control and diabetic mice maintained with and without CDC inhibitor treatment.

<table>
<thead>
<tr>
<th>Variable Group</th>
<th>Body weight (g)</th>
<th>Blood glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Control</td>
<td>24.6 ± 0.4</td>
<td>35.6 ± 1.2</td>
</tr>
<tr>
<td>Control + CDC</td>
<td>25.1 ± 0.3</td>
<td>35.3 ± 1.3</td>
</tr>
<tr>
<td>Diabetic</td>
<td>25.3 ± 0.5</td>
<td>27.9 ± 0.6*</td>
</tr>
<tr>
<td>Diabetic + CDC</td>
<td>25.2 ± 0.6</td>
<td>27.2 ± 0.5*</td>
</tr>
</tbody>
</table>

Data are expressed as Means ± SEM. n = 15 per group. *p < 0.01 vs non-diabetic control group.

Figure 1. Representative Western blot analyses of 12/15-lipoxygenase expression (a), (d), 12/15-lipoxygenase protein contents (densitometry, %, (b), (e)), and 12(S)HETE concentrations (c), (f) in the sciatic nerve and spinal cord of non-diabetic control and diabetic mice maintained with or without cinnamyl-3,4-dihydroxy-α-cyanocinnamate treatment. C-control; D-diabetic; CDC-cinnamyl-3,4-dihydroxy-α-cyanocinnamate. Mean ± SEM, n = 6 - 11 per group. **p < 0.05 and <0.01 vs non-diabetic control group. *p < 0.05 and <0.01 vs untreated diabetic group.
(b)), ERK (Figures 2(d), (e)), and SAPK/JNK (Figures 2(g), (h)) phosphorylation, compared with the corresponding control group. Total p38 MAPK (Figures 2(a), (c)), ERK (Figures 2(d), (f)), and SAPK/JNK (Figures 2(g), (i)) levels were indistinguishable among the groups. CDC treatment did not affect the phosphorylation state of any of three MAPKs in non-diabetic mice. It reduced p38 and ERK phosphorylation by 58% and 23% (p < 0.05 vs corresponding untreated group for both comparisons), but did not affect SAPK/JNK phosphorylation, in diabetic mice. Spinal cord p38 MAPK (Figures 3(a), (b)), ERK (Figures 3(d), (e)) and SAPK/JNK (Figures 3(g), (h)) phosphorylation was elevated in both diabetic untreated mice, compared with the non-diabetic controls. Total p38 MAPK (Figures 3(a), (c)), ERK (Figures 3(d), (f)), and SAPK/JNK (Figures 3(g), (i)) levels were similar in control and diabetic mice maintained with or without CDC treatment. The 12/15-lipoxygenase inhibitor did not affect the phosphorylation state of any of three MAPKs in either non-diabetic or diabetic mice.

3.2. Human Schwann Cells Experiment

MAPK Expression

Phosphorylation of p38 MAPK (Figures 4(a), (b)), ERK (Figures 4(d), (e)), and SAPK/JNK (Figures 4(g), (h)) was increased by 47%, 38%, and 95% in human Schwann cells cultured in 30 mM glucose, compared with those cultured in 5.5 mM glucose. Total p38 MAPK (Figure 4(c)), ERK (Figure 4(d)), and SAPK/JNK (Figure 4(i)) levels were indistinguishable among the experimental groups. CDC treatment did not affect levels of any total and phosphorylated MAPKs in human Schwann cells cultured in 5.5 mM glucose, but completely prevented high glucose-induced increase in human Schwann cells p38 MAPK and ERK, but not SAPK/JNK, phosphorylation.
Figure 3. Representative Western blot analyses of phosphorylated and total p38 MAPK, ERK, and SAPK/JNK expression (a), (d), (g), and phosphorylated (b), (e), (h) and total (c), (f), (i) p38 MAPK, ERK, and SAPK/JNK protein contents (densitometry, %) in the spinal cord of non-diabetic control and diabetic mice maintained with or without cinnamyl-3,4-dihydroxy-α-cyanocinnamate treatment. C-control; D-diabetic; CDC-cinnamyl-3,4-dihydroxy-α-cyanocinnamate. Mean ± SEM, n = 6-8 per group. *, ** p < 0.05 and <0.01 vs non-diabetic control group.

4. DISCUSSION

The findings described herein indicate that pharmacological inhibition of 12/15-lipoxygenase suppresses diabetes-induced excessive p38 MAPK and ERK, but not SAPK/JNK, phosphorylation in mouse sciatic nerve. Furthermore, 12/15-lipoxygenase inhibition blunts high glucose-induced p38 MAPK and ERK, but not SAPK/JNK, phosphorylation in human Schwann cells, thus suggesting the existence of the similar relationship between 12/15-lipoxygenase and MAPK in diabetic peripheral neuropathy in humans.

Evidence for the important role of the enzymes of arachidonic acid metabolism, cyclooxygenase-2 (COX-2, [23,39]) and 12/15-lipoxygenase [24,25], in functional, morphological, and biochemical abnormalities in diabetic peripheral neuropathy is emerging. Increased activity of COX-2 was implicated in motor nerve conduction velocity and sensory nerve conduction velocity deficits, oxidative stress, and inflammation associated with experimental diabetic peripheral neuropathy [23,39], as well as in diabetic cardiac autonomic neuropathy and left ventricular dysfunction [40]. In our previous studies [24, 25], increased activity of 12/15-lipoxygenase was identified as an important contributor to diabetes-induced motor and sensory nerve conduction slowing, thermal and mechanical hypoalgesia, axonal atrophy of large myelinated fibers, and oxidative-nitrosative stress in peripheral nerve and spinal cord. Despite its clear role in oxidative-nitrosative stress, 12/15-lipoxygenase activation was not involved in diabetes-associated reduction in intraepidermal nerve fiber density [24,25]. This makes exploration of the protective and pathobiocchemical processes triggered through 12/15-lipoxygenase in tissue-sites for diabetic peripheral neuropathy particularly interesting.

Studies in animal models of diabetes-associated atherosclerosis and in vascular smooth muscle cells exposed to
the diabetic milieu [41-45] revealed that 12/15-lipoxygenase overexpression and activation is implicated in multiple biochemical changes including increased phosphorylation of ERK and p38 MAPK, Ras activation, cAMP response element-binding protein (CREB) phosphorylation, DNA-binding activity, and transactivation, overexpression of intercellular adhesion molecule-1, monocyte chemoattractant protein-1, and interleukin-6, activation of nuclear factor-κB, Src tyrosine kinase, focal adhesion kinase, and Akt, and histone H3-Lys-9/14 acetylation. The important role for 12/15-lipoxygenase in increased p38 MAPK, ERK, and CREB phosphorylation, and increased activator protein-1 and CREB DNA binding and transcriptional activities, as well as fibronectin overexpression has been identified in renal mesangial cells isolated from diabetic mice [46]. We have been particularly interested in the relationship between 12/15-lipoxygenase and MAPKs, because both p38 MAPK [26-28,47-49] and, recently, ERK [50,51], have been implicated in neuropathic changes in diabetes. In particular, increased p38 MAPK phosphorylation is involved in nerve conduction deficit [27], diabetic erectile autonomic neuropathy and vasculopathy [47], mechanical hyperalgesia [28,48], and tactile allodynia [49]. Increased ERK phosphorylation contributes to tactile allodynia [50] and mechanical hyperalgesia [51]. Furthermore, inhibition of p38 MAPK with its specific inhibitors SB239063 and SB203580 counteracted diabetes-associated GSH depletion in the peripheral nerve and COX-2, inducible nitric oxide synthase, and tumor necrosis factor-α overexpression in dorsal root ganglion neurons [28,52]. Our recent experiments [31] revealed that 12/15-lipoxygenase gene deficiency prevents diabetes-associated excessive p38 MAPK, ERK, but not SAPK/JNK phosphorylation, in peripheral nerve, and p38 MAPK, ERK, and SAPK/JNK phosphorylation in...
Interestingly, spinal cord p38 MAPK, ERK, and SAPK/JNK phosphorylation induced by diabetes, appeared independent of the LO mechanism. In the present study, similar findings in peripheral nerve and spinal cord were obtained with the 12/15-lipoxygenase inhibitor CDC. This is consistent with our previous results [24,25] suggesting that CDC, often considered as a relatively weak and non-specific 12/15-lipoxygenase inhibitor [53-55], can be used to dissect the role for leukocyte-type 12/15-lipoxygenase in diabetic peripheral neuropathy, and that the role for 12/15-lipoxygenase in MAPK activation varies in different tissues-12/15-lipoxygenase products have been found to cause SAPK/JNK activation in fibroblasts [56], but, apparently, this mechanism does not mediate high glucose-induced SAPK/JNK activation in human Schwann cells.

In conclusion, the present pharmacological study dissects the role for 12/15-lipoxygenase in diabetes- and high glucose-induced MAPK activation in tissue-sites for diabetic peripheral neuropathy and human Schwann cells. They support interaction between the two mechanisms in the peripheral nerve, but not in spinal cord. The findings are relevant to understanding the mechanisms of diabetic peripheral neuropathy in humans, and suggest, that together with MAPK inhibitors, 12/15-lipoxygenase inhibitors can be used as pharmacological tool for inhibiting excessive MAPK phosphorylation in experimental, and, potentially, future clinical studies. In addition, our findings suggest that the 12/15-lipoxygenase inhibitor CDC can be used for dissection of the pathobiocchemical mechanisms triggered by leukocyte-type 12/15-lipoxygenase, because the biochemical effects of CDC closely mimic those of leukocyte-type 12/15-lipoxygenase gene deficiency.

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