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ROLE OF 12/15-LIPOXYGENASE IN NITROSATIVE STRESS AND PERIPHERAL PREDIABETIC AND DIABETIC NEUROPATHIES

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Abstract

This study evaluated the role for 12/15-lipoxygenase, which converts arachidonic acid to 12(S)- and 15(S)-hydroxyeicosatetraenoic acids, in nitrosative stress in peripheral nervous system and peripheral prediabetic and diabetic neuropathies. The experiments were performed in C57Bl6/J mice made diabetic with streptozotocin or fed high-fat diet, and human Schwann cells cultured in 5.5 mM or 30 mM glucose. 12/15-lipoxygenase overexpression and activation were present in sciatic nerve and spinal cord of diabetic and high fat diet-fed mice, as well as in human Schwann cells cultured in high concentrations of D-, but not L-glucose. 12/15-lipoxygenase inhibition with cinnamyl-3,4-dihydroxy- α -cyanocinnamate (8 mgkg⁻¹d⁻¹ s.c., for 4 weeks after 12 weeks without treatment) alleviated accumulation of nitrated proteins in sciatic nerve and spinal cord, and large and small nerve fiber dysfunction, but not intraepidermal nerve fiber loss. 12/15-lipoxygenase gene deficiency alleviated nitrosative stress and nerve conduction deficit, but not small sensory fiber neuropathy, in high-fat diet fed mice. In conclusion, 12/15-lipoxygenase is implicated in nitrosative stress and peripheral neuropathy in mouse models of Type 1 and early Type 2 diabetes. Its presence in human Schwann cells and upregulation by high glucose suggest a potential involvement in human disease.

Keywords

human Schwann cell; 12(S)-hydroxyeicosatetraenoic acid; intraepidermal nerve fiber density; 12/15-lipoxygenase; nitrosative stress; nerve conduction; peripheral diabetic neuropathy; tactile allodynia; thermal allodynia

A leukocyte-type 12/15-lipoxygenase (LO) is a cytosolic nonheme iron-containing dioxygenase that oxidizes esterified arachidonic acid in lipoproteins (cholesteryl esters) and phospholipids with formation of the bioactive lipids termed eicosanoids [1,2]. Those include primary unstable 12(S)- and 15(S)-hydroperoxytetraenoic acids which are rapidly transformed to 12(S)- and 15(S)-hydroxyeicosatetraenoic acids [12(S)HETE and 15(S)HETE] and a number of their derivatives. LO is expressed in multiple cell types

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including pancreatic β -cells [3], adipocytes [4], vascular endothelial [5,6] and smooth muscle [6] cells, renal podocytes [7], mesangial [8], and tubular [9] cells, monocytes/macrophages [10], as well as neurons [11], astrocytes [12], and oligodendrocytes [13] of the nervous system.

High glucose- and diabetes-induced LO overexpression and activation in vascular and renal cells have been found associated with increased cytosolic Ca^{++} [14], affected signal transduction mechanisms, including protein kinase C (PKC) and mitogen-activated protein kinase C (MAPK) signaling cascades [15,16], and a pro-inflammatory response [17]. LO-derived 12(S)HETE generates superoxide, a precursor of highly reactive oxidant peroxynitrite, known to play an important role in diabetes and diabetic complications [1,2,18,19].

Recent studies implicated the LO pathway in diabetes-induced β -cell degeneration and insulin resistance [3], as well as endothelial dysfunction [1,2,20], nephropathy [1,2,7-9,18,19,21], and atherosclerosis [1,2,20,22]. The contribution of this mechanism to diabetic retinopathy appeared minor [23]. The role for LO in peripheral diabetic neuropathy (PDN), a severe complication that affects ~ 50% of patients with diabetes mellitus, and is a leading cause of foot amputation [24,25], has never been explored. Here, we describe findings implicating LO in nitrosative stress in the sciatic nerve and spinal cord and neuropathic changes associated with prediabetes and overt diabetes, and demonstrating the presence of LO and its overexpression and activation in high glucose-exposed cultured human Schwann cells (HSC).

METHODS

A. Reagents

Unless otherwise stated, all chemicals were of reagent-grade quality, and were purchased from Sigma Chemical Co., St. Louis, MO, USA. Cinnamyl-3,4-dihydroxy- α -cyanocinnamate (CDC) was obtained from Enzo Life Sciences International, Plymouth Meeting, PA. Mouse monoclonal (clone 1A6) anti-nitrotyrosine (NT) antibody for Western blot analysis of nitrated proteins was purchased from Millipore, Billerica, MA, USA. Rabbit polyclonal (clone H-100) anti-12-lipoxygenase (LO) antibody for Western blot analysis was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. 12-lipoxygenase (murine leukocyte) polyclonal antiserum for immunohistochemistry was purchased from Cayman Chemical, Ann Arbor, MI, USA. For double immuno-histochemistry, mouse monoclonal anti-S-100 antibody was purchased from Santa Cruz, Santa Cruz, CA, USA, and isolectin GS-IB4 from Griffonia simplicifolia, Alexa Fluor(R) 594 conjugate as well as goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 from Molecular Probes, Eugene, OR, USA. For assessment of intraepidermal nerve fiber density (INFD), rabbit polyclonal anti-protein gene product 9.5 (PGP 9.5) antiserum was obtained from UltraClone, Isle of Wight, UK, Alexa Fluor 488 goat anti-rabbit highly cross-adsorbed IgG (H+L) from Invitrogen, Eugene, OR, USA, SuperBlock blocking buffer from Thermo Scientific, Rockford, IL, USA, and the optimum cutting temperature (O.C.T.) compound from Sakura Finetek USA, Torrance, CA, USA. VECTASHIELD Mounting Medium was obtained from Vector Laboratories, Burlingame, CA, USA. Other reagents for immunohistochemistry were purchased from Dako Laboratories, Inc., Santa Barbara, CA, USA. HSC and culture medium were obtained from ScienCell Research Laboratories, Carlsbad, CA, USA.

B. Animals

The experiments were performed in accordance with regulations specified by 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 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, several male mice were used for double fluorescent immunohistochemistry, to localize LO to endothelial and Schwann cells of sciatic nerve. In experiment 2, male mice were randomly divided into two groups. In one group, diabetes was induced by STZ as we described previously [26]. 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 ≥ 13.8 mM were considered diabetic. The control and diabetic mice were maintained for 12 weeks and then divided into three subgroups. One subgroup was euthanized for tissue harvest. Two other subgroups were maintained with or without treatment with CDC, $8 \text{ mg kg}^{-1} \text{d}^{-1}$ s.c., for another 4 weeks. Non-fasting blood glucose measurements were performed at induction of diabetes and at the end of the study. Physiological and behavioral measurements were taken at three time-points i.e., at the beginning of the study and before and after CDC treatment. MNCV and SNCV were measured in mice anaesthetized with a mixture of ketamine and xylazine (45 mg kg^{-1} body weight and 15 mg kg^{-1} body weight, respectively, i.p.). In experiment 3, a colony of LO-deficient mice (LO^{-/-}, C57Bl6/J background, [3,23]) was established from several breeding pairs provided by Dr. Nadler's laboratory. The female wild-type and LO^{-/-} mice were randomly assigned to receive standard mouse chow (NC) or HFD (D12328, 10.5 kcal% fat, and D 12330, 58 kcal% fat with corn starch, respectively, Research Diets, New Brunswick, NJ, USA), for 16 weeks. We previously reported that C57Bl6/J female mice fed HFD for 16 weeks develop obesity, hyperinsulinemia, and impaired glucose tolerance, in the absence of overt hyperglycemia [27]. In the present study, non-fasting blood glucose measurements, and physiological and behavioral tests were performed prior to HFD feeding and at the end of the study.

C. 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 and nitrated protein expression and 12(S)-HETE concentrations. Footpads were sampled for assessment of INF α .

D. Specific Methods

D.1. Double fluorescent immunohistochemistry—The sections of sciatic nerve were deparaffinized in xylene, hydrated in decreasing concentrations of ethanol, and washed in water. The anti-LO antiserum (1:3200 dilution) was applied in combination with isolectin Alexa Fluor(R) 594 conjugate or anti-S-100 antibody (1:100 dilution), for localization of LO to endothelial and Schwann cells, respectively. Then all the sections were washed in PBS containing 0.01% Triton X-100, and a cocktail of secondary Alexa Fluor 488 and 594 antibodies (both in 1:150 dilution) or secondary Alexa Fluor 488 antibody alone (1:150 dilution, for isolectin-stained sections only) were applied for 2 hr at room temperature. After this step, the sections were washed, coverslipped, and mounted in Kaiser's glycerin-gelatine (Merck, Darmstadt, Germany). Low power observations were made using a Zeiss Axioplan 2 imaging microscope. Color images were captured with a Photometric CoolSNAPTM HQ CCD camera at 1392×1040 resolution. Low power images were generated with a 40X acroplan objective using the RS ImageTM 1.9.2 software. The Adobe Photoshop 7.0 program was used to merge the images. To verify specificity of all immunostainings, primary antibody (anti-S-100) or antiserum (LO) were omitted in negative controls.

D.2. Biochemical studies

D.2.1. Western blot analysis of LO and nitrated proteins: To assess LO and nitrated protein expressions by Western blot analysis, sciatic nerve and spinal cord materials (~10–20 mg) were placed on ice in 200 μ l 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 μ g/ml), pepstatin (1 μ g/ml), aprotinin (20 μ g/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 μ g protein for sciatic nerve and spinal cord, respectively) 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 % (nitrated proteins) or 7.5% (LO) 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 [25 mmol/l Tris-HCl, pH 8.3; 192 mmol/l glycine; and 20% (v/v) methanol] [27]. Free binding sites were blocked in 2% and 5% (w/v) BSA (for nitrated proteins and LO, respectively) in 20 mmol/l Tris-HCl buffer, pH 7.5, containing 150 mmol/l NaCl and 0.05% Tween 20, for 1 h, after which LO or nitrotyrosine antibodies were applied for 2 h, for detection of nitrated protein and LO expressions. The horseradish peroxidase-conjugated secondary antibody was then applied for 1 h. After extensive washing, protein bands detected by the antibodies were visualized with the Amersham ECLTM Western Blotting Detection Reagent (Little Chalfont, Buckinghamshire, UK). Membranes were then stripped in the 25 mmol/l glycine-HCl, pH 2.5 buffer containing 2% SDS, and reprobed with β -actin antibody to confirm equal protein loading.

D.2.2. 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).

D.3. Physiological and behavioral tests—Sciatic MNCV, hind-limb digital SNCV, thermal allgesia (paw withdrawal), and tactile response thresholds were measured as described previously [26,27].

D.4. INFD—Footpads were fixed in ice-cold Zamboni's fixative for 3 hr, washed in 100 mM phosphate-buffered saline (PBS) overnight, and then in PBS containing increasing concentrations of sucrose i.e., 10%, 15%, and 20%, 3 hr in each solution. After washing, the samples were snap-frozen in O.C.T. compound and stored at -80°C. Three longitudinal 50 μ m-thick footpad sections from each mouse were cut on Leica CM1950 cryostat (Leica Microsystems, Nussloch, Germany). Non-specific binding was blocked by 3% goat serum containing 0.5% porcine gelatin and 0.5% Triton X-100 in SuperBlock blocking buffer at room temperature, for 2 hr. The sections were incubated overnight with PGP 9.5 antiserum in 1:400 dilution, at 4°C, after which secondary Alexa Fluor 488 IgG (H+L) in 1:1000 was applied at room temperature, for 1 h. Sections were then coverslipped with VECTASHIELD mounting medium. Intraepidermal nerve fiber profiles were counted blindly by three independent investigators under Axioplan 2 microscope (Zeiss) at 40X magnification, and the average values were used. The length of epidermis was assessed on the

microphotographs of stained sections taken at 5X magnification with a 3I Everest imaging system (Intelligent Imaging Innovations, Inc., Denver, CO, USA) equipped with Axioplan 2 microscope (Zeiss), using the NIH ImageJ software (version 1.42q). An average of 2.8 ± 0.3 mm of the sample length was investigated to calculate a number of nerve fiber profiles per mm of epidermis. Representative images of intraepidermal nerve fibers were obtained by confocal laser scanning microscopy at 40X magnification, using Leica TCS SP5 confocal system (Leica Microsystems, Mannheim, Germany).

D.5. Experiment 4: studies in HSC

D.5.1. HCS culture: Experimental evidence in support of the important role of SC in PDN is emerging. In addition to axonal atrophy and myelinated fiber loss characteristic for advanced PDN, SC contribute to early axonopathy and painful sensory neuropathy that develop in the absence of demyelination. The fact that diabetes-associated peripheral nerve oxidative-nitrosative stress originates primarily from SC is supported by several lines of evidence obtained in animal model studies [28-30]. Note, that cultured primary rat HS do not display increased superoxide production even after prolonged (up to 16 d) exposure to high (30 mM) glucose [31]. In contrast, cultured HSC (cell line cat. #1700, ScienCell, Carlsbad, CA) manifest increased superoxide production, accumulation of nitrated and poly(ADP-ribosyl)ated proteins and 4-hydroxynonenal adducts, inducible nitric oxide synthase overexpression, and downregulation of taurine transporter early (1-7 d) after exposure to high glucose [32-34], and therefore represent a good model for studying mechanisms of oxidative-nitrosative stress in the peripheral nerve. In experiment 4, HSC (passages 7-10) were cultured in 6-well plates in commercial media containing 5.5 mM D-glucose. At ~50% confluency, the commercial media were replaced with those containing either 5.5 mM D-glucose or 30 mM D-glucose with or without CDC, 10 μ M (6-8 plates per condition). The cells cultured in 5.5 mM D-glucose + 24.5 mM L-glucose were used as the osmolarity control. After 24 hr, part of the cultures were used for Western blot analysis of LO. The parallel cultures were used for 12(S)HETE measurements.

E. Statistical analysis

The results are expressed as Mean $\pm$ 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 $p \leq 0.05$.

RESULTS

In experiment 1, LO fluorescence was clearly identifiable in endothelial (Fig.1a) and Schwann (Fig.1b) cells of mouse sciatic nerve.

In experiment 2, the initial (prior to STZ 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 STZ administration) non-fasting blood glucose concentrations were 67% and 71% 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. In experiment 3, the initial (prior to HFD feeding) body weights were similar in all experimental groups. A 16-week HFD feeding resulted in 30% and 28% differences in body weights between wild-type and LO^{-/-} mice fed HFD and the corresponding groups fed NC. Non-fasting blood glucose concentrations were similar among wild-type and LO^{-/-} mice fed NC or HFD, both at the beginning and at the end of the study.

In experiment 2, diabetic mice displayed clearly manifest peripheral nerve and spinal cord LO overexpression and activation at the 12-wk time point, prior to CDC intervention. Sciatic nerve and spinal cord LO expressions were increased by 38% and 65%, respectively (Fig.2a-d). Sciatic nerve and spinal cord 12(S)HETE concentrations were increased 2.8-fold and 1.7-fold (Fig.2e,f).

In a similar fashion, increased LO expression (Fig.3a,b and 3c,d) and 12(S)HETE concentrations (Fig.3,e,f) were found in sciatic nerve and spinal cord of untreated mice with 16-wk duration of diabetes (experiment 2). CDC treatment did not affect diabetes-induced sciatic nerve and spinal cord LO overexpression, but significantly reduced 12(S)HETE concentrations, indicative of a reduction of LO activity.

In experiment 3, a 16-wk HFD feeding increased LO expression by 29% and 30% in sciatic nerve and spinal cord of wild-type mice (Fig.4a,b and 4c,d, respectively). 12(S)HETE concentrations were increased by 113% and 27%, respectively (Fig.4e,f). 12(S)HETE was also identified in both sciatic nerve and spinal cord of LO^{-/-} mice. However, 12(S)HETE concentrations, especially those in the sciatic nerve, were significantly lower in LO^{-/-} mice than in the wild-type mice. No differences in either sciatic nerve or spinal cord 12(S)HETE concentrations were found between NC- and HFD-fed LO^{-/-} mice.

In experiment 2, sciatic nerve and spinal cord nitrated protein expressions were increased by 56% and 32% and by 63% and 33%, in mice with 12-wk and 16-wk durations of STZ-diabetes, respectively, compared with non-diabetic controls (Fig.5). CDC treatment reduced nitrated protein expression in sciatic nerve, and normalized this variable in spinal cord of diabetic mice. LO inhibition was not associated with a significant decrease in nitrated protein expression in non-diabetic mice.

In experiment 3, a 16-wk HFD feeding of wild-type mice increased sciatic nerve and spinal cord nitrated protein expressions by 38% and 68% (Fig.6a,b and Fig.6c,d, respectively). There was a tendency towards a modest increase in nitrated protein expression in HFD-fed LO^{-/-} mice, but the difference with the corresponding group fed NC did not achieve statistical significance. Accumulation of nitrated proteins in the spinal cord of HFD-fed LO^{-/-} mice was 31% lower than in the corresponding wild-type group.

In experiment 2, diabetic mice displayed 20 % and 14% MNCV and SNCV deficits at the 12-wk time point i.e., prior to CDC intervention (Table 2). Note, that both MNCV and SNCV were similar in non-diabetic mice at the beginning of the study and at the 12-wk time point which indicates that diabetes-induced nerve conduction slowing did not develop due to affected peripheral nerve growth and maturation. CDC alleviated, but did not completely normalize, MNCV and SNCV in diabetic mice, without affecting those variables in non-diabetic controls. In experiment 3, MNCV and SNCV were similar in all experimental groups at the beginning of the study, prior to HFD feeding. The HFD-fed wild-type mice displayed 20% MNCV deficit and 16% SNCV deficit at the 16-wk time point. In contrast,

HFD-fed LO^{-/-} mice preserved essentially normal SNCV. They did develop MNCV deficit, but it was less severe (13%) than in the HFD-fed wild-type mice.

In experiment 2, the sensitivities to both thermal and tactile noxious stimuli were similar in non-diabetic mice at the beginning of the study and at the 12-wk and 16-wk time points. Diabetic mice displayed thermal hypoalgesia and tactile allodynia at both 12 wks and 16 wks after induction of diabetes. CDC treatment partially reversed both sensory disorders in diabetic mice, without affecting either thermal response latencies or tactile response thresholds in non-diabetic controls. In experiment 3, thermal response latencies and tactile response thresholds were similar in all experimental groups at the beginning of the study. Both wild-type and LO^{-/-} mice displayed thermal hypoalgesia and tactile allodynia after 16 wks of HFD feeding. LO gene deficiency did not alleviate either of these two sensory disorders in HFD-fed mice.

In experiment 2, diabetic mice displayed a 21% reduction in INFD at the 12-wk time point (Fig. 7a,b). This reduction progressed to 40% at the 16-wk time point (Fig. 7c,d). CDC treatment did not affect INFD in non-diabetic mice, and did not induce intraepidermal nerve fiber regeneration or slowed down their degeneration in diabetic mice. In experiment 3, a 16-wk HFD feeding did not affect INFD in either wild-type or LO^{-/-} mice (Fig. 7e,f). INFDs were similar in all experimental groups.

In experiment 4, LO was identified in HSC. LO expression (Fig. 8a,b) and 12(S)HETE accumulation (Fig. 8c) were 29% and 3.5-fold greater in HSC cultured in 30 mM D-glucose, than in those cultured in 5.5 mM glucose (Fig. 7a,b). In contrast, HSC cultured in 5.5 mM D-glucose plus 24.5 mM L-glucose (osmolarity control) did not display increased LO expression or 12(S)HETE accumulation.

DISCUSSION

It has been known for at least a decade, that diabetes is associated with disturbances of arachidonic acid metabolism in peripheral nerve [35]. Arachidonic acid is metabolized by cyclooxygenases (COX)-1 and -2, 5- and 12/15-LOs, and cytochrome p450 epoxygenase. Diabetes does not affect rat sciatic nerve COX-1 expression, and the findings with nonselective COX inhibitor flurbiprofen suggest that COX-1 is essentially required for normal peripheral nerve function [36]. In contrast, sciatic nerve COX-2 overexpression was identified as a pathogenetic factor in PDN [36,37]. The roles for 5-lipoxygenase and cytochrome p450 epoxygenase in PDN have never been explored, although evidence for cell toxicity of their products, leukotrienes and epoxyeicosatrienoic acids, is emerging [23]. The findings reported herein demonstrate an important role of 12/15-LO in MNCV and SNCV deficits and small sensory nerve fiber dysfunction in mouse models of Type 1 diabetes and Type 2 prediabetes and obesity.

Sciatic nerve and spinal cord LO overexpression and 12(S)HETE accumulation were observed in STZ-diabetic and HFD-fed mice. LO inhibition with CDC did not reduce LO protein expression, but normalized 12(S)HETE concentrations, a measure of LO activity, in both tissue targets for PDN. Note, that 12(S)HETE was present in sciatic nerve and spinal cord of both NC- and HFD-fed LO^{-/-} mice, probably due to activities of other, non-leukocyte type, LO(s).

Studies with conventional antioxidants and metal chelators [38-46], inhibitors of superoxide-generating enzymes and superoxide dismutase mimetics [47-49], and so called “indirect antioxidants” such as aldose reductase [50], COX-2 [37], poly(ADP-ribose) ribose polymerase [32] inhibitors, suggest a key role for oxidative stress in neurovascular dysfunction, nerve conduction deficits, axonal atrophy of large myelinated fibers, and small

fiber neuropathy. The role for oxidative stress in PDN has also been confirmed in several transgenic mouse model studies [37,51,52]. The potent oxidant peroxynitrite [53,54], previously shown to play an important role in neurodegenerative synucleinopathies such as Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy [55], as well as Alzheimer disease and other tauopathies [56,57], has recently been implicated in both peripheral [58-60] and autonomic [61] neuropathies. Accumulation of a foot-print of peroxynitrite injury, nitrotyrosine, has been identified in peripheral nerve, *vasa nervorum*, spinal cord, and dorsal root ganglia in animal models of prediabetes and overt diabetes [27,29,30,32,43,47,58-60,62], and in microvasculature of diabetic human subjects [63]. In our recent study in STZ-diabetic rat model [64], nitrotyrosine accumulation was present in endothelial and Schwann cells of the peripheral nerve, spinal cord neurons, astrocytes, and oligodendrocytes, and neuron (perikarya) and glial cells of dorsal root ganglia, and nerve NT concentrations inversely correlated with MNCV, SNCV, and myelin thickness. Others identified correlations between plasma NT content and endothelial dysfunction [65], an important factor in the pathogenesis of PDN [38,40-45,47-50], in human subjects with diabetes. Furthermore, evaluation of phorase as a probe for the determination of plasma total antioxidant capacity, revealed a correlation between quenching of phorase chemiluminescence with peroxynitrite and total neurological impairment score-lower limbs [66]. Mechanisms underlying nitrotyrosine accumulation in PNS and other tissue-sites for diabetic complications are not completely understood, although previous studies suggest involvement of increased AR [67-69], inducible and neuronal nitric oxide synthases [29,30], PARP [32,64,69,70], as well as oxidized lipoproteins [62]. In the present study, nitrated protein accumulation was reduced (sciatic nerve) or normalized (spinal cord) in diabetic mice treated with CDC. HFD-fed LO^{-/-} mice developed less severe nitrosative stress in both tissue targets for PDN, than the HFD-fed wild-type mice. Thus, our findings identify LO as an important contributor to nitrosative stress associated with both prediabetic and diabetic neuropathies.

The afore-mentioned data are consistent with a beneficial effect of LO inhibition or gene deficiency on peripheral nerve function. HFD-fed LO^{-/-} mice were partially protected from MNCV and SNCV deficits that were clearly manifest in HFD-fed wild-type mice. LO inhibition with CDC alleviated motor and sensory nerve conduction slowing in STZ-diabetic mice, and this was not due to correction or amelioration of hyperglycemia. The incomplete correction of MNCV and SNCV by CDC could result from a relatively short duration of treatment after quite extended (12 wks) presence of a severe diabetes. Also note that although CDC treatment normalized 12(S)HETE concentrations in the peripheral nerve *in toto*, it is unclear whether the selected dose was sufficient for *vasa nervorum*. Endothelial cells do contain LO and accumulate 12(S)HETE [1,2,5,6], and endothelial dysfunction leading to decreased nerve blood flow and endoneurial hypoxia is known to play an important role in diabetes-induced MNCV and SNCV deficits [38,40-45,47-50,72-74].

In addition to MNCV and SNCV deficits, human subjects with advanced PDN display increased vibration and thermal perception thresholds [24]. A significant proportion of patients also have tactile allodynia i.e., a condition in which a light touch is perceived as painful [24]. Whereas most investigators agree on the presence of MNCV and SNCV deficits in C57Bl6/J mice made diabetic with STZ [28-30,51,58,59,75,76], the data on thermal and tactile sensitivity in this and other rodent models of PDN are extremely contradictory (reviewed in [25]). Therefore, the behavioral test data should be interpreted with caution and can not be used as reliable endpoints for drug discovery, until the controversies are resolved. We consistently observe tactile allodynia (consistent with findings of C.Toth, personal communication) and thermal hypoalgesia in C57Bl6/J mice with different durations of STZ-diabetes. In the current study, LO inhibition with CDC alleviated thermal hypoalgesia and tactile allodynia in diabetic mice which implicates LO in

small sensory nerve fiber dysfunction in PDN. Alleviation of thermal hypalgesia was achieved despite the absence of intraepidermal nerve fiber regeneration. Together with corneal confocal microscopy, intraepidermal nerve fiber density is emerging as a new approach for detection of small sensory nerve fiber degeneration in human subjects with diabetes and impaired glucose tolerance [24,25,75,76]. Both variables have shown excellent correlations with sensory function [75,76]. In contrast, several animal studies including those of our group revealed dissociations between loss of sensitivity to thermal noxious stimuli and intraepidermal nerve fiber loss ([77], reviewed in [25]). Note, that LO gene deficiency did not protect from development of either thermal hypoalgesia or tactile allodynia in HFD-fed mice.

It has previously been shown [78] that high glucose induced depletion of glycerol-phospholipid arachidonoyl-containing molecular species in HSC, a widely used cell culture model of PDN [32-34,78]. The present study demonstrating LO overexpression and 12(S)HETE accumulation in cultured HSC as early as 24 h after exposure to high glucose suggests that such depletion is at least partially due to increased arachidonate metabolism through the LO pathway. These findings combined with evidence of the contribution of LO to neuropathic changes obtained in two animal models suggest that the LO mechanism may be implicated in human PDN.

In conclusion, LO overexpression and activation contribute to nitrosative stress in peripheral nervous system and development of neuropathic changes associated with prediabetes and overt diabetes. The findings support the rationale for development and future studies of LO inhibitors and LO-inhibitor-containing combination therapies. The presence of LO in HSC and its upregulation early after exposure to high glucose suggest a potential involvement in human disease.

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Abbreviations

CDC	cinnamyl-3,4-dihydroxy-alpha-cyanocinnamate
COX-1 and COX-2	cyclooxygenases-1 and -2
DAB	diaminobenzidine
DRG	dorsal root ganglion
12(S)HETE	12(S)-hydroxyeicosatetraenoic acid
HSC	human Schwann cells
INFD	intraepidermal nerve fiber density
LO	12/15-lipoxygenase
MNCV	motor nerve conduction velocity
NC	normal chow
NT	nitrotyrosine
PBS	phosphate-buffered saline
PDN	peripheral diabetic neuropathy
PGP 9.5	protein gene product 9.5
PKC	protein kinase C
MAPK	mitogen-activated protein kinase
SNCV	sensory nerve conduction velocity
STZ	streptozotocin

Fig.1.

Representative microphotographs of fluorescent immunostaining [or fluorescent staining with isolectin Alexa Fluor(R) 594 conjugate, (*a, center*)] of diabetic mouse sciatic nerve for (*a*) LO (*left*); vascular endothelium (*center*), LO and vascular endothelium (*right*), and (*b*) LO (*left*); S-100 (*center*), LO and S-100 (*right*). Magnification x 150.

Fig.2.

Representative Western blot analyses of LO expression, LO protein contents (densitometry, %), and 12(S)HETE concentrations in the sciatic nerve (a,b,e) and spinal cord (c,d,f) of control mice and those with STZ-diabetes of 12-wk duration. C – control; D – diabetic. Mean $\pm$ SEM, n = 6-8 per group. ** p < 0.01 vs non-diabetic control group.

Fig.3. Representative Western blot analyses of LO expression, LO protein contents (densitometry, %), and 12(S)HETE concentrations in the sciatic nerve (a,b,e) and spinal cord (c,d,f) of control and STZ-diabetic mice maintained with or without CDC treatment. C – control; D – diabetic. Mean $\pm$ SEM, n = 8-12 per group. *, ** p < 0.05 and < 0.01 vs non-diabetic control group. #, ## p < 0.05 and < 0.01 vs untreated diabetic group.

Fig.4.

Upper and middle panels. Representative Western blot analyses of LO expression and LO protein contents (densitometry, %) in sciatic nerve (a,b) and spinal cord (c,d) of mice fed normal chow or high-fat diet. *Lower panel.* Sciatic nerve (e) and spinal cord (f) 12(S)HETE concentrations in wild-type and $LO^{-/-}$ fed normal chow or high-fat diet. NC – normal chow; HFD - high fat diet. Mean $\pm$ SEM, n = 6-9 per group. *, ** p < 0.01 vs mice fed normal chow. §§, §§§ - p < 0.05 and < 0.01 vs wild-type mice fed NC; ## - p < 0.01 vs wild-type mice fed HFD.

Fig.5.

Upper panel: Representative Western blot analyses of nitrated protein expression and their content (densitometry, %) in the sciatic nerve (a,b) and spinal cord (c,d) of control mice and those with STZ-diabetes of 12-wk duration. *Lower panel:* Representative Western blot analyses of nitrated protein expression and their content (densitometry, %) in the sciatic nerve (e,f) and spinal cord (g,h) of control and STZ-diabetic mice maintained with or without CDC treatment. Mean $\pm$ SEM, n = 7-12 per group. ** p < 0.01 vs non-diabetic control group. ## p < 0.01 vs untreated diabetic group.

Fig.6. Representative Western blot analyses of nitrated protein expression and their content (densitometry, %) in the sciatic nerve (a,b) and spinal cord (c,d) of wild-type and LO^{-/-} mice fed normal chow or high-fat diet. NC – normal chow; HFD-high-fat diet. Mean ± SEM, n = 7-12 per group. *, ** p < 0.05 and < 0.01 vs mice fed normal chow; ## p < 0.01 vs high-fat diet fed wild-type mice.

Fig.7.

Intraepidermal nerve fiber profiles in (a,b) control and diabetic mice at the 12-wk time point (prior to CDC intervention); (c,d) control and diabetic mice maintained with or without CDC treatments (a 16-wk time point), and (e,f) LO^{+/+} and LO^{-/-} mice after 16 wks of feeding with normal chow or high-fat diet. (a,c,e) Representative images of intraepidermal nerve fiber profiles, magnification x 40; (b,d,f) Skin fiber density. Mean $\pm$ SEM, n = 10-12 per group. C – control mice, D – diabetic mice, NC- normal chow, HFD-high-fat diet. ** – p < 0.01 vs non-diabetic control group.

Fig.8.

LO expression and 12(S)HETE concentrations in human Schwann cells cultured in 5.5 mM D-glucose (1), 5.5 mM D-glucose plus 24.5 mM L-glucose (2), and 30 mM D-glucose (3). (a) Representative Western blot analysis of LO expression, (b) LO protein content, and (c) 12(S)HETE concentrations. Mean $\pm$ SEM, n = 6 per group. ** p < 0.01 vs cells cultured in 5.5 mM D-glucose.

Table 1

Initial and final body weights and blood glucose concentrations in control and diabetic mice maintained with and without CDC inhibitor treatment (Experiment 2) or fed normal chow or high-fat diet (Experiment 3).

Group	Variable	Body weight (g)		Blood glucose (mmol/l)	
		Initial	Final	Initial	Final
EXPERIMENT 2					
Control + CDC	Control	25.8 ± 0.6	38.5 ± 1.3	9.4 ± 0.7	8.7 ± 0.3
	Control + CDC	25.4 ± 0.5	36.6 ± 1.4	9.4 ± 0.4	9.3 ± 0.3
	Diabetic	25.1 ± 0.4	27.7 ± 0.5**	15.7 ± 1.2**	27.7 ± 1.1**
	Diabetic + CDC	25.5 ± 0.5	26.8 ± 0.6**	16.1 ± 1.5**	28.9 ± 1.0**
EXPERIMENT 3					
Wild-type, HFD	Wild-type, NC	24.8 ± 0.7	35.4 ± 1.0	8.4 ± 0.6	9.3 ± 0.6
	Wild-type, HFD	25.0 ± 0.6	46.0 ± 1.6**	9.1 ± 0.7	9.5 ± 0.7
LO ^{-/-} , HFD	LO ^{-/-} , NC	25.6 ± 0.7	34.9 ± 1.2	9.0 ± 0.5	9.4 ± 0.4
	LO ^{-/-} , HFD	25.3 ± 0.5	44.7 ± 1.5**	8.6 ± 0.4	9.2 ± 0.5

Data are expressed as Means ± SEM. n = 24 per group in Experiment 3 and at the beginning of the study in Experiment 2. n = 10-12 per group after the beginning of CDC treatment in Experiment 2.

** p < 0.01 vs non-diabetic control group (Experiment 2) and vs mice fed normal chow (Experiment 3).

NC – normal chow, HFD- high-fat diet.

Table 2

Motor and sensory nerve conduction velocities, thermal response latencies, and tactile response thresholds in control and diabetic mice maintained with or without CDC inhibitor treatment (Experiment 2) or fed normal chow or high-fat diet (Experiment 3).

Group	Variable	MNCV	SNCV	Thermal response latency (s)	Tactile response threshold (g)
EXPERIMENT 2					
Baseline (prior to induction of diabetes)					
Control		51.2 ± 1.0	37.5 ± 0.3	9.4 ± 0.5	2.18 ± 0.14
12-wk time point (prior to CDC intervention)					
Control		50.2 ± 1.3	38.2 ± 0.4	9.3 ± 0.4	2.23 ± 0.13
Diabetic		40.1 ± 0.9**	32.7 ± 0.85**	18.9 ± 0.6**	0.70 ± 0.1**
16-wk time point					
Control		56.7 ± 1.9	37.8 ± 0.7	11.1 ± 0.5	2.54 ± 0.13
Control +CDC		54.5 ± 1.8	38.1 ± 0.8	11.4 ± 0.4	2.35 ± 0.18
Diabetic		41.2 ± 2.3**	30.4 ± 0.8**	18.0 ± 0.95**	1.23 ± 0.06**
Diabetic+CDC		47.1 ± 2.6**a	34.0 ± 0.5***##	15.3 ± 0.6***##	1.91 ± 0.1***##
EXPERIMENT 3					
Baseline (prior to high-fat diet feeding)					
LO ^{+/+} , NC		52.3 ± 1.3	36.7 ± 1.6	8.5 ± 0.3	2.31 ± 0.12
LO ^{-/-} , NC		52.9 ± 1.4	37.0 ± 1.3	8.1 ± 0.2	2.25 ± 0.13
16-wk time point					
LO ^{+/+} , NC		52.7 ± 2.0	36.2 ± 0.9	7.6 ± 0.2	2.36 ± 0.11
LO ^{+/+} , HFD		42.4 ± 1.5***	30.5 ± 0.7**	14.3 ± 0.2**	1.22 ± 0.05**
LO ^{-/-} , NC		54.5 ± 2.0	36.1 ± 0.5	8.7 ± 0.1	2.34 ± 0.08
LO ^{-/-} , HFD		47.5 ± 1.4*, #	34.2 ± 1.3##	14.1 ± 0.3**	1.12 ± 0.04**

Data are expressed as Means ± SEM, n = 8-12 per group.

*, ** p < 0.05 and < 0.01 vs non-diabetic control group (Experiment 2) and vs corresponding groups of mice fed normal chow (Experiment 3).
 #, ## p < 0.05 and < 0.01 vs untreated diabetic group (Experiment 2) and vs HFD-fed wild-type mice (Experiment 3).

$p_{0.0599}$ vs untreated diabetic group.

NC – normal chow, HFD- high-fat diet.