Dyslipidemia-Induced Neuropathy in Mice: the Role of oxLDL/LOX-1

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Running Title: oxLDL and Neuropathy

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Objective: Neuropathy is a frequent and severe complication of diabetes. Multiple metabolic defects in type 2 diabetic patients result in oxidative injury of dorsal root ganglia (DRG) neurons. Our previous work focused on hyperglycemia clearly demonstrates induction of mitochondrial oxidative stress and acute injury in DRG neurons; however, this mechanism is not the only factor that produces neuropathy \emph{in vivo}. Dyslipidemia also correlates with the development of neuropathy, even in pre-diabetic patients. This study was designed to explore the contribution of dyslipidemia in neuropathy.

Research Design and Methods: Mice (n=10) were fed a control (10% kcal% fat) or high fat (45% kcal% fat) diet to explore the impact of plasma lipids on the development of neuropathy. We also examined oxidized lipid-mediated injury in cultured DRG neurons from adult rat using oxidized low density lipoproteins (oxLDL).

Results: Mice on a high fat diet have increased oxLDL and systemic and nerve oxidative stress. They develop nerve conduction velocity (NCV) and sensory deficits prior to impaired glucose tolerance. \textit{In vitro}, oxLDL lead to severe DRG neuron oxidative stress via interaction with the receptor LOX-1 and subsequent NAD(P)H oxidase activity. Oxidative stress resulting from oxLDL and high glucose is additive.

Conclusions: Multiple metabolic defects in type 2 diabetes directly injure DRG neurons through different mechanisms that all result in oxidative stress. Dyslipidemia leads to high levels of oxLDL that may injure DRG neurons via LOX-1 and contribute to the development of diabetic neuropathy.
Our work is focused on understanding the mechanisms that lead to diabetic neuropathy and developing rational therapeutic interventions. Hyperglycemia clearly leads to peripheral nerve injury through the development of systemic and neuronal oxidative stress (1-6). An emerging idea is that dyslipidemia also contributes to the development of diabetic neuropathy (7; 8). Lipid profiles are commonly abnormal early in the course of type 2 diabetes in a temporal pattern that correlates with the presence of diabetic neuropathy and we recently reported that elevated triglyceride levels predict a more rapid disease course (9; 10). In addition, several large scale trials of type 2 diabetes patients point to early dyslipidemia as a major independent risk factor for the development of diabetic neuropathy (11).

In experimental diabetes, the complex etiology of diabetic neuropathy is difficult to explore due to the multiple sources of nerve injury including hyperglycemia, AGEs, systemic oxidative stress, and altered growth factor availability (12). Furthermore, lipid profiles of mice differ from human patients in that the majority of plasma cholesterol is transported in HDL and LDL levels are constitutively low (13). Mice with genetically increased plasma cholesterol have accelerated atherosclerosis that renders them unsuitable for neuropathy studies (13). Several studies explored the role of a high fat diet in the development of both diabetes and diabetic complications (14-16). Susceptibility to neuropathy is mouse strain-dependent; the constitution of the diet is another important factor. One study suggests that a high fat diet produces neuropathy independent of hyperglycemia (17) and the present study explores a potential mechanism of high fat-induced neuropathy.

Because high fat diets increase plasma LDL and pre-diabetes is associated with systemic oxidative stress (18), we proposed that oxLDL will be elevated in mice fed a high fat diet. Furthermore, we predicted that increased oxLDL may produce DRG neuron injury through binding the receptor LOX-1 in a similar manner to vascular endothelial cells (19) and renal tubular cells (20). The activation of LOX-1 on endothelial cells leads to intracellular oxidative stress and inflammation and a feed-forward cycle of injury in diabetes, since both oxLDL and glucose increase LOX-1 expression (21; 22).

In this study, we employed high fat feeding in the C57/Bl6 mouse strain using a 45 kcal% fat (mostly from lard) diet. We demonstrate morphological and functional evidence of neuropathy prior to loss of glucose regulation in agreement with clinical findings (10; 23; 24). This is associated with significant increases in plasma oxLDL. We assessed oxLDL-mediated injury in cultured DRG neurons from adult rats. oxLDL directly lead to oxidative stress and injury in DRG neurons via LOX-1. DRG neuron injury is partially induced via activation of NAD(P)H oxidase. We conclude that diet-induced plasma oxLDL can produce neuron injury and may be a contributing factor in the development of neuropathy in prediabetes or diabetes.

**RESEARCH DESIGN AND METHODS**

**High fat fed mice.** C57/Bl6 mice (Jackson Laboratories, Bar Harbor, Maine) at 3 wk age were placed on either control AIN5003 (10%kcal%fat) or high fat (45%kcal%fat) chow D12451i from Research Diets (New Brunswick, NJ), with 10 mice/group. Chows were matched for protein and carbohydrate content. Blood glucose was tested every 4 wk following a 6 h fast. One drop of tail blood was analyzed using a standard glucometer (One Touch Profile, LIFESCAN, Inc. Milpitas, CA, #6 strips). Glucose tolerance tests were performed by measuring blood glucose 5, 15, 30, 60, and 120 min after gavage administration of a glucose bolus. Nerve conduction velocity (NCV) studies were performed after 12 and...
34 wk, and neuropathy phenotyping (see below) at termination at 34 wk. Glycated hemoglobin (GHb) was measured using the Helena Laboratories Test Kit, Glyco-Tek Affinity Column Method (Catalog #5351) as previously described (25; 26). Insulin was measured by radioimmunoassay in the MDRTC Chemistry Core.

**Nerve Conduction Studies.** Hind paw thermal latency and NCV were measured per our published protocols (2; 12; 26-28) and in compliance with protocols established by the AMDCC (http://www.amdcc.org). Mice were anesthetized with 30/0.75 mg/kg ketamine/acepromazine by peritoneal injection and body temperatures were maintained at 32-34°C using a heating pad.

**Tissue Harvest.** Mice were euthanized with an overdose of pentobarbital 34 weeks after initiation of control or high fat diets. Sciatic nerves were harvested and submerged in ice-cold antioxidant Buffer A (100 µM diethylene tetramino pentaacetic acid, 50 µM butylated hydroxytoluene, 1% [v/v] ethanol, 10 mM 3-amino-1,2,4-triazole, 50 mM sodium phosphate buffer, pH 7.4), rapidly frozen by immersion in liquid nitrogen, and stored at -80°C until analysis. A blood sample (100-300 µl) was collected into K3 EDTA Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), and centrifuged at 100 g for 15 min at 4°C. Plasma was collected and stored at -80°C.

**Intraepidermal Nerve Fiber Density.** Foot pads were collected from the hind paw plantar surface, postfixed in Zamboni’s (2 % PFA, 1 % picric acid in 0.1 M phosphate buffered saline) solution overnight, rinsed in 5, 10 and 20% sucrose in 50 mM sodium phosphate buffer, cryoembedded, sectioned (35 µm) and processed for PGP 9.5 immunohistochemistry (1:2000 Chemicon, Temecula, CA) per our previous work (25; 26; 28). The data are presented as the number of fibers/mm² across one entire papilla.

**Plasma lipid profiling.** Plasma samples from 5 mice/group were pooled to produce 2 pools/group and each pool was run 5 times on FPLC, then the fractions assayed for cholesterol and triglycerides. These assays were performed as previously published by the MMPC Core at the University of Washington, Seattle (29). LDL were isolated from mouse plasma by discontinuous density ultracentrifugation as described previously (30). 200 µL plasma was overlaid with normal saline (d = 1.006 kg/l) and ultracentrifuged for 60 min at 541,000 g max at 4°C. After removal of VLDL from the top, the density was adjusted with solid KBr to d = 1.300 kg/l, then overlaid with normal saline and ultracentrifuged for 60 min at 541,000 g max at 4°C. LDL were removed by aspiration of the top layer. The sample was desalted using PD10 column (Amersham; Sephadex G-25M) and eluted with 0.01M PBS (pH 7.4).

**HO*DE (hydroxyoctadecadienoic acid), Dityrosine and Nitrotyrosine Levels.** HODE, dityrosine and nitrotyrosine were measured in plasma, sciatic nerve, and isolated LDL as described previously (31-33). HODEs were quantified in lipid extracts by reverse-phase C-18 HPLC (Ultrasphere column; 250 mm×4.6 mm; particle size 5 µm; Beckman). Amino acids were analyzed by reverse phase HPLC (Jasco HPLC; Column; Beckman ODS Ultrasphere C-18 column 250 mm×4.6 mm; particle size 5 µm). The identity and quantity of the compounds was confirmed by identical retention time on standard curves with authentic o,o'-dityrosine and tyrosine by monitoring absorbance at 460 nm. Nitrotyrosine was quantified by isotope dilution mass spectrometry as described previously (33).

**Adult DRG Neuron Cultures.** DRG were collected from euthanized adult female Sprague Dawley rats and dissociated in 0.2 % collagenase for 30 min followed by 1 % trypsin for 15 min. Cells were seeded on collagen-coated plates in DMEM:F-10, 50:50,
containing 1 x B27 additives, 40 µM FUDR, 7 µM aphidicolin and 1,000 U/ml penicillin/streptomycin/neomycin. Cells were re-fed on day 2 and experiments performed on day 3. In this media the basal glucose concentration is 5.7 mM. For high glucose experiments, 20 mM glucose is added to the media, yielding a total of 25.7 mM. For oxLDL experiments, a dose curve from 1-100 µg/ml was selected, based on other in vitro studies (34-37). From LDL isolation, we estimate that there was around 300-400 µg/ml LDL protein in the mouse plasma. The amount of LDL protein was not significantly different between control and high fat mice, but oxidation markers were 1.6-24-fold higher in high fat compared to control mice (Table 1). This plasma LDL concentration is 10-30 X higher than the oxLDL 30 µg/ml dose used in the in vitro experiments. Control experiments were always performed with non-oxidized LDL and these did not produce the cellular responses of oxLDL (data not shown).

**NAD(P)H Oxidase Activity.** NAD(P)H oxidase activity in DRG neuron lysates is assessed as previously described (1). The rate of luminescence increase in the presence or absence of apocynin was calculated to give a measure of superoxide generation by NAD(P)H oxidase activity (luminescence units/min).

**Fragmentation of Genomic DNA.** TdT mediated dUTP-biotin nick end labeling (TUNEL) staining is used to detect cell death in cultured DRG neurons (10, 18, 29). Samples are fixed in 4% paraformaldehyde, labeled with digoxygenin-dUTP, then stained with horseradish peroxidase-conjugated anti-digoxygenin antibody using a kit according to the manufacturer’s instructions (Intergen, Gaithersburg, MD).

**Immunohistochemistry.** DRG neurons were fixed in 4 % paraformaldehyde in 0.1 M phosphate buffered saline solution (PBS) for 5 min. Primary antibodies were applied in 0.1 M PBS/1% Triton X100/5% serum for 16 h at 4°C. Cells were washed for 3x 10 min in PBS, then species-specific secondary antibody conjugated to AlexaFluor 594, (Molecular Probes, Eugene, OR) diluted 1:1000 in 0.1 M PBS containing 1% Triton X100 and 5% serum. After a further 1 h at room temperature, cells were washed 3x 10 min in PBS. For nitrotyrosine, the primary was antibody 244 (10 µg/ml; Upstate Biologicals, Lake Placed, NY). Fluorescence was quantitated in the fluorescent plate reader at 485 ex, 590 em. For LOX-1 the primary was Ox-LDL R-1 (Y-21) from Santa Cruz sc11653, diluted 1:100. Immunofluorescence was examined on an Olympus Fluoview inverted microscope.

**Western blotting.** Western blotting was performed per our previous studies (1-3) with 40 µg protein/lane, separated on a 12.5% polyacrylamide gel.

**Caspase 3.** After 5 h exposure to 20 mM excess glucose, 1 coverslip from each dose was treated with fluorescent CaspaTag reagent per the manufacturer’s protocol.
Statistical Analysis. In cell culture studies, all experimental paradigms were performed in triplicate on three separate occasions with different cell cultures, giving a final n=9 for each data point. In mouse studies, there were 10 mice per group for all in vivo studies. Lipid profiling was performed 3X on each of 2 pools of plasma/group, n=2. Data analyses were performed using Prism, version 3 (GraphPad Software, Inc.). Assumptions about the Gaussian distribution of data and rules for transformation of non-normative data were made as previously described (28). Comparison of dependent variables was performed using factorial analysis of variance (ANOVA) with 95% confidence intervals. All measurements were made by an observer blinded to the experimental condition. Bar graphs illustrate the mean ± standard error of the mean (SEM).

RESULTS

High fat fed mice develop neuropathy. Following 12 weeks on a high fat diet, mice were modestly heavier than mice on a control diet (28.9±1.4 g compared to 25.4±0.5 g) (Fig. 1A) but did not have increased fasting blood glucose (Fig. 1B). Despite the lack of evidence for glucose intolerance, the mice displayed evidence of neuropathy. The latency of hind paw response to a heat stimulus was significantly increased (Fig. 1C; p<0.05) and the sciatic NCV was slowed in the high fat mice (Fig. 1D; p<0.05). Other neuropathy measures at 12 wk (tail flick and sural NCV) showed no difference between control and high fat diet fed mice (not shown).

By 34 wk, mice displayed glucose intolerance with significantly higher blood glucose levels 15 min after applying the glucose bolus, and this difference remained at 2 h (Fig. 2A). Similarly, body weight, plasma insulin, and glycated hemoglobin (GHb) were all significantly increased in the high fat group compared to control diet (Fig. 2B-D), indicating frank diabetes in the high fat mice. NCV measures in sural (p<0.05) and sciatic (p<0.01) nerves were both decreased in the high fat compared to control diet mice (Fig. 2E-F). Sensory neuropathy was evident in the high fat mice through decreased response to a heat stimulus on the hind paw (Fig. 2G) and decreased intraepidermal nerve fiber density (IENFD) in the hind paw skin (Fig. 2H-I).

Oxidative stress was assessed in plasma, isolated LDL, and sciatic nerves (Table 1). Plasma lipids were significantly oxidized, as evidenced by increased HODE, and plasma proteins were both oxidized (dityrosine) and nitrosylated (nitrotyrosine) in high fat fed mice compared to the control diet. We also measured these oxidation markers in LDL isolated from the mice to confirm the elevation of oxLDL (Table 1). Next, we compared plasma lipid profiles between the two groups of mice. HDL were increased and the peak shifted slightly left in the high fat fed group, indicating uptake of cholesterol. There was also a shift in the high fat fed group to increase the population of LDL and these contain significantly elevated triglycerides. Also, a peak of VLDL is evident in the high fat mice, but absent in the control diet mice (Fig. 3A). There were significant increases in the levels of triglycerides associated with the LDL/VLDL samples in the high fat fed mice, as well as free triglycerides in the high fat fed mice only (Fig 3B). Together, data in Table 1 and Fig. 3 confirm that high fat feeding increases plasma lipids and oxLDL.

DRG neurons express LOX-1. We demonstrated that DRG neurons express the receptor for oxLDL, LOX-1, by immunocytochemistry and Western blotting. The Western blot demonstrates that 3 isoforms of LOX-1 are present in adult DRG neurons (Fig. 4A). The 48 kDa glycosylated form is most highly expressed in the neurons. This isoform is glycosylated for transport to the plasma membrane and is considered the...
active isoform (40). Exposure to 5 µg/ml or higher concentrations of oxLDL for 3 h led to an increase in the 48 kDa isoform (Fig. 4B).

Immunocytochemistry in control DRG neurons confirms that LOX-1 is present on the neurons (arrows) and not only on residual Schwann cells in the cultures (Fig. 4C).

**oxLDL generate oxidative stress in DRG neurons via LOX-1.** To define the mechanisms of oxidized lipid-induced neuropathy, we explored the effects of oxLDL on adult DRG neurons in culture. We found a dose-dependent increase in mitochondrial superoxide after 1 h exposure to oxLDL (Fig. 5A). Above 30 µg/ml oxLDL there was a significant increase in mitochondrial superoxide that was additive to the levels produced by high glucose. Using a second probe, DHE, we confirmed that a portion of oxLDL-induced superoxide involved the mitochondria (Fig. 5B). We demonstrate that, similar to MitoSOX oxidation, DHE oxidation increases above 30 µg/ml oxLDL and that around 50% of this signal is blocked in the presence of the mitochondrial uncoupler FCCP (2.5 µM). This dose of FCCP leads to loss of mitochondrial membrane potential and ATP generation and the data suggest that around 50% of the DHE signal is produced via mitochondrial electron transfer chain generation of superoxide. Preloading the cells with α-lipoic acid (100 µM) also blocked DHE oxidation at 10 µM and 30 µM doses. α-lipoic acid decreased (p<0.05) but did not completely prevent DHE oxidation in response to 100 µM oxLDL (Fig. 5B). Confirmation that generation of superoxide leads to oxidative stress in this system was achieved using assays for total radical antioxidant potential (TRAP; Fig. 5C) and cellular nitrotyrosine (Fig. 5D). Using a single dose and time-point where there was substantial oxidative stress in the MitoSOX assay, exposure to 30 µg/ml oxLDL for 3 h significantly decreased the antioxidant potential of DRG neurons by greater than 40%. Doses as low as 10 µg/ml oxLDL increased nitrotyrosine over 24 h in the DRG neurons (Fig. 5D) and 100 µg/ml oxLDL achieved nitrotyrosine levels similar to high glucose. oxLDL-induced, but not high glucose-induced nitrotyrosine was blocked by pre-incubating the DRG neurons with a LOX-1 neutralizing antibody (100 mg/ml).

**oxLDL-LOX-1-induced DRG neuron injury involves NAD(P)H oxidase.** We next examined DRG neuron injury via activation of caspase 3 (Fig. 6A) and degradation of nuclear DNA (Fig. 6B). Activation of caspase 3 after 5 h was significantly increased by at least 3-fold in the presence of high glucose or 10, 30 or 100 µg/ml oxLDL. Pre-treatment with LOX-1 neutralizing antibody (100 mg/ml) significantly blocked oxLDL-induced but not glucose-induced caspase 3 activation. The NAD(P)H oxidase inhibitor apocyanin (1 µM) significantly blocked caspase 3 activation with 10 µg/ml oxLDL, but decreased with increasing concentrations of oxLDL. This may be due to the dose of apocyanin being too low to block high levels of NAD(P)H oxidase activity, but higher doses of apocyanin induce oxidative stress and cell injury (data not shown). The antioxidant α-lipoic acid (100 µM) efficiently blocked caspase 3 activation induced by both high glucose and doses of oxLDL up to 100 µM.

The TUNEL assay was performed after 24 h to determine the total cell death caused by high glucose or oxLDL treatments. High glucose produces 60±4% DRG neuron death after 24 h (Fig. 6B). This death is completely blocked by the antioxidant α-lipoic acid (100 µM) consistent with high glucose injury in these cells via mitochondrial oxidative stress as we previously published in embryonic DRG (and see Fig. 4A). Glucose-induced injury was not altered by anti-LOX-1 or by apocyanin.

At 10 µg/ml oxLDL there was an approximate 10% increase in TUNEL over 24 h. There was no significant effect of antioxidant or apocyanin, but blocking LOX-
1 significantly decreased TUNEL to control levels (Fig. 6B). 30 µg/ml oxLDL increased TUNEL to approximately 60% and 100 µg/ml to 70%. All three inhibitors - apocyanin, anti-LOX-1, and antioxidant - significantly decreased oxLDL-induced cell death to approaching control levels (p<0.01).

Confirmation that oxLDL-LOX-1 interaction activates NAD(P)H oxidase.

Next, we examined the activation of NAD(P)H oxidase at the 1 h and 3 h time-points (Fig. 7). Both p47 and gp91 were localized in the cell soma in untreated control DRG neurons, but 3 h subsequent to exposure to oxLDL, labeling was also identified along the dendrites and the intensity of staining was increased (Fig. 7A). The activity of NAD(P)H oxidase was significantly increased after 1 h and 3 h exposure to high glucose, as we showed previously (1). Following exposure to 30 µg/ml oxLDL, NAD(P)H oxidase activity increased by approximately 100 %, but was decreasing towards control levels by 3 h (Fig. 7B). By Western blotting, the p47 subunit was increased in DRG neurons exposed to high glucose or oxLDL (30 µg/ml) for 3h. The gp91 antibody did not produce a clearly defined band on Western blots (not shown).

DISCUSSION

In this report, we confirm that high fat feeding in mice produces neuropathy and increased tissue and systemic oxidative stress prior to the development of frank diabetes. We postulated that these metabolic changes would generate oxLDL and that these could contribute to neuronal injury mechanisms. We demonstrate that DRG neurons express the oxLDL receptor LOX-1 and that activation of this receptor in vitro leads to NAD(P)H oxidase activity that injures DRG neurons via generation of oxidative stress.

After 12 wk on a high fat diet, weight was modestly increased while fasting blood glucose remained normal. The hind paw latency was increased and sciatic NCV decreased, consistent with peripheral neuropathy. Previous studies established that a high fat diet leads to type 2 diabetes in mice (14; 41; 42). These studies focused on the mechanisms leading to insulin resistance, although one study examined the consequences of a high fat diet on the onset of nephropathy (43) and one on neuropathy (17). No previous study has shown that a high fat (specifically high cholesterol) diet leads to NCV deficits and reduced hind paw withdrawal response prior to impaired glucose tolerance (IGT). Our current data suggest that high fat-induced neuronal deficits may precede the development of diabetes.

By 34 wk on the high fat diet, the mice displayed IGT, a significant increase in plasma insulin levels and an increase in glycated hemoglobin. These increases are small compared to mice with frank type 2 diabetes, where the plasma insulin may be elevated four-fold and glycated hemoglobin by several percent, but the extent of neuropathy is comparable (26). This provides supporting evidence for a role for dyslipidemia in the development of neuropathy. Clinical studies support this finding. In the Eurodiab Trial, of 1,200 subjects who did not have diabetic neuropathy at baseline, hypertension, serum lipids and body mass index were each independently associated with the risk of developing diabetic neuropathy during a 7 year follow-up period. Of these risk factors, dyslipidemia was closely linked with the onset and progression of diabetic neuropathy (44). In parallel, we recently reported that dyslipidemia, not hyperglycemia, was more closely correlated with neuropathy progression in 427 trial participants (10).

We demonstrate not only that the high fat diet produced dyslipidemia, with high levels of HDL and LDL cholesterol and triglycerides, but also that LDL and other circulating lipids and proteins are oxidized. Oxidatively modified proteins and lipids are toxic to complication-prone tissues, such as renal tubules (20) and the retina (45).
particular, oxLDL mediate vascular injury via interaction with LOX-1 on endothelial cells (46). This led us to postulate that oxLDL may injure DRG neurons.

We report strong expression of LOX-1 on the adult DRG neurons by Western blotting and immunocytochemistry. Next, we characterized the effects of high glucose and oxLDL on DRG neuron oxidative stress. Both insults lead to mitochondrial superoxide formation, the key mediator of DRG neuron injury in high glucose (1; 2; 38; 47). The effects of high glucose and oxLDL on mitochondrial superoxide were additive. Furthermore, antioxidant potential is depleted over time by oxLDL. Using a neutralizing antibody to LOX-1, we demonstrate that the effects of oxLDL are largely mediated via the LOX-1 receptor. This protection has been shown for vascular injury in dyslipidemia (48), but not previously for neurons. Our data suggest that multiple metabolic deficits in dyslipidemia and early diabetes combine to produce oxidative stress and accelerate the onset and progression of neuropathy. These data strongly argue for an antioxidant component to therapies for diabetic neuropathy (49). Our work also raises the interesting question of how obesity, immobility and the metabolic syndrome contribute to neuropathy.

High glucose or oxLDL insults increased DRG neuron injury, evidenced by the activation of caspase 3 and by nuclear DNA degradation. The injury produced by either stressor was blocked by the antioxidant α-lipoic acid. These data support the consensus that pathways leading to cellular injury in diabetes (mainly shown for hyperglycemia to date) converge upon oxidative stress. Antioxidant therapies remain the most promising strategy for diabetic neuropathy, but clearly greater understanding of the long-term application of antioxidant therapy needs to be explored.

We also found that inhibition of NAD(P)H oxidase could block oxLDL-induced but not high glucose-induced injury. Supporting evidence that oxLDL led to NAD(P)H oxidase activity was obtained by observing subunit expression and localization and by measuring apocyanin-inhibitable NADPH oxidation. Thus, NAD(P)H oxidase activation and subsequent generation of superoxide is the primary injury mechanism in oxLDL-treated DRG neurons. This activation of NAD(P)H oxidase is mediated via LOX-1 signaling, since blocking LOX-1 abrogates oxLDL-induced DRG neuron injury. Our data agree with studies showing LOX-1-mediated activation of NAD(P)H oxidase in vascular endothelial cells through recruitment of NAD(P)H oxidase subunits (50). Collectively, these data demonstrate a pivotal role for NAD(P)H oxidase in the injury of DRG neurons and the progression of neuropathy (51).

This study demonstrates a mechanism by which dyslipidemia produces DRG neuron injury that may underlie emerging clinical evidence that dyslipidemia is an independent risk factor for diabetic neuropathy. The data suggest why glycemic control alone is insufficient to prevent complications in type 2 diabetes and argue for combination therapies targeting multiple metabolic imbalances and receptor-mediated signaling that leads to oxidative injury. Prevention of oxLDL formation may be one strategy, but targeting oxLDL receptors may be an important alternative approach.

ACKNOWLEDGEMENTS

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Diabetes Association (AMV), the Animal Models of Diabetes Complications Consortium (AMDCC; NIH U01 DK076160, the Animal Models of Diabetes Complications ELF), and the Program for Neurology Research and Discovery.

Table 1  Oxidative stress measures in plasma, LDL, and sciatic nerve. Lipid and protein peroxidation were assessed by reverse phase HPLC. HODE, dityrosine, and nitrotyrosine were all significantly increased in plasma (n=10), sciatic nerve (n=10), and isolated LDL (n=2), *p<0.05.

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Fig. 1. Mild neuropathy after 12 wk high fat diet. Following 12 wk of high fat or control diet, weight (A), blood glucose (B), hind paw withdrawal latency (C) and sciatic NCV (D) were assessed. n=10/group. *p<0.05 compared to control diet.

Fig. 2. Neuropathy and impaired glucose tolerance after 34 wk high fat diet. Complete phenotyping after 34 wk on control (Ctrl) or high fat (HF) diet was performed. The figure displays the glucose tolerance test (A), weight (B), plasma insulin (C), glycated hemoglobin (D), sural NCV (E), sciatic NCV (F), hind paw withdrawal latency (G), and intraepidermal nerve fiber density (IENFD) (H). *p<0.05 compared to the control diet group. In all panels, n=10. (I) Representative IENFD images from one control and one high fat fed sample. Bar = 50 µm; d=dermis, e=epidermis. White dots indicate nerve fibers counted.

Fig. 3. Plasma lipid profiles in the plasma at 34 wk. Pooled plasma samples (2 pools/group, each pool analyzed 3 times) were subjected to fractionation by FPLC, then cholesterol (A) and triglycerides (B) were measured in each fraction. The graph shows the mean and SEM for n=2 pools/group.

Fig. 4. LOX-1 expression on DRG neurons. DRG neurons from adult rat were assessed for LOX-1 by Western blotting (A,B) and immunocytochemistry (C) after 3 d in culture. In A, 40 mg protein from untreated control (C) cultures in lane 1 display the glycosylated 48 kDa isoform of LOX-1 that is predicted to be at the cell surface. The lower molecular weight precursor (40 kDa) and non-glycosylated (32 kDa) isoform are also present. Exposure to high glucose (G; 20mM added, total 25.7 mM glucose) or 1 µg/ml oxLDL for 3 h did not alter LOX-1 expression. 5, 10, or 30 mg/ml oxLDL for 3 h increased the level of the 48 kDa isoform and the two higher doses appear to decrease the lower molecular weight forms. + represents a positive control peptide based on the 40 kDa predicted precursor form of LOX-1. In B, densitometry was performed on three replicates of the representative blot shown in A. Mean pixel densities for the 48 kDa band are normalized against actin. The graph shows the mean and standard error, *p<0.05 compared to the untreated control. In, a representative image of untreated DRG neurons is shown. White arrows indicate neurons labeled for LOX-1. Bar = 50 µm.

Fig. 5. High glucose and oxLDL induce oxidative stress in adult DRG neurons. After 3 d in culture, oxidative stress assays were performed in cultured DRG neurons. In A, DRG neurons were exposed to increasing concentrations of oxLDL in the presence of basal (5.7 mM) or high (25.7 mM) glucose for 1 h, then loaded with MitoSOX for 15 min. n=9, *p<0.01 compared to control (basal glucose, 0 µg/ml oxLDL). In B, DRG neurons were exposed to increasing concentrations of oxLDL alone or in the presence of the mitochondrial uncoupler FCCP (2.5 µM) or the antioxidant α-lipoic acid (LA, 100 µM) for 1 h then loaded with DHE for 15 min. n=9, *p<0.01, +p<0.05 compared to control. In C, TRAP was assessed in untreated DRG neurons and those exposed to 30 µg/ml oxLDL for 3 h, n=3, *p<0.01. In D, nitrotyrosine fluorescence was assessed 24 h following exposure to high glucose or increasing concentrations of oxLDL in the presence or absence of LOX-1 neutralizing antibody (100 mg/ml). n=9, *p<0.01 compared to untreated control, †p<0.01 compared to no antibody.

Fig. 6. High glucose and oxLDL cause cell death in DRG neurons. Adult DRG neurons were exposed to high glucose (25.7 mM) or increasing concentrations of oxLDL and then cell death
was quantitated by caspase 3 activation after 5 h (A) or TUNEL after 24 h (B). DRG neurons were additionally pre-treated with LOX-1 neutralizing antibody (Anti-LOX-1, 100 mg/ml), apocyanin (Apo, 1 µM), or a-lipoic acid (LA, 100 µM). n=9, *p<0.01 compared to untreated control, +p<0.01 compared to no pre-treatment (None).

Fig. 7. High glucose and oxLDL activate DRG neuron NAD(P)H oxidase. In (A), adult DRG neurons were exposed to 30 µg/ml oxLDL and then immunolabeled for NAD(P)H oxidase subunits p47 or gp91. In (B), adult DRG neurons were exposed to high glucose (25.7 mM) or oxLDL (30 µg/ml) for 1 h or 3 h, then lysed for biochemical assays of NAD(P)H oxidase. *p<0.01 compared to untreated control, +p<0.05 compared to untreated control. In (C), adult DRG neurons were exposed to high glucose (G: 25.7 mM) or oxLDL (30 µg/ml) for 3 h, then lysed for Western blotting. The representative image shows the immunoblot for p47 and the loading control actin and the bar graph contains the mean and SEM of three repeats of the illustrated blot. *p<0.01 compared to untreated control (C).
Figure 2
Figure 3

A

- Total Cholesterol (mg/dL)
- Fraction Number
- VLDL - LDL - HDL
- Regular Chow
- High Fat Chow

B

- Triglyceride (mg/dL)
- Triglyceride (mg/dL)
- Fraction Number
- Regular Chow
- High Fat Chow
Figure 4

A:

B:

C:

Ratio LOX-1:Actin

μg/ml oxLDL

C  G  1  5  10  30

![Image of Western blot and bar graph showing the ratio of LOX-1 to actin across different oxLDL concentrations.]

![Images of cell cultures, showing differential staining and cell morphology.]

![Bar graph illustrating the mean ± standard deviation of the ratio LOX-1:Actin at various oxLDL concentrations.]

* Indicates statistically significant difference compared to control.
Figure 7