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The role of miR-146a in dorsal root ganglia neurons of experimental diabetic peripheral neuropathy

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Abstract

Sensory neurons mediate diabetic peripheral neuropathy. Using a mouse model of diabetic peripheral neuropathy (db/db mice) and cultured dorsal root ganglion (DRG) neurons, the present study showed that hyperglycemia downregulated miR-146a expression and elevated interleukin-1 receptor activated kinase (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) levels in DRG neurons. In vitro, elevation of miR-146a by miR-146a mimics in DRG neurons increased neuronal survival under high glucose conditions. Downregulation and elevation of miR-146a in DRG neurons, respectively, were inversely related to IRAK1 and TRAF6 levels. Treatment of diabetic peripheral neuropathy with sildenafil, a phosphodiesterase type 5 inhibitor, augmented miR-146a expression and decreased levels of IRAK1 and TRAF6 in the DRG neurons. In vitro, blockage of miR-146a in DRG neurons abolished the effect of sildenafil on DRG neuron protection and downregulation of IRAK1 and TRAF6 proteins under hyperglycemia. Our data provide the first evidence showing that miR-146a plays an important role in mediating DRG neuron apoptosis under hyperglycemic conditions.

Keywords

peripheral neuropathy; diabetes; mice; sildenafil; mir-146a

INTRODUCTION

Peripheral neuropathy is a common complication of diabetes (Feldman et al., 1997). Dorsal root ganglion (DRG) neurons comprise thermoceptors, mechanoceptors and itch sensors (Patapoutian, 2001, Patapoutian et al., 2003, Liu and Ma, 2011), which are responsible for the complication of diabetic sensory neuropathy (Kishi et al., 2002, Schmeichel et al., 2003, Pickup, 2004). Alteration of gene expression and signals in DRG neurons may contribute to distal axonal damage in diabetic peripheral neuropathy (Scientist 2008, 14, 313). Toll-like receptors (TLRs) are pattern-recognition receptors that are key players of the innate immune responses (Akira et al., 2001, Schnare et al., 2001, Takeda and Akira, 2005). Activation of

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Conflict of Interest: None

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TLRs recruits interleukin-1 receptor activated kinase 1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6), which mediate proinflammatory reaction (Takeda and Akira, 2004). The TLR signaling pathway contributes to the pathophysiology of diabetes. High-glucose upregulates TLR2 and TLR4 expression in human monocytes, and type 2 diabetic patients have increased TLR and IRAK1 activation in monocytes (Dasu et al., 2008, Dasu et al., 2010). However, the effect of TLR activation on DRG neurons in diabetes has not been examined.

MicroRNAs (miRNAs) are small noncoding RNA molecules that negatively regulate gene translation and promote RNA degradation (Behm-Ansmant et al., 2006, Dahiya et al., 2008). Biological function of miRNAs in mediating diabetes and diabetic complications is emerging (Kantharidis et al., 2011, Natarajan et al., 2012). Among miRNAs, miR-146a has been shown to play an important role in developing diabetic retinopathy and diabetic wound healing (Feng et al., 2011, Xu et al., 2012). In the innate immune response, miR-146a negatively regulates inflammation by targeting IRAK1 and TRAF6 (Hou et al., 2009). Type II diabetic mice exhibit substantial reduction of miR-146a expression in skin, whereas elevation of miR-146a promotes wound healing by downregulation of the target gene expression (Xu et al., 2012). DRG neurons express miRNAs (Kusuda et al., 2011, Li et al., 2011). Whether diabetes affects miR-146a in DRG neurons has not been investigated.

In the present study, using a mouse model of diabetic peripheral neuropathy (db/db mice) and cultured DRG neurons, we found that downregulation of miR-146a expression in DRG neurons by hyperglycemia led to DRG neuron apoptosis by increasing IRAK1 and TRAF6 protein levels, whereas sildenafil, a phosphodiesterase type 5 (PDE-5) inhibitor, reversed the effect of hyperglycemia on miR-146a and its target gene expression in DRG neurons.

EXPERIMENTAL PROCEDURES

Animals

All experimental procedures were carried out in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the institutional Animal Care and Use Committee of Henry Ford Hospital. Male BKS.Cg-*m*+/*Lepr*^{db}/*J* (db/db) mice (Jackson Laboratories, Bar Harbor, Maine) aged 20 weeks were used. Age-matched heterozygote mice (db/m), a non-penetrant genotype (Jackson Laboratories), were used as the control animals. For isolation of cultured primary DRG neurons, 1–2 day-old C57BL6 mice (Jackson Laboratories) were be used.

Sildenafil treatment

db/db mice at age 20 weeks were treated daily with sildenafil (10 mg/kg/d, subcutaneous, n=10/each group, Viagra, Pfizer Inc.) for 28 days. For control, db/db mice and db/m mice were treated with the same volume of saline as a vehicle (n=10/each group). All mice were sacrificed 28 days after the treatment. Lumbar 3 (L3) to L6 DRGs were dissected bilaterally. Doses of 10 mg/kg/d of sildenafil were selected based on our previous study (Wang et al., 2011). Sildenafil powder was dissolved in saline.

In situ hybridization for miR-146a

To examine expression of miR-146a in DRG neurons, in situ hybridization was performed with locked nucleic acid (LNA) probes specific for miR-146a according to a published protocol (Zhao et al., 2001). Briefly, mice were sacrificed under anesthesia. DRG were post-fixed by 4% paraformaldehyde. The sections were incubated in hybridization solution (50% formamide, 5× SSC, 200 µg/mL yeast tRNA, 500 µg/mL salmon sperm DNA, 0.4 g Roche blocking reagent, and 5× Denhardt's solution) at room temperature for 2 h. The sections

were incubated overnight in hybridization solution containing 3 pmol of digoxin (DIG)-labeled LNA MiRCURY probes for miR-146a and scramble probes (Exiqon Inc, Woburn, MA, USA) at below -20° predicted T_m value of the probe used. The sections were washed at 55°C for 30 min $1\times$ SSC and for 10 min in 0.1 M Tris-HCl buffer (pH 7.5) and incubated in the blocking solution (10% fetal calf serum in 0.1 M Tris-HCl buffer) for 1 h at room temperature followed by labeling with anti-DIG-FAB peroxidase (POD, Roche Applied Science, Indianapolis, IN, USA) for 1 h at room temperature. The signals were amplified using the Individual Indirect Tyramide Reagent Kit (PerkinElmer Life Science, Waltham, Massachusetts, USA), according to the protocol (Zhao et al., 2001). Alkaline phosphatase was used for the detection of the miRNA signals.

Cultured primary DRG neurons

DRG cell cultures were prepared as previously described (Russell et al., 1999). Whole DRG from 1–2 day old mice were incubated in 0.125% trypsin for 60 minutes at 37°C and mechanically dissociated into single cells. Dissociated DRG cells were plated on lamine coated glass cover slips and cultured in Neurobasal Medium containing 25 mM glucose with 0.5% B27 without antioxidants, 10 ng/ml Nerve Growth Factor, 0.5% Pen/Strep/Neo, and 0.7 mM l-glutamine, and 10 nM uridine and 10 nM 5-fluorodeoxyuridine. In the present study, we define a normal glucose (NG) at 25 mM because medium used in the present study contained 25 mM glucose. This dose is the optimal concentration for primary DRG neuron growth because sensory neurons have high metabolic requirements (Russell et al., 2002, Cnop et al., 2005). A high glucose (HG) medium refers to a medium containing 45 mM glucose, which was chosen to match glucose levels prevalent in uncontrolled diabetic patients (Cnop et al., 2005). These glucose concentrations are generally used for the in vitro hyperglycemic experiments (Russell et al., 2002, Vincent et al., 2004, Cnop et al., 2005).

Transfection of miRNA mimics and inhibitors

To examine the effect of miR-146a on IRAK1 and TRAF6 expression, the DRG neurons were transfected with 10 μl (20 mol/L) of miR-146a mimic, a negative control miRNA mimic (cel-miR-67), miR-146a inhibitor, or a negative control miRNA inhibitor (Dharmacon) via electroporation, using the mouse Neuron Nucleofector kit (Lonza, Basel, Switzerland) according to the manufacturer's protocol.

Quantification NF-kBp65 activity

Nuclear extracts were prepared from DRG neurons using a Nuclear Extracts Kit (Active Motif, USA). To examine NF-kBp65 activity in nuclear extracts, the NF-kB p65 active ELISA (Active Motif) kit was used to measure NF-kBp65 levels according to the manufacturer's instructions.

TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining

Cultured DRG neurons were fixed in 4% paraformaldehyde for 20 minutes and then stained using the Apoptag fluorescein In Situ Detection Kit to detect nuclear fragmentation, as previously described (Delaney et al., 2001; Russell et al., 1999).

Immunohistochemistry and immunocytochemistry

L3–L6 DRGs were isolated, fixed in 4% paraformaldehyde, and embedded in paraffin or OCT compound, according to published protocols (Zhang et al., 1999, Wang et al., 2011). Paraffin embedded (each with 6 μm in thickness) or frozen (each with 20 μm in thickness) sections were used for immunohistochemistry analysis. The following primary antibodies were used: rabbit anti-cleaved caspase-3 (1:200, cell signaling), rabbit anti-IRAK1 (1:50, Santa Cruz Biotech) and rabbit anti-TRAF6 (1: 50, Santa Cruz Biotech). Rabbit anti-IgG

was used as a negative control. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:5000).

Cultured DRG neurons were fixed for 30 minutes with 4% paraformaldehyde in PBS, and then incubated with primary antibodies, rabbit anti-cleaved caspase-3 (1:100), rabbit anti-IRAK1 (1:400) and rabbit anti-TRAF6 (1:400) for 2 hours at 37°C. Cells were then incubated with the secondary antibodies conjugated with Cy3 at room temperature for 1 hour. Cells were counterstained with DAPI (1:5000).

Image analysis and quantification

For in vivo analysis, the DRG sections were digitized under a 40x objective (Zeiss Axiophot) via a Micro Computer Imaging Device (MCID) system (Imaging Research Inc, St. Catharines, ON, Canada) (Zhang et al., 1999). Three sections spaced at 60 μ m intervals from each DRG were used. Three fields of the view per section were randomly imaged. The percentage of immunoreactive neurons was determined by counting the number of immunoreactive neurons and divided by total number of neurons.

For in vitro analysis, slides were digitized under a 20x objective (Zeiss Axiophot) via a MCID system. Immunoreactive cells and total number of cells in each slide were counted in five random fields with each field containing 25–100 cells. Six slides were used for individual experiments. The percent of positive neurons were determined. Each experiment was repeated by at least 3 times.

All analysis was conducted by the examiners blinded to the identity of the samples being studied.

Quantification of mRNA by real-time RT-PCR

Total RNA samples from cells were isolated using the Stratagene Absolutely RNA MicroRNA isolation kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The complementary DNA (cDNA) was reversely transcribed from the same concentrations of total RNA products using random hexamers and M-MLV reverse-transcriptase (Invitrogen, Carlsbad, CA). Using the SYBR Green real-time PCR method (Wang et al., 2006, Wang et al., 2011), quantitative PCR was performed on an ViiA7 Instrument (Applied Biosystems, Foster City, CA) by means of three-stage program parameters provided by the manufacturer, as follows; 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. Each sample was tested in triplicate, and samples obtained from three independent experiments were used for analysis of relative gene expression using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The following primers for real-time PCR were designed using Primer Express software (ABI): Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FWD, AGA ACA TCA TCC CTG CAT CC; REV, CAC ATT GGG GGT AGG AAC AC), IRAK1 (FWD, GAG, ACC, CTT, GCT, GGT, CAG, AG; REV, GCT, ACA, CCC, ACC, CAC, AGA, GT), TRAF6 (FWD, GCC, CAG, GCT, GTT, CAT, AAT, GT; REV, CGG, ATC, TGA, TGG, TCC, TGT, CT).

Quantification of mature miRNAs by real-time RT-PCR

Individual reverse transcription and TaqMan® microRNA assays were performed on an Applied Biosystems ViiA7 Instrument (Applied Biosystem). 15 μ l Reverse transcription reactions consisted of 1–10 ng Total RNA isolated with TRIzol (Qiagen), 5U MultiScribe Reverse Transcriptase, 0.5 mM each dNTPs, 1 $\times$ reverse transcription buffer, 4U RNase Inhibitor, and nuclease free water. Reverse transcription reactions were incubated at 16°C

for 30 min, 42°C for 30 min, 85°C for 5 min, and then stored at 4°C until use in TaqMan assays. 20 µl TaqMan real-time PCR reactions consisted of 1× TaqMan Universal PCR Master Mix No AmpErase UNG, 1× TaqMan miRNA assay, 1.33 µl of undiluted cDNA, and nuclease free water. Each TaqMan assay was done in triplicate for each sample tested. Relative quantities were calculated using the $2^{-\Delta\Delta C_t}$ method with U6 snRNA TaqMan miRNA control assay (Applied Biosystem) as the endogenous control and calibrated to the wild type samples (Livak and Schmittgen, 2001). Three independent experiments were performed. Reactions were run with the Standard 7000 default cycling protocol without the 50°C incubation stage, with reactions incubated at 95°C 10 min, followed by 40 cycles of 95°C 15 sec, 60°C 1 min. Fluorescence readings were collected during the 60°C step.

Western blot analysis

Western blot was performed according to published methods (Wang et al., 2006). Briefly, equal amounts of proteins were loaded on 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes, and the blots were subsequently probed with the following antibodies: rabbit anti-IRAK1 (1:1000), rabbit anti-TRAF6 (1:1000), and mouse anti-phospho-NF-κB p65 (1:1000, Cell Signaling Technology, MA). For detection, horseradish peroxidase-conjugated secondary antibodies were used (1:2000) followed by enhanced chemiluminescence development (Pierce, Rockford, IL). Normalization of results was ensured by running parallel Western blot with an antibody against β-actin as loading and internal control. The optical density was quantified using an image processing and analysis program (Scion Image, Ederick, MA). Densitometric ratios were computed to examine differences in expression between controls.

Statistical analysis

One-way ANOVA was used for multiple group experiments. t-test was used for two comparisons. The data are presented as mean ± SE. A value of $P < 0.05$ was taken as significant.

RESULTS

Diabetes increases protein levels of IRAK1 and TRAF6 and decreases miR-146a expression in DRG neurons

The innate immune system is involved in the development of Type 2 diabetes mellitus, and type 2 diabetic patients have increased TLR and IRAK1 activation in monocytes (Pickup, 2004, Dasu et al., 2010). Diabetic peripheral neuropathy induces DRG neuron damage (Zochodne et al., 2001). To examine whether diabetic mice exhibit altered innate immune gene expression in DRG neurons, we measured levels of IRAK1 and TRAF6 in DRG neurons of diabetic db/db mice. Immunohistochemistry analysis revealed that db/db mice at age of 24 weeks had substantial increases in the number of IRAK1 and TRAF6 positive DRG neurons compared with age matched non-diabetic db/m mice (Fig.1A, B, D, E and V). Western blot analysis of DRG neurons isolated from db/db and db/m mice showed significant elevation of IRAK1 and TRAF6 levels in db/db mice (Fig.1X). These data indicate that diabetes increases IRAK1 and TRAF6 proteins in DRG neurons.

To examine whether the increase in IRAK1 and TRAF6 is related to neuronal damage, we measured caspase-3 immunoreactive cells with an antibody specifically against activated caspase-3. Immunofluorescent staining showed that only few caspase-3 DRG neurons were detected in db/m mice, while the number of caspase-3 positive neurons significantly increased in diabetic db/db mice (Fig.1M, N and W). These data suggest that elevation of IRAK1 and TRAF6 could lead to neuronal apoptosis.

Both IRAK1 and TRAF6 are targets of miR-146a (Taganov et al., 2006, Hou et al., 2009). To examine whether the observed elevation of IRAK1 and TRAF6 in diabetic mice is associated with changes in miR-146a expression, we measured miR-146a levels in DRG neurons. Using LNA probes specific to miR-146a, we first performed in situ hybridization on sections of L3~L6 DRG tissue. Robust miR-146a signals within neuronal soma of small to large DRG neurons were detected in db/m mice (Fig.2A), whereas miR-146a signals were not found in DRG neurons probed with scrambled probes (Fig.2D), indicating, specificity of the miR-146a probes. However, diabetic db/db mice exhibited substantial reduction of miR-146a signals in DRG neurons (Fig.2A and B). To further verify that diabetes downregulates miR-146a expression in DRG neurons, we measured miR-146a levels in DRG tissue with TaqMan primers specific to detect mature miR-146a. Quantitative real-time RT-PCR analysis revealed that DRG tissue from db/db mice had substantial reduction of miR-146a compared to DRG tissue from non-diabetic db/m mice (Fig.2I). DRG neurons express miRNA-133b. However, miR-133b levels did not significantly change in DRG neurons between db/db (1.14 ± 0.11 , $n=4/\text{group}$) and db/m mice (1.03 ± 0.15 , $n=4/\text{group}$). These data indicate that diabetes reduces miR-146a in DRG neurons, which was inversely associated with protein levels of IRAK1 and TRAF6.

Hyperglycemia suppresses miR-146a and increases IRAK1 and TRAF6 levels in DRG neurons

To examine the direct effect of hyperglycemia on expression of miR-146a, IRAK1, and TRAF6 in DRG neurons, we performed in vitro experiments using primary DRG neurons that share many of the characteristics of sensory neurons in vivo and are regularly used to investigate mechanisms underlying diabetic neuropathy (Melli and Hoke, 2009). Real-time RT-PCR analysis showed that DRG neurons incubated with high glucose (45 mM) for 24h exhibited significant reduction of miR-146a and considerable increases in mRNA levels of IRAK1 and TRAF6 compared with the neurons cultured under normal glucose (25 mM) (Fig. 3B and C). Western blot analysis showed that high glucose robustly elevated IRAK1 and TRAF6 levels in DRG neurons (Fig. 3D). Moreover, high glucose significantly increased the number of caspase-3 immunoreactive and TUNEL positive DRG neurons compared with normal glucose (Fig. 3E, F, I, J, K and N). These results indicate that hyperglycemia suppresses miR-146a, and activates IRAK1 and TRAF6 in DRG neurons, as well as increases neuronal apoptosis. These data are parallel with aforementioned in vivo data.

We hypothesized that miR-146a regulates IRAK1 and TRAF6 levels. To test this hypothesis, we transfected DRG neurons with miR-146a mimics and then cultured the neurons under high glucose condition for 48h. Real-time RT-PCR analysis showed that miR-146a mimics suppressed IRAK1 and TRAF6 levels elevated by high glucose compared to mimic control (Fig. 3B and C), although miR-146a mimics did not change IRAK1 (1.3 ± 0.1 vs. 1.0 ± 0.05 in control, $n=6/\text{group}$) and TRAF6 (1.2 ± 0.1 vs. 1.0 ± 0.04 in control, $n=6/\text{group}$) levels in DRG neurons cultured under normal glucose condition. In line with this finding, miR-146a mimics substantially suppressed high glucose-induced neuronal apoptosis (Fig.3F, G, I, K, L and N). We then examined the effect of endogenous miR-146a on IRAK1 and TRAF6 levels in DRG neurons. DRG neurons were transfected with siRNA against miR-146a (siRNA-miR-146a) and then cultured under normal glucose condition. Real-time RT-PCR analysis showed that miR-146a levels were reduced by $47 \pm 1.3\%$ in DRG neurons transfected with siRNA-miR-146a compared to the neurons transfected with the siRNA control under normal glucose (0.43 ± 0.01 vs. 0.92 ± 0.02 in siRNA control, $n=6/\text{group}$), indicating that siRNA-miR-146a decreases endogenous miR-146a. Real time RT-PCR and immunocytochemistry analysis revealed that attenuation of endogenous miR-146a by siRNA-miR-146a considerably increased IRAK1 and TRAF6 mRNA levels, the number of

caspase-3 positive and TUNEL positive cells (Fig.4B). Collectively, these data indicate that exogenous and endogenous miR-146a regulates levels of IRAK1 and TRAF6 under normal and high glucose conditions, respectively, thereby, likely affecting neuronal cell apoptosis.

Sildenafil acts on miR-146a in diabetic DRG neurons

We previously demonstrated that sildenafil improves neurological function in diabetic db/db mice (Wang et al., 2011). To examine whether the therapeutic effect of sildenafil on neurological outcomes acts on miR-146a, we examined miR-146a expression in DRG neurons. Db/db mice at the age of 20 weeks were treated with sildenafil at a dose of 10 mg/kg for 4 weeks. In situ hybridization showed that treatment with sildenafil markedly increased miR-146a signals in DRG neurons compared with db/db mice treated with saline (Fig.2B and C). Quantitative RT-PCR further confirmed elevation of miR-146a by sildenafil (Fig.2I). Moreover, sildenafil dramatically reduced IRAK1 and TRAF6 protein levels in DRG tissue measured by Western blots as well as the number of caspase-3 positive neurons compared to the saline treatment (Fig.1X, N, O and W). Activation of NF- κ B mediates elevation of cytokines in diabetic patients and IRAK1 is one of the signal transducers in activation of the NF- κ B pathway (Heimberg et al., 2001, Cnop et al., 2005, Taganov et al., 2006). Consistently, Western blots showed that DRG tissues harvested from diabetic db/db mice had substantial increases in phosphorylated NF- κ B p65 levels compared with DRG tissue from age-matched non-diabetic db/m mice, whereas treatment of diabetic mice with sildenafil significantly reduced NF- κ B p65 levels (Fig.4C). To establish whether sildenafil acts on miR-146a, we treated primary DRG neurons with sildenafil under high glucose condition and measured levels of miR-146a, NF- κ B p65, IRAK1 and TRAF6. Sildenafil elevated miR-146a levels suppressed by high glucose and reduced high glucose-upregulated mRNA levels of IRAK1 and TRAF6 (Fig.3A, B and C), and high glucose-induced NF- κ B p65 activity in the nuclear fraction of DRG neuron (Fig.4D). However, attenuation of endogenous miR-146a by siRNA-miR-146a abolished the effect of sildenafil on reduction of IRAK1 and TRAF6 in DRG neurons under high glucose condition (Fig.3B and C). We and others have demonstrated that sildenafil elevates cGMP levels (Zhao et al., 2001, Zhang R, 2002, Wang et al., 2011). We therefore, treated DRG neurons cultured under high glucose condition with 8-Br-cGMP (10 μ mol/L), a stable analog of cGMP and then measured levels of miR-146a, IRAK1 and TRAF6. Quantitative RT-PCR analysis showed that 8-Br-cGMP increased miR-146a and decreased IRAK1 and TRAF6 (Fig.3A, B and C). These data suggest that sildenafil, through elevation of cGMP, upregulates miR-146a, leading to downregulation of its target genes of IRAK1 and TRAF6 in DRG neurons under hyperglycemia.

DISCUSSION

In the present study, we demonstrated that diabetes considerably downregulated miR-146a expression in DRG neurons, which was inversely associated with elevation of IRAK1, TRAF6 and caspase-3 in db/db mice. In addition, treatment of diabetic peripheral neuropathy with sildenafil substantially overturned the effect of hyperglycemia on miR-146a and its target gene expression and caspase-3 immunoreactivity in DRG neurons. In vitro, we demonstrated a cause-effect of downregulation of endogenous miR-146a in DRG neurons by hyperglycemia on neuronal apoptosis via augmentation of IRAK1 and TRAF6, whereas sildenafil and cGMP suppressed hyperglycemia-altered expression of miR-146a, IRAK1, and TRAF6. These in vivo and in vitro data provide the first evidence showing that miR-146a plays an important role in mediating DRG neuron apoptosis under hyperglycemic conditions.

Experimental studies have shown that DRG neuron apoptosis is not a primary cause of diabetic peripheral neuropathy. In a model of streptozotocin (STZ)-treated rats, Zochodne et al

reported no significant changes in DRG neuron number even 12 months after STZ (Zochodne et al., 2001). However, a recent study showed approximately 33% loss of DRG neurons in 8 month old db/db mice (Shi et al., 2013). In the present study, we found an increase in caspase-3, a protein involved in apoptotic cell death, immunoreactivity in DRG neurons of 24 week old db/db mice. In vitro, we demonstrated that upregulation of IRAK1 and TRAF6, two adaptor proteins for the TLR signaling pathway, in DRG neurons by hyperglycemia was associated with activation of NF- κ B and DRG neuron apoptosis. IRAK1 and TRAF6 orchestrate the activation of proinflammatory events and induce cell death (Takeda and Akira, 2004, Tamura et al., 2008). Thus, our findings suggest that hyperglycemia-induced innate immune responses may trigger DRG neuron apoptosis observed in 8 month old db/db mice (Shi et al., 2013). In addition to DRG neurons, activation of TLRs, IRAK1, and TRAF6 in type 2 diabetes has been observed in patient and experimental studies (Balasubramanyam et al., 2011, Feng et al., 2011). Patients with type 2 diabetes exhibit elevation of TLR, IRAK1, and TRAF6 expression in monocytes (Dasu et al., 2008, Dasu et al., 2010). Elevation of IRAK1 and TRAF6 exacerbates wound healing impairment in db/db mice (Xu et al., 2012). DRG neurons express miRNAs (Hua et al., 2008, Kusuda et al., 2011, Li et al., 2011). It is well established that miR-146a acts as a modulator of the adaptive immune response by binding to 3'UTR of IRAK1 and TRAF6 (Taganov et al., 2006, Hou et al., 2009, Nahid et al., 2011). The present study showed that DRG neurons under non-diabetic condition expressed miR-146a, while hyperglycemia greatly downregulated miR-146a expression. More important, our findings demonstrated that attenuation of endogenous miR-146a by siRNA against miR-146a and elevation of miR-146a by miR-146a mimics in DRG neurons induced neuronal death under low glucose condition and neuronal survival under high glucose situation, respectively, which was inversely related to IRAK1 and TRAF6 protein levels. These in vitro data suggest that miR-146a through its targets, IRAK1 and TRAF6, mediates DRG neuronal apoptosis in diabetic peripheral neuropathy. Others have shown activation of IRAK1 and TRAF6 by downregulation of miR-146a in skin of db/db mice (Xu et al., 2012). Collectively, the present study along with published data suggest that miR-146a is a potential therapeutic target for type 2 diabetes and its complications, including peripheral neuropathy.

We previously demonstrated that hyperglycemia reduces cGMP levels in Schwann cells by upregulation of PDE5 expression, whereas elevation of cGMP by inhibition of PDE5 with sildenafil substantially improves myelination and reduces motor and sensory conducting velocities and thermal and mechanical noxious stimuli, but does not significantly affect blood glucose levels and animal body weight in diabetic peripheral neuropathy (Wang et al., 2011). The present study showed that treatment of diabetic peripheral neuropathy with sildenafil augmented miR-146a expression and decreased protein levels of IRAK1, TRAF6 and caspase-3 in the DRG neurons. In vitro data further showed that attenuation of endogenous miR-146a in DRG neurons abolished the effect of sildenafil on DRG neuron protection and downregulation of IRAK1 and TRAF6 proteins under hyperglycemia. Thus, the present study provides a molecular mechanism that miR-146a through its target genes mediates the neuroprotective effect of sildenafil on DRG neurons, which may contribute to the reduction of diabetic sensory neuropathy observed in db/db mice treated with sildenafil (Wang et al., 2011). Moreover, our data suggest that cGMP levels play an important role in regulating miR-146a expression under hyperglycemia. Thus, the present data along with published studies showing involvement of miR-146a, IRAK1 and TRAF6 in development of type II diabetes, suggest that regulation of miR-146a levels and its downstream target genes by sildenafil may contribute to the reduction of diabetic sensory neuropathy observed in db/db mice treated with sildenafil (Wang et al., 2011).

There are limitations in the present study. Cultured adult DRG neurons have high metabolic requirements at a basal level (Russell et al., 1999, Russell et al., 2002, Vincent et al., 2004).

Thus, the glucose concentrations used for culturing DRG neurons and for studying the effect of hyperglycemia on DRG neurons are much higher than blood glucose levels under physiological and hyperglycemic conditions in animals (Acheampong et al., 2009). Thus, the relevance of our in vitro findings to diabetic peripheral neuropathy warrants further investigation.

In addition, the protocol used for immunohistochemistry with the NeuN antibody may not be optimal to detect the majority of DRG neurons, which could lead to underestimation of the number of DRG neurons.

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LIST OF ABBREVIATIONS

db/db	BKS.Cg-m+/+ <i>Lepr^{db}</i> /J mice
db/m	heterozygote mice
DRG	dorsal root ganglion
IRAK1	interleukin-1 receptor activated kinase
TRAF6	tumor necrosis factor receptor-associated factor 6
TLRs	Toll-like receptors
MiRNA	MicroRNA
PDE5	a phosphodiesterase type 5
NG	normal glucose
HG	high glucose
MCID	Micro Computer Imaging Device

References

- Acheampong EA, Roschel C, Mukhtar M, Srinivasan A, Rafi M, Pomerantz RJ, Parveen Z. Combined effects of hyperglycemic conditions and HIV-1 Nef: a potential model for induced HIV neuropathogenesis. *Virol J.* 2009; 6:183. [PubMed: 19878567]
- Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature immunology.* 2001; 2:675–680. [PubMed: 11477402]
- Balasubramanyam M, Aravind S, Gokulakrishnan K, Prabu P, Sathishkumar C, Ranjani H, Mohan V. Impaired miR-146a expression links subclinical inflammation and insulin resistance in Type 2 diabetes. *Molecular and cellular biochemistry.* 2011; 351:197–205. [PubMed: 21249428]
- Behm-Ansmant I, Rehwinkel J, Izaurralde E. MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. *Cold Spring Harbor symposia on quantitative biology.* 2006; 71:523–530.
- Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes.* 2005; 54(Suppl 2):S97–107. [PubMed: 16306347]
- Dahiya N, Sherman-Baust CA, Wang TL, Davidson B, Shih Ie M, Zhang Y, Wood W 3rd, Becker KG, Morin PJ. MicroRNA expression and identification of putative miRNA targets in ovarian cancer. *PLoS one.* 2008; 3:e2436. [PubMed: 18560586]

- Dasu MR, Devaraj S, Park S, Jialal I. Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. *Diabetes care*. 2010; 33:861–868. [PubMed: 20067962]
- Dasu MR, Devaraj S, Zhao L, Hwang DH, Jialal I. High glucose induces toll-like receptor expression in human monocytes: mechanism of activation. *Diabetes*. 2008; 57:3090–3098. [PubMed: 18650365]
- Feldman EL, Stevens MJ, Greene DA. Pathogenesis of diabetic neuropathy. *Clin Neurosci*. 1997; 4:365–370. [PubMed: 9358981]
- Feng B, Chen S, McArthur K, Wu Y, Sen S, Ding Q, Feldman RD, Chakrabarti S. miR-146a-Mediated extracellular matrix protein production in chronic diabetes complications. *Diabetes*. 2011; 60:2975–2984. [PubMed: 21885871]
- Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, Darville M, Eizirik DL. Inhibition of cytokine-induced NF-kappaB activation by adenovirus-mediated expression of a NF-kappaB super-repressor prevents beta-cell apoptosis. *Diabetes*. 2001; 50:2219–2224. [PubMed: 11574401]
- Hou J, Wang P, Lin L, Liu X, Ma F, An H, Wang Z, Cao X. MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. *J Immunol*. 2009; 183:2150–2158. [PubMed: 19596990]
- Hua YJ, Tu K, Tang ZY, Li YX, Xiao HS. Comparison of normalization methods with microRNA microarray. *Genomics*. 2008; 92:122–128. [PubMed: 18514480]
- Kantharidis P, Wang B, Carew RM, Lan HY. Diabetes complications: the microRNA perspective. *Diabetes*. 2011; 60:1832–1837. [PubMed: 21709278]
- Kishi M, Tanabe J, Schmelzer JD, Low PA. Morphometry of dorsal root ganglion in chronic experimental diabetic neuropathy. *Diabetes*. 2002; 51:819–824. [PubMed: 11872686]
- Kusuda R, Cadetti F, Ravanelli MI, Sousa TA, Zanon S, De Lucca FL, Lucas G. Differential expression of microRNAs in mouse pain models. *Molecular pain*. 2011; 7:17. [PubMed: 21385380]
- Li X, Gibson G, Kim JS, Kroin J, Xu S, van Wijnen AJ, Im HJ. MicroRNA-146a is linked to pain-related pathophysiology of osteoarthritis. *Gene*. 2011; 480:34–41. [PubMed: 21397669]
- Liu Y, Ma Q. Generation of somatic sensory neuron diversity and implications on sensory coding. *Current opinion in neurobiology*. 2011; 21:52–60. [PubMed: 20888752]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(Delta Delta C(T))} Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Melli G, Hoke A. Dorsal Root Ganglia Sensory Neuronal Cultures: a tool for drug discovery for peripheral neuropathies. *Expert opinion on drug discovery*. 2009; 4:1035–1045. [PubMed: 20657751]
- Nahid MA, Satoh M, Chan EK. Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. *J Immunol*. 2011; 186:1723–1734. [PubMed: 21178010]
- Natarajan R, Putta S, Kato M. MicroRNAs and diabetic complications. *Journal of cardiovascular translational research*. 2012; 5:413–422. [PubMed: 22552970]
- Patapoutian A. Making the pain connection. *Neuron*. 2001; 31:4–6. [PubMed: 11498043]
- Patapoutian A, Peier AM, Story GM, Viswanath V. ThermoTRP channels and beyond: mechanisms of temperature sensation. *Nature reviews Neuroscience*. 2003; 4:529–539.
- Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes care*. 2004; 27:813–823. [PubMed: 14988310]
- Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, Feldman EL. High glucose-induced oxidative stress and mitochondrial dysfunction in neurons. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 2002; 16:1738–1748. [PubMed: 12409316]
- Russell JW, Sullivan KA, Windebank AJ, Herrmann DN, Feldman EL. Neurons undergo apoptosis in animal and cell culture models of diabetes. *Neurobiology of disease*. 1999; 6:347–363. [PubMed: 10527803]
- Schmeichel AM, Schmelzer JD, Low PA. Oxidative injury and apoptosis of dorsal root ganglion neurons in chronic experimental diabetic neuropathy. *Diabetes*. 2003; 52:165–171. [PubMed: 12502508]

- Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. *Nature immunology*. 2001; 2:947–950. [PubMed: 11547333]
- Shi TJ, Zhang MD, Zeberg H, Nilsson J, Grunler J, Liu SX, Xiang Q, Persson J, Fried KJ, Catrina SB, Watanabe M, Arhem P, Brismar K, Hokfelt TG. Coenzyme Q10 prevents peripheral neuropathy and attenuates neuron loss in the db-/db- mouse, a type 2 diabetes model. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:690–695. [PubMed: 23267110]
- Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:12481–12486. [PubMed: 16885212]
- Takeda K, Akira S. TLR signaling pathways. *Seminars in immunology*. 2004; 16:3–9. [PubMed: 14751757]
- Takeda K, Akira S. Toll-like receptors in innate immunity. *International immunology*. 2005; 17:1–14. [PubMed: 15585605]
- Tamura T, Yanai H, Savitsky D, Taniguchi T. The IRF family transcription factors in immunity and oncogenesis. *Annual review of immunology*. 2008; 26:535–584.
- Vincent AM, Olzmann JA, Brownlee M, Sivitz WI, Russell JW. Uncoupling proteins prevent glucose-induced neuronal oxidative stress and programmed cell death. *Diabetes*. 2004; 53:726–734. [PubMed: 14988258]
- Wang L, Chopp M, Szalad A, Liu Z, Bolz M, Alvarez FM, Lu M, Zhang L, Cui Y, Zhang RL, Zhang ZG. Phosphodiesterase-5 is a therapeutic target for peripheral neuropathy in diabetic mice. *Neuroscience*. 2011; 193:399–410. [PubMed: 21820491]
- Wang L, Zhang ZG, Zhang RL, Gregg SR, Hozeska-Solgot A, LeTourneau Y, Wang Y, Chopp M. Matrix metalloproteinase 2 (MMP2) and MMP9 secreted by erythropoietin-activated endothelial cells promote neural progenitor cell migration. *J Neurosci*. 2006; 26:5996–6003. [PubMed: 16738242]
- Xu J, Wu W, Zhang L, Dorset-Martin W, Morris MW, Mitchell ME, Liechty KW. The role of microRNA-146a in the pathogenesis of the diabetic wound-healing impairment: correction with mesenchymal stem cell treatment. *Diabetes*. 2012; 61:2906–2912. [PubMed: 22851573]
- Zhang RWY, Zhang L, Zhang Z, Tsang W, Lu M, Zhang L, Chopp M. Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. *Stroke*. 2002 Nov; 33(11): 2675–80. [PubMed: 12411660]
- Zhang Z, Davies K, Probst J, Fenstermacher J, Chopp M. Quantitation of microvascular plasma perfusion and neuronal microtubule-associated protein in ischemic mouse brain by laser-scanning confocal microscopy. *J Cereb Blood Flow Metab*. 1999; 19:68–78. [PubMed: 9886357]
- Zhao L, Mason NA, Morrell NW, Kojonazarov B, Sadykov A, Maripov A, Mirrakhimov MM, Aldashev A, Wilkins MR. Sildenafil inhibits hypoxia-induced pulmonary hypertension. *Circulation*. 2001; 104:424–428. [PubMed: 11468204]
- Zochodne DW, Verge VM, Cheng C, Sun H, Johnston J. Does diabetes target ganglion neurones? Progressive sensory neurone involvement in long-term experimental diabetes. *Brain: a journal of neurology*. 2001; 124:2319–2334. [PubMed: 11673332]

Highlights

- MiR-146a mediates dorsal root ganglion survival in diabetic peripheral neuropathy.
- MiR-146a targets IRAK1 and TRAF6 in dorsal root ganglion neurons.
- Upregulation of miR-146a contributes sildenafil-improved diabetic neuropathy.

Figure 1. IRAK1 and TRAF6 levels in DRG neurons

Representative microscopic images show IRAK1 (A to C), TRAF6 (D to F) and cleaved caspase3 (M to O) immunoreactive DRG neurons (arrows) in db/m mouse (A, D and M) and db/db mouse treated with saline (B, E and N) or sildenafil (C, F and O). Double immunofluorescent staining shows that IRAK1 (G, I, P and R) and TRAF6 (J, L, S and U) immunoreactivity were colocalized to NeuN (H, I, K and L) and cleaved caspase 3 (Q, R, T and U) positive cells. Quantitative data show the percentage of IRAK1 and TRAF6 (V), cleaved caspase3 (W) immunoreactive DRG neurons in each group. n=6/group. Panel X shows Western blot analyses of IRAK1 and TRAF6 in DRG tissue. n=6/group. β -actin was used as an internal control. * $P < 0.05$ and # $P < 0.05$ versus the db/m mouse and the saline treated db/db mouse, respectively. Values are mean $\pm$ SE. Bar=100 μ m in C, F, I, L, O, R and U. dm=db/m mouse; db=db/db mouse.

Figure 2. Expression of miR-146a in DRG neurons in vivo

Representative microscopic images of in situ hybridization with LNA miR-146a probes show miR-146a signals (arrowheads) in cytoplasm of DRG neurons of db/m mouse (A) and db/db mouse treated with saline (B) or sildenafil (C). However, no signals in DRG neurons were detected by scrambled probes (D). The same pattern of miR-146a signals was observed in 3 individual mice per group. Representative microscopic images (E to H) show that DRG neurons with (E and G, arrowheads) and without (E and G, arrows) miR-146a signals detected by in situ hybridization were IRAK1 and TRAF6 immuno-negative (F and H, arrowheads) and-positive (F and H, arrows), respectively, detected by immunofluorescent staining on an adjacent section. Panel I shows miR-146a levels in each group measured by real-time RT-PCR. n=4/group. *P<0.05 and #P<0.05 versus the db/m mouse and db/db mouse treated with saline, respectively. Bar=100μm in C and E. dm=db/m mouse; db=db/db mouse.

Figure 3. The effect of hyperglycemia on expression of miR-146a, IRAK1 and TRAF6 in DRG neurons in vitro

Real-time RT-PCR data (A to C) show miR-146a (A), IRAK1 (B) and TRAF6 (C) expression in DRG neurons cultured under normal glucose (NG) and high glucose (HG) conditions. Cultured DRG neurons treated with sildenafil (sil) and 8-Br-cGMP (cGMP), or DRG neurons transfected with miR-146a mimic (146^{+/+}) or siRNA-miR-146a (146^{-/-}) under high glucose conditions. Western blot data (D) show protein levels of IRAK1 and TRAF6 in cultured DRG neurons treated with sildenafil under high glucose condition. Representative microscopic images (E to M) show cleaved caspase3 immunoreactive (red, arrow, E to H) and TUNEL positive (red, arrow, J to M) DRG neurons cultured under normal glucose (NG, E and J) and high glucose (HG, F and K) conditions. DRG neurons transfected with miR-146a mimic (146^{+/+}, G and L) or DRG neurons treated with sildenafil (sil, H and M) under high glucose conditions. Quantitative data (I and N) show the percentage of cleaved caspase3 (I) and TUNEL (N) positive cells under different conditions. Bar=100μm in H and M. GAPDH and β-actin were used as internal controls for mRNA and proteins, respectively. n=6/group. *P<0.05, #P<0.05 and \$P<0.05 versus the normal glucose (NG), high glucose (HG) and high glucose with sildenafil, respectively. sil = high glucose and sildenafil, NC^{+/+} and NC^{-/-} = miR146a mimic and siRNA-miR146a controls, respectively.

Figure 4. The effect of attenuation of endogenous miR-146a on DRG neurons and the effect of hyperglycemia activation of NF-κB

Panels A and B show mRNA levels of IRAK1 and TRAF6 measured with real-time RT-PCR (A) and the percentage of cleaved caspase3 and TUNEL positive cells (B) in DRG neurons transfected by siRNA-miR-146a under normal glucose (NG) condition. Western blot data (C) show protein levels of NF-κB p65 in DRG neurons isolated from db/m mice (dm), db/db mice treated with saline (db), or db/db mice treated with sildenafil (+sil). ELISA (D) shows NF-κB levels in DRG neuron cultured under normal glucose (NG), high glucose (HG), or high glucose with sildenafil (+sil). GAPDH and β-actin were used as internal controls for quantification of mRNAs and proteins, respectively. n=6/group. *P<0.05 and #P<0.05 versus normal glucose and the db/m mouse groups, and #P<0.05 versus siRNA miR-146a, db/db mouse treated with saline (db/db) and high glucose (HG) groups.