Diabetic Complications Consortium

Application Title: Identifying Alterations in Mitochondrial Dynamics Associated with Diabetic Neuropathy

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1. Project Accomplishments:

The overall objectives of this proposal were to (1) establish a role for endoplasmic reticulum-mitochondrial contact sites in models of diabetic neuropathy and (2) determine if alterations in mitochondrial trafficking are calcium dependent. The rationale for the proposed research is that identifying ER-Mt interactions and calcium as key regulators of mitochondrial dysfunction associated with diabetic neuropathy will allow us to identify mechanistic therapies for treating diabetic neuropathy.

Support from the Diacomp Pilot and Feasibility grant has allowed us to develop an *in vitro* assay for imaging ER-Mt interactions in live dorsal root ganglion sensory neurons. Initial studies indicate that stimulation of DRG neurons with fatty acids increases ER-Mt interactions in the axons of DRG neurons in culture. During the course of the grant, we determined the appropriate transfection conditions, the confocal imaging parameters, and an analysis program to perform the intracellular ER-Mt assay. In order to evaluate ER-Mt interactions *in vivo*, transmission electron microscopy images were taken of Dorsal root ganglion, sciatic nerve, and sural nerve from control and 60% high fat fed mice to evaluate ER-Mt interactions.

In aim 2, we sought to evaluate fatty acid induced calcium flux as a mechanistic inducer of mitochondrial dysfunction in DRG neurons. As a first step, we found that physiological long chain fatty acid palmitate impairs mitochondrial trafficking in DRG axons. The overall percentage of motile mitochondria and velocity of the moving mitochondria were significantly reduced in palmitate treatments. This impairment in mitochondrial trafficking correlates with a reduction in mitochondrial membrane potential indicative of altered mitochondrial bioenergetics. Finally, we evaluated the axonal calcium level in DRG neurons treated with glucose and palmitate. Interestingly, we did not find consistent increases in intracellular calcium concentration in DRG axons. Further analysis is required to identify the palmitate-induced molecular changes that impair mitochondrial trafficking.

2. Specific Aims:

Specific Aim 1: Establish a role for ER-Mt contact site alterations in DN.

Results:

ER-Mt interactions in live DRG neurons: Cultured DRG neurons from adult C57/Bl6 mice were transfected with mito-GFP and ER-RFP to evaluate colocalization between the ER and mitochondria. Neurons were treated for 24 hours with saturated fatty acid palmitate to model dyslipidemia and glucose to mimic hyperglycemia. Images were taken with live-cell confocal microscopy with a 40X ocular. To evaluate the percentage axonal ER-Mt interactions, we developed an analysis program to identify the percentage of mitochondria (green) colocalized with endoplasmic reticulum (red) to form ER-Mt (yellow) interactions. We observed an increase in the number of axonal ER-Mt interactions in DRG neurons treated with diabetic concentrations

of palmitate (Figure 1; 125 μM and 250 μM palmitate). Interestingly, lower physiological concentrations of palmitate (data not shown) and control conditions with no palmitate exhibit fewer ER-Mt interactions (Figure 1; basal media and 0.25% BSA).

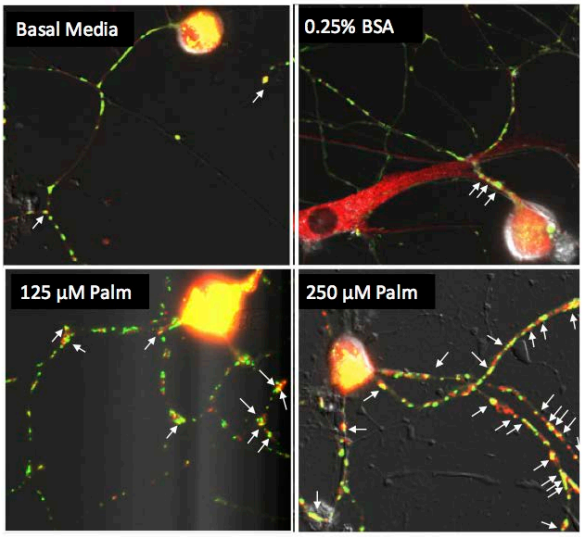

Figure 1. ER-Mt interactions in DRG neurons

ER-Mt interactions in vivo: Tissues including DRG, sural, and sciatic nerve were isolated from five 24 week old C57BL6 mice fed a control chow with 10% fat and five 24 week old C57BL6 mice on a 60% fat chow. Mice on a 60% high fat diet exhibited neuropathy phenotypes relative to mice on a 10% fat standard diet. Nervous tissues were fixed in glutaraldehyde and processed by thin sectioning for electron microscopy. Electron micrographs have been taken for each cohort and tissue but the analysis remains to be completed.

Conclusions and additional studies: Dyslipidemia induces increases in ER-Mt interactions in live DRG neurons *in vitro.* It remains to be determined whether ER-Mt interactions correlate with the progression of diabetic neuropathy *in vivo.* These results were expected and support observations in the literature showing increases in ER-Mt associated with metabolic stress.

<u>Specific Aim 2</u>: Determine if alterations in mitochondrial trafficking are calcium-dependent in DN.

<u>Results:</u>

Mitochondrial Trafficking:

To identify molecular changes in DRG sensory neurons associated with the progression of DN, cultured DRG neurons were transfected with mito-GFP to evaluate mitochondrial dynamics. Neurons were treated with dyslipidemic concentrations of long chain saturated fatty acid palmitate and hyperglycemic concentrations of glucose. Mitochondrial trafficking was assessed by recording time-lapse videos of mitochondrial movement. The videos were analyzed with kymographing analysis to identify the percentage of motile mitochondria, the directionality of mitochondrial movement, and the velocity of motile mitochondria. We found that diabetic concentrations of palmitate induced a dose-dependent reduction in mitochondrial trafficking (Figure 2B) while glucose had no impact on the percentage of motile mitochondria (Figure 2A).

The directionality of mitochondrial movement was not altered by palmitate but the velocity of moving mitochondria exhibited a dose-dependent reduction. These results suggest that DRG neurons respond to dyslipidemic concentrations of palmitate by altering axonal mitochondrial transport.

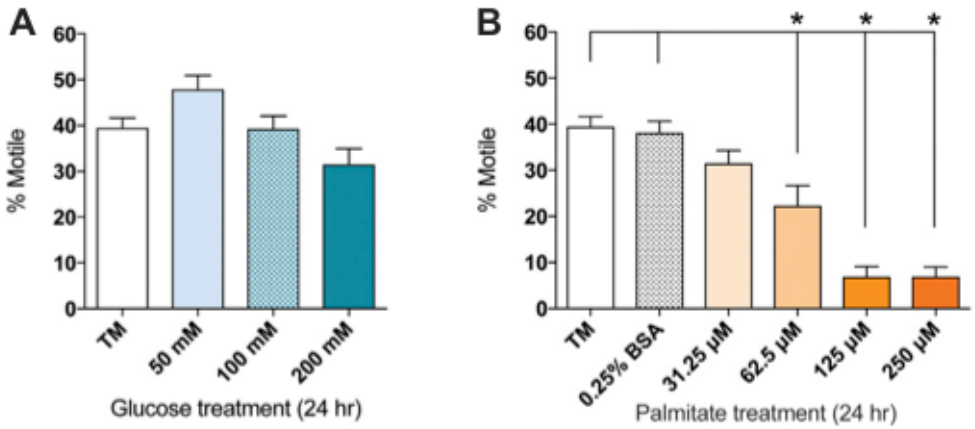

Figure 2. Palmitate reduces the percentage of motile mitochondria in DRG neurons.

Mitochondrial depolarization:
To measure the impact of palmitate and glucose on mitochondrial function, mito-GFP transfected DRG neurons were treated with 31.25-250 µM palmitate and 50-200 mM glucose for 24 hours and stained with tetramethylrhodamine methyl ester (TMRM) prior to live-cell confocal imaging. A TMRM analysis program was used to identify the level of red TMRM stain in each mitochondria. Healthy mitochondria with normal mitochondrial membrane potential show punctate red staining (Figure 3A-C) while depolarized mitochondria exhibit diffuse TMRM (Figure 3D-F). Treatment of DRG neurons with physiological concentrations of palmitate induced dose-dependent increases in mitochondrial depolarization in DRG neurons (Figure 3D-F,G) while glucose had no impact on mitochondrial membrane potential (Figure 3G).

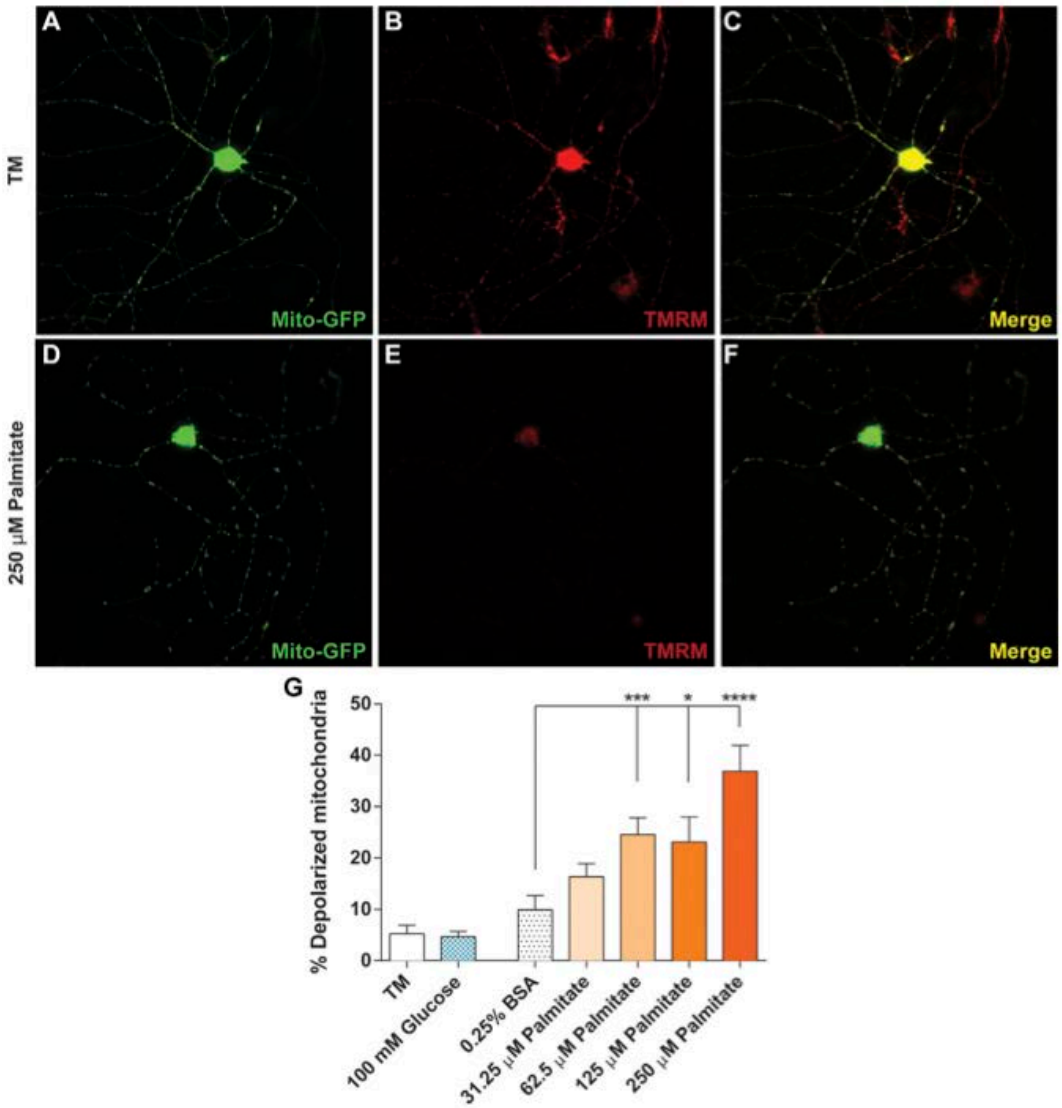

Figure 3. Palmitate leads to a dose-dependent increase in mitochondrial depolarization

Mitochondrial bioenergetics:

Alterations in mitochondrial bioenergetics were assessed by performing a Seahorse analysis on palmitate and glucose-treated DRG neurons. Under 100 mM glucose treatment, DRG neurons showed a reduction in mitochondrial respiration, ATP production, and proton leak relative to the control (Figure 4A,B,D, blue bars). Approximately 80% of the oxygen utilized by mitochondria was used to produce ATP suggesting that the coupling efficiency was retained in glucose treatments (Figure 4C, blue bar). On the other hand, DRG neurons treated with 125 μM palmitate maintained coupling efficiency despite elevated basal mitochondrial respiration and ATP turnover. The increase in ATP turnover and mitochondrial respiration were compensated for by a proportional increase in proton leak. The 250 μM palmitate treated DRG neurons, retained increased mitochondrial respiration and ATP production, however, the coupling efficiency was significantly reduced and proton leak was further increased (Figure 4A-D, orange bars). These results suggest that slight uncoupling of mitochondrial membrane potential in DRG neurons may be an adaptive response to small increases in palmitate but excess palmitate

prevents mitochondria from matching energy production. Therefore, palmitate and glucose have a differential impact on mitochondrial bioenergetics.

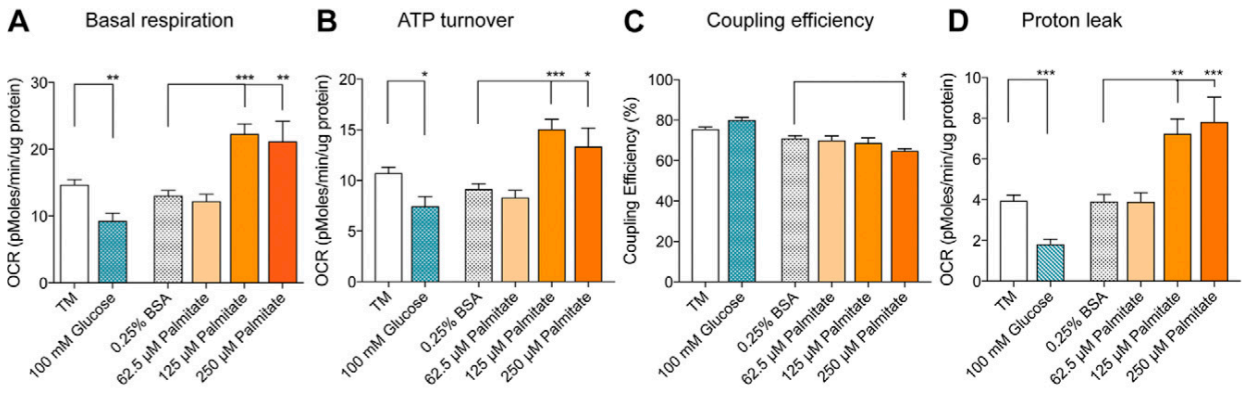

Figure 4. Palmitate and glucose treatments differentially impact mitochondrial bioenergetics in DRG neurons.

Calcium staining:
To evaluate Ca^{2+} flux as a potential underlying molecular change that alters mitochondrial movement and function, we evaluated the level of axonal Ca^{2+} in DRG neurons treated with palmitate and glucose. DRG neurons were transfected with mito-RFP and stained with a Fluo4 Ca^{2+} indicator. DRG neurons were treated with 31.25-250 µM palmitate or 50-200 mM glucose and live cell confocal microscopy was used to obtain images of neurons in each condition. We did not observe a distinct elevation in Ca^{2+} level in palmitate treated cells relative to control cells.

Conclusions:
These studies show that dyslipidemia associated with diabetic neuropathy causes mitochondrial dysfunction and impairs mitochondrial transport which disrupts the bioenergetic capacity of DRG neurons. Hyperglycemia does not impair mitochondrial trafficking or function in DRG neurons. Interestingly, although Ca^{2+} is a well characterized factor that alters mitochondrial transport, it unlikely that Ca^{2+} impairs mitochondrial transport and function in dyslipidemic DRG neurons because there was no apparent increase in axonal calcium level in palmitate treatments.

3. <u>Publications/Presentations:</u>
We recently published a journal article titled “Dyslipidemia alters mitochondrial trafficking and function in sensory neurons” in the FASEB journal. We are also preparing two additional manuscripts for submission to peer-reviewed journals. Furthermore, some of the work completed with the support of the DiaComp Pilot and Feasibility grant was presented at national and international conferences. Below is a list of conferences where the DiaComp grant was acknowledged.

<u>Publications:</u>

1) Amy E. Rumora*, Stephen I. Lentz*, Lucy M. Hinder, Samuel W. Jackson, Andrew Valesano, Gideon E. Levinson, Eva L. Feldman. 2017. Dyslipidemia impairs mitochondrial trafficking and function in sensory neurons. FASEB. (Epub ahead of print- September 13)

*Authors contributed equally

Presentations:

1) Amy E. Rumora, John M. Hayes, Giovanni Lograsso, Julia Haidar, Justin Dolkowski, Stephen I. Lentz, Eva L. Feldman. Differential effect of saturated and unsaturated fatty acids on Mitochondrial Trafficking in Dorsal Root Ganglion Sensory Neurons. Abstract for oral poster presentation, 2017, Peripheral Nerve Society Meeting, Sitges, Spain, July 2017.
2) Amy E. Rumora, Maegan A. Tabbey*, Giovanni Lograsso, Justin Dolkowski, Julia Haidar, Stephen I. Lentz, Eva L. Feldman. Impairment of mitochondrial trafficking in dorsal root ganglion neurons is dependent on hydrocarbon chain length of saturated fatty acids. Abstract for poster presentation, 2017, Peripheral Nerve Society Meeting, Sitges, Spain, July 2017.
   *Presenting author
3) Amy E. Rumora. Diabetic neuropathy is a disorder of trafficking. Oral presentation, 2017, Peripheral Nerve Society Meeting, Sitges, Spain, July 2017.
4) Amy E. Rumora, Maegan A. Tabbey, Giovanni Lograsso*, Justin Dolkowski, Julia Haidar, Stephen I. Lentz, Eva L. Feldman. Impairment of mitochondrial trafficking in dorsal root ganglion neurons is dependent on hydrocarbon chain length of saturated fatty acids. Poster presentation, 2017, Taubman Institute Symposium, October 2017.
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