Diabetic Complications Consortium

Application Title: Longitudinal Intravital Imaging of Biosensor-labeled β-cells and Hepatocytes in Diabetic Mice

Principal Investigator: Amelia K. Linnemann, Ph.D.

1. Project Accomplishments:

The overall goal of this project was to obtain liver and islet-specific measurements of cellular function in diabetic mice using biosensors coupled to intravital microscopy both before and after drug intervention. We encountered unanticipated problems with liver imaging, and are currently still working through those methods to ensure reliability of both window stability and imaging parameters. We have had much more success with pancreatic β-cell imaging, and this is where we have made the most progress, as detailed below.

We have had great success with our analysis of islet β-cell ROS using a roGFP2 with the addition of human glutaredoxin 1 and an insulin promoter (Ins-Grx1-roGFP2), and with our development of intravital microscopy methods to image islets in the endogenous pancreas of live mice under anesthesia. As detailed below, we obtained robust biosensor infection of islets in situ (Fig. 1), and found that metformin infusion acutely stimulates accumulation of ROS in the pancreatic β-cells of *db/db* mice, which is especially pronounced in the pancreata of male mice (Fig.2). This was surprising to us, as we expected a global reduction in ROS in response to metformin. Due to the time it took to refine our methods and generate this data, experiments are ongoing to study the effects of repeated dosing of metformin on longitudinal changes in islet β-cell ROS of diabetic animals.

We have had less success with our autophagy biosensor, though predominantly our issues lie with the analysis of our biosensor over time. As shown in Fig. 3 below, expression of our islet β-cell-selective autophagy biosensor (Ins-LC3-eGFP-TdTomato) is robust in the pancreata of *db/db* mice. However, due to the nature of LC3 distribution (dispersed signal that is rearranged into foci upon autophagosome formation), we have found it difficult to reliably quantify changes in not only formation of puncta that are sufficiently different from the dispersed signal, but also changes in the fluorescent output of the biosensor over time as autophagy proceeds. Refinements in our analysis are ongoing, and we are also beginning to explore alternate biosensor designs to rapidly and accurately measure autophagic flux.

2. Specific Aims:

Specific Aim 1. To evaluate redox dynamics longitudinally in vivo in β-cells and liver.

Results: In this aim, we set out to answer the question: Does islet ROS accumulation precede the development of liver complications *in vivo*, and how do the kinetics change with metformin treatment? To answer these questions, we used 8-12-week old *db/db* mice on the BLKS

background. At this point in time, animals are diabetic and have just begun to develop liver steatosis. The redox-sensitive GFP's (roGFP's) enable the measurement of ROS levels over time in living cells. RoGFP's have been utilized in a variety of systems, from plants to cell lines, and can be modified with additional protein epitopes to increase the dynamic range and make the biosensor organelle specific. Further, we have found that roGFP2 with the addition of human glutaredoxin 1 (Grx1-roGFP2) yields enhanced sensitivity due to the rapid and robust coupling with the glutathione redox cycle, and the addition of an insulin promoter yields β-cell-specificity (see Reissaus *et al., Scientific Reports* 2019). We packaged our biosensor in an AAV8 construct, and injected male and female mice (N=6) with ~10^{11} viral particles of biosensor via IP injection to label islet β-cells *in situ*. Approximately 3 weeks later, animals were subjected to intravital microscopy (IVM), a method to image tissues in live animals. After each animal was placed under inhaled isoflurane anesthesia, we surgically exposed the pancreas then gently placed the exposed pancreas in an imaging dish, using the weight of the animal over top to reduce breathing motion artifacts. As shown in **Figure 1**, we obtained robust infection of islets in situ with the biosensor. We then proceeded to measure the baseline roGFP2 signal ratio at baseline and over time after IV infusion (via retro-orbital injection) of 0.5 mg/kg metformin (**Figure 2**). We ultimately saw an increase in ROS in the β-cells of both male and female mice, though the kinetics were different, and female mice uniquely exhibited a transient decline in ROS prior to a somewhat delayed response relative to their male counterparts. This is starkly different from the lack of signal change we see after saline injection (not shown), suggesting that the responses are indeed due to the metformin. We are currently underpowered to determine if the effects induced acutely by metformin are significant, and what the long-term functional outcomes are. Therefore, additional studies are ongoing to extend our observations in these directions.

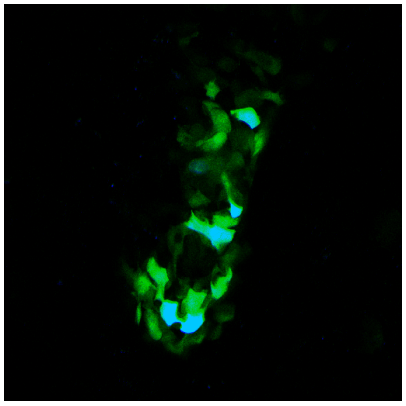

Figure 1. Representative image of an elongated islet *in situ* showing robust infection efficiency of roGFP2 biosensor in β-cells of *db/db* mice.

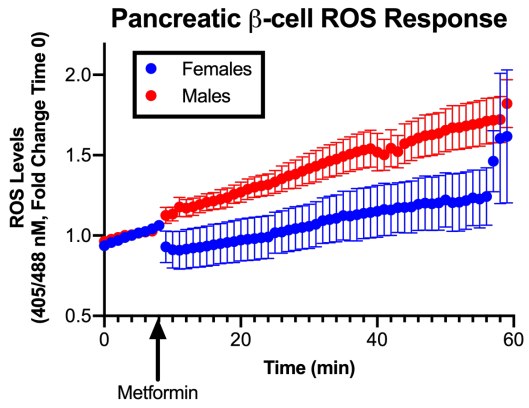

Figure 2. Quantification of ROS in *db/db* mouse islet β-cells *in situ* before and after IV injection of 0.5 mg/kg metformin.

Specific Aim 2. To characterize autophagic flux in vivo in β-cells and liver.

Results: In this aim, our goal was to study autophagic flux in live *db/db* mice using intravital microscopy, and to determine how autophagy is influenced in real time by exposure to 25 mg/kg L-carnitine to stimulate autophagic flux. To do this, we used an AAV8-Ins-GFP-LC3-TdTomato autophagy biosensor that we developed, a dual color β-cell-selective biosensor that

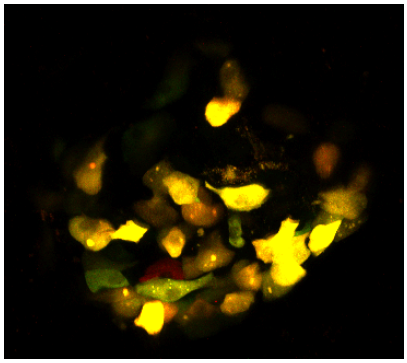

Figure 3. Representative image of an islet *in situ* showing robust infection efficiency of LC3-eGFP-TdTomato biosensor in β-cells of *db/db* mice. Overlapping eGFP and Tomato signal appears in yellow, and autophagosomes have a punctate appearance.

has baseline readouts for both GFP and TdTomato. Upon stimulation of autophagy, and flux through the pathway (i.e., as autophagosomes fuse with lysosomes), GFP is quenched in the acidic environment of the lysosome, whereas TdTomato is less acid-sensitive and signal will be preserved. The use of this construct should be advantageous over a single-color LC3 probe, as it will uniquely allow the monitoring of flux through the pathway with our *in vivo* system.

Our initial experiments with this biosensor were plagued by the formation of fluorescent aggregates that appeared to be artifacts, and not truly representative of autophagosomes. After many months of trial and error to optimize dose and timing of imaging after AAV injection, we were able to obtain reliable and robust biosensor expression in db/db mouse islet β-cells *in situ* as shown in the representative image in Figure 3. However, due to refinement of our approach, and commercial delays in obtaining AAV stocks, we did not meet our endpoint goal of studying response to L-carnitine in the islets of these mice. Despite these setbacks, experiments are now ongoing to complete these experiments, and we anticipate future success in these endeavors.

3. Publications:

No publications have resulted from this work yet, however it is anticipated that data supported by this project will be included in a manuscript that will be forthcoming.