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Biochemical Measures of Neuropathy -- Glutathione S-Transferase

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We use this protocol and it's working

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Abstract

Summary:

Oxidative stress is highly correlated with the metabolic changes caused by hyperglycemia. Increased levels of glucose overload mitochondria and result in the production of reactive oxygen species (ROS). In addition, the flow of excess glucose through cellular pathways decreases the cell's normal ability to detoxify ROS. As a result, the neurons and axons of the peripheral nervous system contain increased levels of ROS and decreased antioxidant capacity. The following assays are used to measure these changes in rodent models of diabetic neuropathy.

Diabetic Complication:

Neuropathy

Materials

MATERIALS

- HPLC-grade water **Fisher Scientific Catalog #**W5-4
- 1-Chloro-2 4-Dinitrobenze CDNB **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**237329-10G
- Potassium phosphate Monobasic **Merck MilliporeSigma (Sigma-Aldrich)**
- Potassium phosphate Dibasic **Merck MilliporeSigma (Sigma-Aldrich)**
- EDTA **Fisher Scientific Catalog #**16 004Y
- Reduced Glutathione **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G6529-1G
- GST Control

Reagent Preparation:

Homogenizing Buffer: To 10 mL 100 mM potassium phosphate **pH to 6.5, add 400 µL** 50 mM EDTA. (Final concentration EDTA is 2mM)

Assay Buffer: Add 20 µL Triton X-100 (0.1%) to 20 mL of 100 mM potassium phosphate buffer pH 6.5. Equilibrate to 25ºC before using.

GST (Control): Solution of rat liver.

Glutathione: Add 6.15 mg to 2 mL HPLC H_2O. (10 mM)(**Final concentration in well 1 mM)** (Habig, The first enzymatic step in mercapturic acid formation. J.Biol. Chem. 249, 7130-7139 (1974))

CDNB: (Upstairs in fume hood.) Add 4 mg CDNB to 1mL ethanol. (20 mM) (Final concentration in well 1 mM)

Note:
Fisher Scientific, RRID:SCR_008452

Sigma-Aldrich, RRID:SCR_008988

Troubleshooting

Sample Preparation: Tissue

1 1. Label 2 sets of 1.5 ml micro-centrifuge tubes and 1 set of 0.5 ml tubes.

2. If not perfused at dissection, rinse tissue with ice cold homogenization buffer to remove RBC's and clots.

3. Sonicate the tissue on ~5 in 0.5–1 mL of cold homogenizing buffer per mg of tissue.

General Equation: **µL Buffer = mg Tissue x 10**

4. Centrifuge at 10,000 x g for 15 minutes at 4°C.

5. Remove 15 µL supernatant and place in a 0.5 ml tube for protein analysis.

6. Transfer supernatant to a new labeled tube for assay and store on ice. Sample may be stored at – 80°C for at least one month.

Sample Preparation: Plasma

2 1. Collect blood in a tube containing an anticoagulant.

2. Centrifuge at 700-1000 x g for 10 min. at 4ºC.

3. Place the top yellow plasma layer in a labeled 1.5ml micro-centrifuge tube.

4. Remove 15 µL supernatant and place in a 0.5 ml tube for protein analysis.

Erythrocyte Lysate:

3 1. Remove the white buffy layer (leukocytes) and discard.

2. Lyse the erthrocytes (RBC) in 4 times its volume of ice-cold **HPLC-grade water**.

3. Centrifuge at 10,000 x g for 15 min. at 4ºC.

4. Place supernatant (erythrocyte lysate) in labeled 1. 5ml micro-centrifuge tube. **Dilute prior to assay**.

5. Remove 15 µL supernatant and place in a 0.5 ml tube for protein analysis.

Performing Assay:

4 *Final volume of the assay is 200µL in all wells*

1. Turn on Multiskan and open file gst.sed and make sure the machine is set to 25°C.

2. Add 20 µL of Assay Buffer to background wells, 20 µL GST control to the control wells and 20 µL sample to sample wells.

3. Add 150 µL Assay Buffer to all wells.

4. Add 20 µL of GSH to each well.

5. Initiate reactions by adding 10 µL of CDNB to all wells as quickly as possible. **Note precise time the reaction is initiated.**

6. Place plate onto Multiskan holder and click **START** .

7. Read absorbance once every minute at 340 nm at least 5 minutes. ***Absorbance increase should be between 0.012 & 0.064/min. If not, dilute or concentrate sample. If initial reading is >0.7, dilute sample.***

8. From the sheet menu, select Process>Organize. Choose the appropriate data to organize (usually Measure1), and click **OK**. This re-arranges the data into columns.

9. Save organized data as an Excel file into the GTx data folder. Use the naming convention GTXXXX.xls, where XXXXXX is the date in yymmdd format.