SRC project report

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Measurement of Mitochondrial NADPH concentration

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Abstract

The main theme of the project my mentor and I carried out this summer was on the measurement of mitochondrial pathways of NADPH production in vitro (in two cancer cell lines) and in vivo (from ground-mice tissue) with the intent to identify any differences that might exist among different tissues/cells: normal and diseased. The oxidative phosphate pentose pathway had been known to be a main production pathway for cytosolic NADPH which is an important metabolite. But production pathways or functions of mitochondrial NADPH have not been well analyzed. The method to detect mitochondrial NADPH assumes there are no mitochondrial transporters of NADPH and uses a metabolite called pyrroline 5 carboxylate. Pyrroline 5 carboxylate (P5C) is an intermediate in mitochondrial-specific protein synthesis that uses one NADPH and that can be utilized as an NADPH reporter. Since pyrroline 5 carboxylate is a relatively unstable compound, a derivatization method involving thioglycolic acid and ammonium bicarbonate was used to transform it into a similarly structured compound to simplify its detection. Our results showed increased P5C/ proline and NADPH labeling in pancreatic tissue: this is made remarkable because of the de novo synthesis of the non-essential amino acid proline in the pancreas and the direct labeling of an infusion of the non-essential amino acids glutamine and serine. Further research could elucidate the pathway for the observed relatively high concentrations of NADPH in the mitochondria of pancreatic tissue. A clinical application for future works might involve pancreatic cancer which is among the hardest to cure upon prognosis.

Scientists are looking for newer and better ways to treat pancreatic cancer and such research might build the base for knowledge on the chemical reactions that make the pancreas special.

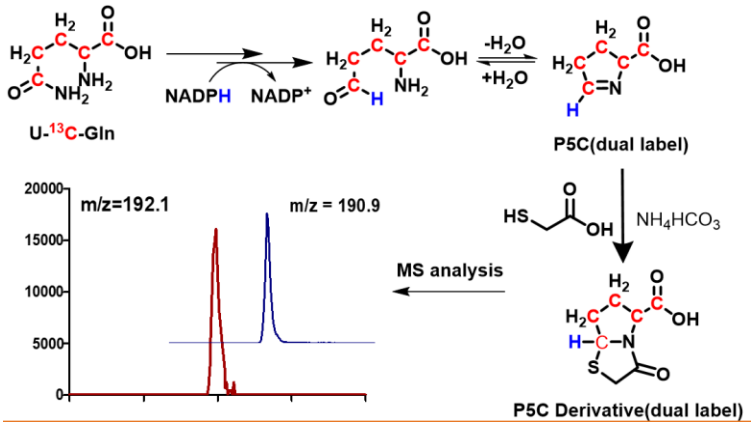


Figure 1 *An overview of a method to detect NADPH from a previous poster project by Zhaoyue, Li Chen and Rabinowitz. A dual tracer strategy using the C-13 and H-2 isotopes had been used to detect pyrroline 5 carboxylate. The figure shows how C-13 labeled glutamine (upper left) is incorporated into P5C upon consumption of one NADPH. The derivatization product is shown at the bottom right. It is produced by treatment with ammonium bicarbonate (NH_4HCO_3) and thioglycolic acid.*

Project Context and Rationale

Metabolism is a very important aspect of the biology of life. Genes for enzymes involved in metabolism account for ~10% of the human genome and ~25% of microbial genome. The basic pathways of metabolism have been known for quite some time. But a quantitative and integrated understanding of the metabolites in these pathways remains to be explored. (Rabinowitz and Vastag)

Rabinowitz Lab uses metabolomics and isotope tracing to study metabolism. Metabolomics (the study of metabolites similar in principle to genomics-the study of the genome- and proteomics- the study of the proteome) uses mass spectrometry to measure the concentration of metabolites while isotope tracing uses stable isotopes to measure metabolic flux. Flux is the change in concentration of the metabolites. Around 100 metabolites, which include amino acids, and nucleotides, are considered high flux and of considerable interest. (Cholsoon et al) “Metabolomics, with or without isotope tracing, involves three basic steps: (1) sample

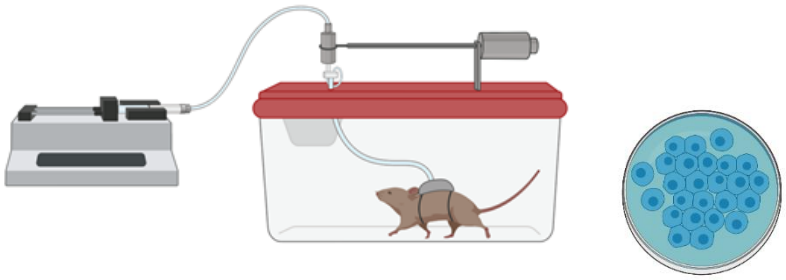
preparation, (2) metabolome measurement, and (3) data analysis" Using the three steps , it has become possible to find cellular and/or organellar concentration of metabolites. Isotope tracing , on the other hand, is used to measure pathway activity by quantifying either the rate of consumption or the rate of formation of a known metabolite downstream or upstream the reaction pathway.(Cholsoon et al)

Methodology

In metabolomics, the sample could be prepared from *biofluids* (serum, urine) and *cells in media* for in vitro study or from ground-tissue for in vivo analysis. The ideal studies for isotope tracing, on the other hand, are in vivo. (Cholsoon et al). For the four weeks of my summer project, I learned how to handle tools needed for both in vitro and in vivo studies. For in vitro studies, our lab utilizes cancer cell lines/ cell culture. I was tasked with establishing two cancer cell lines and maintaining their growth in a specific medium until the time of our experiment. To analyze the metabolites in a cancer cell line/cell culture, there are specific extraction methods that are used. These extraction methods differ on the type of metabolite used and usually involve the use of an extraction buffer to stabilize a metabolite. My mentor and I prepared an extraction buffer containing an 80:20 methanol water solution, 100 microlitres of ammonium bicarbonate , and 10 microliters of thioglycolic acid for every 10ml of the methanol water solution. This extraction buffer was specifically designed for our target metabolite pyrroline 5 carboxylate (P5C). To analyze the metabolites in vivo, our mice were first infused with labeled amino acids. Tissue from dead mice is then preserved (frozen and ground) using a machine called a CryoMillil prior to extraction. We measured a specific mass of tissue and transferred it to a microtube before adding a proportional amount of extraction buffer into our sample. The extraction buffer we used for the tissue sample was the same as the one we used to extract our metabolite in the cancer cell lines.

The main instrument we used for the next step of our experiment was a liquid chromatography mass spectrometer. The liquid chromatography mass spectrometer combines "separation capabilities of liquid chromatography with the mass analysis capabilities of mass

spectrometry." Chromatography and spectrometry together allow us to identify P5C in our prepared samples. We loaded our prepared samples to the LC-MS after running standard P5C and P5C-derivative samples.



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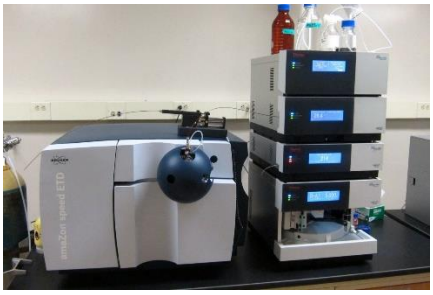


(2) *(3)*

Figure 2 An overview of our methodology: the first step in our metabolic experiment (1) involved infusing mice with isotopically labeled nutrients/amino acids or culturing cell lines in isotopically labeled media. The second step (2) was extracting our desired metabolite from ground-mice tissue or from a fully confluent cell culture. The third step (3) involved using a liquid chromatography mass spectrometer to identify our desired metabolite and measure its concentration.[12]

LC-MS data is analyzed on a software called El-MAVEN. We measured the P5C peaks in our in vivo and in vitro samples and analyzed the retention time (roughly the time it takes for compounds to move through the mass spec) to identify P5C. On the mass spectrum, we are able to see the mass to charge ratio of our target compound for identification and the peaks for comparing abundance.

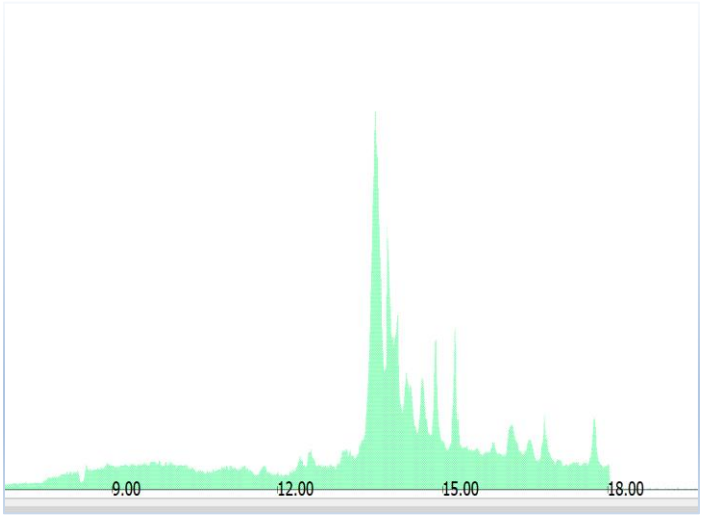
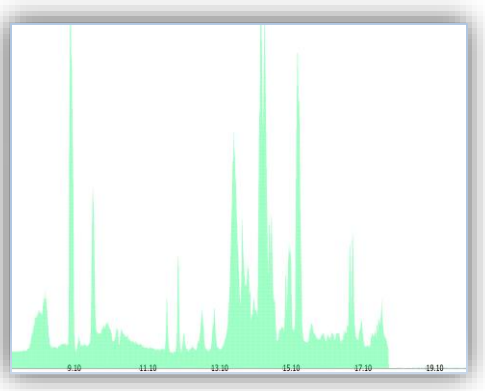


Figure 1 The peaks and retention time of a standard P5C sample and a standard P5C-derivative sample are shown on the figures at the top and bottom left respectively. The graph on the bottom right shows peaks from our in vitro prepared sample. The retention time - shown in minutes on the horizontal axis of the three figures- depends on the mass to charge ratio of the ion/substance detected. The retention time is helpful for identification of a substance. The peaks represent the intensity/concentration of the ion detected. The height of the peaks indicates the intensity(concentration) of the ion/substance identified.



Preliminary Results and Future Directions

Out of twelve tissues examined, preliminary data showed that the pancreas and the small intestine showed significant labeling in P5C and proline concentrations in the mitochondria. Since P5C and proline are reporters/ indicators for NADPH concentration, normal pancreatic and small intestine tissue had increased presence of NADPH in the mitochondria.

Main Findings:

(1) the validity of our methodology based on previous similar data

(2) Evidence of increased NADPH labeling in wild type pancreatic tissue

Upon further examination of pancreatic tissue, SHMT inhibited mice models showed little to no labeling of NADPH when compared to wild-type pancreatic tissue. SHMT (Serine hydroxymethyltransferase) is an important enzyme that is involved in protein/amino acid metabolism – more specifically serine metabolism. Further research could be done in pancreatic cancer cell lines by seeing the effect of SHMT inhibition in their cell growth and division.

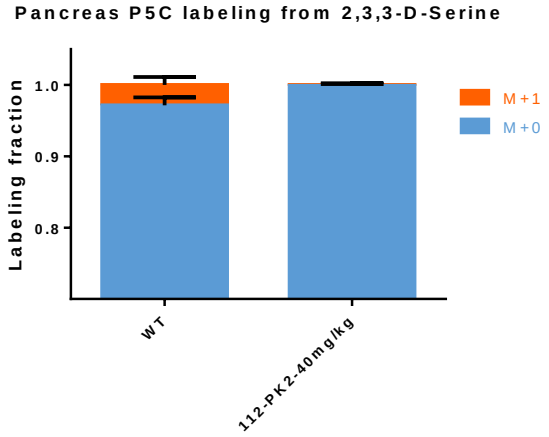


Figure 2 In wild type mice, increased pancreatic labeling of P5C is seen in normal tissue upon serine infusion. This is an indicator of the presence of more NADPH in the pancreas and the role serine has to play in this regard. To fruther establish this role, the wildtype labeling is compared to SHMT inhibited pancreatic tissue. SHMT is an enzyme important for serine metabolism and since no lableing is seen when SHMT is inhibited, the role of serine in mitochondrial NADPH concentration is evident.

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