Detection of serine isotopomers as a measure of mitochondrial function

C. B. Johnson[1], P. Pediaditakis[1], A. Tikunov[1], D. Romney[2], H. Lee[1], E. Holmuhamedov[2,3], M. Gamcsik[4], and J. MacDonald[1]

[1]Biomedical Engineering, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [2]Cell Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [3]The Center of Theoretical Problems of Physico-Chemical Pharmacology, Moscow, Russian Federation, [4]Biomedical Engineering, North Carolina State University, Raleigh, NC, United States

Introduction: The glycine cleavage system, found loosely associated with the mitochondrial inner membrane **(1)**, is responsible for the majority of glycine catabolism in mammals. It converts the 1-C of glycine into CO_2 and transfers the 2-C to tetrahydrofolate (THF), forming methylene THF (mTHF). The formation of mTHF in the mitochondria is tightly coupled to mitochondrial serine hydroxymethyltransferase (mSHMT) activity **(2)**, a reaction that transfers the methyl group obtained from glycine onto a second glycine to form serine. A similar reaction also occurs in the cytosol, catalyzed by cytosolic SHMT (cSHMT). Pasternack et al. first exploited this compartmentation in yeast, feeding the cells labeled 2-^{13}C glycine and observing the pattern of serine isotopomer formation **(3)**. Since GCS is restricted to the mitochondria, serine can only be labeled in the 3 position from 2-^{13}C glycine if it is formed in the mitochondria (Fig 1). As a result, by monitoring formation of serine labeled at the 3 position, it is possible to assess GCS activity. Since GCS activity is stimulated by an increased NAD^+/NADH ratio **(4)**, hormonally activated by glucagon **(5)**, and absent in diseases such as non-ketotic hyperglycinemia (NKH), we hypothesize that analysis of differences in the resulting pools of 2-C serine, the cytosolic pool, and 3-C serine, the mitochondrial contribution, can be used as a non invasive, in vivo detection system of mitochondrial function.

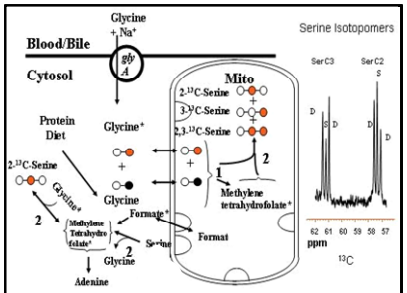

Fig 1. Diagram of serine isotopomer formation from 2-^{13}C glycine. Orange circles indicate ^{13}C label. Enzyme 1 is the GCS and enzyme 2 is SHMT .

Methods: Rat liver cells were isolated using a standard collagenase digestion protocol as described by **(6)**. The hepatocytes, viability ≥ 85%, were then resuspended to a density of 2.0 million/mL in plating media (DMEM high glucose with L-glutamine and pyruvate plus 10% FBS, 100U penicillin/streptomycin, 140nM insulin, and 1uM dexamethasone) and 10mL was plated onto collagen-coated 145cm^2 plates. After 1-2 h the media containing unattached/dead cells was removed and replaced with fresh plating media (37^{o}C). After overnight incubation at 37^{o}C, 5%CO_2, hepatocyte plating media was then replaced with 10mL of appropriate test media for one hour, and then spiked with 5 mM 2-^{13}C glycine for two hours. At this point, two plates were extracted using a 1:1:1 methanol:water:chloroform extraction method and protein was measured using the Bradford assay. Mixture was then shaken vigorously, allowed to separate overnight, lyophilized and pellet was dissolved in phosphate-buffered D_2O containing 1mM TSP and 2.5mM ^{13}C, ^{15}N formamide, pH 8.0. The ^{13}C spectra were obtained using an 11.7T Varian (Palo Alto, CA) INOVA equipped with a 5 mm broadband probe at 25°C. The SW = 32K Hz, AT= 2 sec, and D1 = 2 sec, and ^{1}H decoupling was performed with a WALTZ 16 during acquisition. All spectra were normalized to formamide and peaks were fitted using ACD software.

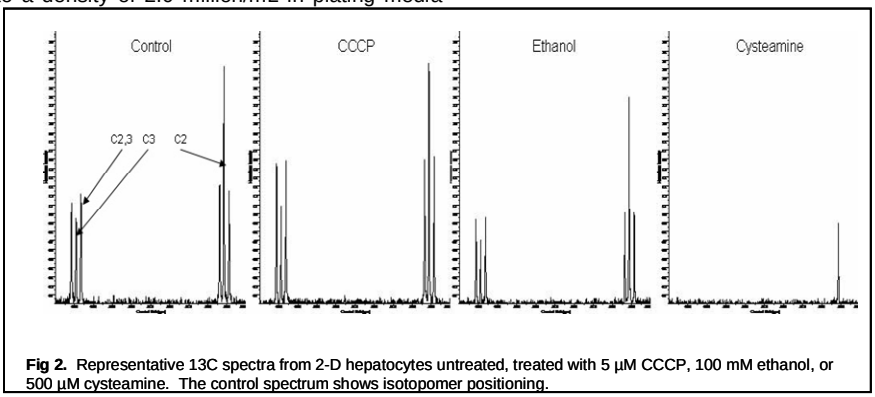

Fig 2. Representative 13C spectra from 2-D hepatocytes untreated, treated with 5 µM CCCP, 100 mM ethanol, or 500 µM cysteamine. The control spectrum shows isotopomer positioning.

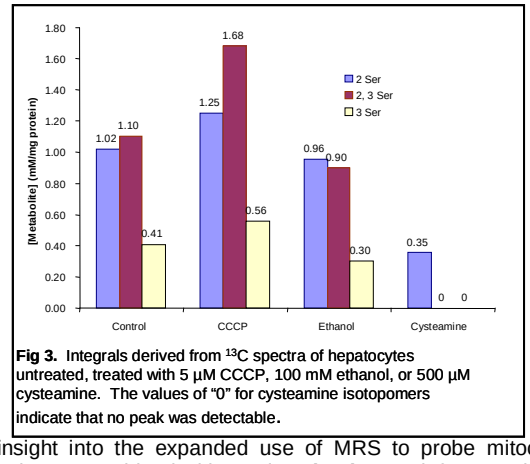

Fig 3. Integrals derived from ^{13}C spectra of hepatocytes untreated, treated with 5 µM CCCP, 100 mM ethanol, or 500 µM cysteamine. The values of "0" for cysteamine isotopomers indicate that no peak was detectable.

Results: Figure 2 presents the characteristic serine isotopomers formed from 2-^{13}C glycine treatment. The chemical shifts of the 2 and 3 positions of serine were 56.6 and 60.4 ppm, respectively. Figure 3 presents the integrated areas of the isotopomers. As proof of concept, the potent GCS inhibitor cysteamine, used at 500µM, prevents formation of mitochondrial serine isotopomers, the 2,3- and 3-^{13}C serine isotopomers, and decreases 2-^{13}C serine by 65%. Treatment with 5µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler that increases the NAD^+/NADH ratio, results in a 36% increase in 3-^{13}C serine, a 52% increase in 2,3-^{13}C serine, and a 23% increase in 2-^{13}C serine compared to control. Treatment with 100mM ethanol, which causes accumulation of NADH, decreased these isotopomers by 18%, 26%, and 6%, respectively. Additionally, 100nM glucagon caused an increase in isotopomers similar to CCCP.

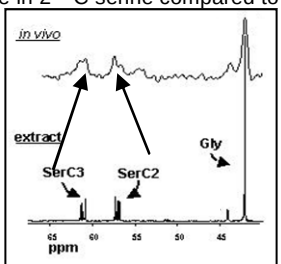

Fig 4. *Top* In vivo spectrum from rat liver infused with 2-^{13}C glycine showing serine isotopomers. *Bottom* is the corresponding liver extract.

Discussion and conclusion: These results present new insight into the expanded use of MRS to probe mitochondrial function. This data, obtained from 2-D hepatocyte cultures, combined with previous **in vivo** work from our lab (Fig 4) **(7)**, suggests that monitoring serine isotopomers after 2-^{13}C glycine infusion is a novel research tool to probe mitochondrial function. Additionally, we also speculate that this approach may be clinically relevant in diagnosing NKH, which currently requires a liver biopsy to definitively diagnose **(8)**.

REFERENCES:

1. Y. Motokawa, G. Kikuchi. Arch. Biochem. Biophys. 146, 461. 1971.
2. V. Prabhu, K. B. Chatson, G. D. Abrams. J. King. Plant Physiology 112(1), 207-216. 1996.
3. L. B. Pasternack, D. A. Laude, Jr., D. R. Appling. Biochemistry 31, 8713. 1992.
4. R. K. Hampson, M. K. Taylor, M. S. Olson. Journal of Biological Chemistry 259(2), 1180-1185. 1984.
5. Jois, M., Hall, B., Fewer, K., and Brosnan, J. T. Journal of Biological Chemistry 264(6), 3347-3351. 1989.
6. Berry et al. Journal of Cell Biology 43, 506-20. 1969.
7. Macdonald et al. Magnetic Resonance in Medicine 48(3), 430-439. 2002.
8. Lang, T. F. et al. Dev Med & Child Neurology 50, 157-159. 2008.

ACKNOWLEDGEMENTS: NIH, GM075941-01, and a UNC Biomedical Engineering Research Initiative Project.