Mass balance phenotyping of primary human hepatocytes in 2D cultures treated with acetaminophen

J. H. Winnike[1], P. Pediaditakis[1], P. B. Watkins[2], and J. M. Macdonald[1]

[1]Biomedical Engineering, University of North Carolina, Chapel Hill, NC, United States, [2]Medicine, University of North Carolina, Chapel Hill, NC, United States

INTRODUCTION: This study applies a new MRS phenotyping metholology to determine acetaminophen (paracetamol, APAP) toxicity in two dimensional cultures of primary human hepatocytes. APAP, an analgesic and antipyretic, has been studied as a model hepatotoxin in humans and animals.[1] A recent clinical trial has shown that one-third of the people receiving the maximum recommended daily dose of 4 g per day of APAP experience moderate elevations in serum alanine aminotransferase (ALT) levels.[2] This ALT increase is indicative of liver injury. While there have been other studies demonstrating APAP toxicity in animals and humans, these studies usually examine hepatotoxic events due to APAP overdose, APAP toxicity potentiated by ethanol administration, or APAP toxicity potentiated by malnourishment. This study examines the sub-lethal effects APAP has on primary human hepatocyte metabolism using a robust new method, mass balance phenotyping, which uses ^{13}C-labeled nutrients to ultimately derive relative fluxes and concentrations. Translation to a clinical diagnostic may be superior to the current measure of ALT as a gross measure of apoptotic or necrotic events produced by extreme APAP toxicity.

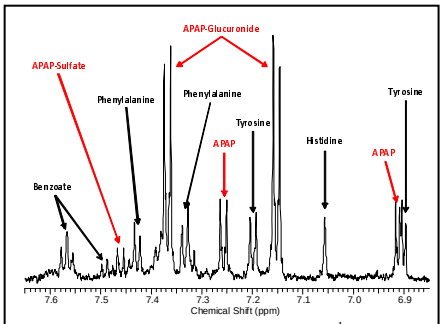

Figure 1- Representative 700 MHz ^{1}H NMR spectrum of human hepatocyte cell extract showing the aromatic region of the spectrum. APAP metabolites can be seen in red and endogenous metabolites can be seen in black.

METHODS: Human hepatocytes were obtained on collagen-coated 6-well plates with a well diameter of 35 mm and a cell density of 10^6 cells per well. All hepatocytes were extracted 24 hours after arrival with a modified cold methanol/H_2O (65:35, stored on dry ice) extraction procedure.[3] This procedure is used to extract the polar intracellular metabolites. Two hours prior to extraction, the media in all of the plates was changed to a u-^{13}C glucose DMEM (Dulbecco's Modified Eagle Medium) formulation so that glucose uptake can be monitored. One of the plates was used as a control and another plate was used as a vehicle control (0.1% dimethylsulfoxide- DMSO). The remaining 2 plates were treated with 1 mM or 10 mM APAP in 0.1% DMSO for two hours prior to extraction.

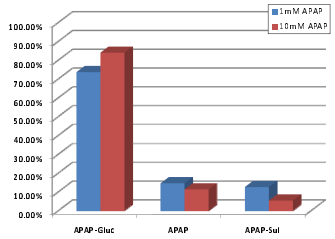

Figure 2- APAP metabolism profile describing intracellular APAP at media APAP concentrations of 1 mM and 10 mM. Values are expressed as a percentage of total intracellular APAP, conjugated or unconjugated.

NMR experiments were performed on a 16.4 T Oxford magnet (Oxford Instruments, Plc, United Kingdom) controlled by a Varian Inova console (Varian Inc., Palo Alto, CA). The spectra were collected with a pulse sequence consisting of a 100 ms d1 delay, 2.0 s of presaturation, acquisition pulse at the Ernst angle, and 3.64 s of acquisition. The free induction decay (FID) was acquired over a sweep width of 8999.9 Hz with 32768 complex points.

The NMR data was processed with ACD NMR Processor, version 11 (Advanced Chemistry Development, Toronto, Canada). The FIDs were zero filled to 32768 real points and a 0.5 Hz exponential decay window was applied prior to Fourier transformation. The resulting spectra were then phased, baseline corrected, and imported into Chenomx NMR Suite 5.0 (Chenomx, Inc., Edmonton, Canada) for metabolite identification and concentration determination.

RESULTS: The aromatic region of a representative NMR spectrum of the hepatocyte extract can be seen in Figure 1. Peaks from the two APAP phase II conjugates: APAP-glucuronide and APAP-sulfate can be seen in this region. In addition, peaks from the unmetabolized parent APAP can be seen in addition to a few endogenous metabolites containing aromatic rings. The relative ratios of the APAP compounds can be seen in Figure 2 at the two APAP concentrations, 1 mM and 10 mM. One can see that the majority of the intracellular APAP is in the form of the glucuronide conjugate. At the lower concentration, there is more of the sulfate metabolite than parent APAP and the opposite is true at the higher dose. Figure 3 shows the endogenous metabolite profile of the hepatocytes at the 4 conditions: control, vehicle control, 1 mM APAP, and 10 mM APAP. The metabolites are grouped according to metabolic function.

Examination of the intracellular metabolites shown in Figure 3 show an approximately 2-fold decrease in acetate, formate, and NADP+ in the high and low APAP treated hepatocytes when compared to the controls. A 2-fold decrease in intracellular fumarate can be seen in the high APAP treated hepatocytes.

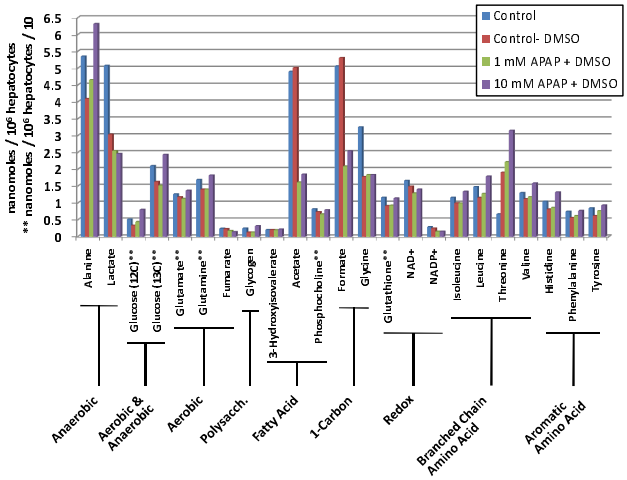

Figure 3- Endogenous intracellular metabolite profile of human hepatocytes. Control, DMSO control, 1 mM APAP, 10 mM APAP treatment conditions are shown.

DISCUSSION: The results in Figure 2 show a decrease in sulfation at the higher APAP treatment. This is likely due to the fact that while the affinity for sulfation is generally high, it has a low capacity and is therefore likely being depleted. However, glucuronidation is a low affinity, high capacity process and therefore, will not be depleted.

The results in Figure 3 show the pharmacodynamic effects APAP has on the metabolome, including decreased aerobic metabolism and increased redox and one-carbon metabolism. These results were similar to the findings of a recent metabolomic analysis of human urine samples obtained in a clinical APAP trial.[4] Both sets of results support the accepted mechanism of toxic action of APAP, which is known to be bioactivated by cytochrome P450, making a toxic intermediate which binds to mitochondrial proteins thus inactivating oxidative phosphorylation and the TCA cycle.[5]

REFERENCES:

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ACKNOWLEDGEMENTS: National Institutes of Health, GM075941-01 and GM038149. Stephen Strom, Ph.D., University of Pittsburgh, PA, for providing primary human hepatocytes.