Application of stable isotope labelling in cell culture experiments: [2-^{13}C]pyruvate as novel and superior substrate for *in vitro* metabolic studies in primary mouse hepatocytes

S. Gottschalk[1], D. Leibfritz[2], C. Zwingmann[1,2], and M. Bilodeau[1]

[1]Département de sciences biomédicales, Université de Montréal, Montréal, Québec, Canada, [2]Department of Organic Chemistry, University of Bremen, Bremen, Germany

Introduction:

Use of stable isotope labeling in cell culture experiments is a widely applied and powerful technique to study metabolic pathways and fluxes. In particular, the incorporation of ^{13}C-labels at specific carbon positions of a given metabolite can provide important information about cellular metabolism and may even reveal unknown metabolic phenomena. Depending on the cellular model and the metabolic pathway of interest, one has to take into consideration which labeled substrate will give the most valuable information.

Primary hepatocyte cultures are a widely used model for studying liver physiology and liver diseases. We have recently shown that labeled glucose (e.g. [U-^{13}C]glucose) was not feasible for the investigation of cellular and energy metabolism in cultured primary hepatocytes. But, we were able to demonstrate that [3-^{13}C]pyruvate is readily metabolized by these cells to yield useful information about a number of different metabolic pathways in the liver. However, some difficulties remained in distinguishing the contributions of specific metabolic pathways due to overlap in the signals from different isotopomers after metabolism of [3-^{13}C]pyruvate through pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC). To overcome these difficulties we applied one- and two-dimensional multinuclear NMR-spectroscopy to characterize the metabolism of labeled [2-^{13}C]pyruvate and to identify its metabolic isotopomers derived through various intermediary pathways in cultured hepatocytes.

Aims:

In this study, our aim was to identify metabolites and isotopomers derived from [2-^{13}C]pyruvate metabolism in cultured murine hepatocytes; in particular: metabolites of pyruvate oxidation through mitochondrial pathways and anaplerosis.

Methods:

Cell culture: Hepatocytes were isolated from male Balb/c mice. The cells were allowed to attach overnight. Afterwards, the medium was changed to glucose- and pyruvate-free Dulbecco's modified essential medium (DMEM) supplemented with [2-^{13}C]pyruvate (2.5mM). After 4 hours of incubation at 37°C, air and 5% CO_2, the cells were extracted. ***Extraction:*** Hepatocytes were flash frozen in liquid nitrogen and subsequently extracted with 5% perchloric acid. After centrifugation, the supernatant was neutralized using potassium hydroxide solution and lyophilized. ***NMR analysis:*** Lyophilized samples were redissolved in 0.5 ml D_2O. One- and two-dimensional ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DRX 600 MHz Spectrometer to identify metabolic isotopomers derived from [2-^{13}C]pyruvate metabolism.

Results:

NMR-spectra showed that after 4 hours of incubation, [2-^{13}C]pyruvate was readily metabolized to various metabolites in the cytosol and by the mitochondrial TCA cycle. The following metabolites and isotopomers were identified in ^{13}C-NMR spectra, given in the order of increasing chemical shifts (GSx = reduced + oxidized glutathione; gln/GSx-glu = signal overlay of glutamine and GSx-glutamate):

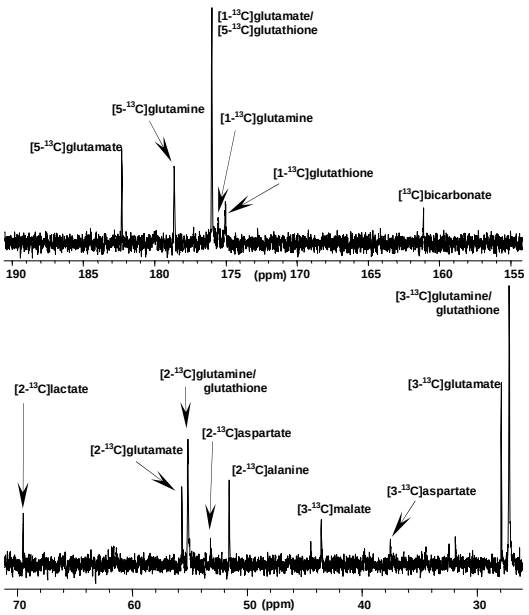
[2-^{13}C]alanine, [2-^{13}C]lactate – directly derived from pyruvate in the cytosol.

[3-^{13}C]gln/GSx-glu, [3-^{13}C]glutamate, [2-^{13}C]gln/GSx-glu, [2-^{13}C]glutamate – these isotopomers are produced solely by PC-flux through the TCA-cycle.

[^{13}C]bicarbonate, [1-^{13}C]GSx-glu, [1-^{13}C]glutamine, [1-^{13}C]glutamate, [5-^{13}C]GSx-glu, [5-^{13}C]glutamine, [5-^{13}C]glutamate – predominantly derived by PDH-flux through the TCA-cycle.

Furthermore, **[3-^{13}C]- and [2-^{13}C]malate** (direct conversion from pyruvate via malic enzyme) as well as **[3-^{13}C]- and [2-^{13}C]aspartate** (conversion of labeled oxaloacetate via aspartate aminotransferase) were identified.

Although after entry of the ^{13}C-labeled carbon into the TCA cycle through PDH, a fraction of the label ends up as [^{13}C]bicarbonate, [2-^{13}C]pyruvate has a definite advantage over [3-^{13}C]pyruvate. The signals of the observed isotopomers have little to no overlap, and the fluxes through PC and PDH can be identified more specifically compared to the use of [3-^{13}C]pyruvate. Therefore the separation of metabolic information towards PC, PDH and other reactions associated with the mitochondrial TCA cycle can be achieved more easily from the ^{13}C-signals of isotopomers from [2-^{13}C]pyruvate.



Sections of ^{13}C-NMR spectra of cell extracts after 4h of incubation with [2-^{13}C]pyruvate. (glutathione = glutamate-residue in reduced+oxidized form)

Conclusions:

Our results clearly showed that [2-^{13}C]pyruvate was readily taken up and metabolized by primary mouse hepatocytes. Our results also demonstrated metabolism of [2-^{13}C]pyruvate by lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, malic enzyme, PC, PDH and subsequent metabolic pathways through the TCA-cycle (glutamate dehydrogenase, glutamine synthetase, pathways of glutathione synthesis). In comparison to our previous findings, [2-^{13}C]pyruvate has proven to be superior over [3-^{13}C]pyruvate for studying and quantifying contributions of metabolic pathways in primary hepatocytes.

We conclude that [2-^{13}C]pyruvate is a versatile physiological substrate to assess hepatocellular pathways and the *de novo* synthesis of major metabolites in primary hepatocytes under normal and pathological conditions.