Isotopic Labeling

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Insights into Neuronal Cell Metabolism Using NMR Spectroscopy: Uridyl Diphosphate *N*-Acetyl-Glucosamine as a Unique Metabolic Marker**

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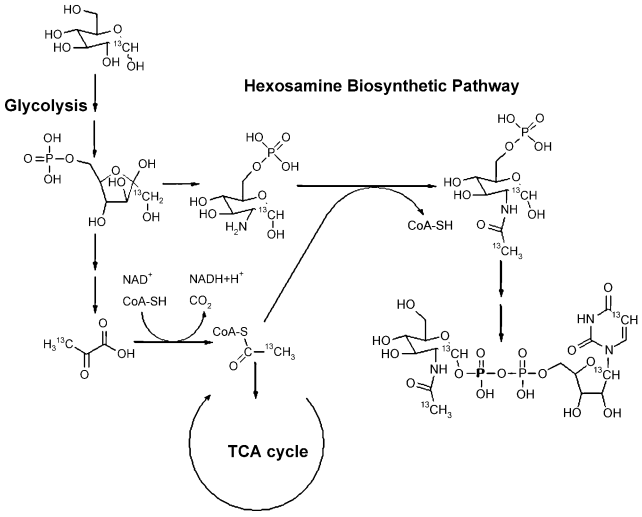
Uridyl diphosphate *N*-acetyl-glucosamine (UDP-GlcNAc) was discovered 1953[1] and is a nucleotide or a so-called activated sugar that is produced by the hexosamine biosynthetic pathway (HBP). It was proposed that such activated sugars provide important information about the metabolic state of a cell,[2] and in particular reflects possible pathological disorders. UDP-GlcNAc delivers GlcNAc units for the biosynthesis of N and O glycans, and it gained even more biological importance as a metabolic precursor along with the discovery of O GlcNAcylation.[3] O GlcNAcylation is a post-translational modification of nuclear and cytosolic proteins wherein UDP-GlcNAc covalently links a GlcNAc residue to a serine or threonine residue, similar to phosphorylation.[4] This modification plays an important role in many fundamental cellular processes and its dysregulation may be associated with human diseases such as cancer or diabetes.[5] In the brain, especially in the neurons, the availability of UDP-GlcNAc is very important because O GlcNAcylation of the Tau protein is assumed to counteract the development of Alzheimer's disease.[6]

In this context neuronal metabolism plays an important role. Although it is known that 2–3% of the intracellular glucose is shuttled into the HBP[7] and that intracellular concentration of UDP-GlcNAc reaches levels as high as that of ATP in some cells,[8] the focus in most metabolic studies of neurons was on metabolites of glycolysis and the tricarboxylic acid (TCA) cycle.[9]

Herein we investigate neural cell metabolism using UDP-GlcNAc as a metabolic marker that is easily detected by NMR spectroscopy. In our study we demonstrate that NMR spectroscopy with a cryogenic probe is sensitive enough to detect ^{13}C-labeled metabolites[10] with moderate effort in terms of neural cell mass. By using 1-^{13}C-D-glucose, a ^{13}C label can be introduced into UDP-GlcNAc[11] as shown in Scheme 1. By employing a simple but effective experiment we switched HT-22 cells, an immortalized neural cell line, into a glycolytic state. Whereas flux through the HBP is not affected and the 1-^{13}C label is propagated into UDP-GlcNAc, a decrease in labeling of the N-acetyl group of UDP-GlcNAc is observed. Thus we can compare glycolytic activity with flux through the HBP using one type of molecule—UDP-GlcNAc.



Scheme 1. Distribution of the ^{13}C label from 1-^{13}C-D-glucose throughout the different metabolic pathways, and the labeling pattern of UDP-GlcNAc.

HT-22 cells were incubated in a medium containing 1-^{13}C-D-glucose as the sole source of energy. ^{13}C labeling was used to reduce the amount of cell mass for NMR detection and at the same time to provide the option for isotope-edited NMR experiments such as ^{1}H,^{13}C-HSQC spectra. The great advantage of these spectra is their reduced complexity compared to proton NMR spectra. By using a cryoprobe it was possible to reduce the required cell mass (according to growth area and number of Petri dishes used) by a factor of up to 38 as compared to prior work.[9b,c,12]

By using 1-^{13}C-D-glucose as a precursor, selective labeling of metabolites is achieved. This fact further reduces the complexity of the spectra. The identification of metabolites in ^{1}H,^{13}C-HSQC spectra of cell extracts was done by comparing the spectra with ^{1}H,^{13}C-HSQC spectra of reference substances. An almost complete identification of the metabolites was achieved (see Figures 1 A and B in the Supporting Information). The peak assignment was performed based on data from the human metabolome database (http://www.hmdb.ca).[13]

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During incubation glucose and lactate concentrations in the medium were monitored (see Figure 2 in the Supporting Information). Under normal incubation conditions glucose consumption and lactate release by the HT-22 cells is linear. Therefore, the rates of consumption and release are normalized to the total protein content, and can therefore be determined. The cells show a glucose uptake rate of (1.16 ± 0.07) μmol glucose $h^{-1} mg^{-1}$ protein and a lactate production rate of (1.82 ± 0.49) μmol lactate $h^{-1} mg^{-1}$ protein.

To challenge our assumption that concurrent metabolic changes in the TCA cycle and in the HBP can be monitored using UDP-GlcNAc as a marker, we used sodium azide to switch the cells to a glycolytic state. Sodium azide is a competitive inhibitor of complex IV in the respiratory chain. It blocks the O_2 binding site, thus causing an inhibition of the electron flux. As a result, the proton gradient cannot be maintained and the ATP production is disrupted. Furthermore, NAD^+ production by complex I, the NADH-dehydrogenase, is stopped and NADH accumulates. Incubating the cells with sodium azide results in an increased glucose consumption. The glucose uptake rate increases to (2.08 ± 0.13) μmol glucose $h^{-1} mg^{-1}$ protein and the lactate production rate increases to (3.74 ± 0.10) μmol lactate $h^{-1} mg^{-1}$ protein.

Upon consuming more glucose the cells try to compensate the stalled ATP production by increased glycolysis, which delivers two ATP molecules from one glucose molecule. As a result of this glycolytic state more pyruvate, the end product of glycolysis, is produced. Under normal conditions pyruvate delivers an acetyl group for the formation of acetyl-CoA, and this step connects glycolysis with the TCA cycle. In our case the increased lactate production rate indicates that the pyruvate is no longer used for the TCA cycle. With the increased production of lactate the cells replenish the NAD^+ pool because NADH is consumed. Therefore, an incubation of the cells with sodium azide should result in a decreased flux through the TCA cycle. This hypothesis is confirmed by the peak pattern of the corresponding $^1H,^{13}C$-HSQC spectra. As shown in Figure 1 the peaks of the metabolites of the TCA cycle, citric acid and malic acid, vanish. This clearly indicates that the TCA cycle has stopped.

In the following we demonstrate that UDP-GlcNAc is a metabolic marker that simultaneously reflects changes in both the TCA cycle and in the HBP through one molecule, and we show that these changes are easily monitored by NMR spectroscopy. As mentioned above, 1-^{13}C-D-glucose as a labeled nutrient leads to selective labeling of metabolites. In the case of UDP-GlcNAc, only four atoms are labeled and observed in the $^1H,^{13}C$-HSQC spectra. C1′ of ribose and C6 of uracil are ^{13}C labeled through the pentose phosphate pathway. The C1 position of the GlcNAc residue receives its label directly from glucose, which is directed to the HBP. Finally, the N-acetyl group is labeled through acetyl-CoA, which is produced at the interface between glycolysis and the TCA-cycle (Scheme 1).[11] We have assigned the corresponding peaks in the $^1H,^{13}C$-HSQC spectra (Figures 1 and 2)

Incubating neuronal cells with sodium azide leads to an altered labeling pattern of UDP-GlcNAc. The intensity of the peak corresponding to the N-acetyl group significantly decreases, whereas labeling of the C1 position of the GlcNAc residue is not affected. To confirm these findings we recorded one-dimensional (1D) $^1H,^{13}C$-HSQC NMR spectra. These spectra, shown in Figure 3, are easier to quantify and demonstrate that labeling of C1 remains the same, and that the peak corresponding to the N-acetyl group almost vanishes. This confirms our hypothesis that UDP-GlcNAc can be used as a dual metabolic marker. Most importantly, the fact that pyruvate is used to produce lactate instead of acetyl-CoA is reflected in the significant decrease of the intensity of the peak corresponding to the N-acetyl group of UDP-GlcNAc. In fact, one should assume that under the present conditions where oxidative phosphorylation is inhibited, cells no longer synthesize UDP-GlcNAc de novo because of the lack of acetyl-CoA. Obviously, this is not the case, and incorporation of natural-abundance ^{13}C-acetyl groups into UDP-GlcNAc is observed. This observation suggests that there must be a source other than pyruvate for the production of acetyl-CoA.

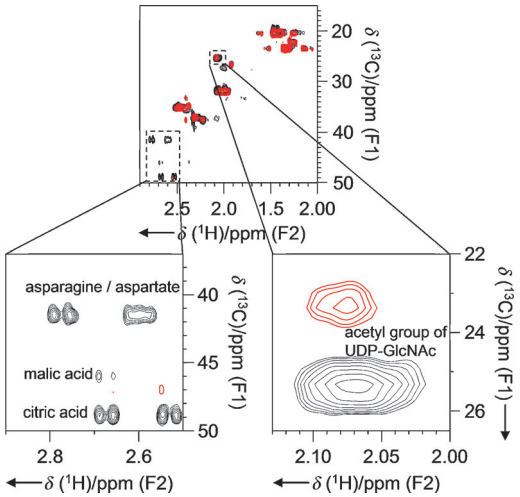

Figure 1. Sections of the $^1H,^{13}C$-HSQC spectra of cell extracts. Black: Normal incubation conditions. Red: Cells were incubated in the presence of 10 mmol L^{-1} sodium azide. Top: Overview of the aliphatic region, spectra superimposed. Bottom: Enlarged regions of the spectra. For clarity the red spectrum has been shifted in F1 by 2 ppm.

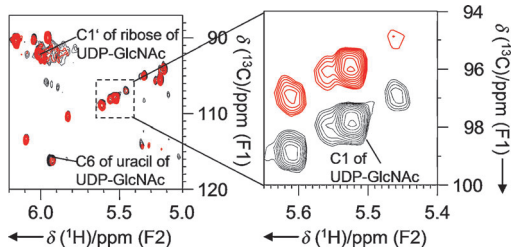

Figure 2. Sections of the $^1H,^{13}C$-HSQC spectra of cell extracts. Black: Normal incubation conditions. Red: Cells were incubated in the presence of 10 mmol L^{-1} sodium azide. Left: Overview of the anomeric region; spectra superimposed. Right: Enlarged region focusing on the anomeric proton/carbon H1/C1 of UDP-GlcNAc with the red spectrum shifted in F1 by 2 ppm. C1 of UDP-Glc: $\delta = 5.61$ ppm, C1 of UDP-GalNAc: $\delta = 5.55$ ppm, C1 of glucose-1-phosphate: $\delta = 5.46$ ppm. The C6 of uracil and the C1′ of ribose are not well suited to the interrogation of the UDP-GlcNAc levels because these signals overlap with corresponding signals from other UDP sugars.

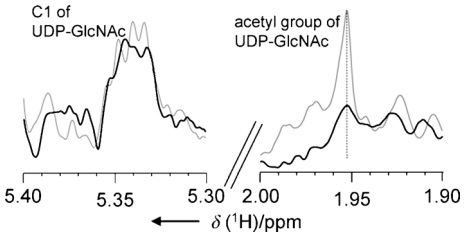

Figure 3. 1D ^{1}H,^{13}C-HSQC NMR spectra (no ^{1}H decoupling) of cell extracts showing the peak corresponding to the N-acetyl group (right), and the proton attached to C1 of UDP-GlcNAc (left). Only the low-field shifts of the doublets are shown because of severe signals overlap for the high-field shifts (chemical shifts are identical with the ones in Figures 1 and 2 where ^{1}H decoupling has been applied). Black: Normal incubation conditions. Grey: Cells were incubated in the presence of 10 mmol L^{-1} sodium azide.

To substantiate the hypothesis that inhibition of NAD^+ production induces an enhanced lactate production and leads to a decreased labeling of the N-acetyl group of UDP-GlcNAc, such that UDP-GlcNAc can be used as a metabolic maker in a control experiment, we incubated HT-22 cells with rotenone, a specific inhibitor of NADH-dehydrogenase.

As before with sodium azide we observed elevated glucose consumption and lactate production rates: (2.04 ± 0.18) μmol glucose h^{-1} mg^{-1} protein and (3.58 ± 0.24) μmol lactate h^{-1} mg^{-1} protein, respectively. Two-dimensional (2D) as well as 1D ^{1}H,^{13}C-HSQC spectra show a decrease in the intensity of the peak corresponding to the N-acetyl group of UDP-GlcNAc, whereas incorporation of the ^{13}C label at C1 was not affected (Figure 4).

In conclusion, we have shown that it is possible to investigate neural cell metabolism by NMR spectroscopy with rather moderate effort in terms of neural cell mass. Essentially, NMR spectroscopy turns UDP-GlcNAc into a dual metabolic marker that allows simultaneous detection of fluxes through two different metabolic pathways, the TCA cycle and the HBP. Our findings suggest that NMR detection of UDP-GlcNAc, a principal metabolite, in conjunction with ^{13}C labeling of precursor metabolites, in this case 1-^{13}C-D-glucose, furnishes new possibilities for studying altered metabolic states, such as those found in diabetes or Alzheimer's disease. We are currently applying this methodology to investigate other cell lines and primary cell cultures.

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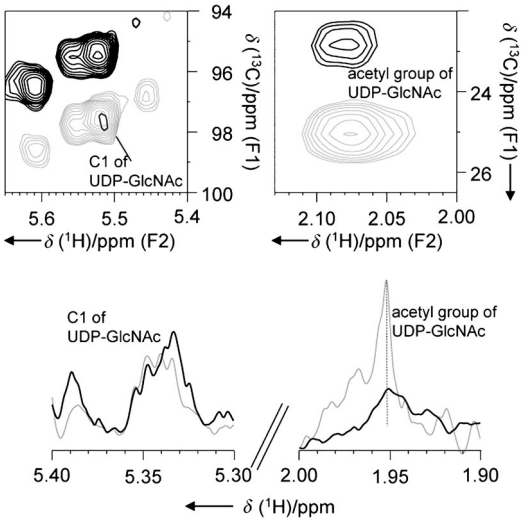

Figure 4. Sections of 2D ^{1}H,^{13}C-HSQC spectra (top; for clarity the grey spectrum has been shifted in F1 by 2 ppm) and 1D ^{1}H,^{13}C-HSQC spectra (bottom; as in Figure 3 only the low-field shifts of the doublets are shown) of cell extracts showing peaks of the N-acetyl group and the C1 proton of UDP-GlcNAc. Black: Normal incubation conditions. Grey: Cells were incubated in the presence of 50 nmol L^{-1} rotenone.

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