UDP-N-Acetylhexosamine Modulation by Glucosamine and Uridine in NCI N-417 Variant Small Cell Lung Cancer Cells: $^{31}$P Nuclear Magnetic Resonance

Results

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Abstract

Small cell lung cancer (SCLC) occurs as two neuroendocrine subtypes, SCLC-C (classic) and SCLC-V (variant). One reported difference is elevations of diphosphodiesterases (DPDE) in the more differentiated SCLC-C subtype. DPDE have been identified as primarily UDP-N-acetylgalactosamine (UDP-NAH) in a variety of tumors, and changes in DPDE levels have been observed during experiments designed to induce cell differentiation. UDP-NAH synthesis is controlled by negative feedback regulation of glutamine:fructose-6-P amidotransferase (EC 2.6.1.16), which can be circumvented by glucosamine. Using $^{31}$P nuclear magnetic resonance analysis of extracts and perfused cells, we have identified UDP-N-acetylgalactosamine and UDP-N-acetylgalactosamine as the primary metabolites in the DPDE spectral region of SCLC-V N-417 cells. Glucosamine addition causes a rapid increase in UDP-NAH levels. At glucosamine: glucose ratios of 1:1 and 10:1 formation of the UDP-NAH intermediates N-acetylgalactosamine 6-phosphate and UDP-N-acetylgalactosamine 1-phosphate is also observed, indicating UTP limitation. Subsequent uridine addition results in depletion of the intermediates and increased UDP-NAH formation. Moreover, N-417 cells retain the capacity to rapidly convert uridine to UTP despite low ATP and phosphocreatine levels. This expansion of the uridine pool may represent an additional metabolic reserve not yet addressed in the design of therapy options.

Introduction

SCLC occurs as two neuroendocrine subtypes characterized by differences in growth rate, morphology, protooncogene amplification, and amine precursor uptake and decarboxylation cell markers (1, 2). SCLC-C subtypes possess high specific growth rates, morphology, protooncogene amplification, and absences in response to changes in the glucose supply to HT-29 colon carcinoma cells (5) and addition of the differentiation-inducing agent dibutyryl cyclic AMP to N-18 neuroblastoma cell lines (6). The $^{31}$P NMR DPDE peaks have been identified as primarily UDP-NAH in HT-29 cell lines (5) and melanoma (BRO) tumors (7). UDP-NAH synthesis (Fig. 1) is typically controlled by negative feedback regulation of GFAT (EC 2.6.1.16) in liver (8) and adipocytes (9). However, this can be circumvented in liver (10) and a variety of tumors (11–13) by exogenous addition of GlcN, which is directly phosphorylated to GlcN-6-P.

As part of our analysis of SCLC cell lines, we use $^{31}$P NMR to identify DPDE compounds as primarily UDP-NAH and show that exogenous GlcN elevates UDP-NAH and its biosynthetic intermediates in SCLC-V in a uridine-dependent manner. We also demonstrate the importance of the GlcN:glucose ratio in modulating these responses.

Materials and Methods

NCI N-417 SCLC-V cells (1) are routinely grown and perfused in glucose- and glutamine-free RPMI 1640 medium supplemented with 2.5% fetal bovine serum, HITES, 28 mM glucose, 1 mM pyruvate, 1% nonessential amino acids, 15 mM (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) sodium salt, and 3 g/liter NaHCO$_3$ (RPMI-HITES). Fresh medium pH is adjusted to 7.4–7.6. The phosphate concentration has been reduced to 1 mM to facilitate NMR identification of intracellular inorganic phosphate. DMPM (Sigma) is added as a stable internal $^{31}$P standard (3 mM, 37.87 ppm) that evenly distributes between intra- and extracellular water space and does not alter cell metabolism (14). Antibiotics are not used in stock cultures, but penicillin G (100 units/ml) and streptomycin sulfate (100 mg/ml) are added to the circulating medium for NMR analysis. N-417 cells are entrapped in agarose beads by using a modification of the method of Nilsson et al. (15). A cell pellet is resuspended in 3% low-melting-point agarose (Becton Research Laboratories) in phosphate-buffered saline at 37°C, transferred to a siliconized round-bottomed glass tube containing mineral oil, vortexed, and quenched in an ice bath. Washed 100–500-μm beads are placed in a spinner flask for 12–24 h, transferred to a 10-mm screw cap NMR tube (Wilmar), and perfused in a sterilized system at 1.3 ml/min from a medium reservoir holding approximately 125 ml of fresh RPMI-HITES oxygenated with 95%O$_2$/5%CO$_2$. Exogenous agents (e.g., GlcN and uridine) are adjusted to pH 7.0–7.4 prior to addition to the perfusion medium.

$^{31}$P NMR spectra of cells at 37°C were obtained at 11.75 T by using a 4.7-tesla spectrometer (Bruker BioSpin). NMR spectra of cells at 37°C were obtained at 11.75 T by using a 4.7-tesla spectrometer (Bruker BioSpin). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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a GN 500 spectrometer with a transmitter frequency of 202.44 MHz, a 60 degree tip angle, a 2-s repetition time, 856 acquisitions, 4K block size, 20,000 Hz sweep width, and 2 Hz line broadening prior to Fourier transformation. In order to be certain that the spectral intensity changes observed accurately reflected changes in the intracellular concentrations, the pulse repetition rate was varied over a wide range. It was found that a recycle time of 2 s with a 60-degree pulse provided the best signal:noise ratio with the shortest period of time. Repetition times as long as 3 and 5 s gave no difference in the relative heights of the detectable DPDE, NTP, and PME peaks. Moreover, inversion recovery experiments in our laboratory indicate that the relaxation rates for DPDE compounds are of the same order as those for NTP compounds. Chemical shifts are assigned by addition of known compounds to extracts, coupling patterns, and chemical shift variation with pH, and are referenced to literature values (10, 16). The phosphorylcholine chemical shift is set at 2.57 ppm (which gives the α-NTP a chemical shift of −11.3 ppm). Integrated peak areas are reported relative to the same DMMP peak height to facilitate comparison. The initial spectrum before GlcN addition is shown in Fig. 2A. Peak assignments in ppm are NTP (−19.8, −11.3, −6.17), PCr (−3.75), glycerophosphorylcholine (−0.78), P, (1.4 internal, 1.6 external at pH 7.4), phosphorylcholine (2.57), and DPDE (−13.65, −11.9). The major spectral differences after perfusion for 1 h with medium containing 28 mM GlcN (Fig. 2B) are an increase in the height of the DPDE peaks (identified as UDP-NAH; see below) and the appearance of a PME intermediate identified by a single peak at 3.48 ppm. Also noted is an upfield shift of the extracellular P, peak. Continued perfusion with GlcN for a total of 7 h produced further increases in the levels of UDP-NAH and PME (Fig. 2C). There is also an increase in a signal overlapping with the upfield side of the P, peak. The results of these experiments indicate that the spectral intensity changes observed accurately reflect changes in the intracellular concentrations of UDP-NAH and PME.

Results

Modulation of DPDE Levels in 31P NMR Spectra of SCLC-V Cells by Glucosamine and Uridine. Fig. 2 shows the responses of perfused N-417 cells entrapped in agarose beads to exogenous GlcN and uridine. All 31P NMR spectra are plotted with the same DMMP peak height to facilitate comparison. The initial spectrum before GlcN addition is shown in Fig. 2A. Peak assignments in ppm are NTP (−19.8, −11.3, −6.17), PCr (−3.75), glycerophosphorylcholine (−0.78), P, (1.4 internal, 1.6 external at pH 7.4), phosphorylcholine (2.57), and DPDE (−13.65, −11.9). The major spectral differences after perfusion for 1 h with medium containing 28 mM GlcN (Fig. 2B) are an increase in the height of the DPDE peaks (identified as UDP-NAH; see below) and the appearance of a PME intermediate identified by a single peak at 3.48 ppm. Also noted is an upfield shift of the extracellular P, peak. Continued perfusion with GlcN for a total of 7 h produced further increases in the levels of UDP-NAH and PME (Fig. 2C). There is also an increase in a signal overlapping with the upfield side of the P, peak. The results of these experiments indicate that the spectral intensity changes observed accurately reflect changes in the intracellular concentrations of UDP-NAH and PME.

Identification of DPDE and PME Constituents. Proton-decoupled 31P NMR spectra of N-417 cell extracts obtained following 14 h of perfusion with GlcN are shown in Fig. 3. The DPDE region, which consists of two broad singlets in the proton-decoupled spectrum, is now resolved into two pairs of doublets in the proton-decoupled extract spectrum (Fig. 3A). Each doublet demonstrates homonuclear coupling constants of 21 Hz (Jpp), which reflects 31P-31P coupling. The taller doublets centered at −12.100 and −13.765 ppm are due to the α and β phosphates of a substituted UDP-Glc, while the smaller doublets centered at −12.048 and −13.590 ppm are the α and β phosphates of a substituted UDP-Gal (16). Comparison of the chemical shifts for a variety of substituted UDP-sugars indicate that the doublets correspond to UDP-GlcNac and UDP-GalNac (10). Moreover, direct addition of UDP-GlcNac and UDP-GalNac (collectively referred to as UDP-NAH) confirmed these peak assignments. In a similar manner it was determined that UDP-
Glc is not present in the extract at detectable levels. The peaks at -12.253 and -12.356 ppm are the upfield peaks assigned to the multiplet of NAD$^+$ (Ref. 18; confirmed by NAD$^+$ addition). The downfield PME region containing the unknown intermediate peaks (Fig. 2, B and C) is shown in Fig. 3B. P; and phosphorylcholine are identified at 1.74 and 2.57 ppm, respectively. By comparison with literature values (10, 16) and direct addition, the two singlets at 3.68 and 3.71 ppm are assigned to the $\alpha$ and $\beta$ anomeric phosphorus atoms of GlcNAc-6-P and the singlet at 1.31 ppm is assigned to GlcNAc-1-P. In a similar manner it was determined that GlcN-6-P is not present at detectable levels.

Effect of Glucosamine:Glucose Ratio and Order of Glucosamine and Uridine Addition (Fig. 4). GlcN is thought to inhibit cell growth via three mechanisms: (a) a decrease in glycolysis due to competition for the glucose transporter; (b) a decrease in UTP and ATP concentrations (see above); and (c) interference with normal glycosylation processing (12, 19). Competition for the glucose transporter suggests that the GlcN:Glc ratio may be the most important variable. Cell responses to GlcN and (10 mM) uridine addition are shown in Fig. 4A-C for various GlcN:Glc ratios. Peak areas (relative to DMMP) are shown for UDP-NAH (upfield peak, $\beta$-phosphate), $\gamma$-NTP, PCr, and GlcNAc-6-P. At a 1:10 ratio (Fig. 4A), GlcN increases UDP-NAH levels with a 15% decrease in NTP and little effect on PCr. The absence of detectable GlcNAc-6-P accumulation is consistent with the small extent of NTP depletion. Uridine addition had little effect on the rate of UDP-NAH formation. An experiment at the same GlcN:Glc ratio without uridine addition showed that UDP-NAH levels continued to rise for at least 14 h, and then leveled off after 18 h (results not shown).

Extract analysis following this experiment showed that detectable UTP levels were still present after 18 h (results not shown). These results suggest that the amounts of GlcN and glucose entering cells at a 1:10 ratio allows GlcN conversion to UDP-NAH without depleting the UTP pool. GlcN addition at a 1:1 ratio (Figs. 2 and 4B) also increases UDP-NAH levels. However, under these conditions there is an accumulation of GlcNAc-6-P, and the UDP-NAH level appears to reach a steady value after 6 h. The increase in GlcNAc-6-P is consistent with a more extensive decrease in NTP and PCr. In contrast to the response shown in Fig. 4A, uridine addition at a 1:1 GlcN:Glc ratio (Fig. 4B) caused a rapid increase in UDP-NAH levels that was accompanied by a decrease in GlcNAc-6-P levels. This is consistent with UTP depletion prior to uridine addition. GlcN addition at a 10:1 ratio (Fig. 4C) resulted in a very rapid increase in GlcNAc-6-P levels, with an increase in UDP-NAH similar to that observed at a 1:1 ratio (Fig. 4B). The large initial decreases and steady declines in NTP and PCr levels (Fig. 4C) indicate that a 10:1 GlcN:Glc ratio places a large metabolic burden on the cells. However, despite the compromised metabolic state of the cells, uridine addition increased UDP-NAH levels by almost 150%. The decrease in GlcNAc-6-P is much greater than the rise in UDP-NAH. However, it should be noted that, since the spectra are not fully relaxed, changes in the levels of different compounds cannot be directly compared. Similar UDP-NAH and GlcNAc-6-P responses to those shown in Fig. 4B were obtained by using 2.5 mM concentrations each of GlcN and glucose with subsequent uridine addition (results not shown). This indicates that the GlcN:Glc ratio, rather than the absolute concentrations, is the controlling factor (at least for total glucose and GlcN concentrations $\geq 5$ mM).

As an additional test of GFAT control of UDP-NAH formation (Fig. 1), we examined the effects of adding GlcN and uridine in the reverse order (Fig. 4D). Exposure to 10 mM uridine in the absence of GlcN increased NTP levels by 60% and decreased PCr levels by one-third. UTP accounted for about two-thirds of the NTP present in cells extracted following uridine addition in a similar experiment (results not shown). DPDE levels increased 2-fold shortly after uridine addition, and then remained constant even in the presence of elevated UTP levels. Extract analysis indicates that the DPDE peaks after uridine addition are primarily UDP-NAH, with additional peaks that may represent UDP-Glc. Subsequent addition of 20 mM GlcN (to medium with a residual glucose concentration of 20 mM after 12 h of perfusion) decreased the NTP peak to its initial value. During the next 6 h there was a rapid increase in UDP-NAH. The final UDP-NAH levels (relative to initial NTP) are comparable to those for GlcN addition prior to uridine addition (Fig. 4B).

Discussion

GlcN addition has been shown to increase UDP-NAH levels (detected via chromatography) in a variety of tumor cells, including Sarcoma 180 (11), HT-29 colon carcinoma (12), and TA3 mammary tumor (13). The dynamics of UDP-NAH formation, as well as that of its phosphorylated intermediates, can be analyzed in situ via $^3$P NMR, as demonstrated by in vivo analysis of rat liver (10). We have applied $^3$P NMR analysis of perfused cells and extracts to demonstrate that GlcN also elevates UDP-NAH in N-417 SCLC-V cells. In agreement with results obtained for rat liver (10), we detected significant amounts of N-acetylated GlcN intermediates (GlcNAc-1-P and GlcNAc-6-P; see Fig. 1), but were unable to detect GlcN-6-P. The low level of UDP-NAH in control cells (Fig. 2A) and the large increase after GlcN addition (Fig. 2, B and C) are consistent with control of UDP-NAH levels by feedback regulation of GFAT (Fig. 1) in SCLC-V cells in the absence of glucosamine. UTP limitation for GlcN:Glc ratios of 1:1 and 10:1 is indicated by the build-up of GlcNAc-6-P and GlcNAc-1-P (Figs. 2C, 3B, and 4, B and C) because the irreversible reaction of GlcNAc-1-P with UTP is the only step needed to

![Fig. 3. Extract analysis of N-417 cells following 14 h of exposure to 28 mM glucosamine. Proton-decoupled spectra are shown for the (A) diphosphodiester, and (B) phosphomonoester regions. Peak assignments are indicated and are discussed in the text.](image-url)

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Form UDP-NAH. UTP trapping by GlcN has been demonstrated in many cell types (19), and is consistent with the decrease in NTP following GlcN addition (Fig. 4, A and C), as well as the loss of intermediates and further increase in UDP-NAH formation after uridine addition (Figs. 2D and 4, B and C). The steady rise in UDP-NAH after exposure to 2.8 mM GlcN without uridine addition (Fig. 4A; results not shown) and the presence of detectable levels of UTP after 18 h (results not shown) suggest that de novo UTP synthesis (Fig. 1) is occurring. This is consistent with increased de novo uridylation synthesis in T47D mammary tumor cells treated with GlcN (13). The absence of GlcN-6-P in cell extracts after glucosamine addition and the rapid increase in UDP-NAH after uridine addition indicate that N-acetylation of GlcN-6-P is not limiting under these conditions.

We varied the GlcN:glucose ratio over a wide range from 0.1 to 10. The effects of this ratio on cell responses to GlcN and uridine (Fig. 4 A-C) indicate that care must be taken when comparing results obtained by different investigators. The rate of GlcNAc-6-P formation was below NMR-detectable levels at a 1:10 ratio (Fig. 4A), was approximately the same as the rate of UDP-NAH formation at a 1:1 ratio (Fig. 4B), and was much greater than the rate of UDP-NAH formation at a 10:1 ratio (Fig. 4C). The smaller effect on NTP at a 1:10 ratio is consistent with the observation that 20 mM GlcN decreases ATP levels in Sarcoma 180 cells cultured in 1 mM glucose, but not in 10 mM glucose (11). The UDP-NAH responses may be useful as a metabolic marker. For example, the rate of increase in UDP-NAH levels in cells exposed to low levels of glucosamine in the absence of uridine may provide an estimate of the rate of de novo UTP synthesis. The increase in UDP-NAH levels after addition of uridine to cells perfused with 28 mM GlcN and 2.8 mM glucose (Fig. 4C) suggests that cells metabolically compromised by high levels of GlcN are still able to rapidly convert uridine to UTP. This may provide the basis for improved treatment of SCLC.

Uridine addition alone more than doubled DPDE (mostly UDP-NAH as noted above) levels (Fig. 4D). This is consistent with a 70% increase in UDP-NAH for HT-29 cells treated with 10 mM uridine and 25 mM glucose (20). The increase in UDP-NAH during the first hour after uridine addition may indicate that GFAT control of UDP-NAH levels is loose. However, the constant UDP-NAH content over the next 8 h, even though extract analysis indicates high levels of UTP, suggests that regulation by GFAT is still operative. GlcN addition to cells previously exposed to uridine produces a slow rise in a compound with chemical shift similar to that of GlcNAc-6-P (Fig. 4D). This appears to contradict the disappearance of GlcNAc-6-P following addition of uridine to cells previously exposed to GlcN. However, it should be noted that in this case uridine levels may be substantially depleted during the 9.5 h of perfusion prior to GlcN addition (Fig. 4D). In contrast, fresh uridine was added for the cases in which GlcNAc-6-P levels declined (Fig. 4, B and C). This explanation is supported by two experiments similar to that shown in Fig. 4D except that the circu-
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Lating medium was replaced with fresh medium containing 28 mm glucose, 28 mm glucosamine, and 10 mm uridine at the point denoted by G. In these experiments we observed large increases in UDP-NAH with no increase in GlcNac-6-P after glucosamine addition. Extract analysis after these latter experiments indicates that UTP accounted for about two-thirds of total NTP even after glucosamine addition (results not shown).

UDP-GlcNac:UDP-GalNac ratios ranging from 2:1 to 3:1, which are consistent with those reported by Wice et al. (20) and Perlman et al. (10). This suggests that the equilibrium created by the UDP-acetylglicosamine 4'-epimerase in N-417 cells is similar to that in HT-29 cells and liver, and that it is not altered by glucosamine addition (10). The GlcNac-6-P:GlcNac-1-P ratio of approximately 7:1 is similar to that reported for liver after glucosamine addition (10), which suggests that the equilibrium distribution between these compounds is also similar in different tissues.

Uridine addition in the absence of GlcN increased NTP levels and decreased PCR levels (Fig. 4D). Despite the large increase in NTP, the observation that UTP accounts for two-thirds of NTP after uridine addition indicates that ATP levels actually decreased. The accompanying decrease in PCR may thus reflect a reestablishment of the equilibrium between ATP and PCR. Similar changes in NTP and PCR were observed after uridine addition to GlcN-treated cells (Fig. 4, A and B). However, if the cells had low PCR levels prior to uridine addition there was a much smaller change in NTP and PCR levels (Fig. 4C; results not shown). Since UDP-NAH formation rates do not correlate with the extent of increase in NTP following uridine addition, it seems likely that elevated UTP levels were present in all cases.

Changes in DPDE levels have been observed for a variety of tumor cells during experiments designed to induce cell differentiation (6, 20, 21). It has also been observed that the more differentiated SCLC-C subtype has elevated DPDE levels, while the SCLC-V subtype does not (4). The DPDE peaks have been identified as primarily UDP-NAH in the HT-29 cell lines (5) and melanoma (BRO) tumors (7) via $^{31}$P NMR, and in HL-60 cells via high-pressure liquid chromatography (21). Our results that DPDE are primarily UDP-NAH in SCLC-V cells treated with uridine suggest that DPDE are also UDP-NAH in untreated cells. Extract analysis of untreated SCLC-V and SCLC-C cells confirms that UDP-NAH are the most prevalent compounds in the DPDE region, with smaller amounts of NAD(H) and UDP-glucuronate (results not shown). UDP-NAH levels may differ significantly with the culture conditions, while those of NAD(H) and UDP-glucuronate are more stable. A better understanding of UDP-NAH regulation may improve understanding of the differences between variant and classic SCLC cells.

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References


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