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ORIGINAL PAPER

O-GlcNAc modification of proteins affects volume regulation in Jurkat cells

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Abstract An increasing amount of recent research has demonstrated that the hexosamine biosynthesis pathway (HBP) plays a significant role in the modulation of intracellular signaling transduction pathways, and affects cellular processes via modification of protein by O-linked *β-N*-acetylglucosamine (O-GlcNAc). Besides the many known and postulated effects of protein O-GlcNAc modifications, there is little available data on the role of O-GlcNAc in cellular volume regulation. Our objective was to test the effect of increased O-GlcNAc levels on hypotonia-induced volume changes in Jurkat cells. We pretreated Jurkat cells for 1 h with glucosamine (GlcN), PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-*N*-phenylcarbamate) an inhibitor of O-GlcNAcase, or a high level of glucose to induce elevated levels of O-GlcNAc. We found that the response of Jurkat cells to hypotonic stress was significantly altered. The hypotonia induced cell-swelling was augmented in both GlcN and PUGNAc-treated cells and, to a lesser extent, in high glucose concentration-treated cells. Evaluated by NMR measurements, GlcN and PUGNAc treatment also significantly reduced intracellular water diffusion. Taken together, increased cell swelling and reduced water diffusion caused by elevated O-GlcNAc show notable analogy to the regulatory volume changes seen by magnetic resonance methods in nervous and other tissues in different pathological states. In conclusion, we demonstrate for the first time that protein O-GlcNAc could modulate cell volume regulation.

Keywords O-GlcNAc · Hexosamine biosynthesis pathway · Volume regulation · Osmotic stress · Apparent diffusion coefficient

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Abbreviations

ADC	Apparent diffusion coefficient
DPH	1,6-Diphenyl-1,3,5-hexatriene
EC	Extracellular compartment
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Glc	Glucose
GlcN	Glucosamine
GlcNAc	*N*-Acetyl-glucosamine
HBP	Hexosamine biosynthesis pathway
Hanks' BSS	Hanks' buffered salt solution
IC	Intracellular compartment
NMR	Nuclear magnetic resonance

O-GlcNAc	O-Linked-*N*-acetylglucosamine
O-GlcNAcase	*O*-GlcNAc hexosaminidase (EC 3.2.1.52)
OGT	UDP-GlcNAc-polypeptide *O*-*β*-*N*-acetylglucosaminyltransferase (EC2.4.1.94)
PBS	Phosphate buffered saline
PI	Propidium Iodide
PUGNAc	*O*-(2-Acetamido-2-deoxy-D-glucopyranosylidene) amino-*N*-phenylcarbamate
PVDF	Poly(vinylidene fluoride)
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UDP-GlcNAc	UDP-*N*-acetylglucosamine

Introduction

Although most glucose taken up by cells enters glycolysis, it is estimated that 2–4% is diverted to the hexosamine biosynthesis pathway (HBP; Zhivkov et al. 1975; Hart 1997). The end-product of the HBP is UDP-*N*-acetylglucosamine (UDP-GlcNAc), which is the substrate for O-glycosylation and N-glycosylation of proteins (Peter-Katalinić 2005). The addition of O-linked *N*-acetylglucosamine (O-GlcNAc) to proteins, which is catalyzed by O-GlcNAc-transferase (OGT), is a dynamic and abundant posttranslational modification that has an important role in the regulation of signal transduction (Jackson and Tjian 1988; Zachara and Hart 2006). In contrast with *N*-type glycosylation, O-GlcNAc modification is reversible and occurs in the cytoplasm or in the nucleus, not in the ER. A number of proteins are susceptible to posttranslational O-GlcNAc modification; to date they include more than 400 identified cellular proteins, for example NF-κB, annexin, endothelial nitric oxide synthase, αB-crystallin, OGT, α-tubulin, c-myc, heat shock protein 70, etc. (Whelan and Hart 2003). In this modification O-GlcNAc binds to the Ser/Thr residues of the proteins, so the most thoroughly investigated role of O-GlcNAc is its interaction with phosphorylation and signal-transduction pathways. Although O-GlcNAc and phosphorylation are usually mutually exclusive, they can also co-exist at multiple sites on the same protein (Butkinaree et al. 2009). Apart from interfering with phosphorylation, O-GlcNAc has been implicated in protein localization processes (Kamemura and Hart 2003), or as an inhibitor of protein degradation (Zhang et al. 2003).

O-GlcNAc is thought to be involved in many cellular processes, for example cell cycling, nutrient sensing, apoptosis, and Ca^{2+} handling (Zachara and Hart 2006). O-GlcNAc has also been suspected of participating in the pathogenesis of insulin resistance and diabetes, inflammation, and malignant diseases (Brownlee 2001; Chou and Hart 2001; Buse et al. 2002; Largo 2003; Zachara et al. 2004; Liu et al. 2006). Although the beneficial or detrimental role that O-GlcNAc might play is controversial, data support the belief that O-GlcNAc contributes to diabetic complications (Brownlee 2001; Buse et al. 2002). On the other hand, recent reports show that an increased level of O-GlcNAc protects against ischemic injury (Champattanachai et al. 2007; Nöt et al. 2007; Chatham and Marchase 2009), protection which might be related to modulation of Ca^{2+} homeostasis (Nagy et al. 2006; Chatham and Marchase 2009).

An often overlooked cellular function is volume regulation, even though, in every cell, the metabolism and transport of osmotically active molecules continually require precise regulation and maintenance of intracellular water homeostasis (Lang et al. 1998). Stress, ischemia, or other stimuli also frequently challenge volume regulation; for example cell swelling is a common consequence of hypoxic damage (Nedelcu et al. 1999; van Pul et al. 2005; Pedersen et al. 2006). Similarly to the above mentioned cellular processes, water handling and volume regulation might also be affected by O-GlcNAc. A recent study (Zachara et al. 2004) found that hypertonic stress increased O-GlcNAc levels, and Heart and Sung (2002) showed that GlcN impaired the glucose transport stimulated by hypertonic shock. Apart from these observations, there is no direct evidence supporting a connection between O-GlcNAc and volume regulation. However, not only by modification of signal-transduction pathways but also by changing the hydrophobicity of key proteins by addition of the hydrophilic *N*-acetylglucosamine molecule, O-GlcNAc could also affect the water homeostasis of the whole cell. Indeed, hemoglobins from various species differing only in a few amino acids but having large variation in hydrophobicity (Bogner et al. 1998) or overexpression of strongly hydrophobic proteins (Kabsch and Alonso 2002) will significantly affect the osmotic response of a cell. Although its effect on protein hydrophobicity seems to be implicit, there are very few data about this aspect of O-GlcNAc modification (Zhang et al. 2003).

The objective of our study was to test the hypothesis that increased O-GlcNAc affects the water regulation and osmotic resistance of Jurkat cells. We found that increasing the levels of O-GlcNAc proteins altered the osmotic tolerance of Jurkat cells. Moreover, this was not because of the indirect effect of altered cell cycle progress or apoptosis. We also show that upon O-GlcNAc elevation, intracellular water diffusion decreased, a phenomenon often seen by magnetic resonance imaging during the diagnosis of certain pathological and pathophysiological

states (O'Shea et al. 2000; Hortelano et al. 2001; Sehy et al. 2004; van Pul et al. 2005). These data reveal, for the first time, a direct link between O-GlcNAc and cell volume regulation.

Methods

Cell culture and sample preparation

Jurkat cells (ATCC TIB 152 human acute T-cell leukemia) were cultured in glucose-free RPMI 1640 medium (R1383) supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL) and 5 mM basal glucose. The cell culture was incubated at 37°C, 5% CO_2, in a humidified incubator. Subculturing was performed every 2–3 days and fresh media was replaced 12–24 h prior to each experiment. On the day of each experiment, cells were resuspended in fresh media containing 5 mM basal glucose and supplemented with one of the following: 5 mM glucose (10 mM total Glc; control), 5 mM glucosamine (GlcN), 100 μM *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-*N*-phenylcarbamate (PUGNAc) or 25 mM additional glucose (30 mM total Glc). Subsequently each group was incubated for 1 h at 37°C before further use. For experiments in which hypotonic stress was applied, after pretreatment, the samples were resuspended in either isotonic (300 mOsmol) or hypotonic (150 mOsmol) saline solution.

Immunoblotting with CTD110.6

Jurkat cells pretreated with 5 mM Glc, 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc were washed 2× in ice-cold PBS, and harvested in modified RIPA buffer (10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) containing protease inhibitor cocktail (1 tablet/10 mL, Roche, Cat. No.:#11836153001) on ice for 30 min and centrifuged for 10 min at 14,000*g*. Protein concentration from the supernatant was measured using Bio-Rad *Dc* Protein Assay Kit. Proteins were separated on 7.5% SDS–PAGE and transferred to a PVDF membrane (Millipore). Equal loading of protein was confirmed by Zink staining (Bio-Rad) on the gel before transfer. Blots were probed with CTD110.6, a monoclonal mouse IgM antibody (Pierce; 1:2,000) that is highly specific for O-glycosylated proteins (Kneass and Marchase 2004) with no cross reactivity to similar carbohydrate antigens (Comer et al. 2001), in casein blocking buffer followed by HRP conjugated rabbit anti-mouse IgM antibody (Pierce; 1:5,000). For development, Femto chemiluminescent substrate was used (Pierce) and the signal was detected with Kodak Image Station 2000R. Densitometry was quantified using Kodak 1D analysis software.

Cell cycle analysis

Approximately 10^6 cells from each group of pretreated Jurkat cells were washed quickly in PBS then 1 mL ice-cold ethanol was added gradually, drop by drop while vortex mixing them thoroughly. The ethanol-fixed cells were kept at 4°C for at least 15 min before washing with PBS 3× and resuspending in propidium iodide (PI) solution (PBS, 0.1% Triton-X 100, 20 μg/ml PI, 0.2 mg/ml RNase A). After 30 min incubation in dark at room temperature, the fluorescence intensity of PI dye per cell was detected at 620 nm (FL3 channel) with a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA, USA). Gating and selection of regions (G_{0-1}, S, and G_2 phases) was performed on control cells and identical selections were utilized for all samples.

Apoptosis detection

Jurkat cells were cultured and pretreated with 5 mM Glc, 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc as described above. Subsequently, each sample was exposed for 10 min to 300 mOsmol isotonic conditions, or 150 mOsmol hypotonic stress. Next, 10^6 cells from each group of cells were stained for PI and FITC Annexin V positivity according to the manufacturer's recommended procedure (BD Pharmingen, Cat. No.: 556547). The fluorescence intensity of PI dye per cell was detected at 620 nm (FL3 channel) and FITC Annexin V intensity was detected at 525 nm (FL1 channel) with a Cytomics FC 500 flow cytometer. Defining the quadrant of live cells (negative for both PI and FITC Annexin V) was performed on control samples and identical boundaries were utilized for all samples.

Osmotic swelling

The volume change of individual cells was measured as follows. Jurkat cells pretreated with 5 mM Glc, 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc were resuspended in isotonic saline (300 mOsmol NaCl). One drop of cell suspension was placed on a glass slide on the stage of a light microscope, cells were allowed to sediment (~30–45 s), and immediately several images were taken from various areas of the cell monolayer. Next, a timer was started and an equal amount of distilled water was added to the isotonic cell suspension and mixed to achieve 150 mOsmol osmotic conditions. One minute before image acquisition, one drop of hypotonic cell suspension was also placed on a glass slide on the stage of the microscope.

Images were recorded 2 and 10 min after hypotonic shock, each time from a new drop of cell suspension. Image acquisition was performed with an Axiovert 35 (Carl Zeiss, Göttingen, Germany) inverted microscope. Image acquisition and measurement of the area of the profile for each cell were conducted with Cell^{D} (Olympus) software. The area of the profile was considered to be proportional to cell volume.

Monitoring changes in transparency during osmotic swelling was performed with an F4500 fluorescence spectrophotometer (Hitachi High-Technologies Europe, Krefeld, Germany) equipped with an absorbance cell holder. An equal amount of Jurkat cells was prepared as above. Briefly, control, GlcN, PUGNAc, or 30 mM Glc pretreated cells were resuspended in isotonic saline and a cuvette containing the cell suspension was placed in the cell holder of the spectrophotometer. Transparency at 500 nm was monitored continuously for 15 min, and at $\sim$1 min an equal amount of distilled water was added and quickly mixed.

Membrane fluidity

Membrane fluidity was measured by a fluorescence polarization technique using 1,6-diphenyl-1,3,5-hexatriene (DPH) as lipid probe. Briefly, Jurkat cells were cultured and pretreated with 5 mM Glc, 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc for 40 min at 37°C. After a quick wash in PBS, all samples were resuspended in Hanks' BSS containing 3 μM DPH and the appropriate pretreatment agent (control, GlcN, PUGNAc, or 30 mM Glc) and incubated at room temperature for an additional 20–30 min. After two wash steps to remove excess DPH dye, the cells were resuspended in isotonic saline. Fluorescence polarization was measured at 25°C by use of an F4500 fluorescence spectrophotometer equipped with polarizers and using an excitation wavelength of 350 nm and an emission wavelength of 433 nm. Fluorescence polarization (P) was defined as $P = (I_0 - I_{90} \times G)/(I_0 + I_{90} \times G)$ where I_0 and I_{90} are the fluorescence intensities detected by parallel and perpendicular polarizers, respectively. The G factor (G) was measured with a blank sample at the beginning of each session. Fluorescence recordings were followed up to 3–5 min to obtain long, steady baselines that were used to determine the average value of P for each samples.

NMR measurements

Jurkat cells were cultured and pretreated with 5 mM Glc, 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc as described above. After a quick wash in PBS, cell pellets were weighed before being resuspended in Hanks' BSS containing 10% bovine serum albumin (BSA) to obtain a 50% cell suspension. Each sample was thoroughly mixed and loaded into a hematocrit capillary immediately before the NMR test. NMR measurements were carried out as described earlier (Kotek et al. 2009). Briefly, NMR was performed on a Varian (Palo Alto, CA, USA) Unity Nova 400 WB spectrometer with an 89-mm vertical bore magnet of 9.4 T (Oxford Instruments, Abingdon, UK). Excitation and read-out were delivered by an ID-PFG probe (Varian) with 5-mm outer sample diameter. Auto-shimming (Vnmr 6.1C gzmap) was utilized before each sample measurement.

The apparent diffusion coefficient (ADC) was determined by using a pulsed gradient spin echo (PGSE) pulse sequence with gradient duration $\delta = 4$ ms, and gradient pair separation $\Delta = 40$ ms. Seven echoes were detected at varied gradient strengths in the range 25–180 mTm^{-1}. Areas under peaks were subjected to nonlinear least-squares fitting. The measurements were carried out at controlled $T = 25.0 \pm 0.1$°C. The error of diffusion coefficients was within 4%.

Data analysis

Data are presented as means ± standard deviations throughout. *Unless otherwise indicated*, comparisons were performed using Student's t test and statistically significant differences between groups were defined as P values < 0.05 and are indicated in the legends to the figures.

Results

Intracellular O-GlcNAc levels

After 1 h incubation with GlcN, PUGNAc, or 30 mM Glc we analysed the levels of O-GlcNAc-positive proteins in Jurkat cells by western blot (Fig. 1). At physiological levels of extracellular glucose (5–10 mM), even without any additional GlcN treatment, Jurkat cells exhibit several characteristic bands of O-GlcNAc-positive proteins and this pattern is very similar to those that have been found in other cell types and species (Zachara et al. 2004; Slawson et al. 2005; Nöt et al. 2007; Taylor et al. 2009).

In the cells treated with 5 mM GlcN or 100 μM PUGNAc, a specific substrate that inhibits O-GlcNAcase and removal of *N*-acetyl-glucosamine from proteins, some of the protein bands increased their intensity while others emerged to the level of detection. Treatment for 1 h with 30 mM Glc on the other hand caused a less significant increase of O-GlcNAc.

We compared the O-GlcNAc levels at physiological osmotic concentration (300 mOsmol NaCl) and at 10 min

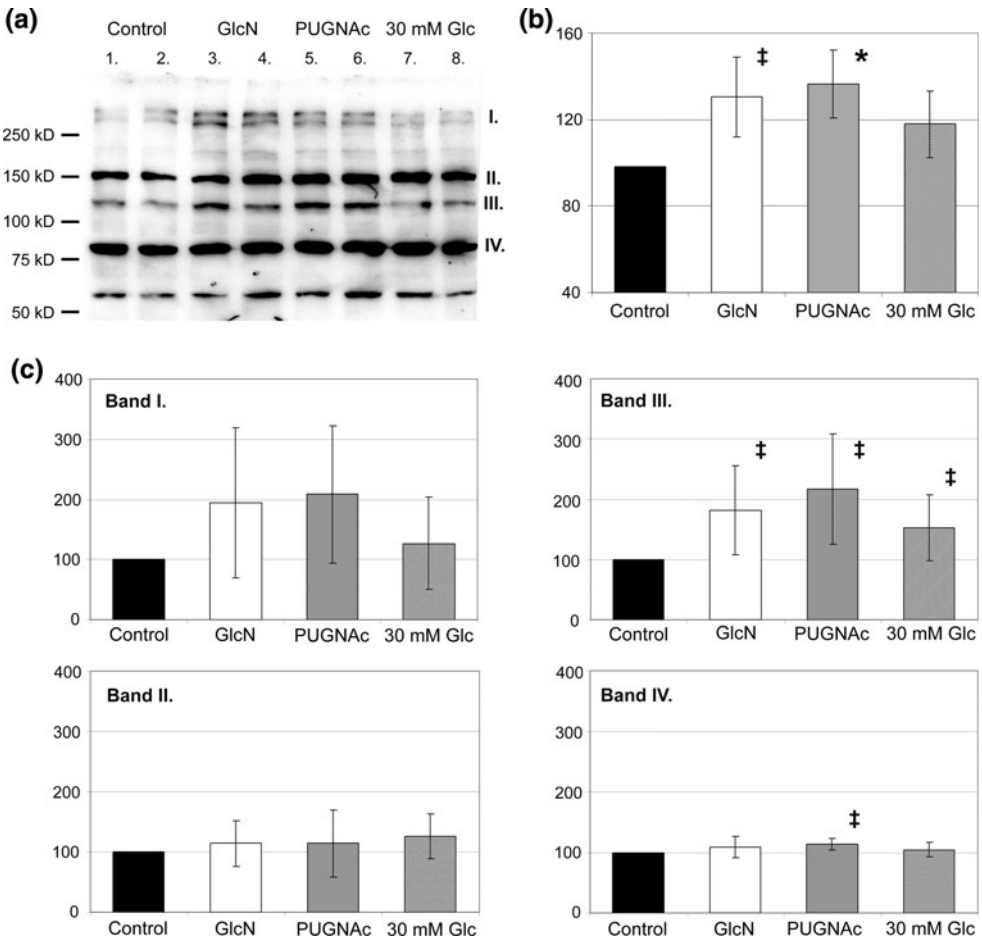

Fig. 1 Protein O-GlcNAc levels assessed via immunoblotting with CTD110.6 antibody in protein extracts from Jurkat cells pretreated for 1 h with *1*, *2*, 5 mM Glc (control), *3*, *4*, 5 mM GlcN, *5*, *6*, 100 μM PUGNAc, *7*, *8*, 30 mM Glc. Samples *1*, *3*, *5*, and *7* were further incubated in 300 mOsmol isotonic saline whereas samples *2*, *4*, *6*, and *8* were incubated in 150 mOsmol hypotonic salt solution. **a** Western blot; cellular extracts (30 μg/lane) were separated by SDS–PAGE and levels of O-GlcNAc were determined by immunoblot with CTD110.6. **b** Densitometry of total CTD110.6 staining was performed on immunoblots from three separate experiments. **c** Densitometry of four individual bands as indicated on (**a**). Levels of O-GlcNAc are expressed as a percentage of control (±SD); $^{*}P < 0.01$ versus control; $^{\ddagger}P < 0.05$ versus control

after changing to 150 mOsmol as this was also the procedure we followed in each subsequent experiment. After 10 min of hypo-osmotic shock, we could not detect any change in O-GlcNAc levels compared with isotonic conditions (*data not shown*). Zachara et al. (2004) showed that osmotic stress (in that case: hyper-osmotic stress) increased O-GlcNAc levels; this, however, was achieved by use of a significantly longer exposure time (8 h).

Osmotic swelling

To assess the effect of O-GlcNAc on volume regulation, we measured the osmotic swelling of Jurkat cells—pre-treated as described above—exposed to hypo-osmotic conditions. Jurkat cells are spherical, therefore the cell volume is proportional to the cross-sectional view. We measured in vivo the average cell-size (area of the cross-sectional view) before and after hypo-osmotic stress (Table 1). The cell size at physiological osmotic concentration was almost identical in each group, compared with control. Hypotonic stress of 150 mOsmol caused a rapid increase in cell size from the average 146 to ~220 μm^2 after 10 min, which corresponds to an increase in cell volume from 1,330–2,450 fL (84% increase). However, GlcN, PUGNAc, and even 30 mM Glc pre-treated cells behaved differently; the average cell size was bigger than

Table 1 Osmotic swelling of Jurkat cells measured as the average cross-sectional area of the cells

	300 mOsmol	150 mOsmol	
	Area (μm^2)	2 min Area (μm^2)	10 min Area (μm^2)
Control	148 (±30)	215 (±43)	217 (±46)
GlcN	146 (±32)	231 (±48)*	226 (±48)*
PUGNAc	146 (±31)	223 (±54)‡	222 (±49)‡
30 mM Glc	143 (±29)*	222 (±50)‡	230 (±52)*

Jurkat cells were pretreated for 1 h with 5 mM Glc (control), 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc. Cell size was measured after resuspending in isotonic saline and 2 or 10 min after exposing the cells to 150 mOsmol hypotonic salt solution. The data are the mean values (±SD) from three independent experiments, at least 350 cells were counted for every condition. $^{*}P < 0.001$ versus control; $^{\ddagger}P < 0.05$ versus control. Because the distribution of the data was not Gaussian, to validate the Students *t* test, we performed the Kolmogorov–Smirnov test and the Mann–Whitney test, with similar results

in the control. Since the standard deviation was larger than the difference between treated and control cells, we plotted the cell-size distribution on a histogram (Fig. 2). At normal osmotic concentration, all preconditioning resulted in an identical and symmetrical distribution. However, as can be clearly seen, GlcN, PUGNAc, and elevated Glc treatments caused a right shift on the histogram plotted after hypo-osmotic stress, indicating that pretreated cells had higher volume than control cells.

To study the dynamics of cell swelling we recorded the changes in transparency of the cell suspensions after exposing the cells to 150 mOsmol hypotonic stress (Fig. 3a). As a result of this hypotonic stress, a rapid increase in transparency, due to the cell-swelling, occurred within seconds and reached its peak after 2–3 min. GlcN and PUGNAc, and, to a smaller extent, 30 mM Glc pre-treatment caused a moderate increase in this process (inset). The most prominent change caused by either GlcN or PUGNAc was that the speed of cell swelling was significantly elevated; peak volumes were reached almost 1 min earlier than in the case of control cells (Fig. 3b). This indicates increased water permeabilities.

Use of 5 mM GlcN or 30 mM Glc as osmotically active metabolites by themselves might have interfered with our cell swelling experiments. To exclude this possibility we applied 5 mM Glc (10 mM total) as control, moreover; PUGNAc had a similar effect on cell volume regulation at an osmotically negligible concentration (100 μM). GlcN, as an osmotically active agent has been tested before by others (Matthews et al. 2007) at a similar concentration (8 mM) and did not have any significant osmotic effect. These observations suggest that the GlcN or PUGNAc-induced changes in volume regulation were not due to the osmotically active metabolites but rather because of increased levels of protein O-GlcNAc.

NMR

Because O-GlcNAc modification might alter the hydrophobicity of proteins, it was plausible to measure the rate

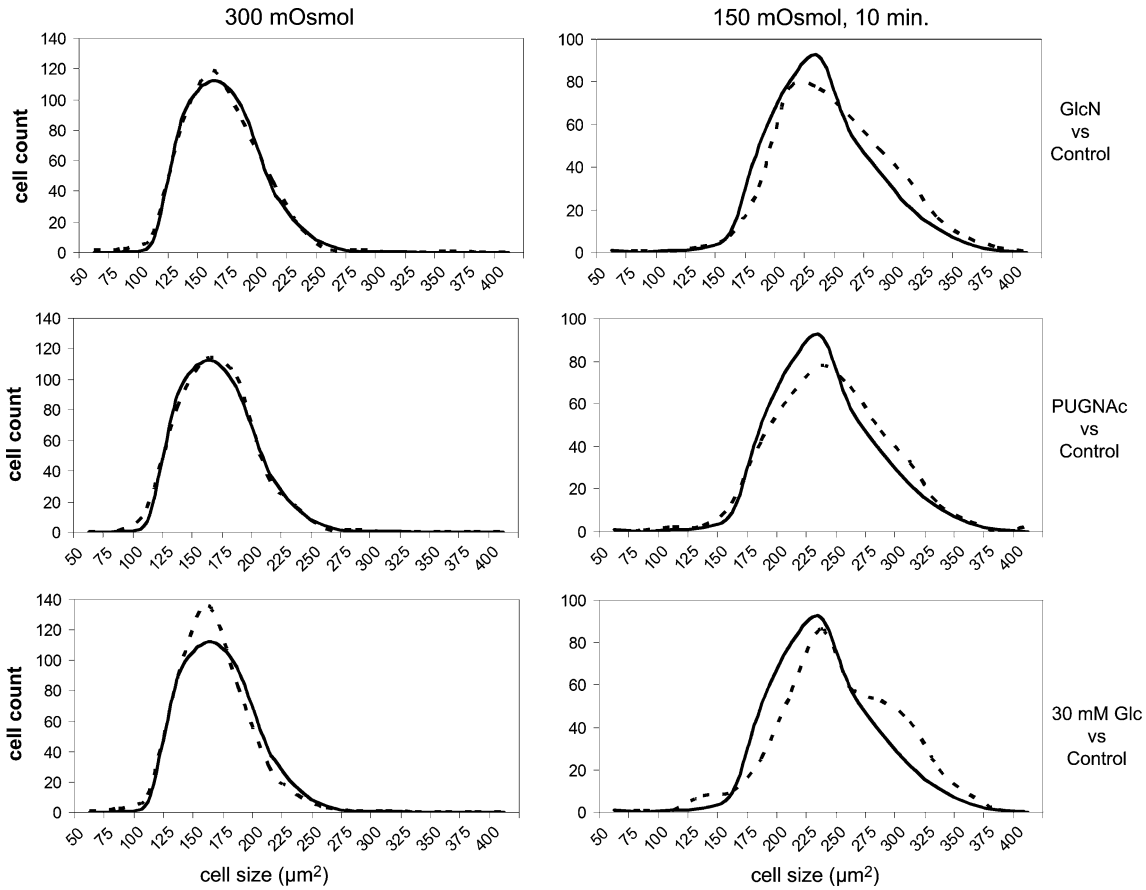

Fig. 2 Cell size distribution of Jurkat cells assessed by bright-field microscopy. Jurkat cells were pretreated for 1 h with 5 mM Glc (control), 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc. Cell size was measured after resuspending in isotonic saline for 10 min after exposing the cells to 150 mOsmol hypotonic salt solution. Each pretreatment (*dotted trendline*) was compared with control (*continuous trendline*). The data are the cumulative sum from three independent experiments, at least 350 cells were counted for every condition. *Trendlines* were generated after ranking the cells in incrementing subgroups according to their cell size (incrementing step: 25 μm^2)

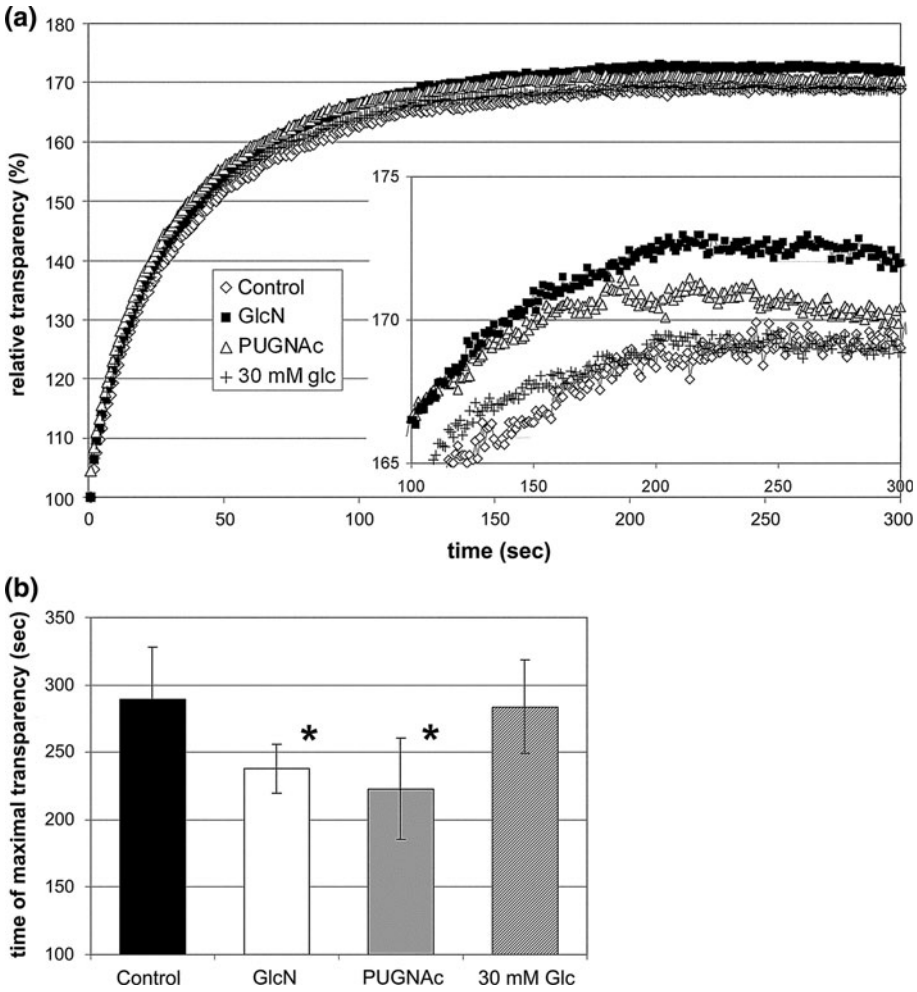

Fig. 3 Relative change of transparency as a measure of cell swelling. Jurkat cells were pretreated for 1 h with 5 mM Glc (control), 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc, resuspended in isotonic saline then diluted to 150 mOsmol with an equal volume of deionized water. **a** Representative curves of relative changes in transparency during hypotonic stress. *Inset* Magnified part of the curves including the peak levels of transparency. **b** Average time (s) needed to reach peak levels of transparency. Data are the mean values (±SD) from at least four independent experiments; $^{*}P < 0.01$ versus control

of intracellular water diffusion by NMR (Fig. 4.). The apparent diffusion coefficient (ADC) depends on both the extracellular (EC) and the intracellular (IC) compartments, a complex relationship which was investigated by Kotek et al. (2009). For practical reasons, we have chosen a 50% cell suspension thus IC water had a significant effect on the diffusion coefficient while the even distribution of the cells still could be maintained. The EC media alone had a diffusion value of 16.8 (±0.33) × 10^{-2} μm^2/s while the diffusion coefficient of the cell suspensions varied between 6.28 and 10.98 × 10^{-2} μm^2/s. We found that compared with control (9.19 ± 0.9), GlcN or PUGNAc treatment significantly reduced the rate of water diffusion under isotonic conditions (7.27 ± 1.2, 7.61 ± 0.7, respectively), whereas 30 mM Glc pre-treated cells were similar to control (9.98 ± 0.8) (Fig. 4).

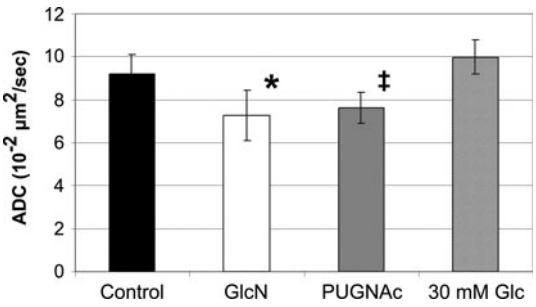

Fig. 4 Water diffusibility measured by NMR. Jurkat cells were pretreated for 1 h with 5 mM Glc (control), 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc, then resuspended in Hanks' BSS (supplemented with 10% BSA) to obtain a 50% cell suspension. The effective diffusion coefficient was measured by NMR within 30 min at 25.0 ± 0.1°C. Data are the mean values (±SD) from at least four independent experiments; $^{*}P < 0.01$ versus control; $^{‡}P < 0.05$ versus control

Apoptosis

To test that neither GlcN nor PUGNAc induces apoptosis that might interfere with volume regulation, we stained the cells with PI and FITC conjugated anti-annexin V. In all cases, more than 89% of the cells were confirmed to be alive without any sign of apoptosis. There was no significant elevation of apoptosis or increased number of dead cells after GlcN, PUGNAc, or 30 mM Glc treatment, and

we also demonstrated that 10 min osmotic stress was not sufficient to induce apoptosis (Fig. 5a).

Cell cycle

Affecting O-GlcNAc levels could lead to altered cell cycle processes, as was shown by Slawson et al. (2005), and altered cell cycle might cause the disturbances in cell volume and water regulation (Lang et al. 1998). Therefore we measured the cell-cycle distribution of Jurkat cells pretreated with GlcN, PUGNAc, and 30 mM Glc. All conditions had similar effects; ~53% of the cells were in G_0 or G_1 phase, ~28% were in S and ~16% in G_2 with no significant difference between control and glucosamine, PUGNAc, or 30 mM Glc treatment (Fig. 5b).

Membrane fluidity

Because membrane fluidity might have an effect on membrane permeability, the level of membrane polarization of GlcN, PUGNAc, or 30 mM Glc-treated Jurkat cells were measured by DPH staining. Although we found a slight decrease of polarization (increase of fluidity) in the pretreated samples (most prominent in the 30 mM Glc-treated sample), it was not a significant change (Table 2).

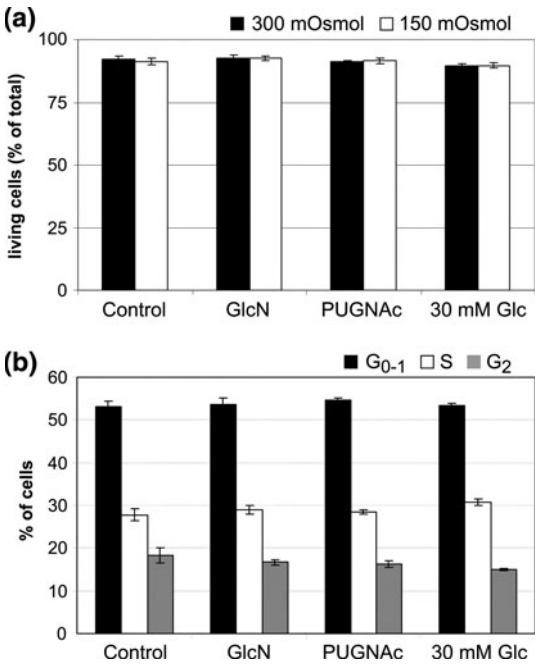

Fig. 5 **a** Percentage of live cells 1 h after treatment with 5 mM Glc (control), 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc. Subsequently, all samples were incubated in either isotonic saline (*solid bars*) or 150 mOsmol hypotonic salt solution (*open bars*) for 10 min. The cells were stained in vivo with anti-annexin-V-FITC and propidium iodide and measured by flow cytometry. Only double negatively stained cells were regarded as live cells. **b** Cell-cycle distribution of Jurkat cells pretreated as described above. Ethanol-fixed cells were stained with propidium iodide and DNA content was evaluated by flow cytometry. Data are the mean values (±SD) from at least three independent experiments

Discussion

HBP and O-GlcNAc participate in a wide range of cellular functions (Wells 2003) and can be triggered by many events, for example nutrient sensing, cell cycle, diabetes, or stress. O-GlcNAc, similarly to phosphorylation, modifies proteins on Ser/Thr residues, however O-GlcNAc's interaction with other signaling mechanisms and its regulation is far from fully understood. Accumulating data suggest that regulation of O-GlcNAc is an important part of the cell's response to various stress situations (Hanover et al. 2009). In our study we demonstrated, for the first time, that increased protein O-GlcNAc levels as a result of GlcN and PUGNAc treatment affected the volume regulatory response of Jurkat cells to a common experimental stimulus—osmotic stress. We also showed that this difference in osmotic resistance was not indirectly caused by apoptosis, cell cycle changes, or altered membrane fluidity. Moreover, we found by NMR measurements that elevated levels of O-GlcNAc significantly reduced intracellular water diffusion. Based on this, we propose that O-GlcNAc has an effect on intracellular water regulation.

In accordance with previous reports, we found that Jurkat cells have abundant O-GlcNAc proteins that were further enhanced by GlcN or PUGNAc treatment. The extent of this increase is comparable with other's findings for both GlcN (Huang et al. 2007) and PUGNAc (Golks et al. 2007) treatment. GlcN selectively increases the flux through the HBP, even a short period of treatment elevates UDP-GlcNAc and O-GlcNAc levels (Nagy et al. 2006). In order to identify the distinct effects of O-GlcNAc, we used PUGNAc as a specific inhibitor of O-GlcNAcase. *While PUGNAc is also recognized as inhibiting*

Table 2 Fluorescence polarization values of DPH-labeled Jurkat cells

	Fluorescence polarization
Control	0.162 (±0.0099)
GlcN	0.160 (±0.0065)
PUGNAc	0.153 (±0.0093)
30 mM Glc	0.151 (±0.0112)

Jurkat cells were pretreated for 1 h with 5 mM Glc (control), 5 mM GlcN, 100 μM PUGNAc, or 30 mM Glc. Membrane polarization was measured at ambient temperature after resuspending the cells in isotonic saline. Data are the mean values (± SD) from at least four independent experiments. No significant changes were found compared with control, $P > 0.05$ versus control

β-hexosaminidases (Stubbs et al. 2006), *it is extensively used to inhibit O-GlcNAcase* (Chatham and Marchase 2009). A high level of extracellular glucose has also been reported to increase O-GlcNAc (Brownlee 2001) although in our experimental setup only a slight elevation could be observed, which indicates that glucose needs longer exposure time to significantly increase the flux through the HBP.

Exposing cells to hypotonic condition triggers rapid swelling. Similar swelling occurs following ischemic injuries in the brain (Nedelcu et al. 1999; van Pul et al. 2005) or heart (Wright and Rees 1997) thus hypo-osmotic conditions are often used experimentally to emulate hypoxia-induced changes in volume regulation. Here we found that augmented O-GlcNAc modification caused faster, more prominent cell swelling under hypotonic condition than control. To our knowledge, this is the first report that O-GlcNAc affects volume regulation. On the other hand, among other stimuli (hypoxia, heat stress, etc.), osmotic stress has already been successfully used to elicit increased levels of O-GlcNAc (Zachara et al. 2004). The role of O-GlcNAc in stress-related situations is not fully understood, although increased O-GlcNAc during stress response is essential (Zachara and Hart 2006; Chatham and Marchase 2009), moreover, augmenting O-GlcNAc in advance of stress is advantageous for cell survival (Zachara et al. 2004; Liu et al. 2006; Champattanachai et al. 2007; Nöt et al. 2007).

In clinical practice, ischemia could be detected by reduced ADC with magnetic resonance imaging and similar decrease of ADC can be observed experimentally after hypotonic stress (O'Shea et al. 2000). ADC is inversely proportional to cell swelling; while cells are swelling upon osmotic (or ischemic) stress, ADC paradoxically decreases (van Pul et al. 2005). Our results showed remarkable resemblance to this model: both GlcN and PUGNAc pretreatment enhanced osmotic swelling and at the same time reduced ADC. Treatment with 30 mM Glc, despite being the highest hyperosmotic precondition, did not cause any changes in ADC in our experimental setup, supporting the idea that increased flux through HBP and elevated O-GlcNAc is required for the modulation of ADC instead of the accumulation of osmotically active metabolites.

As of now, there is no single theory to explain the mechanism underlying the decrease of ADC in osmotic/ischemic stress. It is feasible that the diffusion changes are predominantly intracellular, however multiple theories, for example decreased intracellular streaming (Neil et al. 1996), increased tortuosity (van Der Toorn et al. 1996), changes in sol–gel fractions (Branco 2000; Schwarcz et al. 2004), or altered membrane permeability (Ford et al. 1998), have been proposed. Lang et al. (1998) summarized all known factors that regulate cell volume; although O-GlcNAc is not mentioned by them, several other mechanisms are described that could mediate or explain the effects of O-GlcNAc: the cytoskeletal matrix, cell membrane potential, intracellular Ca^{2+}, macromolecular crowding, etc. O-GlcNAc could target any of those—tubulin is a known O-GlcNAc protein (Walgren et al. 2003) and ion channels, aquaporin proteins in the cell membrane, could be activated by O-GlcNAc. As we have reported earlier, intracellular Ca^{2+} levels are modulated by O-GlcNAc in cardiomyocytes (Nagy et al. 2006). Another possible mechanism we propose here is altered protein hydrophobicity/hydrophilicity. Earlier, our group investigated the relationship between protein hydrophilicity and hydration properties using mammalian erythrocytes and hemoglobins. Significant alterations in hydrophobicity resulted in augmented water binding, closer hemoglobin packaging, and, consequently, in decreased ADC and increased osmotic resistance (Bogner et al. 2005, 1998). Here, elevated O-GlcNAc, could change the hydrophobicity of many proteins thus significantly modifying the intracellular conditions for bound/unbound hydration water also.

Elevation of O-GlcNAc has been reported to affect apoptosis. Some of the studies found pro-apoptotic properties (Wells 2003; Park et al. 2007; Kang et al. 2008); others showed that O-GlcNAc protected from ischemia-provoked apoptosis (Champattanachai et al. 2007). Similar to apoptosis, manipulation of O-GlcNAc levels had been demonstrated to affect the cell-cycle (Slawson et al. 2005). Because both processes (cell-cycle progression and apoptosis) might affect the intracellular milieu, including water regulation, it was important to reveal any changes caused by increased O-GlcNAc level. We found that GlcN, PUGNAc, and high levels of glucose did not induce detectable differences in cell viability or cell cycle distribution. In our experimental setup, 1 h treatment was a relatively short incubation time, thus we assumed that any effect involving water regulation would be the immediate consequence of protein O-GlcNAc.

Alterations of membrane fluidity affect membrane permeability (Lande et al. 1995) and thus might have affected our osmotic swelling experiments. While diabetes can change membrane fluidity in red blood cells or in neutrophil cells (Seres et al. 2006; Adak et al. 2008), we found no significant change in membrane fluidity that could be attributed to elevated O-GlcNAc levels. Significant lipid peroxidation (Negre-Salvayre et al. 2008) and advanced glycation end-products (Yamaguchi et al. 1998) in diabetes needs considerably more time to build-up and to affect membrane fluidity. However, we cannot exclude the possibility that O-GlcNAc might increase membrane permeability by other means.

It has been reported that O-GlcNAc levels are increased after various stresses (Zachara et al. 2004). Here we

demonstrated, for the first time, that raising O-GlcNAc in advance of hypotonic stress affects the osmotic swelling of Jurkat cells. Additionally, while cell-cycle progress, apoptosis, or membrane fluidity seems undisturbed, water diffusion is altered by elevated O-GlcNAc. Activation of O-GlcNAc is already shown to be both deleterious (e.g. mediating diabetic complications) or beneficial, (e.g. in cardiac stress response; Chatham and Marchase 2009). As in these situations, the role of O-GlcNAc in volume regulation is probably double-edged and depends on the circumstances. It is conceivable that protein O-GlcNAc are part of a complex system that maintains and regulates intracellular water homeostasis.

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