Increased Expression of β-N-Acetylglucosaminidase (O-GlcNAcase) in Erythrocytes from Individuals with Pre-diabetes and Diabetes

Running title: Increased O-GlcNAcase in Pre-diabetic Erythrocytes

Kyoungsook Park¹, Christopher D. Saudek², and Gerald W. Hart¹

1: Department of Biological Chemistry, Johns Hopkins University, School of Medicine, Baltimore, MD, 21205
2: Department of Medicine, Division of Endocrinology and Metabolism, Johns Hopkins University, School of Medicine, Baltimore, MD, 21205

Corresponding author:
Gerald W. Hart
Email: gwhart@jhmi.edu

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Objective: O-linked-β-N-acetylglucosamine (O-GlcNAc) plays an important role in the development of insulin resistance and glucose toxicity. O-GlcNAcylation is regulated by O-GlcNAc transferase (OGT), which attaches O-GlcNAc to serine and/or threonine residues of proteins and by O-GlcNAcase, which removes O-GlcNAc. We investigated the expression of these two enzymes in erythrocytes of human subjects with diabetes, or pre-diabetes.

Research design and methods: Volunteers with normal condition, pre-diabetes and diabetes were recruited through an NIH (NIDDK) study and at the Johns Hopkins Diabetes Center. Erythrocyte proteins were extracted and hemoglobins were depleted. Global O-GlcNAcylation of erythrocyte proteins was confirmed by western blotting using an O-GlcNAc specific antibody. Relative OGT and O-GlcNAcase protein amounts were determined by western blot analysis. Relative expression of O-GlcNAcase was compared to that of glycated hemoglobin A1c (HbA1c).

Result: Erythrocyte proteins are highly O-GlcNAcylated. O-GlcNAcase expression is significantly increased in erythrocytes from both individuals with pre-diabetes and with diabetes, compared to normal controls. Unlike O-GlcNAcase, protein levels of OGT did not show significant changes.

Conclusions: O-GlcNAcase expression is increased in erythrocytes from both individuals with pre-diabetes and in individuals with less well-controlled diabetes. These findings, together with the increased site-specific O-GlcNAcylation of certain erythrocyte proteins (Diabetes, Wang et al. 2009), suggest that the upregulation of O-GlcNAcase might be an adaptive response to hyperglycemia-induced increases in O-GlcNAcylation, which are likely deleterious to erythrocyte functions. In any case, the early and substantial upregulation of O-GlcNAcase in individuals with pre-diabetes may eventually have diagnostic utility.
O-GlcNAcylation is a posttranslational modification in which single O-linked N-acetylglucosamine residues (O-GlcNAc) are attached to the hydroxyl groups of serine and/or threonine moieties of proteins within the nucleus and cytoplasm. O-GlcNAcylation is an abundant, inducible and reversible modification. O-GlcNAc serves as a nutrient/stress sensor to modulate signaling, transcription, proteasomal activity, cytoskeletal assemblies, and cellular activities (for reviews, (1; 2)). Recent studies have established that the dynamic crosstalk between O-GlcNAcylation and phosphorylation is extensive (3; 4).

Unlike phosphorylation, O-GlcNAcylation is regulated by only two known enzymes, uridine diphospho-N-acetylglucosamine:polypeptide β-N-acetylglycosaminyltransferase (O-GlcNAc transferase, OGT)(5; 6) which transfers O-GlcNAc from UDP-GlcNAc to proteins, and O-linked β-N-acetylglycosaminidase (O-GlcNAcase, OGA) (7), which removes O-GlcNAc from proteins. However, both enzymes have many transient binding partners within cells, thus creating many different holoenzyme complexes, presumably with different specificities and expression patterns. OGT is essential for life in mammals at the single cell level (8). Over-expression of OGT in muscle or adipose tissue of mice causes hyperleptinemia and insulin resistance (9).

O-GlcNAcase, a nucleocytoplasmic β-N-acetylhexosaminidase is distinct from lysosomal hexosaminidases (7; 10). The O-GlcNAcase gene was found to be identical to a meningioma-expressed antigen 5 (MGEA5)(11). O-GlcNAcase also plays a role in mediating insulin signaling and insulin resistance. Competitive inhibition of O-GlcNAcase using O-(2-acetoamido-2 deoxy-D-glucopyranosylidene)-amino-N-phenylcaramate (PUGNAC, Ki=54nM) results in decreased glucose uptake in response to insulin in adipocytes, termed insulin resistance, the hallmark of type 2 diabetes (12). A single mutation in the O-GlcNAcase gene (MEGA5 gene) results in higher susceptibility to diabetes in a Mexican American population (13). This mutation causes an early termination of translation of O-GlcNAcase and leads to a decrease in the expression of active O-GlcNAcase, suggesting that mutations in O-GlcNAcase may be a risk factor for type 2 diabetes in certain populations, but not in others.

One of the earliest studies of O-GlcNAc was performed in human erythrocytes (14). However, the role of O-GlcNAcylation in erythrocytes is still unknown. Recent proteomic studies have revealed that erythrocytes have a complex cellular system to regulate their physiology (15-17).

Herein, we examined the protein levels of OGT and O-GlcNAcase, as well as O-GlcNAcylation in human erythrocytes from subjects with normal, pre-diabetic and diabetic conditions.

**MATERIALS AND METHODS**

**Samples** - Human blood samples were collected from two sources of volunteers. One set was obtained through National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) and clinical data are shown in the supplemental material in the online appendix at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org). Another set was obtained from the Johns Hopkins Diabetes Center (JH). Clinical data collected in this pilot phase was restricted to age, body weight, a casual plasma glucose and hemoglobin A1c. In JH subjects, the diagnosis of diabetes had been established by accepted clinical criteria for >1 year, with documented hyperglycemia and elevated hemoglobin A1c. Subjects designated as normal had no personal history suggesting...
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Blood samples were drawn and separated. Erythrocytes were washed with cold PBS three times and stored at –80 °C. JH samples were obtained by same procedure except using Histopaque-1077(Sigma) reagent according to the manufacturer’s protocol. Subjects gave written consent, approved by the applicable institutional review boards.

Hemoglobin depletion - Erythrocytes were lysed in NP40 lysis buffer (PBS, 0.5% NP-40, protease inhibitors). Samples were briefly sonicated and centrifuged for 10 min at 13,000 rpm at 4 °C. Lysate was recovered and hemoglobins were depleted using HemogloBind Resin (Biotech Support Group) according to the manufacturer’s protocol. Depletion process was repeated twice to remove up to ~90 % of hemoglobin.

Western blot analysis - Hemoglobin-depleted lysate subjected to SDS-PAGE gels and blotted to PVDF membranes. Membranes were blocked in TBST (0.1 % (v/v) Tween-20) with either 3% (w/v) BSA or 5 % (w/v) non-fat milk and incubated overnight at 4 °C with the appropriate primary antibodies; O-GlcNAc (CTD110.6) (Covance), OGT (AL28), O-GlcNAcase (18), actin (Sigma), GAPDH (SantaCruz). Detection was performed by enhanced chemiluminescence.

Expression and purification of recombinant O-GlcNAcase - Human O-GlcNAcase cDNA was subcloned and protein expression and purification procedure is carried as previously described (18).

Statistical analysis - Densitometry data were obtained by ImageJ program (NIH), and analysis was performed using student t-test. P value <0.05 (two tailed) was considered significant. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

The characteristics of the subjects are summarized in Table 1 and S2. The JH cohort was selected to be more hyperglycemic than the NIDDK group. The differences in pathogenesis between type 1 and type 2 diabetes were not considered important for this study, since both cause chronic hyperglycemia, which affects O-GlcNAcylation.

Erythrocytes Have Many GlcNAcylated Proteins - One of the earliest studies of O-GlcNAc showed the presence of O-GlcNAcylated proteins in human erythrocytes (14) and our recent study showed that the site-specific O-GlcNAcylation of certain erythrocyte proteins increases in individuals with diabetes (19). The Supplemental Figure shows examples illustrating O-GlcNAcylation on many human erythrocyte proteins.

Expression of O-GlcNAcase Increases in Erythrocytes from Both Individuals with Pre-diabetes and Diabetes - O-GlcNAcase protein expression was determined by western blot and analyzed by densitometry. O-GlcNAcase protein levels increased in pre-diabetes and diabetes by 1.25-, 1.5- fold, respectively (P <0.05) (Fig.1A, B). The difference in O-GlcNAcase levels between normal and diabetes were even more pronounced in JH samples, which were selected to have higher differences in blood glucose levels, and was found to be consistently 2.0 – 2.5 fold increased (Fig.1C, D). We attempted to examine the enzymatic activity of O-GlcNAcase from both sample sets, however, due to the liability of O-GlcNAcase to freezing (20), such assays were not reliable.

Measurement of O-GlcNAcase in Erythrocytes - To measure the amount of O-GlcNAcase protein in erythrocytes, we performed western Blot analyses, using recombinant O-GlcNAcase as a standard. Normal and diabetic samples had 6.1 ±1.93 ng and 18.7 ±5.91 ng of O-GlcNAcase per 100 g of hemoglobin-depleted erythrocyte proteins, respectively (p < 0.05)(Fig. 1E, F). Thus, diabetic erythrocyte samples had an average three-fold more O-GlcNAcase than did normal samples.
Protein level of OGT does not change in diabetic condition - Protein expression of OGT was also examined in erythrocytes by western blotting. OGT showed a slight but statistically insignificant increase in pre-diabetic and diabetic samples when compared to normal samples (Fig. 2A, B). Similar results were observed in JH samples (Fig.2C, D). Thus, while these data show that human erythrocytes do contain both enzymes, only O-GlcNAcase expression is upregulated in pre-diabetic and diabetic erythrocytes.

Comparison of O-GlcNAcase and HbA1c - Since the expression of O-GlcNAcase increases in pre-diabetes prior to elevation in HbA1c, we directly compared O-GlcNAcase expression and HbA1c levels in the samples. HbA1c value was, as expected, increased in samples from people with overt diabetes, but unlike O-GlcNAcase, HbA1c was not significantly different between normal and pre-diabetic samples (Fig.3A, B). The level of O-GlcNAcase expression varied more widely in pre-diabetes than normal (possibly reflecting more variable glycemia), and the mean was significantly higher in pre-diabetes. In the NIDDK samples, the range of O-GlcNAcase expression in the diabetic samples largely overlapped with that of samples from individuals with pre-diabetes. Similar patterns were found in JH samples (Fig.3C, D), which were selected to compare individuals with more severely hyperglycemic diabetes to normal controls. In these comparisons, the range of O-GlcNAcase protein expression was comparable to the range seen for HbA1c. This suggests that the severity of hyperglycemia and the resulting increased global O-GlcNAcylation are related to the increased amount of O-GlcNAcase expression in the erythrocytes, perhaps as an adaptive response to maintain erythrocyte functionality.

DISCUSSION

Currently there are several criteria to diagnose diabetes, with the oral glucose tolerance test (OGTT) often considered the gold standard. However, OGTT is a challenge in clinical practice, because it requires overnight fasting and a 2-hour test. Therefore, another diagnostic test for diabetes, simpler and equally sensitive, would be an important advance.

Pre-diabetes is defined on the basis of the OGTT, as a condition with Impaired Fasting Glucose (IFG) and/or Impaired Glucose Tolerance (IGT). HbA1c is routinely used to assess glycemic control (21), and has recently been recommended as a relatively sensitive and specific method and a criterion for diagnosis of diabetes by a group of experts (22) and an international expert committee (23). However, HbA1c is likely not as sensitive in detecting pre-diabetes. Furthermore, Derr et al. showed that HbA1c does not significantly reflect the variance of glycemia, but only the mean (24). This suggests the limitations of the HbA1c assay, since liability of glycemia is a crucial factor in diabetic management and possibly in avoiding diabetic complications (25). This supports the interest in developing a new screening method, which is less subject to current limitations.

Unlike non-enzymatic chemical glycation of proteins, O-GlcNAcylation is a specific and regulated enzymatic process. Thus, it could reflect fluctuations in glycemia in broader dynamic ranges. We hypothesized that hyperglycemia may cause elevated O-GlcNAcylation and/or alterations of the protein levels of OGT and O-GlcNAcase in erythrocytes of pre-diabetes, as well as in fully developed diabetes.

We recently demonstrated striking site specific increases in O-GlcNAcylation on several erythrocyte proteins in response to the diabetic state (22). Interestingly, O-GlcNAcase expression levels increased significantly in subjects with pre-diabetes and
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diabetes (Fig.1). More pronounced increases in O-GlcNAcase expression were observed in JH samples. Subjects of JH samples were deliberately selected for a pilot study from individuals with less well-controlled diabetes and severe hyperglycemia.

O-GlcNAc has been recognized as a stress responsive modification in many studies and O-GlcNAcylation has a biphasic modal effect on cellular survival mechanisms (for a review, (26)). O-GlcNAcylation modulates the function of proteins upon stress such as increased stability, differential targeting and activity changes by increasing global O-GlcNAcylation. Once cells are stabilized, O-GlcNAcylation levels recover to normal range. This phenomenon may explain our observation in pre-diabetic and diabetic erythrocytes. We speculate that increased expression of O-GlcNAcase is an adaptative response to hyperglycemia-induced hyper-O-GlcNAcylation. Increased O-GlcNAcase may remove excess O-GlcNAcylation on proteins to stabilize and maintain the function of erythrocytes. Additionally, the observation that OGT expression levels did not change suggests that the cells are trying not to over-O-GlcNAcylate proteins under chronic hyperglycemia (Fig.2). However, it remains unclear whether the OGT enzymatic activity or substrate targeting in erythrocytes could be disturbed due to hyperglycemia. The concentration of UDP-GlcNAc, the donor of O-GlcNAc, was not determined in this study because the long-term storage of samples would result in the degradation of UDP-GlcNAc at unpredictable rates. In search of a new diagnostic method to screen for diabetes early, these data suggest that analysis of O-GlcNAcase protein levels could be a potential candidate. Protein expression levels of O-GlcNAcase showed significant increases in pre-diabetes and diabetes (Fig.3). It is important to note that changes in HbA1c levels were not sensitive enough to distinguish normal and pre-diabetic samples, however, O-GlcNAcase showed more significant distinction between normal and pre-diabetic samples. This suggests that O-GlcNAcase adapts to cellular stress at an earlier stage of disease progression. Erythrocytes do not synthesize new proteins once they are mature. Thus, we presume that this adaptation occurs during hematopoiesis in bone marrow and continues until the reticulocytes complete their differentiation. This pilot study suggests avenues for further research. The correlation between hyperglycemia and the O-GlcNAc parameters measured needs further definition. The time kinetics over which changes in these parameters occur, its relationship to HbA1c and metabolic side needs elucidation. Only a much larger clinical trial will determine if increased O-GlcNAcylation has enough sensitivity and specificity to have value as a diagnostic for pre-diabetes. Nonetheless, these pilot studies encourage further investigation.

ACKNOWLEDGEMENT

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FIGURE LEGENDS

Figure 1. Elevation of protein levels of O-GlcNAcase in pre-diabetic and diabetic erythrocytes

Protein levels of O-GlcNAcase were determined by Western blotting in NIDDK samples (A, B) and JH samples (C, D). A: Representative Western blot analysis showing that protein levels of O-GlcNAcase are elevated 2-folds in the pre-diabetic (n=13, p<0.01) and diabetic samples (n= 9, p<0.01) compared to normal samples (n=13). B: Quantitation of Western blot analysis (A). C: Representative Western blot analysis showing that protein levels of O-GlcNAcase are elevated 2.5-folds in the diabetic (n=14, p<0.01) compared to normal samples (n=8). D: Quantitation of Western blot analysis (C). Actin was used as a loading control. E: Protein levels of O-GlcNAcase were estimated in JHSamples by Western blotting with recombinant O-GlcNAcase as standard. F: Quantitation based on Western blot analysis. Diabetic samples (18.70 ±5.91 ng/ 100 g of proteins, n=10, p<0.05) have 3-fold more O-GlcNAcase than normal samples (6.10 ± 1.93 ng/100 g of proteins, n=10). Data are mean ± SEM.

Figure 2. Protein levels of OGT in pre-diabetic and diabetic samples

Protein levels of OGT were determined by Western blotting in NIDDK samples (A, B) and Hopkins samples (C, D). A: Representative Western blot analysis showing that protein levels of OGT did not significantly change in the pre-diabetic (n=13) and diabetic samples (n=9) compared to normal samples (n=13). B: Quantitation of Western blot analysis (A). C: Representative Western blot analysis. Changes of protein levels of OGT were not significantly different between the normal (n=14) and diabetic (n=8) samples. D: Quantitation of Western blot analysis (C). Actin was used as a loading control. Data are mean ± SEM.

Figure 3. Comparison of HbA1c and O-GlcNAcase

The ranges of O-GlcNAcase and HbA1c were visualized and compared in NIDDK samples (normal= 13, pre-diabetes= 13, diabetes= 9) (A, B) and JH samples (normal= 10, diabetes = 10) (C, D). A, C: The range of O-GlcNAcase. B, D: The range of HbA1c values. *p<0.05, ** p<0.01

REFERENCES


Table 1. Baseline characteristics of NIDDK and JH subjects. More detailed clinical information of NIDDK samples is provided in the supplemental data 2. Data are mean ± SEM.

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<td><strong>Type of DM (type1/type2)</strong></td>
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Figure 1. O-GlcNAcase increases in pre-diabetic and diabetic erythrocytes.

A. | Normal | Pre-diabetes | Diabetes |
---|---|---|---|
OGA |  |  | |
actin |  |  |

B. 

![Graph showing OGA/Actin (fold)]

C. | Normal | Diabetes |
---|---|---|
OGA |  |  |
actin |  |

D. 

![Graph showing OGA/Actin (fold)]

E. | Recombinant OGA (ng) | 300 µg | 100 µg |
---|---|---|---|
50 |  |  |
25 |  |  |
125 |  |  |
62.5 |  |  |
31.25 |  |  |
15.625 | N | N |
7.8125 | D | N |
3.90625 | D | D |

F. 

![Bar graph showing Amount of OGA (ng) per 100 ng)]

![Graph showing OGA/Actin (fold)]
Figure 2. OGT does not change in protein level.

A.

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B.

![Graph showing OGT/Actin ratio changes](image)

C.

<table>
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D.

![Graph showing OGT/Actin ratio changes](image)
Figure 3. Correlation of HbA1c and O-GlcNAcase