Increased Expression of β-*N*-Acetylglucosaminidase in Erythrocytes From Individuals With Pre-diabetes and Diabetes

Kyoungsook Park,[1] Christopher D. Saudek,[2] and Gerald W. Hart[1]

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 a National Institutes of Health (National Institute of Diabetes and Digestive and Kidney Diseases) study and at the Johns Hopkins Comprehensive 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 with the level of A1C.

RESULTS—Erythrocyte proteins are highly O-GlcNAcylated. O-GlcNAcase expression is significantly increased in erythrocytes from both individuals with pre-diabetes and diabetes compared with normal control subjects. 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 individuals with less well-controlled diabetes. These findings, together with the previous study that demonstrated the increased site-specific O-GlcNAcylation of certain erythrocyte proteins, 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. ***Diabetes*** **59:1845–1850, 2010**

O-GlcNAcylation is a posttranslational modification in which single O-linked *N*-acetylglucosamine (O-GlcNAc) residues 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*-acetylglucosaminyltransferase (O-GlcNAc transferase [OGT]) (5,6), which transfers O-GlcNAc from UDP-GlcNAc (uridine diphospho-*N*-acethylglucosamine) to proteins, and O-linked β-*N*-ace tylglucosaminidase (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*-acetylglucosaminidase, is distinct from lysosomal hexosaminidases (7,10). The O-GlcNAcase gene was found to be identical to 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 = 54 nmol/l) 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.

RESEARCH DESIGN AND METHODS

Human blood samples were collected from two sources of volunteers. One set was obtained through the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and clinical data are shown in the supplemental material (available in the online appendix at http://diabetes.diabetesjournals.

From the [1]Department of Biological Chemistry, School of Medicine, Johns Hopkins University, Baltimore, Maryland; the [2]Department of Medicine, Division of Endocrinology and Metabolism, School of Medicine, Johns Hopkins University, Baltimore, Maryland.

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

Received 24 July 2009 and accepted 11 April 2010. Published ahead of print at http://diabetes.diabetesjournals.org on 22 April 2010. DOI: 10.2337/db09-1086.

org/cgi/content/full/db09-1086/DC1). Another set was obtained from the Johns Hopkins Comprehensive Diabetes Center (JH Center). Clinical data collected in this pilot phase was restricted to age, body weight, a causal plasma glucose, and A1C. In JH Center subjects, the diagnosis of diabetes had been established by accepted clinical criteria for >1 year with documented hyperglycemia and elevated A1C. Subjects designated as normal had no personal history suggesting diabetes. Blood samples were drawn and separated. Erythrocytes were washed with cold PBS three times and stored at −80°C. JH Center samples were obtained by the same procedure except using Histopaque-1077 (Sigma-Aldrich) reagent according to the manufacturer's protocol. Subjects gave written consent, which was 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. The depletion process was repeated twice to remove up to ~90% of hemoglobin.

Western blot analysis. Hemoglobin-depleted lysate was subjected to SDS-PAGE gels and blotted to polyvinylidene fluoride membranes. Membranes were blocked in Tris-buffered saline with Tween (0.1% [v/v] Tween-20) with either 3% (weight/volume) BSA or 5% (weight/volume) nonfat milk and incubated overnight at 4°C with the appropriate primary antibodies O-GlcNAc (CTD110.6) (Covance), OGT (AL28), O-GlcNAcase (18), actin (Sigma), and glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology). Detection was performed by enhanced chemiluminescence.

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

Statistical analysis. Densitometry data were obtained by the ImageJ program (National Institutes of Health), and analysis was performed using Student *t* test. *P* value <0.05 (two-tailed) was considered significant. Data are presented as means ± SEM.

RESULTS

The characteristics of the subjects are summarized in Table 1 and the supplemental table. The JH Center 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 individuals with pre-diabetes and diabetes by 1.25- and 1.5-fold, respectively (**$P < 0.01$) (Fig. 1*A* and *B*). The difference in O-GlcNAcase levels between normal and diabetes was even more pronounced in JH Center samples, which were selected to have higher differences in blood glucose levels and were found to be consistently 2.0–2.5-fold increased (Fig. 1*C* and *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 recom-

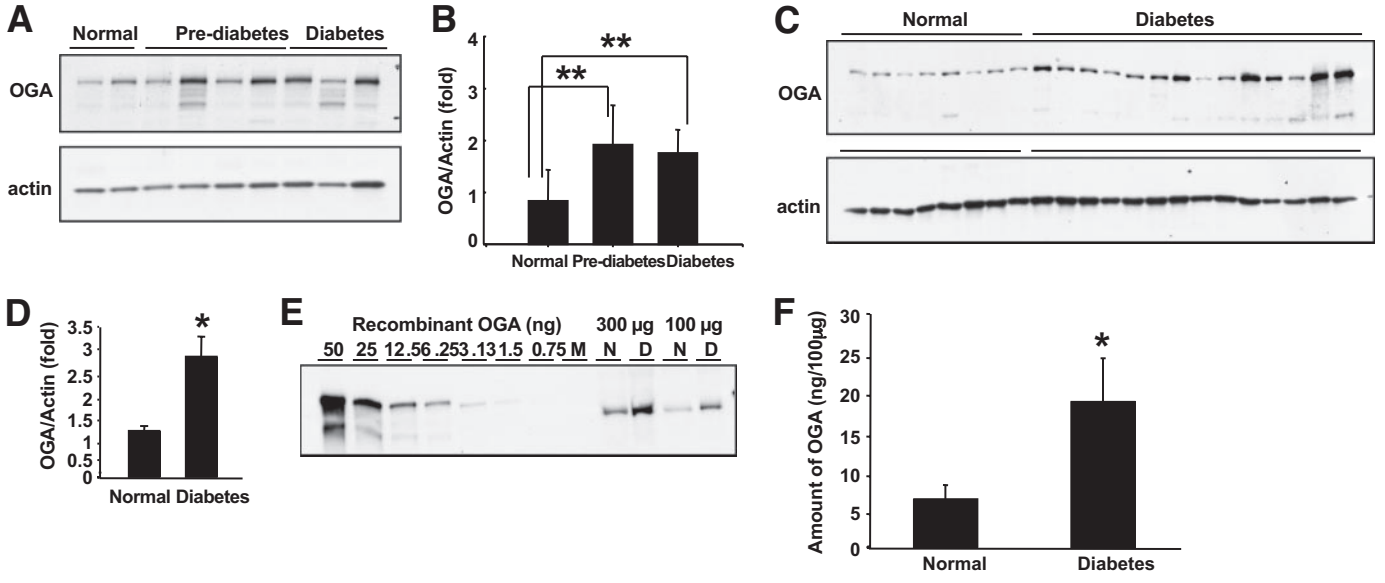

FIG. 1. Elevation of protein levels of O-GlcNAcase in pre-diabetic and diabetic erythrocytes. Protein levels of O-GlcNAcase were determined by Western blotting in NIDDK samples (*A* and *B*) and JH Center samples (*C* and *D*). *A*: Representative Western blot analysis showing that protein levels of O-GlcNAcase are elevated twofold in the pre-diabetic ($n = 13$, **$P < 0.01$) and diabetic samples ($n = 9$, $P < 0.01$) compared with 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-fold in the diabetic ($n = 14$, $P < 0.01$) compared with 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 JH Center samples by Western blotting with recombinant O-GlcNAcase as standard. *F*: Quantitation based on Western blot analysis. Diabetic samples (18.70 ± 5.91 ng/100 µg of proteins, $n = 10$, *$P < 0.05$) have threefold more O-GlcNAcase than normal samples (6.10 ± 1.93 ng/100 µg of proteins, $n = 10$). Data are means ± SEM.

TABLE 1
Baseline characteristics of NIDDK and JH Center subjects. More detailed clinical information of NIDDK samples is provided in the supplemental table in the online appendix.

	NIDDK			JH Center	
	Normal	Pre-diabetes	Type 2 diabetes	Normal	Diabetes
n	13	13	9	23	44
Age (years)	51.2 ± 6.6	54.5 ± 9.6	53.8 ± 9.2	31 ± 8.2	52.9 ± 12.4
Sex (male/female)	2/11	3/10	4/5	11/12	21/23
Fasting glucose (mg/dl)	87.8 ± 4.2	98.2 ± 9.3	128.9 ± 18.6	NA	NA
2-h glucose (mg/dl)	81.0 ± 11.4	166.0 ± 11.7	228.6 ± 28.8	NA	NA
Causal glucose	NA	NA	NA	83.1 ± 17.9	154.2 ± 74.1
A1C (%)	5.0 ± 0.3	5.3 ± 0.3	6.1 ± 0.7	5.5 ± 0.4	8.3 ± 1.7
Duration of diabetes (years)	—	—	NA	—	15.4 ± 13.9
Type of diabetes (type 1/type 2)	—	—	—	—	20/24

Data are means ± SE. NA, not applicable.

binant 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 μg of hemoglobin-depleted erythrocyte proteins, respectively (*$P < 0.05$) (Fig. 1*E* and *F*). Thus diabetic erythrocyte samples had on 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 with normal samples (Fig. 2*A* and *B*). Similar results were observed in JH Center samples (Fig. 2*C* and *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 A1C. Since the expression of O-GlcNAcase increases in pre-diabetes prior to elevation in A1C, we directly compared O-GlcNAcase expression and A1C levels in the samples. A1C value was,

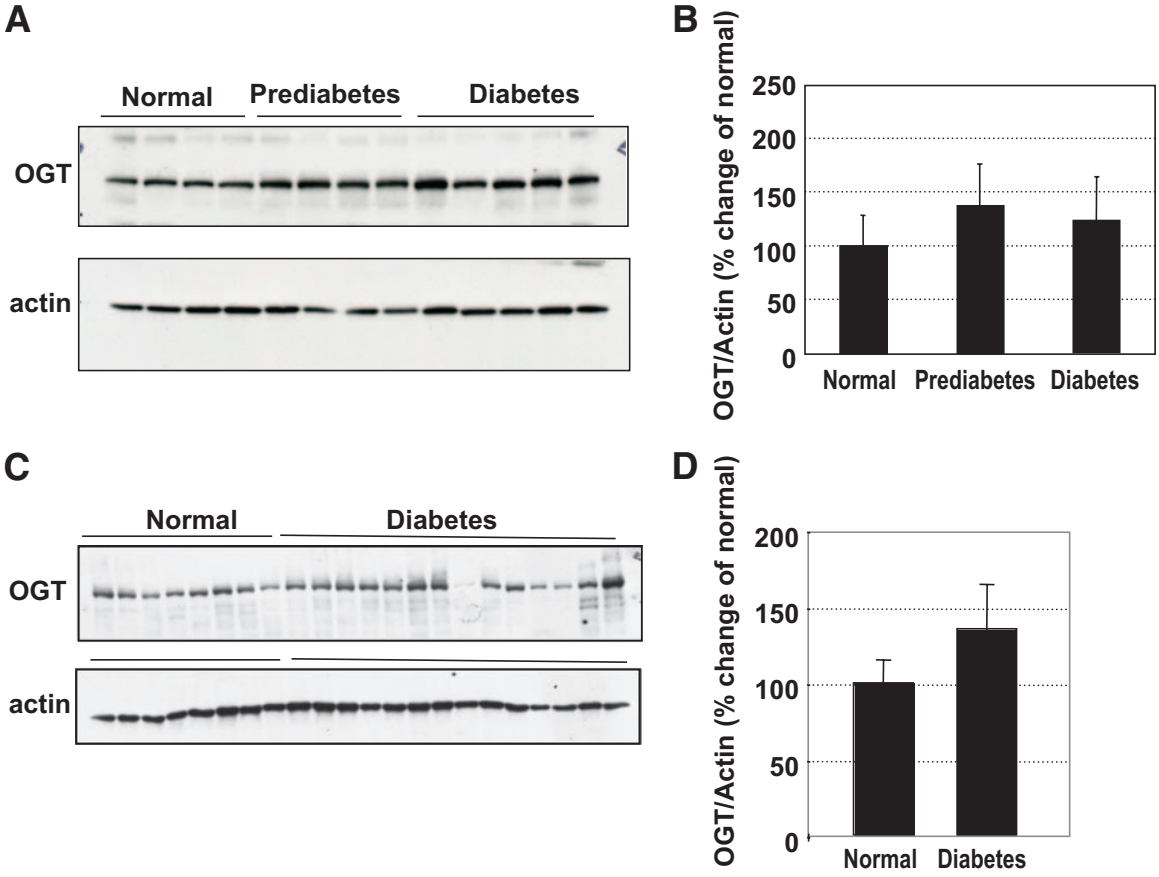

FIG. 2. Protein levels of OGT in pre-diabetic and diabetic samples. Protein levels of OGT were determined by Western blotting in NIDDK samples (*A* and *B*) and JH Center samples (*C* and *D*). *A*: Representative Western blot analysis showing that protein levels of OGT did not significantly change in the pre-diabetic ($n = 13$) and diabetic samples ($n = 9$) compared with 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 means ± SEM.

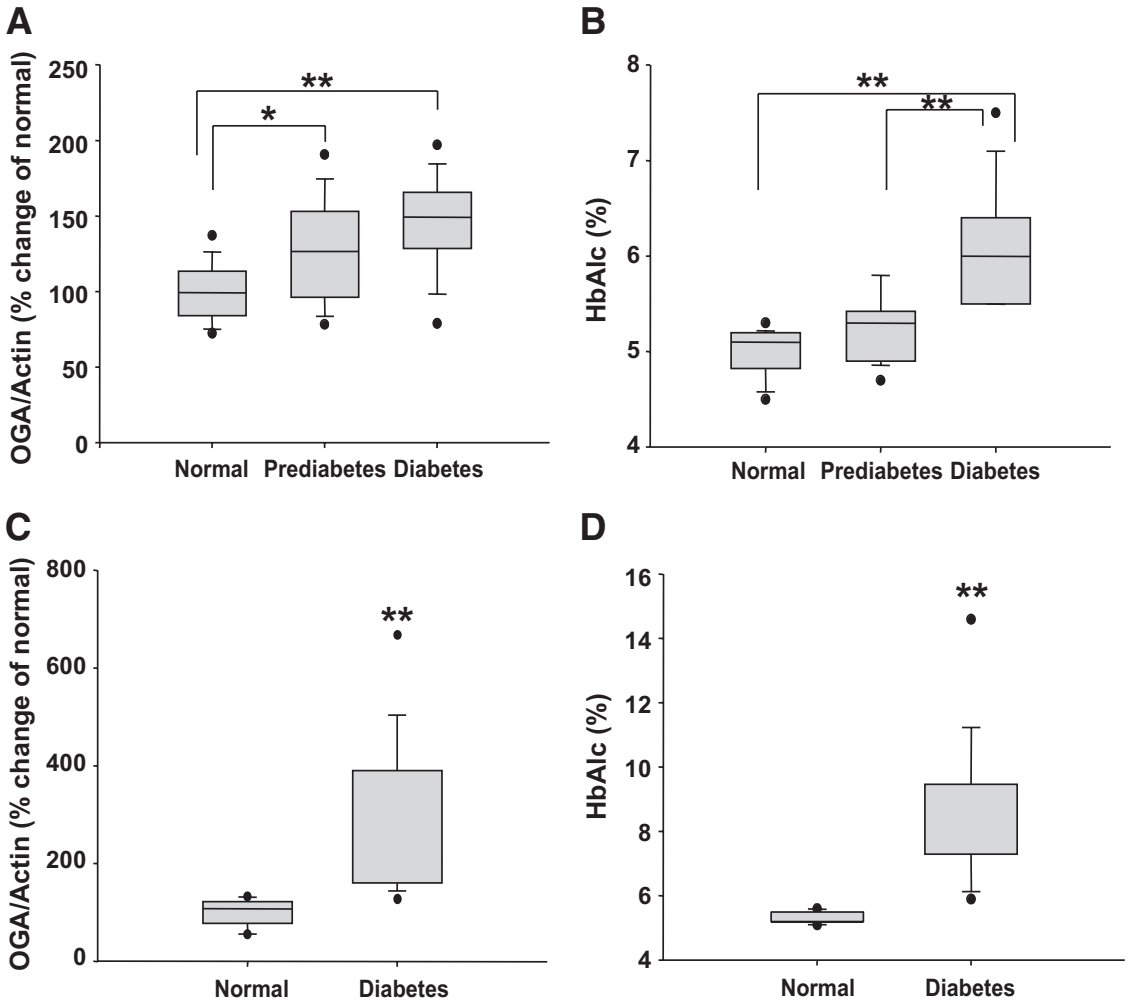

FIG. 3. Comparison of A1C and O-GlcNAcase. The ranges of O-GlcNAcase and A1C were visualized and compared in NIDDK samples (normal = 13, pre-diabetes = 13, diabetes = 9) (*A* and *B*) and JH Center samples (normal = 10, diabetes = 10) (*C* and *D*). *A* and *C*: The range of O-GlcNAcase. *B* and *D*: The range of A1C values. *$P < 0.05$, **$P < 0.01$. Filled circle indicates the highest and the lowest value of each sample.

as expected, increased in the samples from people with overt diabetes but unlike O-GlcNAcase, A1C was not significantly different between normal and pre-diabetic samples (Fig. 3*A* and *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 the samples from individuals with pre-diabetes. Similar patterns were found in the JH Center samples (Fig. 3*C* and *D*), which were selected to compare individuals with more severely hyperglycemic diabetes with normal control subjects. In these comparisons, the range of O-GlcNAcase protein expression was comparable with the range seen for A1C. 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, the OGTT is a challenge in clinical practice because it requires overnight fasting and a 2-h test. Another diagnostic test for diabetes that is 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 and/or impaired glucose tolerance. A1C is routinely used to assess glycemic control (21) and has recently been recommended by a group of experts (22) and an international expert committee (23) as a relatively sensitive and specific method and a criterion for the diagnosis of diabetes. However, A1C is probably not as sensitive in detecting pre-diabetes. Furthermore, Derr et al. (24) showed that A1C does not significantly reflect the variance of glycemia, only the mean. This suggests the limitations of the A1C 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 that is less subject to current limitations.

Unlike nonenzymatic 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 diabetes (Fig. 1). More pronounced increases in O-GlcNAcase expression were observed in the JH Center samples. Subjects of the JH Center 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, ref. 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 the 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 the search for a new early diagnostic method to screen for diabetes, 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 A1C 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 measured O-GlcNAc parameters needs further definition. The time kinetics over which changes in these parameters occur—its relationship to A1C 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.

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No potential conflicts of interest relevant to this article were reported.

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