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Determination of Membrane Protein Glycation in Diabetic Tissue

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ABSTRACT Diabetes-associated hyperglycemia causes glycation of proteins at reactive amino groups, which can adversely affect protein function. Although the effects of glycation on soluble proteins are well characterized, there is no information regarding membrane-associated proteins, mainly because of the lack of reproducible methods to determine protein glycation in vivo. The current study was conducted to establish such a method and to compare the glycation levels of membrane-associated proteins derived from normal and diabetic tissue. We present a detailed sample preparation protocol based on the borohydride-periodate assay, modified to allow manipulation of animal tissue. Assay noise associated with extraction protocols and nonproteinaceous buffer components was eliminated by the using 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) as a membrane detergent, applying desalting columns, and including a protein precipitation step. The glycation level of membrane proteins from diabetic rats is elevated to 4.89 nmol/mg protein (standard deviation [SD] 0.48) compared with normoglycemic control tissue (2.23 nmol/mg protein, SD 0.64). This result is consistent with and correlated to the total glycated hemoglobin levels in diabetic and normoglycemic rats. Using <100 μg protein, the described methods allow further study of protein glycation effects on the function of individual transporter proteins and the role of these modifications in diabetes.

KEY WORDS: Glycation, Membrane Protein, Diabetes.

ABBREVIATIONS: CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; GHb, glycated hemoglobin; HEPES, 4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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INTRODUCTION

Hyperglycemia, the clinical hallmark of poorly controlled diabetes, is known to cause protein glycation, also known as nonenzymic glycosylation (1). Protein glycation takes place when elevated levels of circulating reduced sugars react with amino groups in proteins, forming a labile aldimine Schiff base, which rearranges to form a stable oxoamine sugar adduct known as an Amadori product. The reactive amino group can be either an N-terminal α-NH_2 or a lysyl ε-NH_2 group, depending on its accessibility and environment. Glycation has been found to occur both in vitro and in vivo. In fact, this reaction is responsible for the formation of glycated hemoglobin (GHb) during diabetic hyperglycemia, and the quantification of this Amadori product is used clinically as an index of diabetic control. Other long-lived proteins have been found to undergo glycation in vivo, such as lens crystallins, collagen, low-density lipoprotein, albumin, and fibronectin (2).

Glycation can drastically affect protein function, exemplified by the increased activity of the enzyme ribonuclease A upon glycation of its functional lysine residues (3). Furthermore, glycation of lens crystallin induces a conformational change in the protein that increases SH-group susceptibility to oxidation (4).

Nutrient transporters are proteins with 7-14 membrane-spanning domains and are, therefore, intrinsically bound to the cell membrane. They perform an indispensable function in the uptake and excretion of nutrients and cellular metabolites in virtually all cells throughout the human body. Furthermore, it has been recognized that nutrient transporters play an invaluable role in overall drug absorption, distribution, metabolism, and excretion. Since nutrient transporters are intrinsic membrane proteins, their transmembrane protein regions will likely have a limited exposure to reducing sugars, but their extracellular domains will not be shielded. Bilan and Klip (2) have previously shown glycation

and altered function of the glucose transporter on isolated erythrocytes in vitro, but data on transporter glycation in tissues in vivo are not available, most likely because of the lack of reproducible methods for measuring membrane protein glycation in vivo. The need for a protocol to assess hyperglycemia-associated modifications of membrane transporters during the course of diabetes prompted the work described in this article.

Among the existing protein glycation assays, quantitation of the Amadori product via periodate oxidation remains the most convenient. This method was first proposed by Gallop et al. (5) to quantify the glycation level of hemoglobin and was further adapted by using a microplate reader(6) with sensitivity enhancement (0.1 mg protein). Another important improvement was achieved by reducing proteins with sodium borohydride before periodate oxidation, allowing the successful measurement of the glycation level of serum glycoproteins, which are heavily glycosylated and thus contain terminal reducing sugars (7) that would normally cause excessive background noise in the typical periodate assay. However, when we applied this method to membrane proteins extracted from the intestine of diabetic and normoglycemic rats, we found the assay to be highly sensitive to nonproteinaceous endogenous substances. Furthermore, the method appeared to be sensitive to buffer components used in common membrane protein extraction and isolation protocols. Here, we describe a strategy to overcome technical difficulties that may serve as a general way to determine the glycation level of membrane proteins extracted from tissues by using the borohydride-periodate assay. This technique should be of particular importance for investigating the role of glycation of membrane proteins in the pathogenesis of diabetes.

MATERIALS AND METHODS

Chemicals

A GHb assay kit was purchased from Sigma (St. Louis, MO). Chemicals were obtained from Acros/Fisher Scientific and Sigma and were used without further purification.

Animal Treatment

Diabetes was induced in Sprague-Dawley rats by intraperitoneal injection of 60 mg/kg streptozotocin as described previouslY (8). The rats were killed 6 weeks after injection when plasma glucose levels were >500 mg/dL. Total GHb in control and diabetic rats was measured using a GHb assay kit following the manufacturer's protocol. This assay uses an affinity chromatography procedure for specifically isolating glycated hemoglobins as described by Oremek et al. (9).

Extraction of Membrane Proteins From Isolated Crude Membrane Fragments

The following procedure was used to specifically isolate the membrane protein fraction from other lipid-containing cellular fractions, such as subcellular membranes (mitochondria, peroxisomes, lysosomes, or *trans*-Golgi network) or the cell nucleus. Whole intestinal mucosa was removed from diabetic and normoglycemic (control) rats. The crude membrane fractions were isolated using the method described by Marshak (10), with slight modifications. In brief, the mucosa was minced in ice-cold homogenization buffer (250 mM sucrose, 10 mM 4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 1 mM ethylenediaminetetraacetic acid [EDTA] and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and transferred to a Dounce homogenizer. After 15 strokes with pestle A, the homogenate was centrifuged at 8,000*g* (10 minutes, 4°C, Beckman model J2-21). The pellet was discarded, and the supernatant was centrifuged at 100,000*g* for 20 minutes at 4°C in a Beckman ultracentrifuge (model L5-75). Subsequently, the supernatant was discarded and the pellet was resuspended in homogenization buffer, homogenized using a Dounce homogenizer, and ultracentrifuged using the protocol described above. The supernatant was discarded, and the pellet was mixed thoroughly and incubated with 1.0 mL phosphate-buffered saline (PBS) containing 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 1 mM PMSF for 30 minutes at 4°C. After the next centrifugation step (10,000 rpm, 30 minutes, 4°C, using a micromax RF microcentrifuge [IEC], which was used for all subsequent centrifugation steps), the pellet was discarded, and the supernatant was collected. The

supernatant fraction contained total membrane protein extract and was used for the subsequent sample preparation steps.

Sample Preparation

The fraction containing total membrane fragments, collected via the procedure described above, was adjusted to 2.5 mL in volume with extraction buffer and loaded onto a PD-10 column (Pharmacia, which was pre-equilibrated with PBS containing 0.2% CHAPS. The first 3 mL of eluant was collected, and 500 µL of this fraction was transferred to a new 1.5 mL microcentrifuge tube. Subsequently, 400 µL distilled water and 100 µL of 72% trichloroacetic acid (TCA) solution were added. After mixing thoroughly, the protein precipitate was obtained by centrifugation at 10,000 rpm for 10 minutes at 4°C. The protein pellet was washed twice with 1 mL water and redissolved in 1 mL of 0.1 M sodium phosphate buffer containing 1% sodium dodecyl sulfate (SDS) (pH 8.0). The protein concentration in the redissolved sample was quantified using the bicinchoninic acid method (11), and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% acrylamide gels in a Gibco-BRL vertical gel apparatus under reducing conditions using the Laemmli (12) buffer system. Gels were stained with Coomassie brilliant blue R-250, and separate gels were run for subsequent immunoblotting procedures. Six 100-µL aliquots of this final sample with a protein concentration of 1-2 mg/mL were used for the borohydride-periodate assay.

Immunoblotting

SDS-PAGE gels were subjected to Western blotting according to the method of Towbin et al. (13), and the proteins on the gel were transferred to a polyvinylidine difluoride (PVDF) membrane. Immunoblotting was carried out using a rabbit polyclonal antibody directed against the carboxy-terminal 14 amino acids of the hamster ileal Na^{+}-bile acid cotransporter (kindly provided by Dr. Paul Dawson, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC). Shneider et al. (14) have previously shown that this polyclonal antibody exerts cross-reactivity to detect the rat transport protein. The rabbit antibody was visualized using a horseradish peroxidase-;conjugated goat anti-rabbit antibody and an enhanced chemiluminescence detection system.

Borohydride-Periodate Assay

A previously described borohydride-periodate assay (7) was used with several modifications. Triplicate aliquots of each sample (100 µL) were incubated for 1 hour at room temperature with 20 µL of 0.2 M sodium borohydride in ice-cold 0.01 mM NaOH, using 20 µL of 0.01 mM NaOH as a control (triplicates). This step ensures reduction of protein glycosylation residues before conversion of glycation residues by periodate oxidation. The reduction reaction was stopped by adding 20 µL of 0.2 M HCl. After this step, each sample was incubated with 20 µL of 0.1 M sodium periodate for 30 minutes at room temperature. The oxidation was terminated by cooling the samples on ice for 10 minutes, followed by adding 40 µL each of ice-cold 0.7 M NaOH and 15% zinc sulfate water solution. Precipitate was removed by two consecutive 10-minute centrifugations at 13,000 rpm. From each sample, 200 µL supernatant was transferred to a microcentrifuge tube, and 100 µL color reagent (92 µL acetylacetone in 10 mL of 6.6 M ammonium acetate) was added to each tube. The mixtures were incubated at 37°C for 1 hour. During the incubation, the presence of excess SDS induced flocculation, which can be overcome by a brief high-speed spin, e.g., 10 minutes at 15,000 rpm. Finally, 270 µL supernatant from each tube was transferred to a regular 96-well microplate, and the detection was accomplished at 405 nm in microplate reader (DYNEX). Fructose solution (0-;40 nmol) was used for calibrating the periodate assay as described previously (7). Redissolving buffer (100 µL) was incorporated in both reduction and oxidation reactions as blank solution.

Assay Validation

The current protocol was validated according to the methods of Kennedy et al. (7), using mixtures of native and in vitro glycated fetal calf fetuin as internal standard. Fetuin, a highly glycosylated 48-kd protein, is an appropriate standard because ~20% of its molecular weight comprises enzymatically linked sugars, with many terminal sialic acids. Briefly, fetal calf fetuin (5 g/L) was incubated with 0.5 M glucose in PBS (pH 7.4) for 10 days at 37°C. Before

incubation, buffer solutions were sterilized by filtration (0.22 μm filter) and contained 6 mM sodium azide to prevent microbial growth. Free sugar was removed (<10 μM) by extensive dialysis against distilled water containing 6 mM NaN_3. During dialysis, the labile Schiff base dissociates and is removed, leaving only Amadori products bound to the protein.

Since fetuin is a soluble protein, the protein was spiked into tissue samples after the membrane protein extraction step. The accuracy of the method was demonstrated by the ability of the assay to give consistent measurements on a series of protein samples containing glycated fetuin (0, 25, 50, 75, and 100%) mixed proportionally with unglycated fetuin. To further verify the accuracy of the method, we spiked an appropriate amount of glycated fetuin with known glycation level into the membrane protein extract and determined the glycation level of this protein mixture.

The precision of the assay was assessed by within-run and between-run validations in which we analyzed the same protein sample extracted from diabetic intestine within the same day or over 3 consecutive days, respectively.

RESULTS

Three diabetic rats with plasma glucose levels >500 mg/dL and three healthy (normoglycemic) age-matched control rats were killed, and their small intestine tissues were isolated. Enterocytes were collected, and the apical membrane fractions were isolated using an isolation and sample preparation protocol illustrated in Figure 1.

To ensure equal protein loading and composition, samples from both diabetic and control tissues were subjected to SDS-PAGE, followed by Coomassie blue staining. Representative data obtained from healthy and hyperglycemic samples are shown in Figure 2.

Gel electrophoresis results (Figure 2) indicate that redissolved protein samples from diabetic and normoglycemic rats exhibit similar molecular weight and protein distribution patterns. To ascertain that these isolated protein samples uniquely comprise membrane proteins, Western blotting was carried out using 0.2 μg/mL of a specific rabbit polyclonal

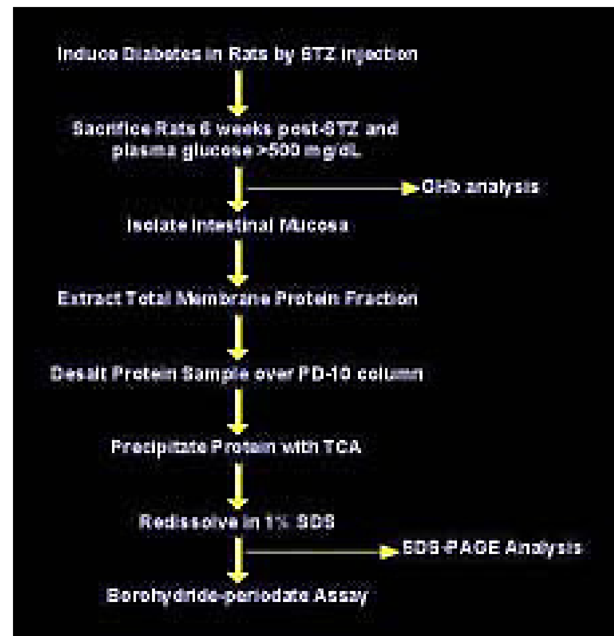

Figure 1. Procedures and techniques for determining membrane protein glycation levels in diabetic tissue.

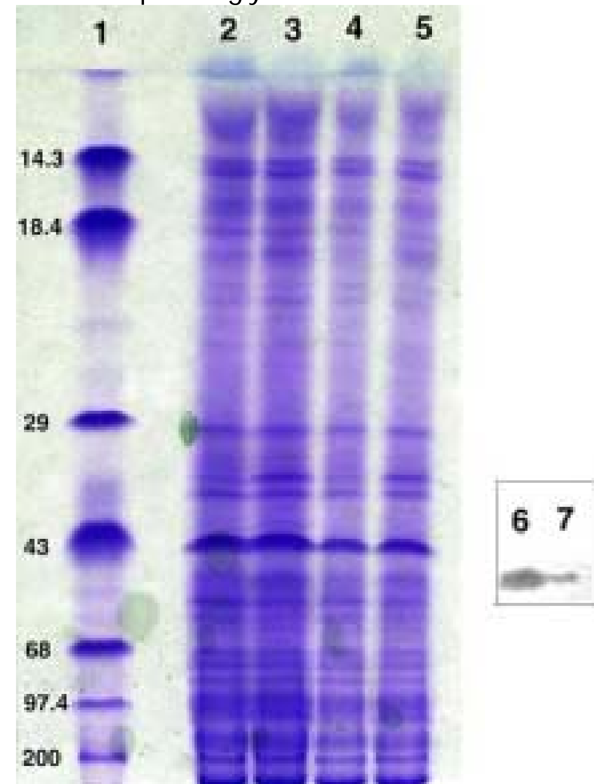

Figure 2. Coomassie blue-;stained 10% SDS-PAGE of redissolved protein samples derived from diabetic and normoglycemic rat intestine. Lane 1 shows the molecular weight standard (19.3-;200 kd). Lanes 2 and 3 show the samples from two normoglycemic rats. Lanes 4 and 5 show membrane protein samples extracted from hyperglycemic tissue. Lanes 6 and 7 (inset) show the results of immunostaining for the ileal bile acid transporter (48 kd) in control and diabetic tissue, respectively.

antibody raised against the carboxy-terminal 14 amino acids of the ileal Na^+-bile acid cotransporter (15). Bands of similar electrophoretic mobility (48 kd) were detected in both normoglycemic and diabetic samples using a chemiluminescence detection system. Previous studies have shown that the 48-kd band corresponds to the fully glycosylated interscapular brown adipose tissue (IBAT) protein (16). Diabetic tissues show a ~2.5-fold decrease in IBAT protein expression at equal protein loading densities (calculated by average pixel density measurements of protein bands using the program NIH Image, version 1.61). The possibility exists, however, that the affinity and specificity of our antibody are reduced because of glycation of the lysine residue in the 14-;amino acid carboxy terminus it was originally raised against. We are currently investigating the (potential) difference in binding affinity of our antibody between unglycated and synthetically glycated peptide. Regardless, these results show the presence of a representative nutrient transport protein in our isolated membrane protein fraction.

A calibration graph for the periodate method was prepared using a fructose standard (Figure 3). Fructose resembles the Amadori product form of a glycated protein in having a C-2 carbonyl group in the straight chain configuration but existing predominantly in the ring form. Like the Amadori product, it liberates 1 mole of HCHO per mole on periodate oxidation and, therefore, can be used as a standard for the Amadori product. Figure 3 shows that absorbance is linear in the range of 0-;50 nmol of fructose per well, and amounts of 2 nmol can be readily detected at absorbance values of 0.05. At lower concentrations, the detection limit is partly prescribed by the short light path and the yield of HCHO from fructose.

In vitro glycated fetuin yielded a glycation level of 2.04 ± 0.08 glycation sites per molecule. This number closely matches the values measured by Kennedy et al. (7) using the original borohydride-periodate assay (number of glycation sites: 1.98 ± 0.07/molecule). The current method also gives comparable and linear measurements on a series of protein mixtures containing both glycated and nonglycated fetuin in varying proportions (data not shown). Further confirmation of the accuracy of the method was achieved by determining the glycation level of

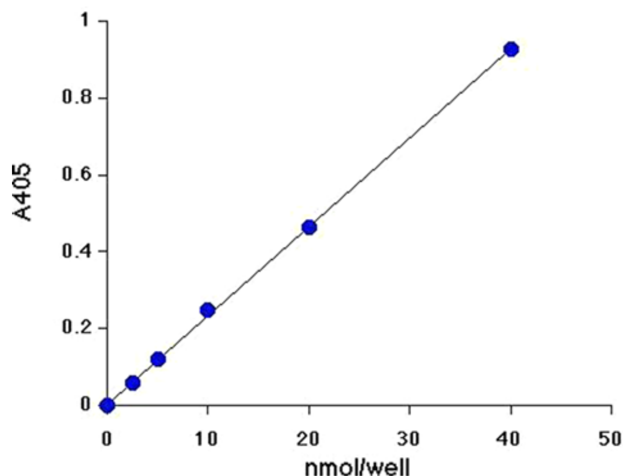

Figure 3. Calibration graph showing the spectrophotometric absorbance at 405 nm against the amount (nmol) of fructose per well. Linear regression of the standard curve yields a straight line with an intercept of 0.0052 nmol fructose/well and a slope of 0.0231 absorbance units per nmol fructose. The regression coefficient R is 0.9998.

membrane protein extract spiked with a known amount of glycated fetuin. The determined glycation level of the protein mixture is not significantly different from the theoretical value (data not shown; $P < 0.05$).

Increased glycation of several soluble proteins has been observed in diabetic individuals with attendant hyperglycemia. Normoglycemic individuals usually display 4%-;6% glycation of total hemoglobin, whereas diabetic patients with poor glycemic control have hemoglobin glycation values of 10% or more, a 2-fold increase over healthy individuals. In rats, we found that the level of total GHb in diabetic (n = 6) and normoglycemic (n = 6) subjects was 16.6% (SD 0.1) and 5.2% (SD 0.1), respectively, which is in good agreement with the data reported by Junod et al. (8). The glycation extent of total membrane protein from the intestine of diabetic rats was 4.89 nmol/mg protein (SD 0.48), whereas the corresponding value for normoglycemic rats was 2.23 nmol/mg protein (SD 0.64). The elevated glycation level of membrane proteins from diabetic rats was consistent with their plasma GHb levels (Figure 4). The percent variance of the assay between days and among different subjects was 4.5%. The within-run and between-run coefficients of variance were 3.9% and 4.5%, respectively.

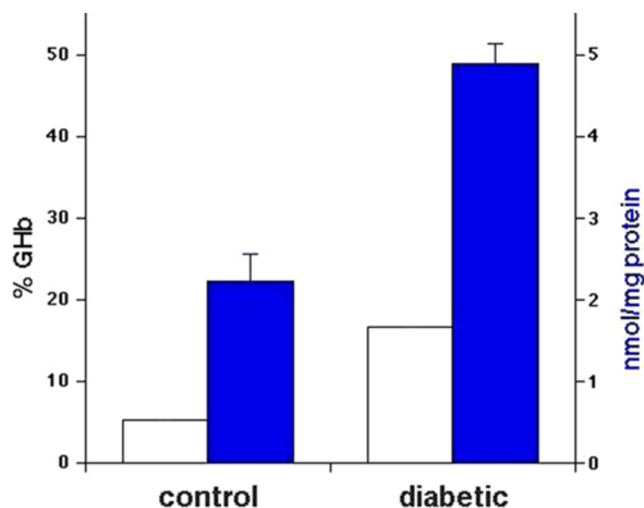

Figure 4. The glycation level (nmol/mg protein) of total membrane protein (blue bars) extracted from diabetic and normoglycemic rat intestine and the corresponding total GHb levels (% GHb, white bars) in their red blood cells. ($P < 0.01$, unpaired *t* test, $n = 6$, error bar = SD). Standard deviations on white bars may not be visible.

Discussion

The present study describes the development and application of a reproducible method for assessing the glycation level of membrane proteins in mammalian tissue. To date, studies describing the biochemical effects of nonenzymic glycation in the pathogenesis of diabetes have mainly focused on purified soluble proteins. Only a handful of studies focus on membrane-associated proteins, mainly by exposing isolated cell systems to high concentrations of glucose for prolonged periods of time in vitro. Using isolated erythrocytes, for example, Bilan and Klip (2) showed that glycation alters the maximal transport rate (J_{max}) of the glucose transporter for cytochalasin B, a well-characterized competitive inhibitor of this carrier protein.

The lack of data on membrane-bound proteins is likely due to the absence of a protein extraction protocol in combination with a sensitive assay. The established method for measuring protein glycation by Kennedy et al. (7) was originally based on several purified model proteins, i.e., bovine serum albumin, human immunoglobulin G and fetal calf fetuin. The initial protein isolation protocol contained one straightforward dialysis step that was sufficient for sample preparation. In contrast, assessing the glycation level of membrane proteins requires multiple sample preparation procedures: isolation of membrane fragments, extraction of membrane protein, and additional purification protocols. Any of these steps may introduce new substances or fail to extract endogenous substances that can either increase background levels or interfere with the detection method itself. Unique identification of all potentially interfering sources is virtually impossible given the complexity of biological systems, but three distinct groups can be recognized. First, many endogenous substances, such as cellular free glucose and other reducing sugars, can interfere with the borohydride-periodate detection method. Second, the hydrophobic nature of membrane proteins requires the presence of detergent throughout sample preparation. However, we found that most nonionic detergents for recovery of membrane components under nondenaturing conditions, such as the widely used Triton X 100, dramatically decrease assay sensitivity. Finally, some commonly used buffer components in membrane extraction and isolation protocols, Tris and HEPES, can cause excessive background levels that negatively affect the sensitivity of the assay.

Our strategies to overcome these problems include the following: *1*) The use of CHAPS for membrane protein extraction. Although Triton X 100 is often the preferred choice for extracting proteins from membranes, CHAPS has a solubilizing efficiency comparable to that of Triton X 100, according to our SDS-PAGE and protein quantification data (not shown). In our experience, multiple washing and redissolving steps are not sufficient to completely remove Triton X 100 from the protein pellet, most likely because of the tight intrinsic association of Triton X 100 with membrane protein fractions. *2*) The use of a desalting PD-10 column to eliminate ions and small molecules that we found caused high background readings in the subsequent assay. *3*) The introduction of a protein precipitation step using TCA and a consequent redissolving step to obtain reproducible results. These treatments remove nonproteinaceous macromolecules that we found interfered with the assay. *4*) The use of buffers containing 1% SDS in 0.1 M sodium phosphate to

redissolve the protein pellet. Conventional buffers contain Tris or HEPES that may interfere with the assay because of high absorbance of these compounds at the assay detection wavelength. The combination of these steps, as illustrated in Figure 1, allowed us to reproducibly determine the glycation level of total apical membrane protein.

Using the above method, we assessed the glycation level of total membrane protein from diabetic tissue to be 2.2-fold higher than that from control tissue. This result is considerably lower than the ratio of 3.2 we detected for GHb levels in diabetic and control animals, but further studies are necessary to fully explain this phenomenon. Interestingly, Bilan and Klip (2) found glycation ratios (glycated:control) in erythrocyte glucose transporters ranging from 1.8-;2.8 following exposure of isolated erythrocytes to 80-;200 mM glucose for 3-;6 days. The J_{max} value for this system was decreased to ~75% of its control value, which demonstrates the adverse effects of glycation on transport protein function. Future efforts are directed toward isolating single nutrient transporters from the pool of total membrane proteins to directly study the effects of glycation on transport function and expression. The currently described glycation assay will serve as a benchmark for assessing glycation levels in single proteins.

To our knowledge, the method described in this paper is the first to directly determine the degree of glycation of membrane proteins extracted from intestinal tissue using <100 μg protein. Because membrane proteins play an important role in transporting nutrients and drugs in the intestine and various other organs in the body, the current protocol will be particularly useful to researchers investigating the role of membrane protein glycation in the pathology of aging and metabolic diseases such as diabetes.

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