Actin and Annexins I and II Are Among the Main Endothelial Plasmalemma-Associated Proteins Forming Early Glucose Adducts in Experimental Diabetes

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An immunochemical and biochemical study was performed to reveal which of the endothelial plasma membrane proteins become glycated during the early phases of diabetes. The blood front of the lung microvascular endothelial plasmalemma was purified by the cationic colloidal silica method from normal and diabetic (streptozotocin-induced) rats and comparatively analyzed by two-dimensional electrophoresis. No major qualitative differences in the general spectrum of endothelial plasmalemmal proteins were recorded between normoglycemic and hyperglycemic animals. By probing with anti-glucitollysine antibodies, we found that at 1 month after the onset of diabetes, several endothelial membrane polypeptides contained glucose covalently linked to their lysyl residues. Ten days of insulin treatment restored euglycemia in the diabetic animals and completely abolished the membrane nonenzymatic glycosylation. All the glycated polypeptides of the endothelial plasma membrane belong to the peripheral type and are associated with its cytoplasmic face (cell cortex). They were solubilized by buffers of high pH and were not detected in the lung cytosolic fraction (100,000 g). By microsequencing, the major proteins labeled by the anti-glucitollysine have been identified as being actin, annexin I, annexin II, the p34 subunit of the Arp2/3 complex, and the Ras suppressor protein-1. Conversely, the intrinsic endothelial membrane proteins do not seem to be affected by hyperglycemia. This defines the internal face of the endothelial plasma membrane, particularly the cortical cytoskeleton, as a preferential target for nonenzymatic glycosylation in diabetes, with possible consequences on the fluidity of the endothelial plasmalemma and impairment of the endothelial mechanotransducing ability. *Diabetes* 50:1666–1674, 2001

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Received for publication 3 April 2000 and accepted in revised form 20 March 2001.

2D, two-dimensional; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; IPG, immobilized pH gradients; rsp-1, Ras suppressor protein-1; PVDF, polyvinylidine fluoride; TFA, trifluoroacetic acid.

The leading cause in the morbidity and mortality of diabetic patients is related to vascular complications such as atheroma, hypertension, and microangiopathy. Abnormalities induced by diabetes are found at the level of circulating plasma proteins and blood cells, but they equally affect the components of the vessel walls (1). The most obvious of these injuries are encountered in the microcirculation of the retina, skin, nerves, and kidney, although it is widely accepted that none of the segments of the vasculature are spared (2,3). It is therefore plausible to consider that on a general background of alterations affecting all blood vessels, local factors exacerbate the disease, conferring clinical and morphologic particularities to the vasculopathies encountered in certain tissues.

Most of the endothelial biological responses are triggered by events taking place at the interface between the blood and the vessel wall, namely at the luminal aspect of the endothelial plasma membrane. On the premise that pathological conditions induce modifications at this level, we have undertaken a study aiming to detect putative alterations in the endothelial plasmalemmal proteins during the early phase of diabetes.

The hallmark of diabetes is hyperglycemia. Depending on the type of glucose transporters in each tissue, this can be accompanied by elevated intracellular concentrations of glucose and glycolytic intermediates. The mechanisms by which hyperglycemia exerts its detrimental effects have not yet been elucidated, and several hypotheses are currently considered (4). Among these, a possible explanation of the injuries induced to cells and extracellular material by hyperglycemic conditions stems from the ability of glucose, as a reducing sugar, to bind to the amino groups in the proteins. These glucose adducts spontaneously evolve toward a heterogeneous population of molecules, generically termed "advanced glycation end products" (AGEs), mostly characterized by extensive intra- and intermolecular cross-linking, acquired fluorescence, and chromogenicity. It is clearly documented that both early glycated proteins, as well as AGEs, exert deleterious effects (5,6). A large number of studies have been dedicated to the consequences of the nonenzymatic glycosylation of circulating proteins such as albumin, immunoglobulins, and lipoproteins (7). Similarly, the structural and functional changes of the connective tissue and extracellular matrix have been extensively analyzed,

particularly in the renal glomerulus and lens crystallins (5,6).

Considerable effort is currently being made to define the alterations induced by diabetes at the level of cell proteins. A reduction in the efficacy but not in the number of the glucose transporters was reported to occur in diabetic rat brain microvessels (8); conversely, in skeletal muscle sarcolemma, it was the number of transporters that decreased by two-thirds (9). In the rat liver plasma membrane, a one-half reduction in serine proteinase activity and in epidermal growth factor receptor number was recorded in experimental diabetes (10,11). This reduction was reversed by insulin treatment. Intra-endothelial glycation of the basic fibroblast growth factor was accompanied by the loss of its mitogenic activity (12). All these results indicate that as a consequence of hyperglycemia and/or hypoinsulinemia, cell proteins (or at least some of them) do change.

Nonenzymatic glycosylation of erythrocyte and platelet membrane proteins in diabetes has been detected by chemical assays (13), but so far only a single attempt has been made to identify these proteins (14) using tritiated borohydride to reduce and label their early glycosylation adducts. The ability of the agarose-immobilized aminophenylboronic acid to bind carbohydrates containing a pair of adjacent *cis*-configurated hydroxyl groups has been tentatively used to assess the existence of glycated polypeptides in the liver and kidney membranes (15) and in the liver cytosol (16) of diabetic animals. However, this method is not free of interference from the naturally occurring glycoconjugates, which are particularly abundant in plasma membrane preparations.

Consequently, we have decided to reveal the endothelial membrane proteins modified by early glycation by an alternative method using a monoclonal antibody that specifically recognizes the glucitollysine residue, irrespective of the carrier protein (17).

RESEARCH DESIGN AND METHODS

Experimental animal model. Twenty-seven Sprague Dawley male rats, 125 g body weight (Charles River, St. Constant, QB, Canada), were used throughout the experiment. One-third was kept as a control, and the rest was rendered diabetic by a single intraperitoneally injected 9-mg dose of streptozotocin. All injected rats became diabetic, as revealed by their glycemia, glycosuria, and ketonuria (Miles, Rexdale, ON, Canada). The animals were fed ad libitum with standard laboratory diet. After 1 month, three rats from the diabetic lot were intraperitoneally injected once a day with Humulin U (ultraslow) insulin (Eli Lilly). The amount of injected insulin (between 5 and 15 U/dose) was adjusted for each animal so that its glycemia, taken 2 h after each injection, fell to between 2.8 and 5.2 mmol/l (close to the normal range). This treatment was continued for 10 days. At 40 days after the initial injection of streptozotocin, all animals were killed, and the lungs were harvested according to the procedure described below. Glycemic control and response to the treatment was further monitored by measuring the extent of plasma protein glycation in blood samples drawn just before death (using the GlycoTest II kit from Pierce Chemical). The experiments were approved by the Committee of Deontology for Experimentations on Animals, Université de Montréal.

Purification and electrophoretic analysis of the endothelial plasma membranes. The blood front (luminal domain) of the lung microvascular endothelial plasma membrane was isolated by the cationic colloidal silica method as described in detail previously (18). Basically, this method involves the perfusion of the lung blood vessels with positively charged 20- to 40-nm colloidal silica particles, adhering by electrostatic interactions to the anionic sites present in high density on the luminal front of the endothelial cells. The cationic colloidal silica was prepared according to the method of Channey and Jacobson (19). The continuous layer of silica attached to the endothelial surface was hindered from other interactions by a second coat, obtained by infusing anionic sodium polyacrylate through the vasculature. The endothelial membranes, rendered very heavy by the attached silica, were separated from any other cellular organelles by two centrifugation steps of the lung homogenate through dense layers of Nycodenz (Life Technologies). The purity of the isolated membrane fractions, labeled P_2, was assessed by electron microscopy. Throughout the purification protocol and subsequent electrophoretic procedures, a cocktail of protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 5 mmol/l benzamidine, and Complete [Boehringer]) was present.

The protein concentration was measured by the bicinchoninic method (Sigma Chemical). The membranes were solubilized in 1% SDS, sonicated, boiled, and microfuged, and the supernatants, free of interfering colloidal silica, were used for the protein assay. As a control for the reproducibility of the results, endothelial plasma membranes, as well as the cytosol, were separately purified for each animal and run as individual distinct samples throughout the subsequent analysis.

The global polypeptide composition of the membranes purified from normal diabetic and insulin-treated diabetic animals was compared by two-dimensional (2D) electrophoresis. Immobilized linear pH gradient (IPG) 18-cm pH 3-10L strips (Amersham Pharmacia Biotechnology) were used for the first dimension, followed by SDS-PAGE in 10% acrylamide (20-cm gel) reducing conditions (20). For the isoelectric focusing step, the samples were solubilized in IPG buffers containing 4% CHAPS, 7 mol/l urea, and 2 mol/l thiourea (21) and loaded concomitantly to the rehydration of the IPG strips (22). The proteins were revealed by silver staining (23).

For further fractionation, 100 μg of each P_2 membrane preparation was sequentially incubated for 1 h at 4°C with 500 μl of 50 mmol/l Na carbonate, pH 11.0, and 500 μl of 1.0 mol/l NaCl in 25 mmol/l HEPES, pH 7.2, and microfuged to recover the supernatants. Each of these steps solubilized a distinct set of cytoplasmically attached peripheral membrane proteins (labeled S_{carb} and S_{NaCl}, respectively), leaving as a pellet a membrane fraction still attached to the silica, named P_3 and highly enriched in integral plasmalemmal proteins (24). In preparation for electrophoresis, the soluble fractions were precipitated with TCA and washed with ethanol/ether; the silica-attached membranes were concentrated by microcentrifugation. The cytosol was obtained by spinning 1.5 ml of each lung homogenate at 100,000*g* for 1 h in a Beckman SW60 rotor (Beckman).

Immunochemical detection of nonenzymatically glycated proteins. First, a comprehensive immunochemical screening of all the animals used throughout the experiment was performed to detect whether a consistent pattern of nonenzymatic glycosylation of endothelial membrane proteins could be defined for each experimental condition. For each animal, 20 μg lung homogenate, cytosol, and endothelial plasma membrane fractions was resolved by SDS-PAGE in reducing conditions (20) using a Minigel system (Bio-Rad) and electrotransferred to nitrocellulose membranes. The membranes were incubated in 100 mmol/l Na borohydride for 1 h to irreversibly transform the glucose-derived early glycation products (ketoamines and aldimines) into a stable glucitollysine. Alternatively, the borohydride treatment was performed before preparing the samples for electrophoresis to avoid a possible breakdown of the labile lysyl-glucose adducts during the electrophoretic run. No differences were recorded between the results obtained with these two variations of the protocol. After quenching with 1% nonfat dry milk in Tris-buffered saline, the nitrocellulose membranes were probed with a mouse monoclonal anti-glucitollysine antibody (clone G8C11; supplied by Drs. L. Curtis and J. Witztum, Scripps Research Institute and University of California at San Diego, respectively), followed by a horseradish peroxidase–conjugated anti-mouse immunoglobulin (Ig) (Amersham, Ontario, Canada), and developed by enhanced chemiluminescence (ECL).

The specificity of the antibody, although previously demonstrated (25), was further tested by performing immunoblotting on samples of native serum albumin and glycated bovine serum albumin (BSA)—the latter prepared according to a published protocol (26). The glycated BSA contained an average of 2.5 mol glucose residues per 1 mol BSA, as measured by a thiobarbituric assay.

Once the consistency of the endothelial plasma membrane glycation pattern was demonstrated in one dimension, the affected polypeptides were further pinpointed in the 2D electrophoretic spectrum. For this, 125 μg of the Scarb fraction of endothelial membranes from diabetic rats was precipitated with methanol/chloroform (27), solubilized in the IPG buffer, and loaded on 13-cm IPG 3-10L strips. After transfer on nitrocellulose, the proteins were stained with a 15-nm colloidal gold suspension containing 0.1% Tween-20 (28) and were submitted to the immunoblotting protocol as previously described. At the end of the immunoblotting sequence, the pattern of proteins on nitrocellulose was intensified by a silver amplification step (29) and aligned with the image generated by immunoblotting and ECL.

Identification of the glycated proteins by microsequencing. The major glycated proteins contained in the Western blotting image were unambiguously identified in the 2D pattern of the Scarb proteins from normal animals.

This was possible because of the perfect reproducibility of the 2D electrophoretic spectra generated by the IPG technique and because no major qualitative differences were found between the 2D patterns of endothelial membranes from diabetic and normal rats.

For microsequencing, 1 mg Scarb proteins from the endothelial membranes of normoglycemic animals was resolved by 2D electrophoresis and transferred on ProBlott polyvinylidine fluoride (PVDF) membranes (Applied Biosystems, Foster City, CA) using a 3-(cyclohexylamino)-1-propanesulfonic acid buffer (30). Selected spots corresponding to major glycated proteins were cut and submitted to the automated Edman degradation to obtain the NH_2-terminal amino acid sequences. This was performed on a 494-cLC-Procise HS sequencer using the general protocol of Hewick et al. (31). For proteins found to be NH_2-terminally blocked, a protocol of digestion "in gel" and peptide separation were applied. Protein spots were cut out of the Coomassie Brilliant Blue R-250 lightly stained 2D gel, reduced with dithiothreitol, and alkylated with iodoacetamide before trypsin digestion (32,33). The enzyme used was the modified sequencing grade trypsin from Promega. The peptides were extracted from the gel and separated on a Brownlee HPLC microbore C18 column (OD-300, 7 μm, 1 × 50 mm) using an applied Biosystem 130A Separation System. Peptides were eluted at 150 μl/min with the following gradient program: 0–40 min (0–80% solvent B) and 40–57 min (80–100% solvent B), with solvent A being 0.1% trifluoroacetic acid (TFA)/H_2O and solvent B being 0.08% TFA in 70% acetonitrile/H_2O. The peptides were detected by their absorbance at 220 nm. Fractions were adsorbed on a TFA-treated glass fiber filter disk coated with 0.374 mg polybrene and 0.025 mg NaCl (Biobrene Plus; ABI) before analysis by Edman degradation as described previously. The sequence fragments found were compared with the primary structures of known proteins using the BLAST system (34).

Immunochemical confirmation of the microsequencing data. The identity of the main glycated species was further confirmed by performing sequential immunoblotting with relevant antibodies on the same nitrocellulose membrane carrying the spots of interest, resolved by 2D electrophoresis. Besides anti-glucitollysine, the following antibodies were used according to the previously described protocol: rabbit anti–β-actin (Sigma Aldrich Canada, Oakville, ON, Canada) and goat anti–annexins I and II (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies conjugated to horseradish peroxidase were from Amersham Pharmacia Biotechnology (anti-mouse and anti-rabbit Ig) and Dako (Carpinteria, CA) (anti-goat Ig). The immunostaining was revealed by ECL, and between the incubations with different primary antibodies, the membranes were stripped in 0.1 mol/l HCl-glycine for 1 h. At the end, the gold staining was silver-amplified to facilitate the alignment of the immunoblots with the global spectrum of the proteins.

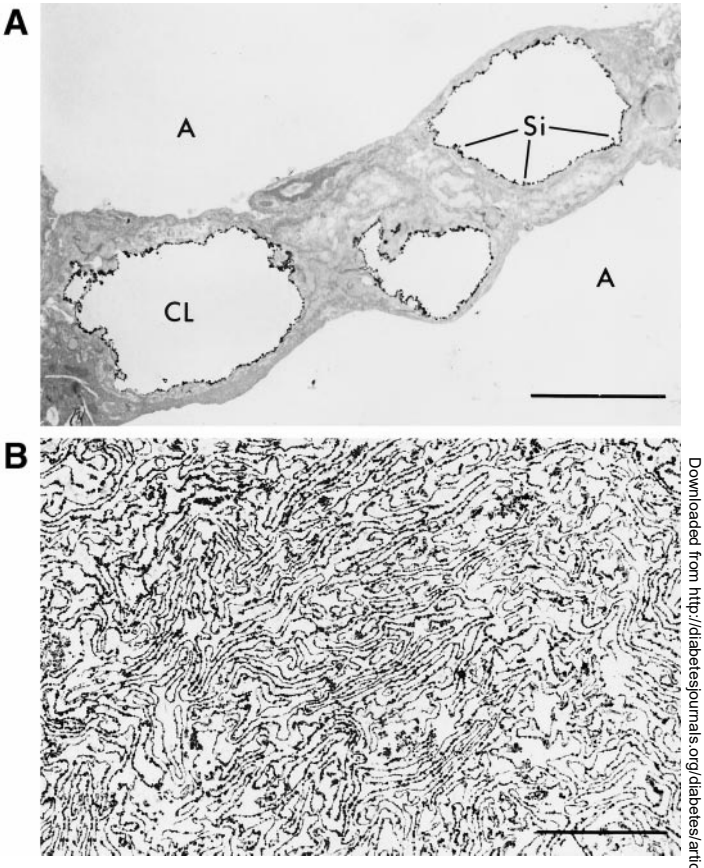

FIG. 1. *A*: Rat lung perfused with cationic colloidal silica and Na polyacrylate. Note that silica particles decorate the luminal front of the endothelial cells only. *B*: Purified lung endothelial plasma membranes (fraction P_2). Note the lack of contaminants from other cellular compartments. A, alveolar space; CL, capillary lumen; Si, silica particles. Bar equals 2.5 μm.

RESULTS

The level of plasma protein glycation is generally taken as an assessment of the effect of the glycemic condition, averaged for periods up to 2 weeks. In our experimental setting, at the moment of death, glycated plasma proteins amounted to 3.03 ± 0.84% ($n = 3$) for the diabetic group vs. 0.96 ± 0.23% in the control rats ($P < 0.01$). In the third group, comprised of diabetic insulin-treated animals, the proportion of glycated plasma proteins fell to 1.54 ± 0.41% ($P < 0.05$ vs. diabetic nontreated rats).

The positively charged colloidal silica particles that were perfused through the vasculature attached themselves to the luminal front of the endothelial cells only (Fig. 1*A*, black dots); they did not cross the endothelial barrier. Consequently, the plasma membrane fractions obtained by this technique were virtually free of contamination, purely endothelial, and lacked detectable endomembranes, as confirmed in the present work by the electron microscope examination (Fig. 1*B*) and, in previous articles, by enzyme and immunochemical assays (18,35).

This purity translates into a remarkably reproducible 2D electrophoretic pattern of the membranes (fraction P_2) isolated from individual animals. A systematic comparison between normal and diabetic animals revealed that no major alterations were induced by diabetes in the global 2D electrophoretic pattern of lung endothelial plasma membrane proteins. An example of this is presented in Fig. 2.

By performing Western blotting with anti-glucitollysine antibodies, we have searched for glycated proteins in the purified endothelial membranes of the diabetic animals. The specificity of these antibodies for the reduced form of glucose conjugated to the epsilon amino group of lysine was demonstrated by an immunoblotting test in which only the glycated albumin, but not its native nonglycated variant, is labeled (Fig. 3). A clear signal was elicited from only 0.37 fmol glucose bound to albumin and loaded on gel (glucose/BSA molar ratio = 2.5). This gives a measure of the sensitivity with which glucitollysine was detected throughout this study.

In all diabetic animals examined, several P_2 polypeptides (220, 74, 57, 42, 36, 34, and 22 kDa of apparent MW) were found to react with the antibody (Fig. 4, *lanes* $D_{1–3}$), indicating that these membrane proteins are the target for the glucose attachment during the early phases of hyperglycemia. Insulin treatment in the diabetic animals completely abolished the glycation of the membrane proteins (Fig. 4, *lanes* $T_{1–3}$) that was also absent in the membranes isolated from normoglycemic animals (Fig. 4, *lanes* C_1 and C_2).

The glycated proteins found in the endothelial membrane of diabetic animals belong to the cell cortex—namely the polypeptides electrostatically attached to the

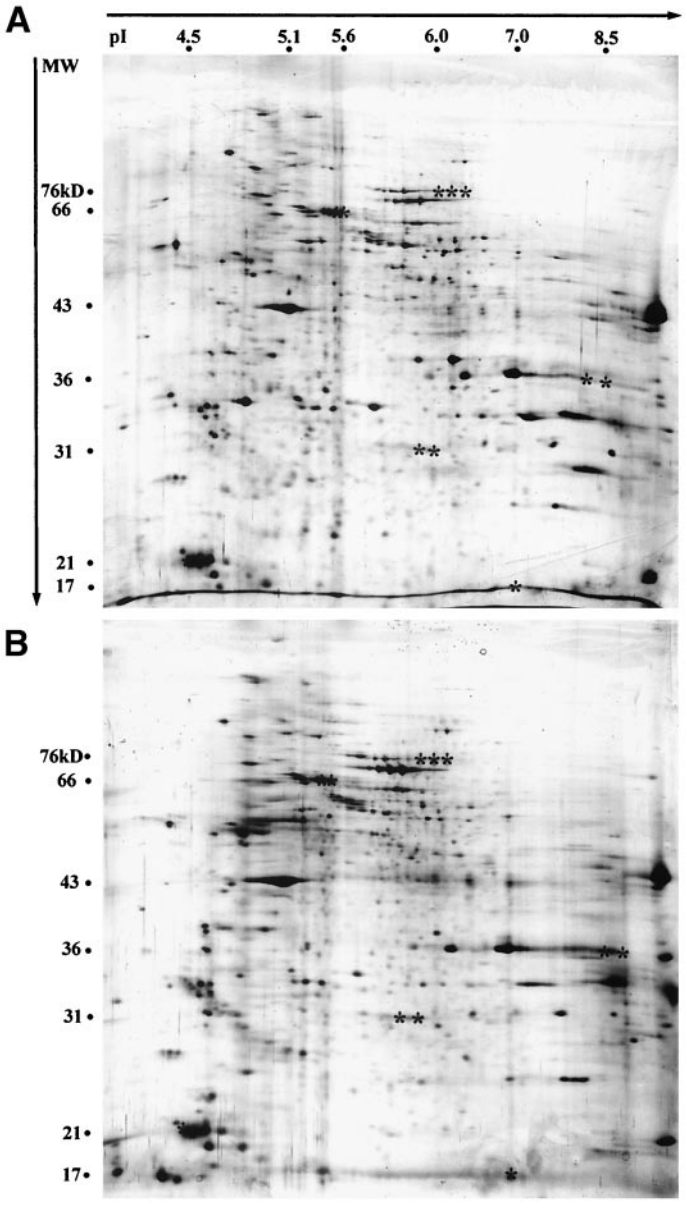

FIG. 2. Comparative 2D electrophoretic profiles of the endothelial P_2 membrane proteins (100 μg/sample) purified from normal (*A*) and diabetic (*B*) rats. Molecular weight (MW) and pI standards (Bio-Rad kit) have been incorporated in the sample (*, **, *) for alignment, comparison of the patterns, and MW/pI scale drawing.**

cytoplasmic face of the plasma membrane. They were virtually completely solubilized by the high pH conditions known to detach peripheral membrane proteins (Fig. 5). As previously shown (24), none of these carbonate-soluble proteins are labeled in situ by membrane impermeable tracers (sulfo-*N*-hydroxysuccinimidyl ester-biotin [sulfo-NHS-biotin]), a fact that demonstrates their localization on the internal cytoplasmic face of the plasmalemma. Surprisingly, no specific glycated species were observed among the intrinsic endothelial membrane polypeptides, except for the faint residues left by carbonate solubilization (Fig. 5, *lane* P_3).

When the distribution of glycated proteins in diabetic rats was comparatively assessed in the whole lung homogenate, in the cytosolic fraction, and in the purified endothelial membranes (fraction P_2), it was observed that the

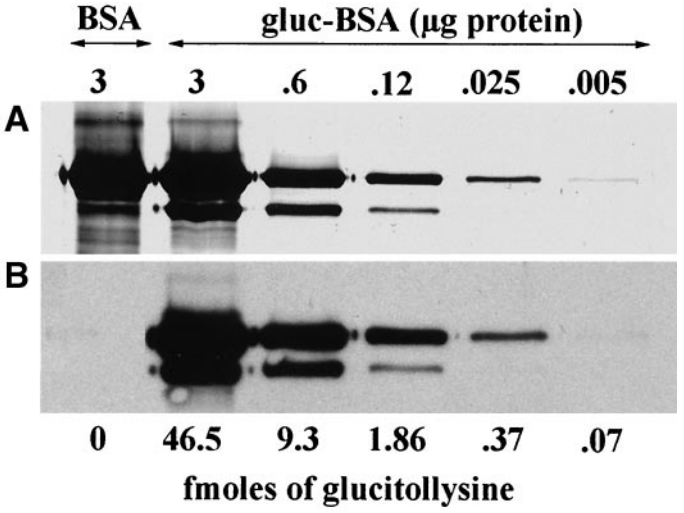

FIG. 3. Test for the specificity of anti-glucitollysine antibodies toward the glucose (gluc)-modified proteins. BSA (3 μg) and decreasing amounts of nonenzymatically glycated BSA (glucose/protein molar ratio of 2.5) were submitted to SDS-PAGE (*A*), transferred to nitrocellulose, and probed with the anti-glucitollysine antibodies (*B*). Only the protein containing glucitollysine was labeled.

glucose-modified species in the cytosol and in the plasma membrane are distinct and complementary to each other in respect to the whole homogenate (Fig. 6).

Spreading the endothelial membrane proteins by 2D electrophoresis before the immunoblotting step revealed that some of the major anti-glucitollysine–reactive bands previously recorded contain a relatively complex distribution of proteins of similar apparent molecular weight (Fig. 7*A* and *B*). To facilitate the comparison of the traditional immunoblotting pattern with that generated by the 2D technique, samples of total Scarb proteins, either directly solubilized in Laemmli's buffer (Fig. 7*A*) or previously resolved by isoelectric focusing in IPG strips (Fig. 7*B*), were simultaneously run in the same 2D gel, transferred,

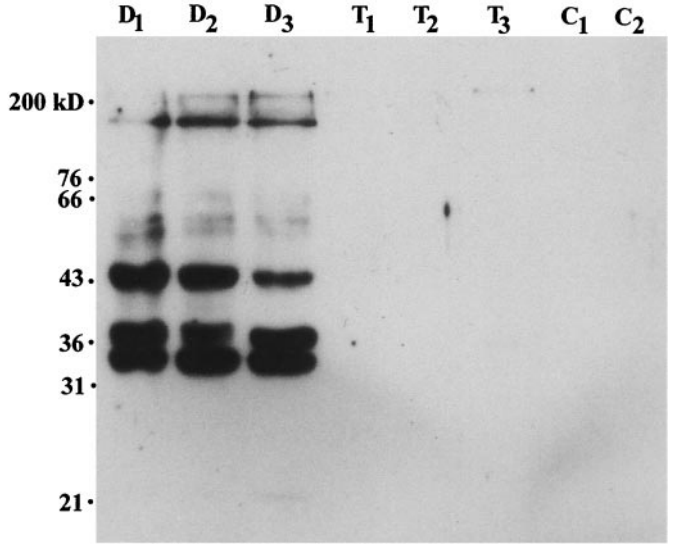

FIG. 4. Nonenzymatically glycated proteins of the endothelial membranes (P_2 fraction) purified from diabetic rats (*lanes* D_{1-3}) revealed by Western blotting with the anti-glucitollysine antibody: P_2 protein (20 μg) per lane. Note the absence of glycated species in diabetic animals treated with insulin (*lanes* T_{1-3}) as well as in the control euglycemic rats (*lanes* C_1 and C_2).

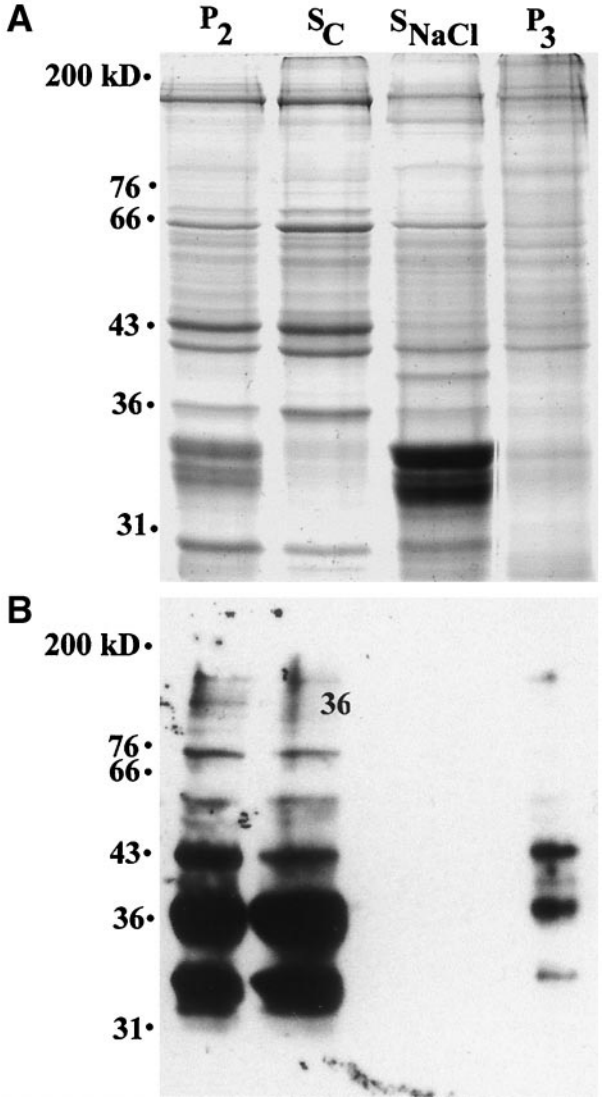

FIG. 5. The glycated proteins of the endothelial membranes are peripheral proteins solubilized by the membrane treatment with a high pH buffer. Forty micrograms of the endothelial membrane P_2 fraction, of the proteins solubilized by Na carbonate (S_C) and by 1 mol/l NaCl (S_{NaCl}), and of the endothelial membrane stripped of the peripheral proteins (fraction P_3) were resolved by SDS-PAGE. Coomassie blue staining (*A*) and Western blotting with anti-glucitollysine (*B*) are shown.

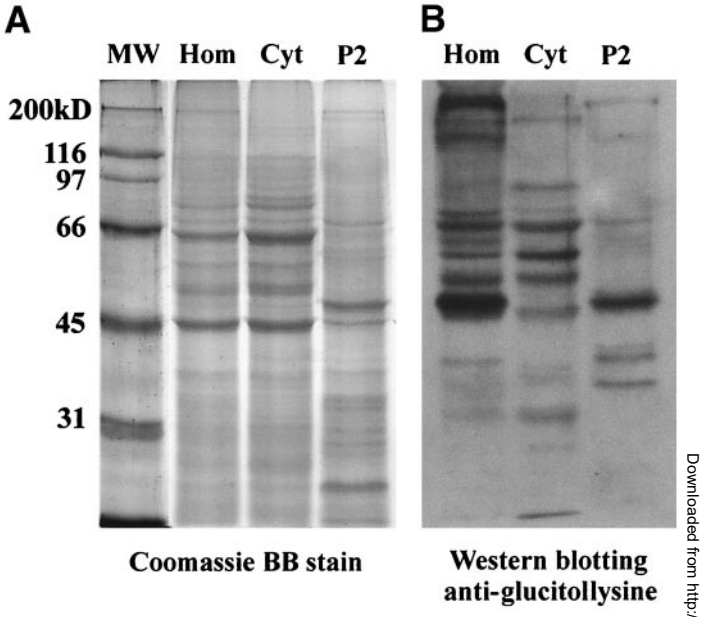

FIG. 6. Forty micrograms of protein/lane of homogenate (Hom), cytosol (Cyt), and endothelial plasmalemma (P_2) of diabetic untreated animals were separated by SDS-PAGE. Coomassie blue staining (*A*) and Western blotting with glucitollysine (*B*) are shown. Note that in respect to the homogenate, the cytosolic and plasma membrane glycated proteins exhibit complementary but clearly distinct patterns. MW, molecular weight.

and probed together with the anti-glucitollysine antibody. The picture obtained demonstrates that the spectrum of glycated proteins revealed by SDS-PAGE/immunoblotting is reproduced without losses and further enriched in resolution by the 2D electrophoretic approach.

Several major glycated polypeptides, marked from 1 to 7 in Fig. 7*B*, were pinpointed in the Coomassie-stained PVDF transfers or in gel, cut out, and identified by microsequencing. The internal or NH_2-terminal sequence fragments found for these proteins presented a high homology or were identical (Fig. 8) to those of the following known polypeptides: rat actin (protein 1) (36), rat annexin I (protein 2) (37), rat annexin II (protein 3) (38), annexin fragments (proteins 4 and 5) (37), the subunit p34 of the human actin-related protein (Arp2/3) complex (protein 6) (39), and human Ras suppressor protein (protein 7) (40). To further confirm the identity of the glycated species, two segments of a nitrocellulose membrane carrying the whole 2D spectrum of endothelial Scarb proteins of a diabetic animal were cut as outlined in Fig. 7 and stained with colloidal gold. The two membrane fragments were incubated sequentially with anti-glucitollysine antibodies and anti–β-actin (Fig. 7*C*) or anti-glucitollysine, anti–annexin II, and anti–annexin I (Fig. 7*D*), with membrane stripping in between. The perfect alignment of the spots positive for these antibodies validated the identities of the glycated proteins established by microsequencing. Moreover, it allowed us to conclude that three more spots, positive for glucitollysine, located on the acidic side of proteins 2 and 3 and previously unchecked by their amino acid sequence, were also fragments of annexins I and II, respectively (Figs. 7*B* and *D*).

DISCUSSION

The first step of this study addresses the question of whether diabetes induces major alterations in the spectrum of endothelial membrane proteins. Because the nuclear factor κB, a factor that plays a pivotal role in early gene responses, was shown to be activated by hyperglycemia in cultured endothelial cells (41), we expect that the endothelial cells from diabetic animals might express new proteins at their surface. As illustrated in Fig. 2, at least during the early phases of streptozotocin-induced diabetes, the spectrum of the proteins making the endothelial surface does not change significantly. However, it must be stressed that the simple comparison of silver-stained gels, although entirely adequate for revealing qualitative differences, has limitations in assessing quantitative variations, particularly for the low-abundance proteins. In fact, the literature contains several reports regarding quantitative variations in the expression of certain endothelial mem-

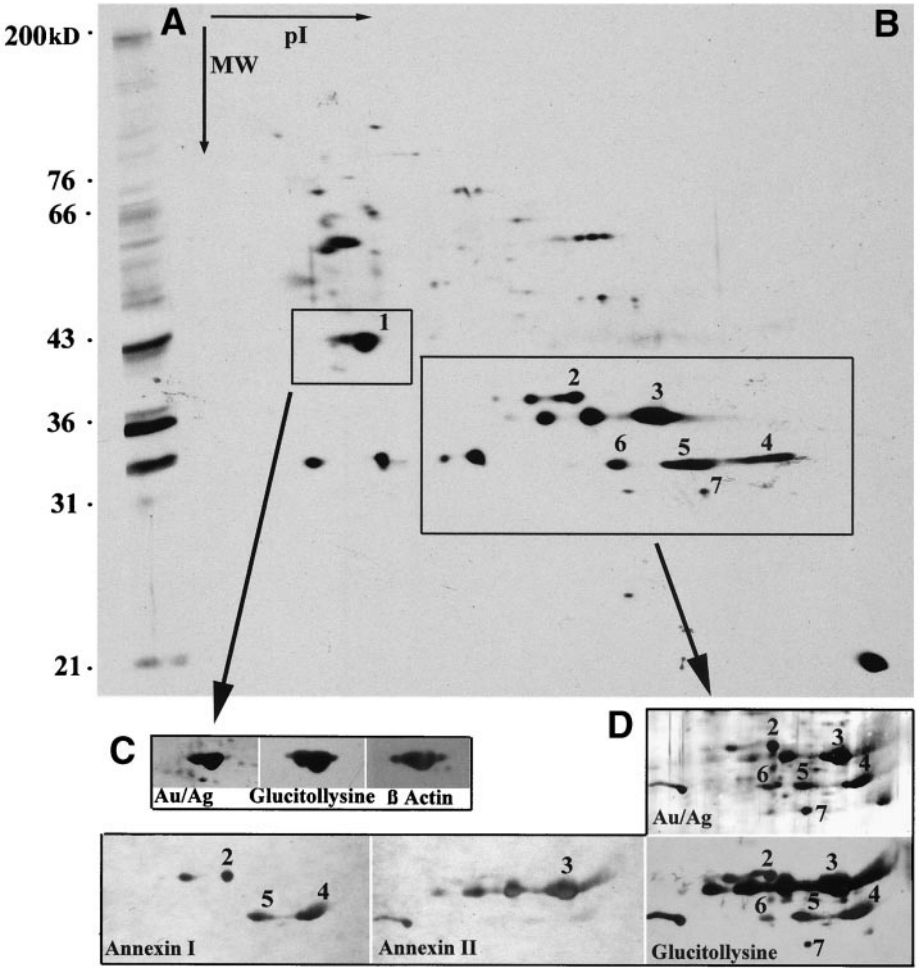

FIG. 7. A total of 200 μg of S_{carb} proteins from a diabetic rat, previously resolved by isoelectric focusing (*B*), were loaded on the same 2D SDS-PAGE gel (*B*) with another 50-μg S_{carb} solubilized directly in Laemmli's buffer (*A*). After electrophoresis, the whole gel was transferred on nitrocellulose (NC) stained with colloidal gold and submitted to immunoblotting with anti-glucitollysine antibody. The numbers label the glucitollysine-positive spots subsequently submitted to identification by microsequencing. From a similar NC membrane, areas of interest were cut, stained with colloidal gold, and sequentially probed with antibodies in the following order: anti-glucitollysine and anti–β-actin (*C*), and anti-glucitollysine, anti–annexin II, and anti–annexin I (*D*), respectively. Note the identity of the position of the proteins identified by microsequencing and by immunoblotting with the corresponding antibodies. MW, molecular weight.

brane proteins. Elevated expression of the cell adhesion molecules intracellular adhesion molecule-1 and P-selectin (42) and the endothelin A receptor (43) was recorded in endothelia from diabetic animals. It was also suggested that in diabetes, the activity of the endothelial Ca^{2+}-dependent protein kinase C is increased (44)—a fact believed to imply the enzyme translocation from the cytosol to the plasma membrane.

A second aspect explored in this study regards the possibility that several of the endothelial membrane polypeptides might be covalently modified by glucose during an early phase of hyperglycemia and that these modifications alter the endothelial membrane functions. The glycated proteins were detected by immunoblotting with anti-glucitollysine antibodies. No significant glycation of total lung or endothelial plasmalemmal proteins was noticed in normoglycemic animals. On the contrary, in diabetic rats, clear and reproducible patterns of protein glucose adducts were revealed (Fig. 4). Some of these were traced in the purified endothelial plasma membrane fraction (P_2) only but not in the cytosol, recommending them as specific plasmalemmal polypeptides (Fig. 6). Decreasing glucose concentration in the blood by insulin treatment was very efficient in canceling nonenzymatic glycosylation (Fig. 4)—a fact that could be explained by the turnover of these proteins (<10 days, the duration of insulin treatment).

All the glycated proteins detected in the P_2 fractions from diabetic animals were solubilized by buffers of high pH (Fig. 5). This places them in the category of peripheral membrane proteins, which accommodates factors of the membrane signal transduction machinery as well as the elements of the cortical actin cytoskeleton. The identification by microsequencing of seven of the major glycated polypeptides (Fig. 8) has shown that all of these are or are believed to be related to the organization of the cortical cytoskeleton. Actin constitutes the very building blocks of this peri-plasmalemmal network. Annexin I is a calcium-dependent phospholipid binding protein known to interact with actin and profilin (45) and is considered an active player in the regulation of membrane-cytoskeleton interactions. We do not yet know whether the two fragments of annexin I found among the glycated species (proteins 4 and 5) and beginning at residues 24 and 28, respectively, are functionally significant or just artifacts produced despite the continuous presence of protease inhibitors

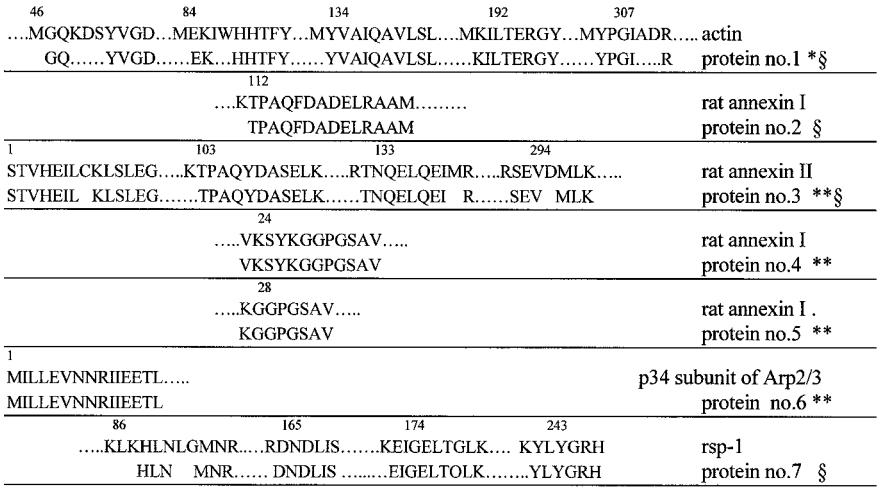

FIG. 8. Matching of the sequence fragments found for the glycated proteins with primary structures of known proteins found in databases. Rat annexin II and the p34 subunit of the Arp2/complex were identified by NH_2-terminal microsequencing. The others, which were NH_2-terminally blocked, were digested with trypsin, except protein 1, which was fragmented by cyanogen bromide (*). All sequences beginning after K, R (trypsin cutting site), or M (CNBr cut) are internal sequences (§). The others represent NH_2-terminal sequences (**).

throughout the experimental procedures. Annexin II, a third major glycated polypeptide, belongs to the same family of Ca^{2+}-, phospholipid-, and actin-binding proteins and has been shown to localize to detergent-resistant cholesterol-rich membrane microdomains, currently equated to the caveolae (46). It is considered to be implicated in the regulation of the vesicular traffic (47). Along with these major glycated species in the lung endothelial plasma membrane, two other proteins, which are significantly less abundant, have been identified as targets for nonenzymatic glycosylation. The actin-related protein (Arp2/3) complex is recognized as a multifunctional actin organizer. It caps the pointed end of actin filaments and provides nucleation sites for actin polymerization (48). The Arp2/3 complex contains seven subunits, and one of these, p34-Arc, was found to be glycated (protein 5). Finally, a relatively novel peptide, Ras suppressor protein-1 (rsp-1), was also identified as carrying glucitollysine residues (protein 7). Although relatively little is known about the functional relationships of this molecule within the cell, rsp-1 is highly homologous to a *Drosophila* fli-1 gene product possessing a gelsolin-like domain (48). It has been suggested that rsp-1 modulates Ras signal transduction (49), and this can be, at least speculatively, related to the fact that Ras proteins are implicated in the regulation of the actin cytoskeleton.

Glucitollysine represents a marker for only the first stage of a continuous process. Before being removed through protein turnover, the intracellular Amadori products can evolve into AGEs—some of them intermediates and others genuine end products. AGEs are known to exert deleterious effects on proteins by altering their charge and therefore their conformation, by promoting free radical–mediated oxidation leading to molecular fragmentation or blocking of metal-associated enzymes, and by forming irreversible intra- and intermolecular cross-links. AGE content increases dramatically in endothelial cells after only 1 week of growth in hyperglycemic conditions (12). The rapidity of this process was attributed to the involvement of glycolytic intermediates, which are significantly more reactive than glucose. Although AGEs could be formed through pathways that do not involve Amadori products (50,51), it has been shown that the latter are the most significant precursor of AGEs in vivo (52). It is therefore plausible to infer that the glycated membrane proteins detected with anti-glucitollysine antibodies during the early stage of diabetes also contain intermediate and late glycation products. At least at this stage, they do not seem to contain extensive intermolecular cross-links, as suggested by the lack of shifts toward the high molecular weight of actin or annexin I– and annexin II–positive bands. The disappearance of the immunocytochemical signal for glucitollysine in diabetic rats treated for 10 days with insulin might be interpreted as the result of the protein turnover as well as the effect of the gradual evolution of these toward AGEs at the expense of early glycation products.

The picture emerging from our data is that the proteins involved in the organization of the cortical actin cytoskeleton represent the preferential target for nonenzymatic glycosylation in early diabetes. The link between this finding and the reported decrease in the plasma membrane fluidity of several cell types in diabetes (53,54) appears logical, particularly in light of the fact that the diabetes-induced rigidity of plasmalemma could not be explained by meaningful modifications of its lipid composition (13). Membrane fluidity is very important in the physiology of endothelial cells, as it modulates the sensitivity of the stretch-activated ion channels in mechanotransduction and controls the mechanism of nitric oxide synthase activation by the shear stress. An alternative hypothetical mechanism might be drawn from the colocalization of actin, annexin II, and nitric oxide synthase at the caveolar microdomains of the endothelial plasma membrane. Amadori products (in this case from actin and annexin) can release superoxide anion (55), which in turn can generate other highly reactive free radicals that are able to induce protein fragmentation and lipid peroxidation in the proximity of their site of genesis. Moreover, superoxide anion as well as actin- and annexin II–based AGEs could quench nitric oxide directly. We therefore advance the idea that in hyperglycemic conditions, the glycation of the actin cytoskeleton associated with the endothelial plasma membrane might be responsible for the diabetes-induced impairment of the endothelial cell's ability to regulate vascular tone.

ACKNOWLEDGMENTS

This work was supported by the Natural Science and Engineering Research Council of Canada and Association Diabète Québec grants to L.D.G.

The excellent technical help of Rozica Bolovan, Diane Gingras, and Jean Leveillé is gratefully acknowledged. The anti-glucitollysine antibody was supplied by Drs. Linda Curtis (Scripps Research Institute, La Jolla, CA) and Joseph Witztum (University of California at San Diego). We also thank Dr. Moise Bendayan (Université de Montréal) for his encouragement and for critically reading the manuscript.

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