Protein Kinase C β2–Dependent Phosphorylation of Core 2 GlcNAc-T Promotes Leukocyte-Endothelial Cell Adhesion

A Mechanism Underlying Capillary Occlusion in Diabetic Retinopathy

Rakesh Chibber,[1] Bahaedin M. Ben-Mahmud,[1] Giovanni E. Mann,[1] Jin J. Zhang,[2] and Eva M. Kohner[3]

Increased leukocyte-endothelial cell adhesion is a key early event in the development of retinopathy and atherogenesis in diabetic patients. We recently reported that raised activity of glycosylating enzyme [β]1,6 acetylglucosaminyltransferase (core 2 GlcNAc-T) is responsible for increased leukocyte-endothelial cell adhesion and capillary occlusion in retinopathy. Here, we demonstrate that elevated glucose increases the activity of core 2 GlcNAc-T and adhesion of human leukocytes to retinal capillary endothelial cells, in a dose-dependent manner, through diabetes-activated serine/threonine protein kinase C β2 (PKCβ2)-dependent phosphorylation. This regulatory mechanism, involving phosphorylation of core 2 GlcNAc-T, is also present in polymorphonuclear leukocytes isolated from type 1 and type 2 diabetic patients. Inhibition of PKCβ2 activation with the specific inhibitor, LY379196, attenuated serine phosphorylation of core 2 GlcNAc-T and prevented increased leukocyte-endothelial cell adhesion. Raised activity of core 2 GlcNAc-T was associated with a threefold increase in O-linked glycosylation of P-selectin glycoprotein ligand-1 on the surface of leukocytes of diabetic patients compared with age-matched control subjects. PKCβ2-dependent phosphorylation of core 2 GlcNAc-T may thus represent a novel regulatory mechanism for activation of this key enzyme in mediating increased leukocyte-endothelial cell adhesion and capillary occlusion in diabetic retinopathy. *Diabetes* 52: 1519–1527, 2003

From the [1]Centre for Cardiovascular Biology & Medicine, Guy's, King's and St. Thomas' School of Biomedical Sciences, King's College London, London, U.K.; the [2]Guy's, King's and St. Thomas' Department of Ophthalmology, the Rayne Institute, St. Thomas' Hospital, London, U.K.; and the [3]Department of Endocrinology, Diabetes & Internal Medicine, St. Thomas' Hospital, London, U.K.

Address correspondence and reprint requests to Dr. Rakesh Chibber, Centre for Cardiovascular Biology & Medicine, 2nd Floor, New Hunt's House, GKT School of Biomedical Sciences, King's College London, London, SE1 1UL, U.K. E-mail: rakesh.chibber@kcl.ac.uk.

Received for publication 26 August 2002 and accepted in revised form 11 March 2003.

core 2 GlcNAc-T, [β]1,6-acetylglucosaminyltransferase; ICAM, intracellular adhesion molecule; MBP, myelin basic protein; PKCβ2, protein kinase C β2; PMN, polymorphonuclear leukocyte; PSGL-1, P-selectin glycoprotein ligand-1.

Diabetic retinopathy, a leading cause of severe visual loss in type 1 and type 2 diabetic patients (1) is characterized in its early stage by areas of capillary nonperfusion and microvascular damage (2,3). As in atherogenesis (4), increased leukocyte-endothelial cell adhesion is a key early event in the development of capillary occlusion in retinopathy (5–10). Leukocytes from diabetic patients are more adhesive to endothelial cells (11), and in experimental diabetes, their increased entrapment in retinal capillaries leads to areas of capillary nonperfusion and endothelial cell damage (12). Increased adhesion of leukocytes in diabetic patients may result from an increased expression of intracellular adhesion molecule (ICAM)-1 on endothelial cells and/or expression of integrins (CD11a, CD11b, and CD18b) on leukocytes (13–16).

We recently reported that raised activity of the glycosylating enzyme [β]1,6-acetylglucosaminyltransferase (core 2 GlcNAc-T) is responsible for increased leukocyte-endothelial cell adhesion and capillary occlusion in retinopathy (17). This Golgi enzyme plays a crucial role in the biosynthesis of O-linked glycans by converting core 1 (i.e., Gal [β] 1,3GalNAc[α]-O) to core 2 (i.e., Gal [β] 1,3[GlcNAc [β] 1,6] GalNAc [α]-O) structures (18,19) and represents an important regulatory step for the extension of O-linked sugars with poly (*N*-acetyllactosamine). These O-linked sugars synthesized by core 2 GlcNAc-T are associated with cellular adhesion (20) and disease states, such as malignant transformation (21), T-cell activation (22), inflammation (23), myocardial dysfunction (24,25), capillary morphogenesis (26), and myeloblastic leukemia (27). On the basis that O-linked sugars are also involved in cell-cell interactions (28), we proposed that their modification by raised activity of core 2 GlcNAc-T, together with glucose-induced expression of adhesion molecules on endothelial cells (29–31), is the underlying mechanism in increased leukocyte-endothelial cell adhesion and capillary occlusion in diabetic patients.

Here we explored the possibility that activity of core 2 GlcNAc-T in leukocytes is regulated at the posttranslational level by protein kinase C β2 (PKCβ2)-dependent phosphorylation in leukocytes of diabetic patients. Ele-

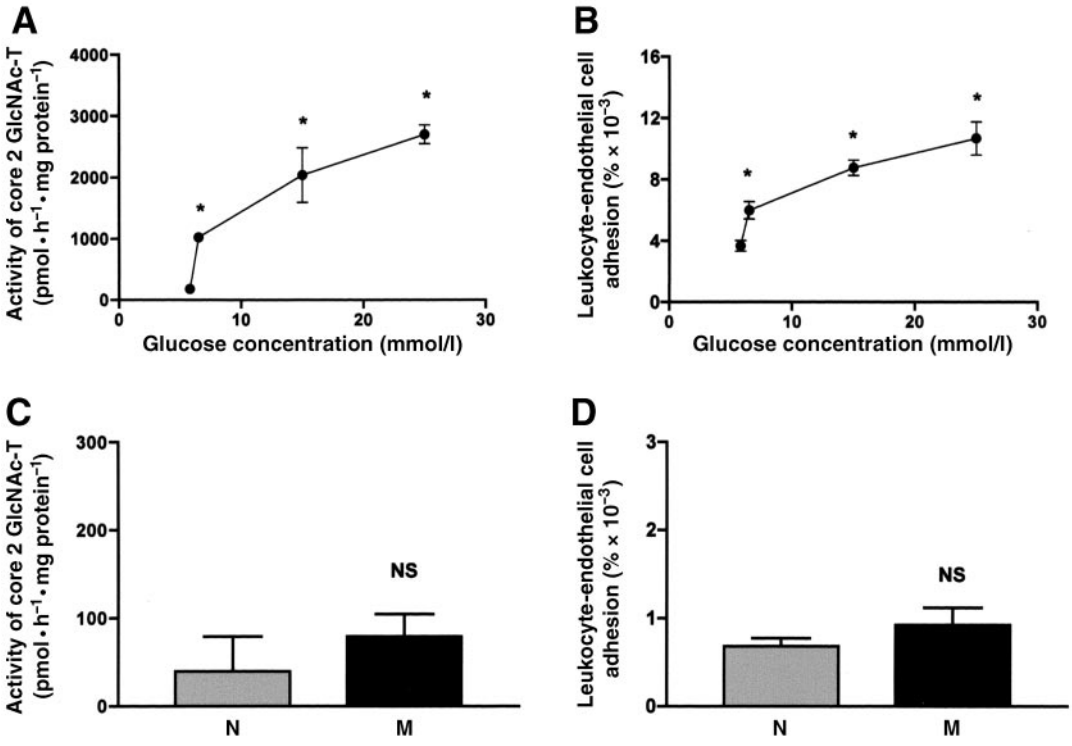

FIG. 1. Elevated glucose increases core 2 GlcNAc-T activity and adherence of human leukocytes to retinal capillary endothelial cells. *A*: Human leukocytes (U937) were exposed to D-glucose at various concentrations (5.8, 6.5, 15, and 25 mmol/l) for 24 h, and activity of core 2 transferase was determined in lysates. Data are mean ± SE (n = 3–5, *P < 0.05). *B*: After exposure to glucose level of leukocyte adhesion to cultured bovine retinal capillary endothelial cells was determined by labeling leukocytes with carboxyfluorescein. Data are mean ± SE (n = 15, *P < 0.05). *C*: Human leukocytes (U937) were exposed to 10 mmol/l mannitol (M) for 24 h, and activity of core 2 GlcNAc-T was measured in lysates. Data are mean ± SE (n = 3, *P = 0.0002). *D*: Effect of 10 mmol/l mannitol on adherence of leukocytes to endothelial cells. Data are mean ± SE (n = 3, *P = 0.0001). N, normal.

gant studies by King and coworkers (32,33) have strongly implicated activation of PKCβ2 in the pathogenesis of diabetes complications such as nephropathy and retinopathy. Their work over many years has led to clinical testing of the PKCβ2 inhibitor, LY333531, for treatment of retinopathy and maculopathy. In the present study, we found that elevated glucose increases the activity of core 2 GlcNAc-T through PKCβ2-dependent phosphorylation, leading to an increased adhesion of leukocytes to retinal capillary endothelial cells.

RESEARCH DESIGN AND METHODS

Subjects. This study included type 1 and type 2 diabetic patients recruited from the Diabetes Outpatient Clinic and Eye Unit at St. Thomas' Hospital (London). The diabetic patients were age-matched with healthy control subjects recruited from family members and friends accompanying the patients to the clinic or from hospital employees. The HbA_{1c} levels for type 1 and type 2 diabetic subjects were 8.4 ± 1.51 and 9.33 ± 0.3% (mean ± SE), respectively. The duration of type 1 and type 2 diabetes was 19.1 ± 1.5 and 11.9 ± 1.1 years, respectively. The overall levels of severity of retinopathy were determined according to the ETDRS (Early Treatment Diabetic Retinopathy Study): levels of 20–47, mild and moderate nonproliferative retinopathy and macular edema; levels of 53 and worse, severe nonproliferative retinopathy, proliferative retinopathy, and advanced eye disease.

Measurement of core 2 GlcNAc-T activity. To measure core 2 GlcNAc-T activity, leukocytes were washed in PBS, frozen, and lysed in 0.9% Triton X-100 at 0°C. The activity of core 2 GlcNAc-T was then measured as described previously (17). Briefly, the reaction was performed in a reaction mixture containing, 50 mmol/l 2(*N*-morpholino) ethanesulfonic acid (MES; Sigma, Dorset, UK), pH 7.0, 1 mmol/l UDP-6 [^{3}H]-*N*-acetylglucosamine (16,000 dpm/nmol) (NEN Life Science Products, Hounslow, UK), 0.1 mol/l GlcNAc (Sigma), 1 mmol/l Galβ1–3-D-GalNAcα-*p*-nitrophenol (Sigma) as substrate, and 16 μl of cell lysate (100–200 μg protein) for a final volume of 32 μl. After incubating the mixture for 1 h at 37°C, the reaction was terminated with 1 ml ice-cold distilled water and processed on a C18 Sep-Pak column (Waters-Millipore, Watford, UK). After washing the column with 20 ml distilled water, the product was eluted with 5 ml methanol. Radioactivity in samples was determined by liquid scintillation counting. Endogenous activity of core 2 GlcNAc-T was measured in the absence of the added acceptor. The specific activity was expressed as picomoles per hour per milligram cell protein. In each case, the protein concentration was determined using the Bio-Rad protein assay (BioRad, Hertfordshire, UK).

Culture of bovine retinal capillary endothelial cells. Bovine retinal capillary endothelial cells were established from bovine retinas dissected from eyes of freshly slaughtered cattle, as previously described (34). Briefly, the isolated retinas were homogenized in serum-free minimal essential medium (Gibco, Paisley, UK) and filtered through a 85-μm nylon mesh. The trapped micro vessels were digested with collagenase dispase (1 mg/ml) for 90 min at 37°C and filtered through a 53-μm nylon mesh. The digested microvessels were then plated in gelatin-coated tissue culture flasks and maintained in minimal essential medium supplemented with 10% pooled human serum, 2 mmol/l glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and used at passage 2–3. The cells were characterized using morphological criteria and by immunostaining with an antibody against factor VIII–related antigen.

Culture of human myelocytic cell line (U937). This leukocytic cell line (35) was cultured in glucose-free RPMI medium (Sigma, Poole, UK) supplemented with 10% FCS, 2 mmol/l glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5 mmol/l glucose. For experimentation, the cells were centrifuged, washed in PBS, and exposed to varying concentrations of D-glucose (5.8, 6.5,15, 25 mmol/l) and mannitol (15 mmol/l) in RPMI. In some experiments, the PKCβ2 inhibitor, LY379196 (Eli Lilly) was added at concentrations of 25 and 50 nmol/l during incubation with elevated glucose. After a 24-h incubation at 37°C, the cells were centrifuged and used in the measurement of the activity of core 2 GlcNAc-T and leukocyte-endothelial cell adhesion assay.

Leukocyte-endothelial cell adhesion assay. Adhesion of leukocytes to endothelial cells was examined by labeling with (5,6)-carboxyfluorescein diacetate succinylmidyl (Molecular Probe, Cambridge, UK). Briefly, endothelial cells were grown to a confluent state to provide an endothelial cell surface for the adhesion of the carboxyfluorescein-labeled leukocytes (U937). After

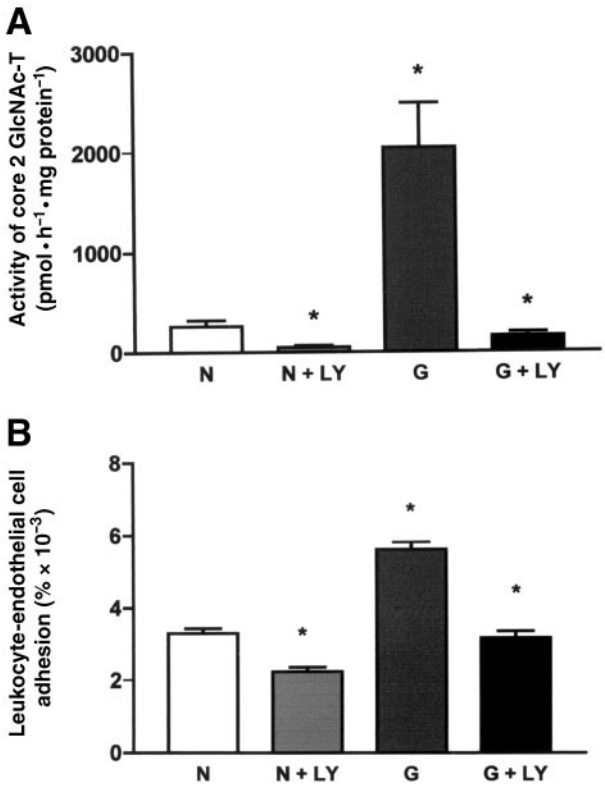

FIG. 2. Specific inhibitor of PKCβ2, LY379196, prevents glucose-induced activity of core 2 GlcNAc-T and adherence of leukocytes to endothelial cells. *A*: Leukocytes were exposed to normal (N, 5.8 mmol/l) and elevated (G, 15 mmol/l) glucose for 24 h in the absence and presence of 50 nmol/l LY379196 (N + LY, G + LY). Activity of core 2 transferase was measured in lysates, and data are presented as mean ± SE (n = 18, *P < 0.05). *B*: Effect of LY379196 on glucose-induced leukocyte-endothelial cell adhesion. Data are presented as the mean ± SE (n = 18, *P < 0.05).

treatment with elevated glucose and mannitol leukocytes were centrifuged (14,000*g* for 1 min) and washed twice with serum-free RPMI. The cells were then resuspended in 1 ml of serum-free RPMI containing 50 μg/ml carboxyfluorescein. The cells were counted with hemocytometer and a known number added to endothelial cells. After a 30-min incubation at 37°C, nonadherent leukocytes were removed by washing with serum-free RPMI and the dishes fixed in 3.7% formalin in PBS. Attached leukocytes were counted in 10 random high-powered fields (×100) by fluorescence microscopy. The results were expressed as percent of adherent leukocytes/field.

Measurement of PKC activity. Total PKC activity was measured in cell extracts using a PKC assay kit (Gibco) according to the manufacturer's instructions. The assay is based on the measurement of phosphorylation of myelin basic protein (MBP) (36).

Immunoprecipitation and immunoblot analysis. For core 2 GlcNAc-T immunoprecipitation, as well as for Western blots, a polyclonal antibody against core 2 GlcNAc-T was used (kindly provided by Dr. A. Datti, Glycodesign, Toronto, Canada). Cells were lysed on ice in lysis buffer (20 mmol/l Tris-HCl, pH 7.4, 1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l sodium vandate, 1 mmol/l PMSF (phenylmethylsulfonyl fluoride), 1 μg/ml aprotinin, and 10 μg/ml leupeptin). The lysate was incubated at 4°C for 20 min with constant agitation and insoluble material removed by centrifugation (14,000*g* for 5 min at 4°C). The clarified lysate was incubated with staphylococcal protein A-Sepharose Cl-4B–conjugated primary antibody for 2 h with constant agitation at 4°C. The immunoprecipitates were washed with Tris-buffered saline (10 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl) containing 0.5% Triton X-100, resuspended in two times SDS/PAGE sample buffer and analyzed by SDS/PAGE. After immunodetection of core 2 GlcNAc-T, the membranes were stripped in a buffer containing 50 mmol/l Tris, 2% SDS, and 100 mmol/l mercaptoethanol at 55°C for 30 min, washed, and immunoblotted with antiphosphoserine antibody (Chemicon, Hampshire, UK). Immunoreactive bands were quantified by scanning densitometrically and calculating the density of individual bands using ImageQuant software (Molecular Dynamics, Kemsing, UK). The level of serine phosphorylation was expressed as a ratio of intensity of phosphorylation immunoreactive band/intensity of core 2 GlcNAc-T immunoreactive bands.

For glycosylation experiments, PSGL-1 was immunoprecipitated using a specific polyclonal antibody (kindly provided by Dr. Kumar, Small Molecule Drug Discovery, Genetics Institute, Cambridge, MA) complexed to staphylococcal protein A-Sepharose Cl-4B and separated by SDS-PAGE. After immunodetection of PSGL-1, the membranes were stripped (as above), washed, and immunoblotted with antibody against "core 2 structures" (Cambridge Biosciences, Cambridge, UK). Immunoreactive bands were quantified and level of O-linked glycosylation expressed as a ratio of intensity of "core 2" immunoreactive band/intensity of PSGL-1 immunoreactive band.

In vitro phosphorylation of core 2 GlcNAc-T. Core 2 GlcNAc-T immunocomplexes were phosphorylated in vitro by incubating with 1.0 unit recombinant PKCβ2 (Upstate Biotechnology, Buckingham, UK) in a buffer system containing 100 mmol/l HEPES, pH 7.9, 10 mmol/l $MgCl_2$, 0.1 mmol/l EDTA, 1 mmol/l dithiorietol, 0.1 mmol/l $ZnCl_2$, 0.2 mmol/l sodium orthovanadate, 5 mmol/l NaF, 1 mmol/l PMSF, and 20 μmol/l ATP. A total reaction volume of 20 μl was incubated for 30 min at 30°C. Samples were centrifuged (12,000*g* for 1 min), washed five times with immunoprecipitation buffer, pellet resuspended in SDS/PAGE sample buffer, and fractionated by SDS/PAGE using 7.5% polyacrylamide gel. Phosphorylated proteins were detected by Western blot analysis.

In addition, core 2 GlcNAc-T was immunoprecipitated, eluted from protein A-Sepharose Cl-4B beads with two times SDS/PAGE sample buffer, and dialysed extensively overnight with PBS. Phosphorylation of the partially purified core 2 GlcNAc-T was then carried out using the PKC assay kit (Gibco) with human recombinant PKCβ2. Briefly, core 2 GlcNAc-T was added to the reaction mixture containing 20 mmol/l Tris pH 7.5, 20 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, [γ-^{32}P]ATP (20–25 μCi/ml) (Amersham, Buckinghamshire, UK), and 20 μmol/l ATP. After incubation for 30 min at 30°C, 25 μl was spotted onto phosphocellulose discs that were subsequently washed in 1% (vol/vol) phosphoric acid and water. Scintillation fluid was added and radioactivity determined. Results were expressed as picomoles phosphate incorporated per minute.

Transfection. Transient transfections of U937 cells were carried out by TransFast kit (Promega, Southampton, UK) using a mixture of PKCβ2 expression plasmid (kind gift from Prof. Alan Fields and Dr. Nicole Murphy (Sealey Center for Cancer Biology, University of Texas Medical Branch, Galveston, TX) and liposome reagent, according to the manufacturer's protocol. The cells were cultured in fresh RPMI medium supplemented with 10% FCS. After 72 h growth, the cells were harvested to monitor PKCβ2 expression by Western blot analysis.

Subcellular fractionation: preparation of Golgi membrane fraction. Golgi fraction was prepared according a modified procedure of Balch et al. (37). Briefly, cells were homogenized in 0.25 mol/l sucrose, 10 mmol/l Tris-HCl, pH 7.4, and 1 mmol/l EDTA. The homogenate was passed through a 21-guage needle and then adjusted to 1.4 mol/l by the addition of ice-cold 2.3 mol/l sucrose containing 10 mmol/l Tris-HCl, pH 7.4, and 1 mmol/l EDTA. The samples were vortexed vigorously to ensure uniform mixing and then loaded into a Beckman polyallomer (Beckman, UK) ultracentrifuge tubes and overlaid with 0.8 mol/l sucrose and 10 mmol/l Tris-HCl, pH 7.4. The gradients were centrifuged for 2.5 h at 25,000 rpm (90,000*g*) (Beckman TL-100 Tabletop Ultracentrifuge) in the TLS-55 swinging bucket rotor. The turbid band at the 0.8 mol/l/1.2 mol/l sucrose interface was harvested by syringe puncture, precipitated using 10% trichloroacetic acid and used for Western blot analysis.

Protein measurement. Total protein was measured using the BCA protein assay kit (Sigma).

Statistical analysis. The statistical software Graphpad Prism version 3.0 was used. A two-tailed Student's t test was used to test the significance of variables. Linear regression and correlation were used to evaluate the relationship between two variables. Data include SEs, which were converted to percentiles where necessary. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Activity of Core 2 GlcNAc-T is modulated by elevated D-glucose. Previous work established a crucial role for raised activity of core 2 GlcNAc-T in increased leukocyte-endothelial cell adhesion and capillary occlusion in diabetic retinopathy (17). Here, we explored whether elevated D-glucose could modulate leukocyte-endothelial cell adhesion by increasing the activity of core 2 GlcNAc-T. In agreement with recent observations reported by Nishio et al. (24) on cultured cardiomyocytes, we found that ele-

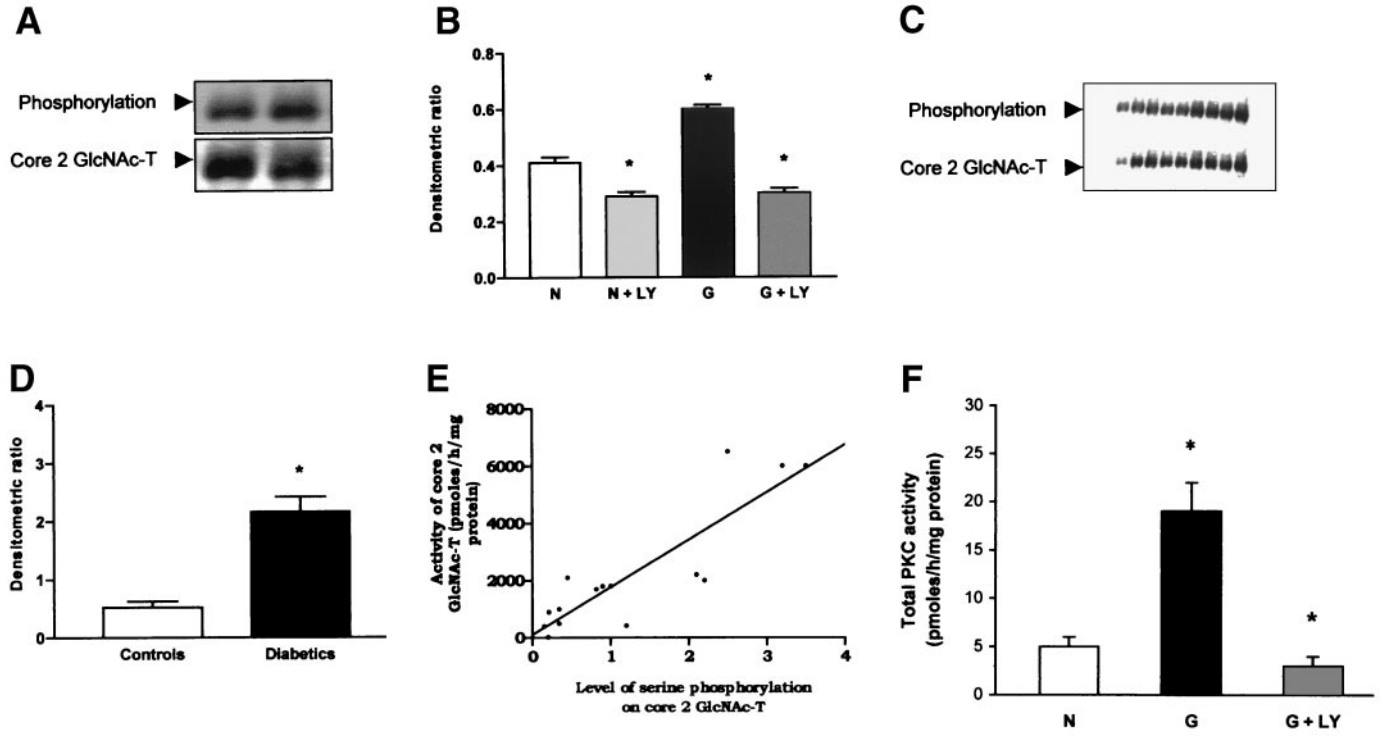

FIG. 3. Elevated glucose increases serine phosphorylation of core 2 GlcNAc-T, and total PKC activity in human leukocytes (U937). *A*: After exposure to normal (N, 5.8 mmol/l) and elevated (G, 15 mmol/l) D-glucose for 12 h at 37°C. Core 2 GlcNAc-T was immunoprecipitated, followed by immunoblotting with antiphosphoserine antibody. Blots were reprobed with anti–core 2 GlcNAc-T antibody. *B*: Phosphorylation of core 2 GlcNAc-T in leukocytes exposed to normal glucose (N, 5.8 mmol/l) and elevated D-glucose (G, 15 mmol/l) for 24 h, in the absence and presence (N + LY, G + LY) of 50 nmol/l LY379196. Phosphorylation level is expressed as densitometric ratio (intensity of phosphorylation immunoreactive band/intensity of core 2 GlcNAc-T immunoreactive band). Data are presented as the mean ± SE ($n = 5$, $^*P < 0.05$). *C*: Phosphorylation level of core 2 GlcNAc-T in leukocytes of isolated from different diabetic patients (type 1 and type 2 diabetes). Core 2 GlcNAC-T was detected in immunoprecipitates of whole cell lysates with antiphosphoserine antibody and then reprobed with anti–core 2 GlcNAc-T antibody. *D*: Phosphorylation level of core 2 GlcNAc-T in PMNs from diabetic patients ($n = 15$) and age-matched healthy control subjects ($n = 6$). The blots were analyzed densitometrically and level of phosphorylation expressed as a ratio (intensity of phosphorylation immunoreactive band/intensity of core 2 GlcNAc-T immunoreactive band). *E*: Significant relationship between activity and level of phosphorylation of core 2 GlcNAc-T in leukocytes of diabetic patients (type 1 and type 2 diabetes; $n = 18$, $r = 0.88$, $P = 0.0001$). *F*: PKC activation in leukocytes (U937) by elevated D-glucose. Leukocytes were exposed to normal glucose (N, 5.8 mmol/l), high glucose (G, 15 mmol/l), and high glucose in the presence of 50 nmol/l LY379196 (G + LY). After a 24-h incubation, U937 were permeabilized with 50 μg/ml digitonin and PKC activity measured using MBP as a specific substrate for PKC (Gibco). Data are presented as the mean ± SE ($n = 4$–5, $^*P < 0.05$).

vated glucose raised the activity of core 2 GlcNAc-T in leukocytes (Fig. 1*A*) and increased leukocyte-endothelial cell adhesion (Fig. 1*B*) in a dose-dependent manner. These glucose-mediated effects were observed with a glucose concentration as low as 6.5 mmol/l (Fig. 1*A* and *B*), but not with mannitol (Fig. 1*C* and *D*).

PKCβ2-dependent phosphorylation of core 2 GlcNAc-T. To explore the role of PKC signaling pathway(s) in core 2 GlcNAc-T activity, we used the specific PKCβ2-inhibitor, LY379196 (36). At a concentration that specifically inhibits the β isoform of PKC (38), LY379196 completely blocked glucose-induced activity of core 2 GlcNAc-T (Fig. 2*A*) and leukocyte-endothelial cell adhesion (Fig. 2*B*). These observations indicated that elevated glucose might regulate the activity of core 2 GlcNAc-T through PKCβ2-dependent phosphorylation of the enzyme. This hypothesis is consistent with the increased serine phosphorylation of core 2 GlcNAc-T in leukocytes exposed to elevated glucose (Fig. 3*A*). Moreover, this increased phosphorylation of core 2 GlcNAc-T was attenuated by the addition of LY379196 (Fig. 3*B*).

Having established in vitro the possibility that activity of core 2 GlcNAc-T is regulated at the posttranslational level through PKCβ2-dependent phosphorylation, we sought to determine whether this novel regulatory mechanism is functional in leukocytes of diabetic patients. Immunoblot analysis revealed that core 2 GlcNAc-T is phosphorylated in leukocytes of diabetic patients (Fig. 3*C*). We detected increased phosphorylation of core 2 GlcNAc-T in leukocytes of diabetic patients (type 1 and type 2 diabetes) compared with age-matched healthy control subjects (Fig. 3*D*), as well as found a significant relationship between the level of phosphorylation and activity of core 2 GlcNAc-T (Fig. 3*E*).

Activation of PKC in leukocytes. The marked inhibition of glucose-induced activity of core 2 GlcNAc-T by LY379196 suggested that the PKCβ2 signaling pathway was important for regulating cellular activity of this glycosylating enzyme. To confirm activation of PKC in leukocytes exposed to elevated glucose, we measured phosphorylation of MBP. As reported in other cell types (39–41), total PKC activity was higher in leukocytes exposed to elevated glucose compared with normal glucose (Fig. 3*F*). Our observations that at concentration of 50 nmol/l, LY379196 prevented PKC activation by glucose appear to suggest the involvement of the β2 isoform of PKC (Fig. 3*F*).

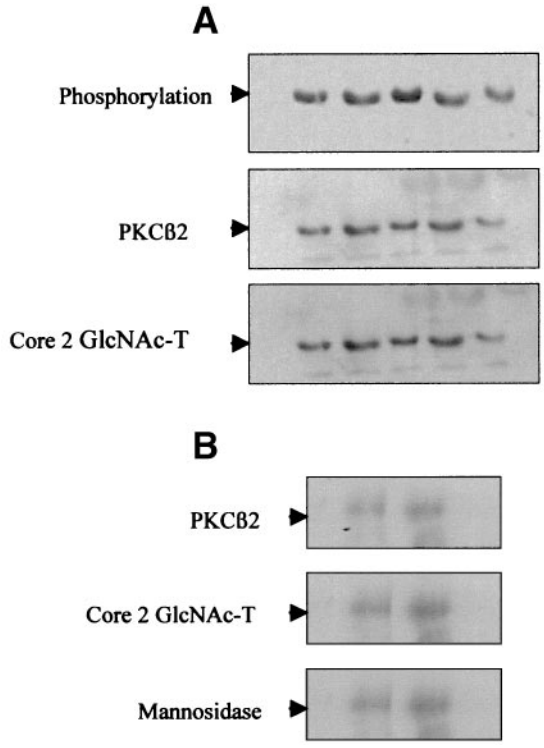

FIG. 4. PKCβ2 colocalizes with core 2 GlcNAc-T in the Golgi. *A*: Colocalization of PKCβ2 with core 2 GlcNAc-T in leukocytes of diabetic patients. Core 2 GlcNAc-T was immunoprecipitated, followed by immunoblotting with antiphosphoserine antibody. Blots were reprobed with anti–core 2 GlcNAc-T antibody and then with anti-PKCβ2 antibody. Data presented are of five diabetic patients and are representative of three separate experiments. *B*: Colocalization of PKCβ2 and core 2 GlcNAc-T in the Golgi of human leukocytes (U937). Golgi was isolated by density gradient centrifugation and the purity confirmed by Western blot analysis using antibody against mannosidase 1. Blot was then immunostained with anti-PKCβ2 and anti–core 2 GlcNAc-T antibody. Representative of two separate experiments.

Colocalization of core 2 GlcNAc-T and PKCβ2 in Golgi fraction. Western blot analysis further demonstrated a close association between PKCβ2 and core 2 GlcNAc-T in leukocytes of diabetic patients (Fig. 4*A*). To demonstrate colocalization of core 2 GlcNAc-T and PKCβ2, we isolated Golgi fraction using density gradient centrifugation. The purity of the isolated Golgi was confirmed by Western blot analysis using antibody against mannosidase 1 (Fig. 4*B*). Immunoblot analysis also confirmed that PKCβ2 and core 2 GlcNAc-T are colocalized in the Golgi of U937 cells (Fig. 4*B*).

Overexpression of PKCβ2 increases phosphorylation of core 2 GlcNAc-T. To further explore the mechanistic link between core 2 GlcNAc-T and PKCβ2, we examined whether overexpression of PKCβ2 could directly increase the activity of core 2 GlcNAc-T. The addition of an expression plasmid carrying PKCβ2 increased the expression of PKCβ2 in transfected cells (Fig. 5*A*). Overexpression of PKCβ2 increased the phosphorylation of core 2 GlcNAc-T (Fig. 5*B*), the association of PKCβ2 with core 2 GlcNAc-T (Fig. 5*C*), and the activity of core 2 GlcNAc-T (Fig. 5*D*) in the transfected cells.

Based on our observation of prominent colocalization of PKCβ2 and core 2 GlcNAc-T in leukocytes, we examined whether the two proteins interacted biochemically in the transfected cells. Core 2 GlcNAc-T was immunoprecipitated from normal (Fig. 5*E*, *lane 1*) and transfected U937 cells (Fig. 5*E*, *lane 2*) using an anti–core 2 GlcNAc-T antibody. The interaction between core 2 GlcNAc-T was markedly diminished when the coimmunoprecipitate was maintained in 1 mol/l NaCl before separation by SDS-PAGE (Fig. 5*E*).

Seeking more definitive evidence that activity of core 2 GlcNAc-T is regulated by PKCβ2-dependent phosphorylation, we performed an in vitro phosphorylation reaction using human recombinant PKCβ2 and core 2 GlcNAc-T immobilized on protein A-Sepharose beads. The addition of human recombinant PKCβ2 increased the phosphorylation (Fig. 5*F*) and activity of core 2 GlcNAc-T (Fig. 5*G*) that was prevented by LY379196. In addition, in vitro phosphorylation experiments demonstrated that human recombinant PKCβ2 increases incorporation of ^{32}ATP into partially purified core 2 GlcNAc-T that was significantly prevented by LY37919 (Fig. 5*H*).

O-linked glycosylation of P-selectin glycoprotein ligand-1. To investigate whether raised activity of core 2 GlcNAc-T increases the level of O-linked glycosylation in leukocytes of diabetic patients, we focused on P-selectin glycoprotein ligand-1 (PSGL-1). Our reason for this is that core 2 GlcNAc-T is known to play a crucial role in the binding of PSGL-1 to P-selectin (42–44). Immunoprecipitation and Western blot analysis demonstrated an almost threefold increase in the level of O-linked glycosylation of PSGL-1 on leukocytes of diabetic patients compared with age-matched healthy control subjects (Fig. 6).

DISCUSSION

We have identified a unique and previously unrecognized mechanism by which the cellular activity of the key glycosylating enzyme, core 2 GlcNAc-T, is regulated at the posttranslational level through PKCβ2-dependent phosphorylation. Several lines of evidence support this. Increased serine phosphorylation of core 2 GlcNAc-T in leukocytes by elevated glucose was associated with increased enzyme activity and increased leukocyte-endothelial cell adhesion. LY379196, a specific PKCβ2-inhibitor (38), attenuated serine phosphorylation and prevented elevated glucose-induced core 2 GlcNAc-T activity and leukocyte adhesion to retinal capillary endothelial cells. Core 2 GlcNAc-T acted as substrate for PKCβ2, and its phosphorylation led to an increase in enzyme activity that was reversed by LY379196. PKCβ2 is closely associated with core 2 GlcNAc-T in leukocytes of diabetic patients. In human leukocytes (U937), PKCβ2 and core 2 GlcNAc-T are colocalized in the Golgi. Overexpression of PKCβ2 in transfected leukocytes significantly increased the phosphorylation and activity of core 2 GlcNAc-T. Furthermore, core 2 GlcNAc-T exhibited an increased phosphorylation in leukocytes of diabetic patients compared with healthy control subjects, and there was a direct correlation between enzyme activity and the level of phosphorylation.

Our finding that PKCβ2 is localized in the Golgi is consistent with previous observations (45–47). Interestingly, activation of PKC by a phorbol ester (phorbol myristic acid) has been reported to induce PKCβ2 to move from the cytoplasm to the Golgi apparatus (48). The possibility that core 2 GlcNAc-T could be a target for PKCβ2 has important implications, since abnormal activa-

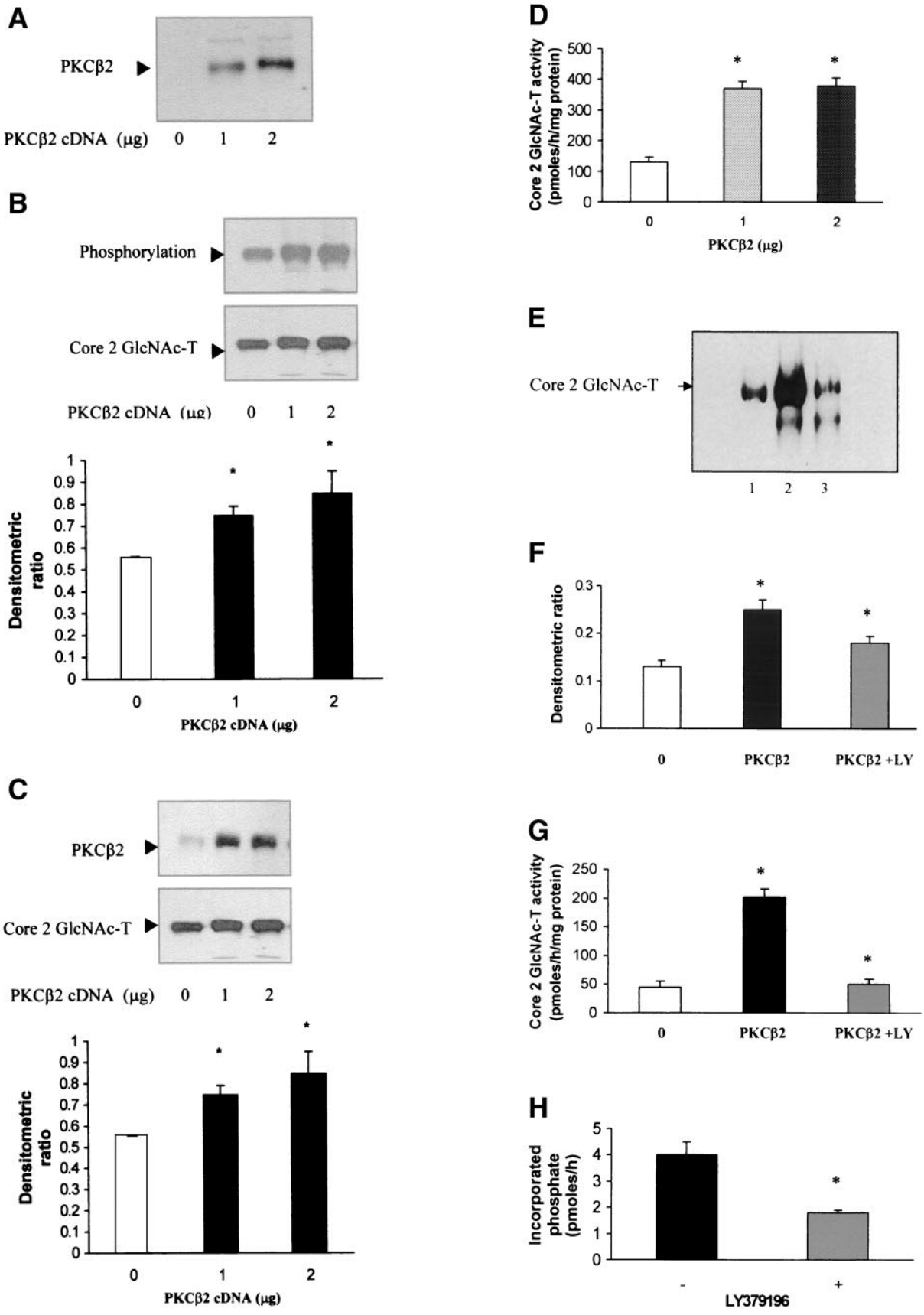

FIG. 5. Overexpression of PKCβ2 increases phosphorylation and activity of core 2 GlcNAc-T. *A*: Leukocytes (U937) were transfected with 0, 1, and 2 μg PKCβ2 cDNA expression plasmid. PKCβ2 expression was monitored by immunoprecipitation and Western blot analysis. *B*: Phosphorylation level of core 2 GlcNAc-T in transfected leukocytes is expressed as densitometric ratio (intensity of phosphorylation immunoreactive band/intensity of core 2 GlcNac-T immunoreactive band). Data are presented as the mean ± SE ($n = 3$, $^*P < 0.05$). *C*: Association

tion of PKCβ2 is now strongly associated with development of vascular complications in diabetes (32,33). Elevated glucose is an important transient signal for the translocation of PKCβ2 to various subcellular proteins. Once bound, PKCβ2 becomes activated, and more importantly, remains relatively resistant to downregulation; therefore, cellular PKC activity is increased for prolonged periods of time. Translocation and association of PKCβ2 is thought to control many cellular functions, including signal transduction, gene expression, cellular differentiation, contractility, and cellular proliferation (49).

From our observations, we propose that in diabetes, the tethering of PKCβ2 to core 2 GlcNAc-T increases the steady-state phosphorylation and activity of core 2 GlcNAc-T in leukocytes of diabetic patients. This increased activity of core 2 GlcNAc-T might cause the functional modification of O-linked glycans and thereby lead to increased leukocyte-endothelial cell adhesion observed in diabetic patients. This possibility is supported by our observation of increased O-linked glycosylation of PSGL-1 on the surface of leukocytes of diabetic patients compared with those from age-matched control subjects. Previous work has already demonstrated a crucial role for core 2 GlcNAc-T in the binding of PSGL-1 to P-selectin (41–44). PSGL-1 supports neutrophil rolling through P-selectin in vitro (50), as well as mediates neutrophil rolling in vivo (51,52). Our observations with PSGL-1 would suggest that there is increased leukocyte rolling in the diabetic state, and this is consistent with a recent report showing increased leukocyte rolling, and adhesion, through mesenteric microvasculature of rats given an intraperitoneal injection of elevated D-glucose (53). Moreover, binding of P-selectin to PSGL-1 triggers tyrosine kinase–dependent mechanisms that lead to CD11b/CD18 activation in polymorphonuclear leukocytes (PMNs) (54). It can be speculated that PKCβ2-dependent phosphorylation of core 2 GlcNAc-T increases enzyme activity by inducing some conformational change, but the exact phosphorylation sites of core 2 GlcNAc-T remain to be determined by mutagenesis experiments of all the PKCβ2 phosphorylation sites of core 2 GlcNAc-T.

PKCβ2-dependent phosphorylation of core 2 GlcNAc-T could be the underlying mechanism for its raised activity and, together with diabetes-induced expression of adhesion molecules such as ICAM-1 on endothelial cells (29–31), may be responsible for increased leukocyte-endothelial cell adhesion in diabetic patients (17). This possibility is indicated by recent reports showing *1*) activation of PKCβ2 in leukocytes and platelets of diabetic patients (55–57), and *2*) prevention of increased leukocyte entrapment in retinal capillaries of diabetic rats by the PKCβ2-inhibitor, LY333531 (58). However, direct evidence for this regulatory mechanism in leukocytes of diabetic patients will only be provided by the results of an ongoing phase 3 multicenter clinical trial with the PKCβ2 inhibitor, LY333531. The regulation of activity of core 2 GlcNAc-T through posttranslational modification of the enzyme by PKCβ2-mediated–dependent phosphorylation may also partly explain the observed paradox that the activity of core 2 GlcNAc-T varies markedly in different tissues, even though these tissues express similar enzyme levels (59).

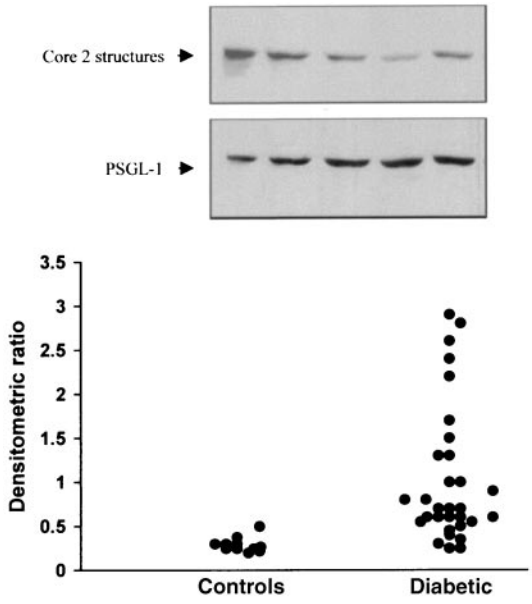

FIG. 6. Increased glycosylation level of PSGL-1 on leukocytes of diabetic patients. *A*: PSGL-1 was immunoprecipitated from five different diabetic PMNs, followed by immunoblotting with anti–PSGL-1 antibody. Blots were reprobed with anti–core 2 antibody. *B*: Level of O-glycosylation of PSGL-1 on leukocytes of diabetic patients (type 1 and type 2 diabetes; $n = 30$) and age-matched control subjects ($n = 15$). Level of glycosylation is expressed as densitometric ratio (intensity of "core 2 structure" immunoreactive band/intensity of PSGL-1 immunoreactive band).

In summary, we have established a novel functional link between diabetes-sensitive PKCβ2 and core 2 GlcNAc-T in promoting increased leukocyte-endothelial cell adhesion and capillary occlusion in diabetic retinopathy. It is also conceivable that this regulatory mechanism could play a role in disease states that are associated with O-glycans synthesized by core 2 GlcNAc-T, such as cancer (60), T-cell activation (22), inflammation (23), and diabetic cardiomyopathy (24,25). In this context, some of these disease states are also characterized by an abnormal activation of PKCβ2 (61–63). If we accept the importance of increased leukocyte-endothelial cell adhesion in capillary occlusion in retinopathy (5–10), then our data will have serious implications because, taken together, they

of PKCβ2 with core 2 GlcNAc-T in leukocytes transfected with 0, 1, and 2 μg PKCβ2 cDNA. Core 2 GlcNAc-T was immunioprecipitated, followed by Western blotting with anti-PKCβ2 antibody. Blots were reprobed with anti–core 2 GlcNAc-T antibody. Level of PKCβ2 association is expressed as a densitometric ratio (intensity of PKCβ2 immunoreactive band/intensity of core 2 GlcNAc-T immunoreactive band). Data are presented as the mean ± SE ($n = 3$, $^*P < 0.05$). *D*: Activity of core 2 GlcNAc-T in leukocytes transfected with 0, 1, and 2 μg PKCβ2 cDNA. Activity of core 2 GlcNAc-T was measured in lysates. Data are mean ± SE ($n = 3$, $^*P < 0.05$). *E*: Core 2 GlcNAc-T was immunoprecipiated from cell lysates of normal (*lane 1*) and leukocytes transfected with 2 μg PKCβ2 cDNA expression plasmid (*lane 2*). The interaction between core 2 GlcNAc-T and PKCβ2 is predominately disrupted in the presence of 1 mol/l NaCl wash (*lane 3*). Representative of two separate experiments. *F*: Human recombinant PKCβ2 increases in vitro phosphorylation of core 2 GlcNAc-T. After phosphorylation, core 2 GlcNAc-T was separated on SDS-PAGE and Western blotted with antiphosphoserine antibody. The blots were reprobed with anti–core 2 GlcNac-T antibody. Data are presented as mean ± SE ($n = 3$, $^*P < 0.05$). *G*: In vitro phosphorylation by PKCβ2 increases activity of core 2 GlcNAc-T. After in vitro phosphorylation, core 2 GlcNAc-T activity was determined. Data are presented as mean ± SE ($n = 3$, $^*P < 0.05$). *H*: Phosphorylation (picomoles of core 2 GlcNAc-T–incorporated phosphate) of core 2 GlcNAc-T in the absence (−) and presence (+) of 50 nmol/l LY379196. Core 2 GlcNAc-T was immunoprecipitated and used as a substrate for PKC. Data are presented as the mean ± SE ($n = 3$, $P < 0.05$).

suggest that the specific PKCβ2-inhibitor, LY333531, could be given much earlier to diabetic patients.

ACKNOWLEDGMENTS

This study was supported by the Juvenile Diabetes Foundation International.

We thank Dr. Allassandro Datti (Glycodesign, Toronto, Canada) for supplying the antibody against core 2 GlcNAc-T. We also thank Dr. Kumar (Small Molecule Drug Discovery, Genetics Institute, Cambridge, MA), Prof. Alan Fields and Dr. Nicole Murphy (Sealey Center for Cancer Research, Galvaston, TX) for the PKCβ2 cDNA plasmid for transfection experiments, and John Schilling (Eye Unit, St. Thomas' Hospital) for blood samples from diabetic patients.

Part of this study was presented at the 61st American Diabetes Association Annual Meeting held in Philadelphia, PA, and has appeared in abstract form [Chibber R, Mahmud BM, Mann GE, Kohner EM: Glycosylating enzyme, β1,6-acetylglucosaminyltransferase (core 2 GlcNAc-T) promotes leukocyte-endothelial adhesion through post-translational PKC β11-dependent phosphorylation (Abstract). *Diabetes* 50:A17, 2001]).

REFERENCES

1. Klein R, Klein BEK, Moss SE, Davis MD, DeMets DL: The Winconsin epidemiology study of diabetic retinopathy. X. Four-year incidence and progression of diabetic retinopathy when age at diagnosis is 30 or more years. *Arch Ophthalmol* 107:244–249, 1989
2. Davis MD: Diabetic retinopathy: a clinical overview. *Diabetes Care* 15: 1844–1873, 1993
3. Kohner EM, Chibber R: Diabetic retinopathy. In *Diabetic Angiopathy*. J.E. Tooke, Ed. Oxford, UK, Oxford University Press, 233–247, 1999
4. Bevilacqua MP, Nelson RM, Mannori G, Cecconi O: Endothelial-leukocyte adhesion molecules in human disease. *Annu Rev Med* 45:361–378, 1994
5. Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clermont AC, Aiello LP, Ogura Y, Adamis AP: Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. *Proc Natl Acad Sci U S A* 96:10836–10841, 1999
6. Miyamoto K, Ogura Y: Pathogenetic potential of leukocytes in diabetic retinopathy. *Semin Ophthalmol* 1:233–239, 1999
7. Kaplar M, Kappelmayer J, Veszpremi A, Szabo K, Udvardy M: The possible association of in vivo leukocyte-platelet heterophilic aggregate formation and the development of diabetic angiopathy. *Platelets* 12:419–422, 2001
8. Lutty GA, Cao J, McLeod DS: Relationship of polymorphonuclear leukocytes to capillary dropout in the human diabetic choroid. *Am J Pathol* 151:707–714, 1997
9. Schröder S, Palinski W, Schmid-Schönbein GW: Activated monocytes and granulocytes, capillary nonperfusion, and neovascularization in diabetic retinopathy. *Am J Pathol* 139:81–100, 1991
10. Barouch FC, Miyamoto K, Allport JR, Fujita K, Bursell SE, Aiello LP, Luscinskas FW, Adamis AP: Integrin-mediated neutrophil adhesion and retinal leukostasis in diabetes. *Invest Ophthalmol Vis Sci* 41:1153–1158, 2000
11. Miyamoto K, Hiroshiba N, Tsujikawa A, Ogura Y: In vivo demonstration of increased entrapment in retinal microcirculation of diabetic rats. *Invest Ophthalmol Vis Sci* 39:2190–2194, 1998
12. Joussen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell SE, Adamis AP: Leukocyte-mediated endothelial cell injury and death in the diabetic retina. *Am J Pathol* 158:147–152, 2001
13. Baumgartner-Parzer SM, Wagner L, Pettermann M, Gessl A, Waldhausl W: Modulation by high glucose of adhesion molecule expression in cultured endothelial cells. *Diabetologia* 38:1367–1370, 1995
14. Rao KM, Hatchell DL, Cohen HJ, De La Paz MA: Alterations in stimulus-induced integrin expression in blood neutrophils of patients with diabetic retinopathy. *Am J Med Sci* 313:131–137, 1997
15. Park CW, Kim JH, Lee JH, Kim YS, Ahn HJ, Shin YS, Kim SY, Choi EJ, Chang YS, Bang BK, Lee JW: High glucose-induced intercellular adhesion molecule-1 (ICAM-1) expression through an osmotic effect in rat mesangial cells is PKC-NF-kappa B-dependent. *Diabetologia* 43:1544–553, 2001
16. Manduteanu I, Voinea M, Serban G, Simionescu M: High glucose induces enhanced monocyte adhesion in valvular endothelial cells via a mechanism involving ICAM-1, VCAM-1 and CD18. *Endothelium* 6:315–324, 1999
17. Chibber R, Ben-Mahmud BM, Coppini D, Christ E, Kohner EM: Activity of core 2 GlcNAc (β1,6) transferase, is higher in polymorphonuclear leukocytes from diabetic patients compared with age-matched control subjects. *Diabetes* 49:1724–1730, 2000
18. Williams D, Schachter H: Mucin synthesis: detection in canine submaxillary glands of an N-acteylglucosaminyltransferase which acts on mucin substrates. *J Biol Chem* 255:11247–11252, 1980
19. Schachter H, Brochausen I: In *Glyconjugates: Composition, Structute and Function*. A.H.J. Allen, E.C. Kisailus, Eds. Marcel Dekker, New York, 1992, p. 263–332
20. Leferte S, Dennis JW: Glycosylation-dependent collagen-binding activities of two membrane glycoproteins in MDAY-D2 tumour cells. *Cancer Res* 48:4743–4748, 1988
21. Yousefi S, Higgins E, Daoling Z, Pollex-Kruger A, Hindsgaul O, Dennis JW: Increased UDP-GlcNAc:Gal[beta]1–3GalNAc-R(GlcNAc to GalNAc) [beta]-1,6-acetylglucosaminyltransferase activity in metastic murine tumour cell lines: control of polylactosamine synthesis. *J Biol Chem* 266:1772–1782, 1991
22. Higgins EA, Siminovitch KA, Zhuang D, Brockhausen I, Dennis JW: Aberrant O-linked oligosaccharide biosynthesis in lymphocytes and platelets from patients with the Wiskott-Aldrich syndrome. *J Biol Chem* 266:6280–6290, 1991
23. Piller F, Piller V, Fox RI, Fukuda M: Human T-lymphocyte activation is associated with changes in O-glycans biosynthesis. *J Biol Chem* 263: 15146–15150, 1988
24. Koya D, Dennis JW, Warren CE, Takahara N, Schoen FJ, Nishio Y, Nakajima T, Lipes MA, King GL: Overexpression of core 2 N-acetylglycosaminyltransferase enhances cytokine actions and induces hyperetropic myocardium in transgenic mice. *FASEB J* 13:2329–2337, 1999
25. Nishio Y, Warren CE, Buczek-Thomas JA, Rulfs J, Koya D, Aiello LP, Feener EP, Miller TB Jr, Dennis JW, King GL: Identification and characterization of a gene regulating enzymatic glycosylation which is induced by diabetes and hyperglycemia specifically in rat cardiac tissue. *J Clin Inv*est 96:1759–1767, 1995
26. Nguyen M, Strubel NA, Blschoff JA: Role of sialyl Lewis-X/A glycoconjugates in capillary morphogenesis. *Nature* 365:267–269, 1993
27. Brockhausen I, Kuhns W, Schachter H, Matta KL, Sutherland DR, Baker MA: Biosynthesis of O-glycans in leukocytes from normal donors and from patients with leukemia: increase in O-glycan core 2 UDP-GlcNAc: Gal[beta]3GalNAc[alpha]-R(GlcNAc to GalNAc)[beta](1,6)-N-acetylglucosaminyltransferase in leukemic cells. *Cancer Res* 51:1257–1263, 1991
28. Li F, Wilkins PP, Crawley S, Weinstein J, Cummings RD, McEver RP: Post-translational modifications of recombinant P-selectin glycoprotein ligand-1 required for binding to P- and E-selectin. *J Biol Chem* 271:3255–3264, 1996
29. Morigi M, Angioletti S, Imberti B, Donadelli R, Micheletti G, Figliuzzi M, Remuzzi A, Zoja C, Remuzzi G: Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycaemia in a NF-kB-dependent fashion. *J Clin Invest* 101:1905–1915, 1998
30. Dosquet C, Weill D, Wautier JL: Molecular mechanism of blood monocyte adhesion to vascular endothelial cells. *Nouv Rev Fr Haematol* 34:S55–S59, 1992
31. Puente Navazo MD, Chettab K, Duhault J, Koenig-Berard E, McGregor JL: Glucose and insulin modulate the capacity of endothelial cells (HUVEC) to express P-selectin and bind a monocytic cell line (U937). *Thromb Haemost* 86:680–685, 2001
32. Koya D, King G: Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859–866, 1998
33. Meier M, King GL: Protein kinase C activation and its pharmacological inhibition in vascular disease. *Vasc Med* 5:173–185, 2000
34. Chibber R, Molinatti PA, Kohner EM: Intracellular protein glycation in cultured retinal capillary pericytes and endothelial cells exposed to high-glucose concentration. *Cell Mol Biol* 45:47–57, 1999
35. Jain S, K., Kannan K, Lim G, McVie R, Bocchini JA Jr: Hyperketonemia increases tumor necrosis factor-alpha secretion in cultured U937 monocytes and type 1 diabetic patients and is apparently mediated by oxidative stress and cAMP deficiency. *Diabetes* 51:2287–2293, 2002
36. Yasuda, I. Kishimoto A, Tanaka S, Tominaga M, Sakurai A, Nishizuka Y: A synthetic peptide substrate for selective assay of protein kinase C. *Biochem Biophys Res Commun* 166:1220–1227, 1990
37. Balch WE, Dunphy WG, Braell WA, Rothman JE: Reconstitution of the

transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell* 39:405–416, 1984
38. Jirousek MR, Gillig JR, Gonzalez CM, Heath WF, McDonald JH 3rd, Neel DA, Rito CJ, Singh U, Stramm LE, Melikian-Badalian A, Baevsky M, Ballas LM, Hall SE, Winneroski LL, Faul MM: (S)-13-[(monomethylamino) methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1H,13H-dibenzo [E,K] pyrrolo- [3,4-H] [1,4,13] oxadiza-cyclohexadecin-1,3(2H)-dione (LY333531) and related analogues: isozyme selective inhibitors of protein kinase Cβ (PKCβ). *J Med Chem* 39:2664–2671, 1996
39. Hull JL, Matter CM, Wang X, Gibbons GH: Hyperglycemia inhibits vascular smooth muscle cell apoptosis through a protein kinase C-dependent pathway. *Circ Res* 87:574–580, 2000
40. Igarashi M, Wakasaki H, Takahara N, Ishii H, Jiang ZY, Yamauchi T, Kuboki K, Meier M, Rhodes CJ, King GL: Glucose or diabetes activates p38 mitogen-activated protein kinase via different pathways. *J Clin Invest* 103:185–195, 1999
41. Park JY, Takahara N, Gabriele A, Chou E, Naruse K, Suzuma K, Yamauchi T, Ha SW, Meier M, Rhodes CJ, King GL: Induction of endothelin-1 expression by glucose: an effect of protein kinase C activation. *Diabetes* 49:1239–1248, 2000
42. Kumar R, Camphausen RT, Sullivan FX, Cumming DA: Core 2 [beta]-1,6-N-acetylglucosaminyltransferase enzyme activity is critical for P-selectin glycoprotein ligand-1 binding to P-selectin. *Blood* 88:3872–3879, 1996
43. Sperandio M, Thatte A, Foy D, Ellies LG, Marth JD, Ley K: Severe impairment of leukocyte rolling in venules of core 2 glucosaminyltransferase-deficient mice. *Blood* 97:3812–3819, 2001
44. Snapp KR, Heitzig CE, Ellies LG, Marth JD, Kansas GS: Differential requirements for the O-linked branching enzyme core 2 beta1–6-N-glucosaminyltransferase in biosynthesis of ligands for E-selectin and P-selectin. *Blood* 97:3806–3811, 2001
45. Kiley SC, Parker PJ: Differential localization of protein kinase C isozymes in U937 cells: evidence for distinct isozyme functions during monocyte differentiation. *J Cell Sci* 108:1003–1016, 1995
46. Saito N, Kose A, Ito A, Hosoda K, Mori M, Hirata M, Ogita K, Kikkawa U, Ono Y, Igarashi K: Immunocytochemical localization of beta II subspecies of protein kinase C in rat brain. *Proc Natl Acad Sci U S A* 86:3409–3413, 1989
47. Kose A, Ito A, Saito N, Tanaka C: Electron microscopic localization of gamma- and beta II-subspecies of protein kinase C in rat hippocampus. *Brain Res* 518:209–217, 1990
48. Ron D, Jiang Z, Yao L, Vagts A, Diamond I, Gordon A: Coordinated movement of RACK1 with activated betaIIPKC. *J Biol Chem* 274:27039–27046, 1999
49. Park JY, Ha SW, King GL: The role of protein kinase C activation in the pathogenesis of diabetic vascular complications. *Perit Dial Int* 19 (Suppl. 2):S222–S227, 1999
50. Moore KL, Patel KD, Bruehl RE, Fugang L, Johnson DA, Lichenstein HS, Cummings RD, Bainton DF, McEver RP: P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J Cell Biol* 128:661–671, 1995
51. Norman KE, Katopodis AG, Thoma G, Kolbinger F, Hicks AE, Cotter MJ, Pockley AJ, Hellewell PG: P-selectin glycoprotein ligand-1 supports rolling on E- and P-selectin in vivo. *Blood* 96:3585–3591, 2000
52. Yang J, Hirata T, Croce K, Merrill-Skoloff G, Tchernychev B, Williams E, Flaumenhaft R, Furie BC, Furie B: Targeted gene disruption demonstrates that P-selectin glycoprotein ligand 1 (PSGL-1) is required for P-selectin-mediated but not E-selectin-mediated neutrophil rolling and migration. *J Exp Med* 190:1769–1782, 2001
53. Booth G, Stalker TJ, Lefer AM, Sealia R: Elevated ambient glucose induces acute inflammatory events in the microvasculature: effects of insulin. *Am J Physiol* 280:E848–E856, 2001
54. Evangelista V, Manarini S, Sideri R, Rotondo S, Martelli N, Piccoli A, Totani L, Piccardoni P, Vestweber D, de Gaetano G, Cerletti C: Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: role of PSGL-1 as a signaling molecule. *Blood* 93:876–885, 1999
55. Ceolotto G, Gallo A, Miola M, Trevsan R, Del Prato S, Baritono E, Semplicini A, Avogaro A: Protein kinase C activity is acutely regulated by plasma glucose concentration in human monocytes in vivo. *Diabetes* 48:316–1322, 1999
56. Assert R, Scherk G, Bumbure A, Pirags V, Schatz H, Pfeiffer AF: Regulation of protein kinase C by short term hyperglycaemia in human platelets in vivo and in vitro. *Diabetologia* 44:188–195, 2001
57. Kimura M, Ishizawa M, Miura A, Itaya S, Kanoh Y, Yasuda K, Uno Y, Morita H, Ishizuka T: Platelet protein kinase C isoform content in type 2 diabetes complicated with retinopathy and nephropathy. *Platelets* 12:138–143, 2001
58. Nonaka, A. Kiryu J, Tsujikawa A, Yamashiro K, Miyamoto K, Nishiwaki H, Honda Y, Ogura Y: PKC-beta inhibitor (LY333531) attenuates leukocyte entrapment in retinal microcirculation of diabetic rats. *Invest Ophthalmol Vis Sci* 41:2702–2706, 2000
59. VanderElst IE, Datti A: Beta1,6 N-acetylglucosaminyltransferase (core 2 GlcNAc-T) expression in normal rat tissues and different cell lines: evidence for complex mechanisms of regulation. *Glycobiology* 8:731–740, 1998
60. Renkonen J, Rabina J, Mattila P, Grenman R, Renkonen R: Core 2 beta1,6-N-acetylglucosaminyltransferase and alpha1,3-fucosyltransferase regulate the synthesis of O-glycans on selectin ligand on oral cavity carcinoma cells. *APMIS* 109:500–506, 2001
61. Gokmen-Polar Y, Murray NR, Velasco MA, Gatalica Z, Fields AP: Elevated protein kinase C beta1 is an early promotive event in colon carcinogenesis. *Cancer Res* 61:1375–1381, 2001
62. Leitges M, Schmedt C, Guinamard R, Davoust J, Schaal S, Stabel S, Tarakhovsky A: Immunodeficiency in protein kinase C β-deficient mice. *Science* 273:788–791, 1996
63. Long A, Kelleher D, Lynch S, Volkov Y: Cutting edge: protein kinase C beta expression is critical for export of Il-2 from T cells. *J Immunol* 167:636–640, 2001.