Tumor Necrosis Factor-α in Diabetic Plasma Increases the Activity of Core 2 GlcNAc-T and Adherence of Human Leukocytes to Retinal Endothelial Cells

Significance of Core 2 GlcNAc-T in Diabetic Retinopathy

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A large body of evidence now implicates increased leukocyte-endothelial cell adhesion as a key early event in the development of diabetic retinopathy. We recently reported that raised activity of the glycosylating enzyme core 2 β 1,6-*N*-acetylglucosaminyltransferase (GlcNAc-T) through protein kinase C (PKC)β2-dependent phosphorylation plays a fundamental role in increased leukocyte-endothelial cell adhesion and capillary occlusion in retinopathy. In the present study, we demonstrate that following exposure to plasma from diabetic patients, the human promonocytic cell line U937 exhibits a significant elevation in core 2 GlcNAc-T activity and increased adherence to cultured retinal capillary endothelial cells. These effects of diabetic plasma on enzyme activity and cell adhesion, mediated by PKCβ2-dependent phosphorylation of the core 2 GlcNAc-T protein, were found to be triggered by increased plasma levels of tumor necrosis factor (TNF)-α. Levels of enzyme activity in plasma-treated U937 cells were closely dependent on the severity of diabetic retinopathy, with the highest values observed upon treatment with plasma of patients affected by proliferative retinopathy. Furthermore, we noted much higher correlation, as compared with control subjects, between increased values of core 2 GlcNAc-T activity and cell adhesion properties. Based on the prominent role of TNF-α in the development of diabetic retinopathy, these observations further validate the significance of core 2 GlcNAc-T in the pathogenesis of capillary occlusion, thereby enhancing the therapeutic potential of specific enzyme inhibitors. *Diabetes* 53:2968–2976, 2004

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BREC, bovine retinal capillary endothelial cell; GlcNAc-T, *N*-acetylglucosaminyltransferase; IL, interleukin; PKC, protein kinase C; TNF, tumor necrosis factor.

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Diabetic retinopathy is a progressive vision-threatening complication of diabetes, characterized by capillary occlusion, formation of microvascular lesions, and retinal neovascularization adjacent to ischemic areas of the retina (1–2). The exact pathogenic mechanism by which capillary nonperfusion, as demonstrated by fluorescein angiography, occurs is still unclear, but recent experimental animal studies (3–5) demonstrate that increased leukocyte entrapment in retinal capillaries of diabetic rats is an early event associated with areas of capillary nonperfusion and the development of endothelial cell damage.

We recently reported (6) that increased leukocyte-endothelial cell adhesion and capillary obstruction in diabetic retinopathy are accompanied by a modification of O-linked carbohydrate side chains on the surface of leukocytes. In this respect, mucin-type O-glycans expressed on the cell surface of leukocytes were previously shown to play a crucial role in leukocyte-endothelial cell adherence through selectins and integrins (7,8). The biosynthesis of such carbohydrate side chains is mainly controlled by the Golgi enzyme core 2 1,6-*N*-acetylglucosaminyltransferase (core 2 GlcNAc-T), which substitutes "core 1" O-linked glycans (i.e., Galβ1,3GalNAcα-R) to produce "core 2" structures (i.e., Galβ1,3[GlcNAcβ1,6] GalNAcα-R) (9). We have previously evaluated the importance of core 2 GlcNAc-T in increased leukocyte-endothelial cell adhesion and found significant increases in the activity of this enzyme in leukocytes of diabetic patients (type 1 and type 2), as compared with age-matched healthy control subjects (6). More recently, we demonstrated that the activity of core 2 GlcNAc-T is regulated by posttranslational modification of the enzyme via serine/threonine protein kinase C (PKC)β2-dependent phosphorylation (10). Interestingly, the abnormal activation of PKCβ2 is associated with the development of vascular complications in diabetes (11), and there is evidence that the PKCβ2 inhibitor, LY333531, prevents leukocyte entrapment in retinal capillaries of diabetic rats (12). Previous studies (11,13) focused on the biology of core 2 GlcNAc-T in diabetes have reported the role of glucose in the regulation of enzyme activity. However, there is currently a total lack of information

TABLE 1
Baseline clinical characteristics of type 1 and type 2 diabetic patients

	Type 1 diabetes	Type 2 diabetes
n	22	33
Sex (F/M)	10/12	11/21
Age (years)	43.8 ± 3.2	61.6 ± 1.9
Known duration of diabetes (years)	16.7 ± 1.7	9.4 ± 1.1
Systolic blood pressure (mmHg)	128.3 ± 7.0	147.0 ± 3.5
Diastolic blood pressure (mmHg)	72.9 ± 2.9	82.7 ± 2.2
HbA_{1c} (%)	8.7 ± 0.3	8.7 ± 0.3
BMI (kg/m^2)	27.9 ± 1.6	31.6 ± 1.1
Diagnosed hypertension	4	18
Diabetic retinopathy		
Absent	6	3
NPDR	10	16
PDR	6	14
Diabetic nephropathy		
Absent	19	28
Incipient	1	3
ESRD	2	2
Diabetic neuropathy	5	12
Ischemic heart disease	3	5
Peripheral vascular disease	0	5

Data are means ±SE, unless noted otherwise. ESRD, end-stage renal disease; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

regarding the signal pathway leading to higher levels of enzyme activity in diabetic patients.

In this report, we demonstrate that upregulation of core 2 GlcNAc-T via phosphorylation of the protein is controlled, directly or indirectly, by tumor necrosis factor (TNF)-α. In light of the role played by TNF-α in the pathogenesis of diabetic retinopathy and ocular inflammation (14–19) and, more generally, during the course of the inflammatory cascade (20–23), the functional link between this cytokine and the activity of core 2 GlcNAc-T further validates the role of O-glycans in cell-adhesion events and brings closer the prospect of specific enzyme inhibitors as valid therapeutics for the treatment of overzealous inflammatory responses.

RESEARCH DESIGN AND METHODS

This study included a total of 22 type 1 and 33 type 2 diabetic patients recruited from the Diabetes Outpatient Clinic and the Eye Unit at St. Thomas' Hospital, London, U.K. Age-matched, nondiabetic, healthy control subjects were found among the accompanying relatives of the patients or from hospital employees. Table 1 shows the baseline clinical characteristics of our study diabetic population. The levels of severity of retinopathy were determined according to lesions, mild and moderate nonproliferative retinopathy, macular edema, severe nonproliferative retinopathy, and proliferative retinopathy.

Collection of blood and preparation of plasma. Whole blood was drawn and collected in heparinized tubes. Plasma was isolated after density gradient centrifugation over Ficoll-Paque (Pharmacia, Milton Keynes, U.K.). Briefly, 10 ml of whole blood were layered onto an equal volume of Ficoll-Paque and centrifuged (Heraeus, Brentwood, U.K.) at 400*g* for 30 min. Plasma above the polymorphonuclear leukocyte-rich buffy coat was carefully removed under sterile conditions and stored at 4°C until used for experiments.

Culture of bovine retinal capillary endothelial cells. Bovine retinal capillary endothelial cells (BRECs) were established from bovine retinas dissected from eyes of freshly slaughtered cattle as described previously (24).

Culture of human myelocytic cell line (U937 cells). This leukocytic cell line (25) was cultured in glucose-free RPMI medium (Sigma, Poole, U.K.), as previously described (10). For experimentation, the cells were centrifuged, washed in PBS, and exposed to 5% plasma in RPMI from either diabetic patients or age-matched control subjects. In some experiments, the PKCβ2-inhibitor, LY379196 (Eli Lilly, Indianapolis, IN), was added at a concentration of 50 nmol/l during incubation with plasma. After a 24-h incubation at 37°C, the cells were centrifuged and used for the measurement of core 2 GlcNAc-T activity and leukocyte-endothelial cell adhesion assay.

In some experiments, U937 cells were exposed to plasma from diabetic patients and age-matched control subjects following a 10-min pretreatment with a TNF-α antibody (Chemicon, Hampshire, U.K.). In other experiments, U937 cells were exposed to various concentrations of human recombinant TNF-α (Chemicon). After a 24-h incubation, the level of core 2 GlcNAc-T activity and leukocyte-endothelial cell adhesion were determined.

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% NaCl and 0.4% Triton X-100 at 0°C. The activity of core 2 GlcNAc-T was then measured as described previously (6). The 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 of cell protein. In each case, the protein concentration was determined using the BCA (bicinchoninic acid) protein assay kit (Sigma, Poole, U.K.).

Leukocyte-endothelial cell adhesion assay. Adhesion of the U937 cell line to bovine endothelial cells was examined via (5,6)-carboxyfluorescein diacetate succinyimidyl ester labeling (Molecular Probe, Eugene, OR) according to our established method (10). Results were expressed as the percentage of adherent leukocytes per field (×100) by fluorescence microscopy.

Measurement of PKC activity. Total PKC activity was measured in cell extracts using a PKC assay kit (Life Technologies, Paisley, U.K.) according to the manufacturer's instructions. The assay is based on the measurement of phosphorylation of myelin basic protein (26).

Immunoprecipitation and immunoblot analysis. For core 2 GlcNAc-T immunoprecipitation, as well as for Western blots, a human, polyclonal antibody was used against the core 2 GlcNAc-T protein (27). Immunoprecipitation, Western blot analysis, and quantification of the level of serine phosphorylation of core 2 GlcNAc-T were carried out as described previously (10).

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. An unpaired 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 are expressed as means ± SE of measurements in the different experiments. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Activity of core 2 GlcNAc-T is modulated by diabetic plasma. To identify possible factors associated with the modulation of core 2 GlcNAc-T activity, we initially treated U937 cells with plasma of diabetic patients before determining enzyme activity and, in parallel, cell adhesion properties. In all instances, we used plasma from age-matched healthy subjects as a control. A 24-h exposure to 5% diabetic plasma (type 1 and type 2) was followed by a sevenfold increase in mean core 2 GlcNAc-T activity (Fig. 1*A*) and a fivefold increase in leukocyte-endothelial cell adhesion (Fig. 1*B*).

Plasma from diabetic patients with retinopathy (nonproliferative and proliferative) consistently induced higher levels of core 2 GlcNAc-T activity (Fig. 2*A*), as well as greater leukocyte-endothelial cell adhesion (Fig. 2*B*), when compared with plasma from patients without retinopathy. Additionally, a close correlation was also observed between the effects of diabetic plasma on the activity of core 2 GlcNAc-T (Fig. 2*C*) and the extent of leukocyte-endothelial cell adhesion (Fig. 2*D*) in relation to the severity of diabetic retinopathy. Furthermore, additional data analysis confirmed that in diabetic plasma-treated U937 cells, elevated core 2 GlcNAc-T activity correlated well with enhanced leukocyte-endothelial cell adhesion (Fig. 2*E*). On the contrary, random values of core 2 GlcNAc-T activity obtained from control studies, ranging between <50 and 500 $pmol \cdot mg^{-1} \cdot h^{-1}$, showed minimal

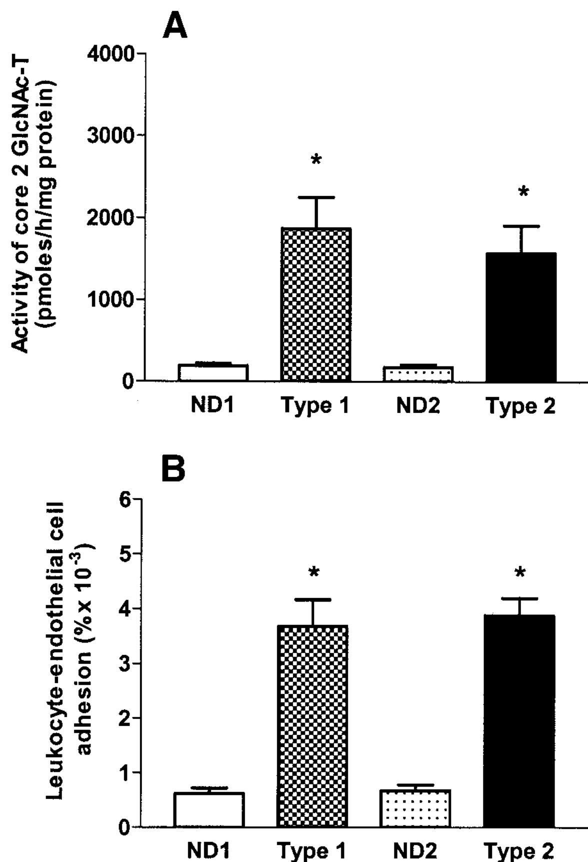

FIG. 1. Plasma from diabetic patients increases the activity of core 2 GlcNAc-T and adherence of human leukocytes to retinal capillary endothelial cells. ***A*: Human leukocytes (U937) were exposed to growth medium containing 5% plasma from diabetic patients (type 1 diabetes, $n = 22$, and type 2 diabetes, $n = 33$) and age-matched, nondiabetic, healthy control subjects (ND1 for type 1 diabetic patients, $n = 22$; ND2 for type 2 diabetic patients, $n = 23$) for 24 h. Activity of core 2 GlcNAc-T was measured in lysates. Data are means ± SE. *$P < 0.0009$. *B*: Effect of plasma from diabetic patients (type 1 diabetes, $n = 19$, and type 2 diabetes, $n = 30$) and age-matched healthy control subjects (ND1, $n = 16$, and ND2, $n = 15$) on the adherence of carboxyfluorescein-labeled leukocytes to cultured BRECs. Labeled leukocytes were added to a confluent monolayer of BRECs for 1 h. After washing, the number of adherent cells was counted using a fluorescence microscope. Data are presented as means ± SE. *$P < 0.0001$.**

correlation with the adhesive properties of the cell line (Fig. 2*F*).

Effect of the PKCβ2 inhibitor, LY379196, on plasma-induced core 2 GlcNAc-T activity. To confirm the role of PKC signaling pathway(s) in diabetic plasma-induced activity of core 2 GlcNAc-T, we used the specific PKCβ2 inhibitor, LY379196 (11). At a concentration of 50 nmol/l that was previously shown (28) to specifically inhibit the β-isoform of PKC, LY379196 caused a significant reversal of both diabetic plasma-induced activity of core 2 GlcNAc-T (Fig. 3*A*) and leukocyte-endothelial cell adhesion (Fig. 3*B*).

Role of TNF-α in diabetic plasma-induced activity of core 2 GlcNAc-T. Based on the higher levels of TNF-α in plasma of diabetic patients compared with control subjects (29–31), we investigated a possible involvement of this cytokine in controlling the activity of the core 2 enzyme. The addition of plasma from diabetic patients (type 1 and type 2) significantly induced the activity of core 2 GlcNAc-T in human leukocytes (U937 cells) after 24 h of exposure (Fig. 4*A*).

The role of TNF-α in the control of core 2 GlcNAc-T activity was initially revealed by the action of a specific TNF-α polyclonal antibody that was incubated with U937 cells 10 min before addition of diabetic plasma. Presence of the TNF-α antibody completely blocked the induction of core 2 GlcNAc-T activity (Fig. 4*A*); consequently, the adhesive properties of the U937 cell line could not be distinguished from those of control subjects (Fig. 4*B*).

Conversely, incubation of human recombinant TNF-α with U937 cells for 24 h was followed by a dose-dependent increase of core 2 GlcNAc-T activity to the levels observed upon treatment with diabetic plasma (Fig. 5*A*). A similar trend was revealed by the corresponding cell adhesion studies (Fig. 5*C*). The effect of recombinant TNF-α was however significantly reduced at higher concentrations, likely due to the occurrence of other cellular responses evoked by the cytokine (Fig. 5*A*). Activation of core 2 GlcNAc-T was maximal within 2 h of incubation in the presence of 8 pg/ml TNF-α (Fig. 5*B*) and required PKC activity, as shown by the fact that 50 nmol/l LY379196 significantly reversed both TNF-α–induced activity of core 2 GlcNAc-T (Fig. 5*C*) and leukocyte-endothelial cell adhesion (Fig. 5*D*).

Plasma-induced PKCβ2-dependent phosphorylation of core 2 GlcNAc-T. To confirm the occurrence of a phosphorylation step associated with the TNF-α–mediated induction of core 2 GlcNAc-T activity, Western blot analyses were performed in series using an anti-phosphoserine polyclonal antibody and polyclonal anti–core 2 GlcNAc-T antibody raised against the human protein (Fig. 6*A*). Serine phosphorylation of core 2 GlcNAc-T was increased in U937 cells treated with diabetic plasma compared with the effect of plasma from age-matched healthy control subjects (Fig. 6*B*). Moreover, phosphorylation of core 2 GlcNAc-T was significantly attenuated by the addition of 50 nmol/l LY379196 (Fig. 6*B*). In order to reveal additional evidence of a link between TNF-α and PKC and core 2 GlcNAc-T, we incubated U937 cells for 24 h with human recombinant TNF-α (8 pg/ml), after which a significant increase was found in the phosphorylation of core 2 GlcNAc-T protein (Fig. 6*C*).

Activation of PKC in leukocytes by diabetic plasma and TNF-α. The marked inhibition of diabetic plasma–induced activity of core 2 GlcNAc-T by LY379196 suggested that the PKCβ2 signaling pathway is involved in the regulation of core 2 GlcNAc-T activity. To confirm activation of PKC in leukocytes exposed to diabetic plasma, we measured phosphorylation of myelin basic protein. Diabetic plasma and 8 pg/ml of human recombinant TNF-α increased the total PKC activity in human leukocytes (U937 cells), and this was significantly reversed by the addition of 50 nmol/l LY379196 (Table 2).

DISCUSSION

There is growing evidence that increased leukocyte-endothelial cell adhesion in the retina is closely associated with vascular nonperfusion and other events leading to the development of diabetic retinopathy (4–6). We recently reported (10) that glucose-mediated upregulation of core 2 GlcNAc-T activity through PKCβ2-dependent phosphorylation contributes to increased leukocyte-endothelial cell adhesion and capillary occlusion in retinopathy. The

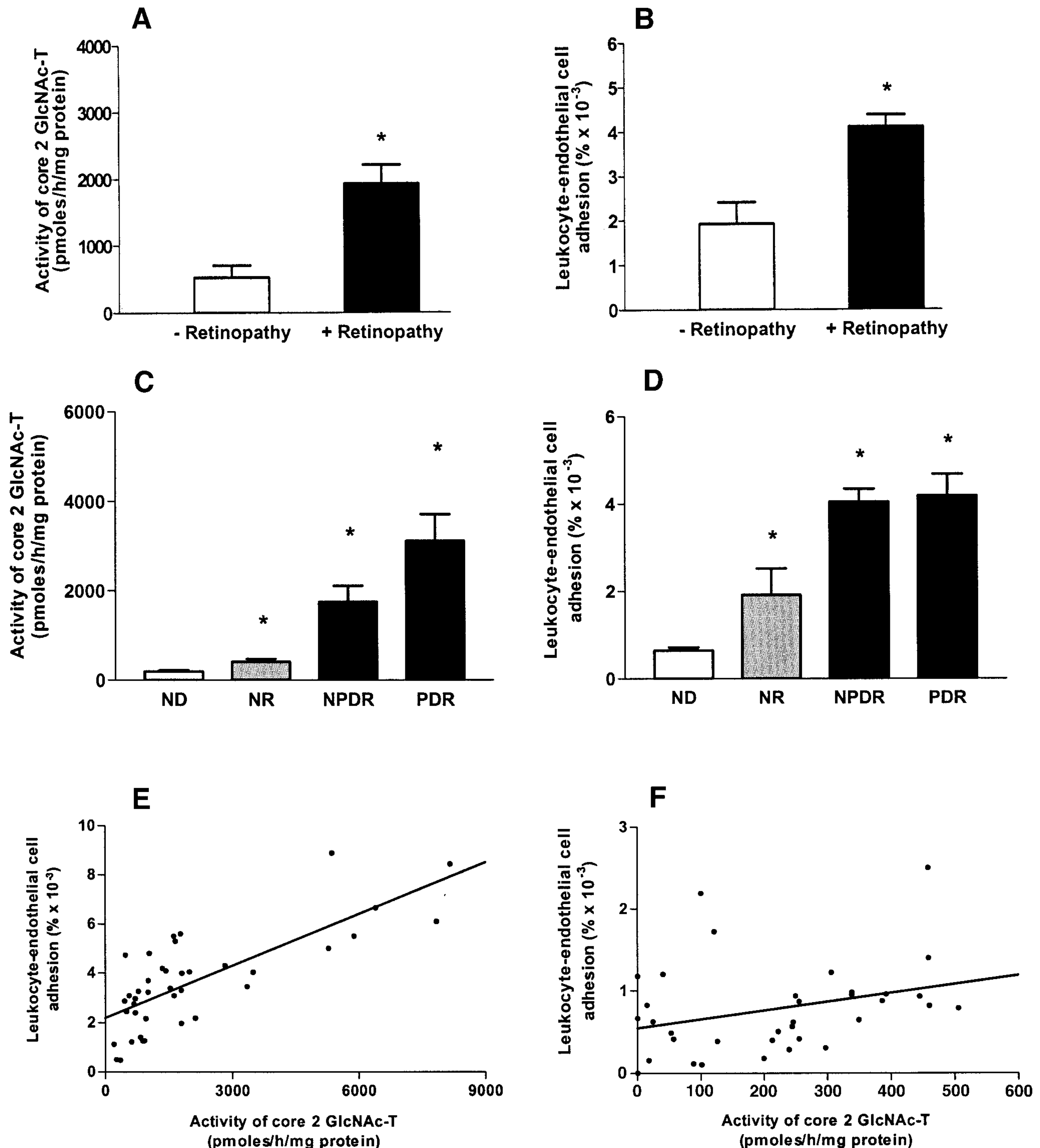

FIG. 2. Plasma-induced activity of core 2 GlcNAc-T correlates with presence of retinopathy. *A*: Core 2 GlcNAc-T activity in human leukocytes (U937 cells) exposed to plasma from diabetic patients (type 1 and type 2) with (+) retinopathy (nonproliferative and proliferative; $n = 47$) and without (-) retinopathy ($n = 9$). Data are means ± SE. *$P < 0.004$. *B*: Adhesion of carboxyfluorescein-labeled leukocytes exposed to plasma from diabetic patients (type 1 and type 2) with (+) retinopathy (nonproliferative and proliferative retinopathy, $n = 42$) to cultured BRECs. Labeled leukocytes were added to a confluent monolayer of BRECs for 1 h. After washing, the number of adherent cells was counted under a fluorescence microscope. Data are means ± SE. *$P < 0.001$. *C*: Activity of core 2 GlcNAc-T in human leukocytes (U937 cells) induced by plasma of patients with different severities of diabetic retinopathy (ND, nondiabetic healthy control subjects, $n = 45$; NR, no retinopathy, $n = 9$; NPDR, nonproliferative diabetic retinopathy, $n = 26$; and PDR, proliferative diabetic retinopathy, $n = 20$). Data are means ± SE. *$P = 0.0003$. *D*: Adhesion of human leukocytes (U937 cells) induced by plasma of patients with different severities of diabetic retinopathy (ND, nondiabetic healthy control subjects, $n = 31$; NR, no retinopathy, $n = 7$; NPDR, nonproliferative diabetic retinopathy, $n = 24$; and PDR, proliferative diabetic retinopathy, $n = 18$). Data are means ± SE. *$P = 0.0002$. *E*: Correlation between activity of core 2 GlcNAc-T and leukocyte-endothelial cell adhesion induced by plasma from diabetic patients (type 1 and type 2, $n = 39$; $r = 0.756$, $P < 0.0001$). *F*: Relationship between activity of core 2 GlcNAc-T and leukocyte-endothelial cell adhesion induced by plasma from age-matched healthy control subjects ($n = 35$; $r = 0.301$, $P < 0.079$).

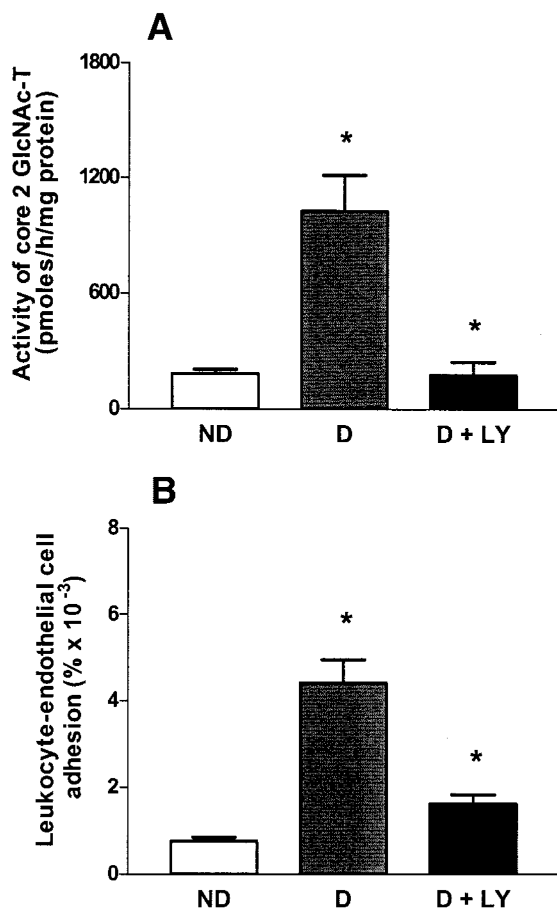

FIG. 3. Specific inhibitor of PKCβ2, LY379196, prevents plasma-induced activity of core 2 GlcNAc-T and adherence of leukocytes to endothelial cells. *A*: Human leukocytes (U937 cells) were exposed to medium containing 5% plasma from type 1 and type 2 diabetic patients (D) and age-matched nondiabetic control subjects (ND) for 24 h in the presence (D + LY) of LY379196 (50 nmol/l). Activity of core 2 GlcNAc-T was measured in lysates. Data are means ± SE ($n = 18$). *$P = 0.002$. *B*: Effect of LY379196 (50 nmol/l) on diabetic plasma-induced adhesion of carboxyfluorescein-labeled leukocytes (U937 cells) to cultured BRECs. Cells were exposed to medium containing 5% plasma from type 1 and type 2 diabetic patients (D) and age-matched nondiabetic control subjects (ND) for 24 h in the presence (D + LY) of LY379196 (50 nmol/l). Labeled leukocytes were added to a confluent monolayer of BRECs for 1 h. After washing, the number of adherent cells was counted under a fluorescence microscope. Data are means ± SE ($n = 18$). *$P < 0.0001$.

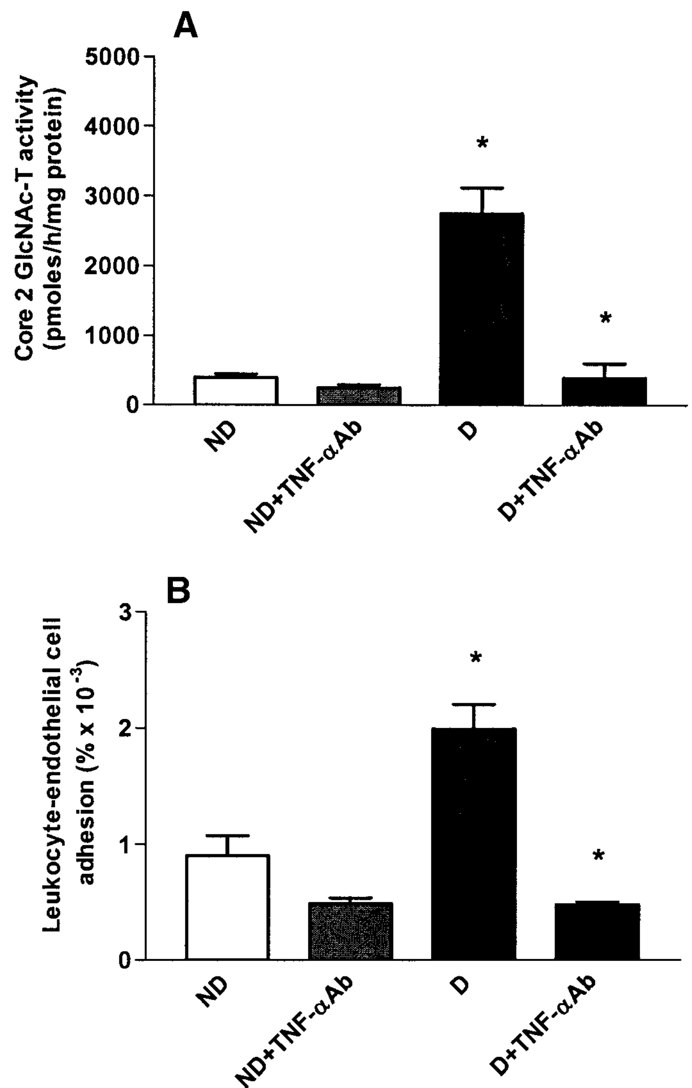

FIG. 4. TNF-α in diabetic serum induces activity of core 2 GlcNAc-T and leukocyte-endothelial cell adhesion. *A*: Human leukocytes (U937 cells) were exposed to plasma from type 1 and type 2 diabetic patients (D) and age-matched nondiabetic healthy control subjects (ND) after a 10-min pretreatment with TNF-α antibody (D + TNF-αAb and ND + TNF-αAb). After 24 h of incubation, the activity of core 2 GlcNAc-T was measured in lysates. Data are means ± SE ($n = 5$). *$P < 0.002$. *B*: Effect of plasma from type 1 and type 2 diabetic patients (D, $n = 8$) and age-matched nondiabetic healthy control subjects (ND, $n = 31$) after pretreatment with TNF-α antibody (D + TNF-αAb and ND + TNF-αAb) on the adherence of carboxyfluorescein-labeled leukocytes to cultured BRECs. Labeled leukocytes were added to a confluent monolayer of BRECs for 1 h. After washing, the number of adherent cells was counted under a fluorescence microscope. Data are means ± SE. *$P < 0.002$.

present study shows that leukocytic cells exhibit significant increases in core 2 GlcNAc-T activity when treated with plasma from type 1 and type 2 diabetic patients due to the same phosphorylation-based mechanism (10). Leukocyte-endothelial cell adhesion is likewise enhanced.

As a model of circulating human leukocytes, we used U937 cells, a human leukocyte cell line, which under normal culture conditions express levels of core 2 GlcNAc-T similar to those observed in normal human leukocytes (32–34). These cells did not show any morphologic changes under our experimental conditions, thereby ruling out differentiation as a cause of modulation of core 2 GlcNAc-T activity.

Rather, elevation in enzyme activity compared well with physiological changes that lead to cell adhesion events observed in normal white cells upon activation (32–34).

The strong correlation between severity of retinopathy and plasma-induced core 2 GlcNAc-T activity suggests that one or more systemic factors associated with progression of retinopathy lead to the sharp elevation of enzyme activity in leukocytes of type 1 and type 2 diabetic patients. Since anti-human TNF-α antibody completely counteracts the effect of diabetic plasma, we postulate an important role for this cytokine in modulating the activity of core 2 GlcNAc-T. This is further supported by the direct effect of human recombinant TNF-α on core 2 GlcNAc-T activity and leukocyte-endothelial cell adhesion. Because TNF-α is thought to be involved in the pathogenesis of diabetic retinopathy and ocular inflammation (14–19), the direct functional link between this cytokine and core 2 GlcNAc-T

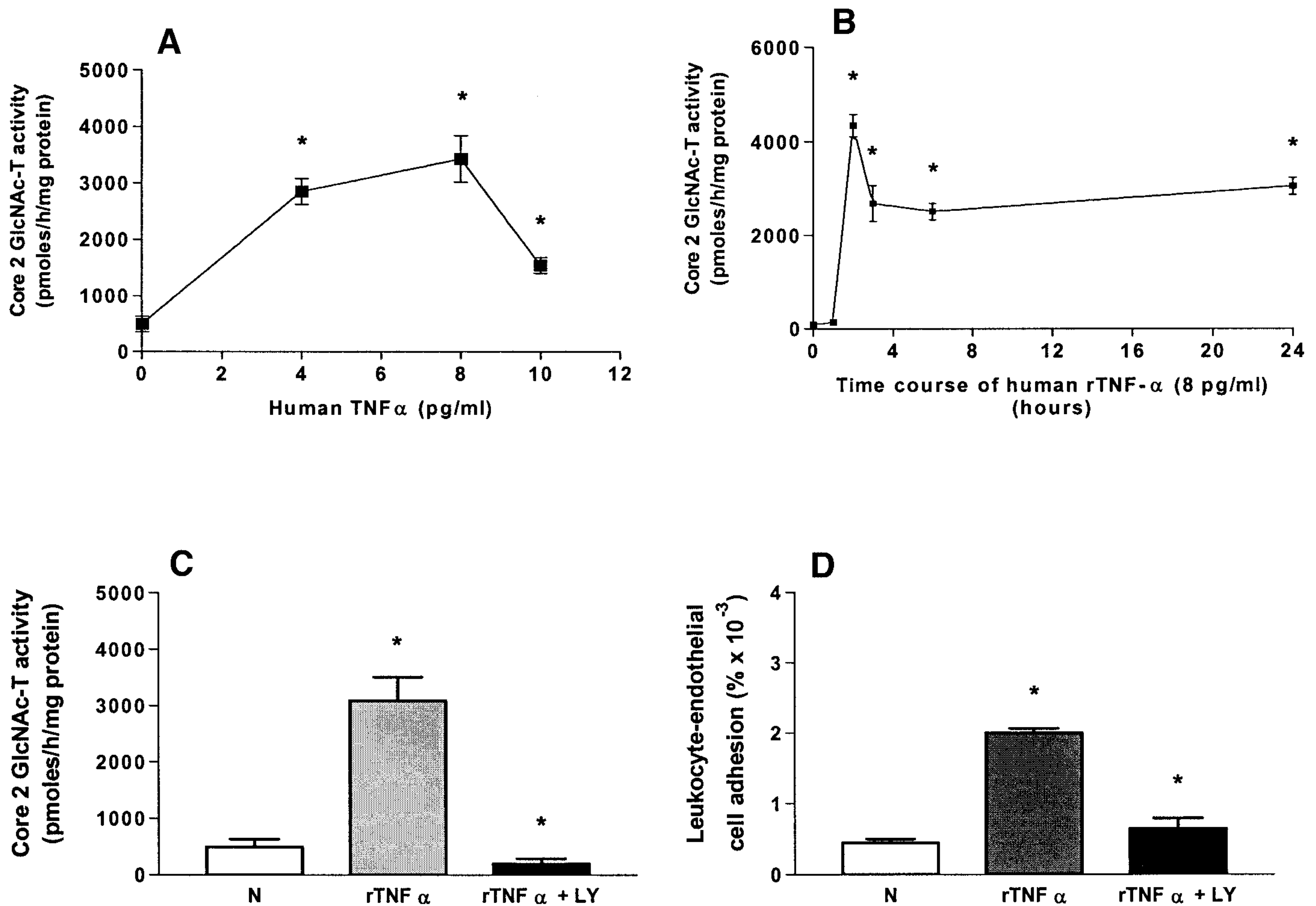

FIG. 5. Human recombinant TNF-α increases the activity of core 2 GlcNAc-T and leukocyte-endothelial cell adhesion. *A*: Effect of human recombinant TNF-α (rTNF-α) on the activity of core 2 GlcNAc-T in human leukocytes (U937 cells). The cells were exposed to different concentrations (0, 4, 8, and 10 pg/ml) of rTNF-α for 24 h at 37°C. Activity of core 2 GlcNAc-T was measured in lysates. Data are means ± SE (n = 5). **P* < 0.004. *B*: Time-dependent effect of human rTNF-α on the activity of core 2 GlcNAc-T in U937 cells. The cells were exposed to 8 pg/ml of rTNF-α for 0, 1, 2, 3, 6, and 24 h at 37°C. Data are means ± SE (n = 5). **P* < 0.009. *C*: Effect of LY379196 on rTNF-α–induced activity of core 2 GlcNAc-T. The cells were exposed to 8 pg/ml of TNF-α in the presence and absence of 50 nmol/l LY379196 for 24 h at 37°C, and activity of core 2 GlcNAc-T was measured in lysates. Data are means ± SE (n = 5). *P* < 0.002. *D*: Effect of LY379196 on rTNF-α–induced adherence of carboxyfluorescein-labeled leukocytes to cultured BRECs. The cells were exposed to 8 pg/ml of rTNF-α in the presence and absence of 50 nmol/l LY379196 for 24 h at 37°C. Labeled leukocytes were added to a confluent monolayer of BRECs for 1 h. After washing, the number of adherent cells was counted under a fluorescence microscope. Data are means ± SE. *P* < 0.001.

activity suggests important implications for the physiological role of core 2 branched O-glycans in the development and/or progression of this pathological condition. TNF-α, a monocyte/macrophage-derived proinflammatory factor, is produced by macrophages and other cell types in response to various stimuli (35,36). TNF-α exerts a variety of biological effects, including upregulation of adhesion molecules, proliferation, differentiation, and cell death (37).

Others have observed that the production of TNF-α is significantly increased during long-term hyperglycemia in spontaneously diabetic rats and mice, as well as in streptozotocin-induced diabetic rats (38,39). Furthermore, although levels of TNF-α in the blood of diabetic patients vary (30–32), a strong correlation between plasma levels of TNF-α and severity of diabetic retinopathy has been described (19). Raised plasma levels of TNF-α can cause induction of proinflammatory cytokines and adhesion molecules and thereby increase monocyte-endothelial cell adhesion, which is now accepted as the key early event in the development of vascular disease and arteriosclerosis (40).

The source of TNF-α in human plasma has yet to be determined; however, it has been shown (41) that macrophages from diabetic patients release more TNF-α than control macrophages. Furthermore, high glucose can activate monocytes and induce the expression of TNF-α via oxidant stress and nuclear factor-κB transcription factor (42).

Our studies with the PKC inhibitor LY379196 suggest that TNF-α induces the activation of PKCβ2 in U937 cells. In this regard, TNF-α has been shown (43) to cause changes in the activities of different PKC isoforms in other cell types. For example, Radeff et al. (44) demonstrated the potential of TNF-α to induce the translocation of PKCβ to the plasma membrane in UMR-106 rat osteoblastic cells, while such translocation was inhibited by LY379196.

Our observation that TNF-α can regulate core 2 GlcNAc-T activity in U937 cells through PKCβ2-dependent

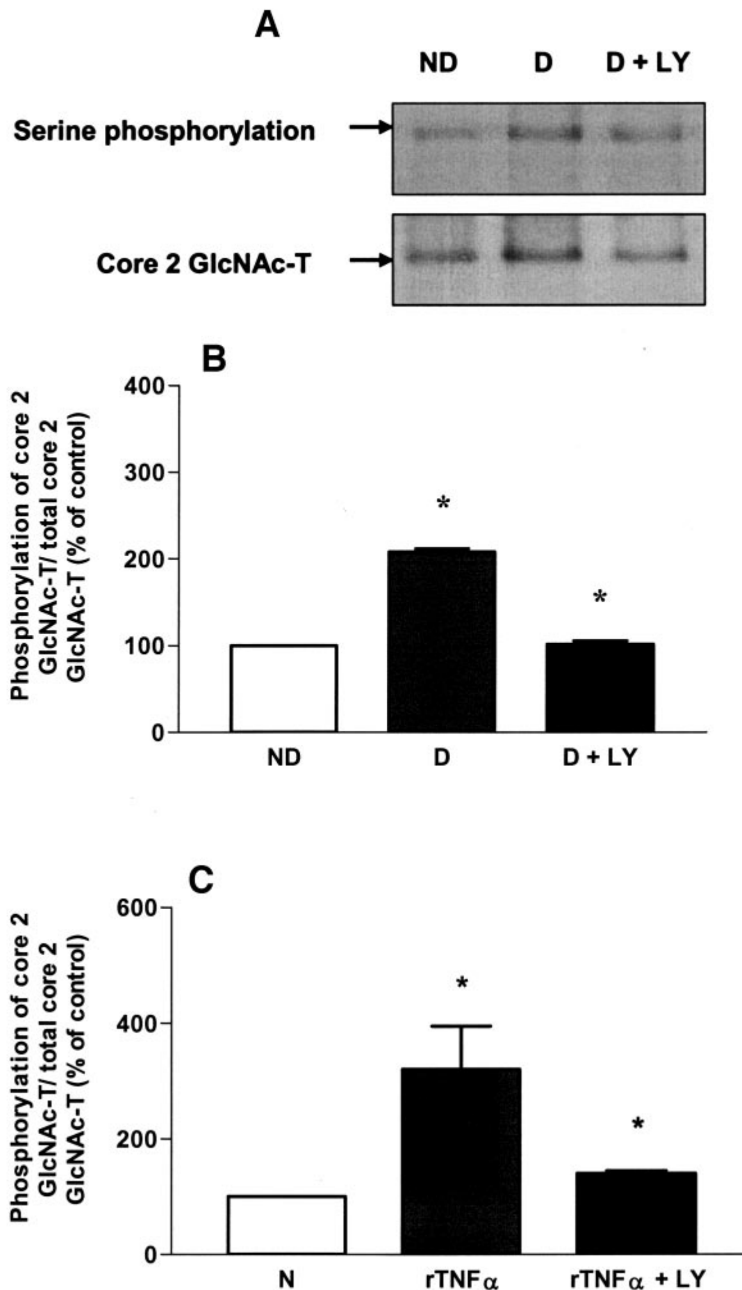

FIG. 6. Diabetic plasma increases serine phosphorylation of core 2 GlcNAc-T in leukocytes. *A*: After exposure to plasma from nondiabetic healthy control subjects (ND) and diabetic patients in the presence (D + LY) and absence (D) of 50 nmol/l LY379196 for 24 h at 37°C, core 2 GlcNAc-T was immunoprecipitated and then immunoblotted using anti-phosphoserine as the primary antibody. The same blots were then reprobed with anti–core 2 GlcNAc-T antibody. Pilot experiments showed proportionality of the signal with amounts of enzyme protein under the experimental conditions used. *B*: Level of phosphorylation of core 2 GlcNAc-T in leukocytes exposed to 5% plasma from nondiabetic healthy control subjects (ND), diabetic plasma (D), and diabetic plasma in the presence (D + LY) of 50 nmol/l LY379196. Levels of phosphorylation are expressed using arbitrary units obtained from densitometric analyses of Western blots. Each value is the ratio between the intensity of phosphorylation and the intensity of the core 2 GlcNAc-T immunoreactive band. Data are means ± SE (n = 6). *P < 0.0001. *C*: Medium supplemented with 8 pg/ml human recombinant TNF-α for 24 h in the absence and presence of 50 nmol/l LY379196. Data are the expressed as described in *B* and represent the mean ± SE (n = 3). *P < 0.05.

phosphorylation is consistent with previous evidence for posttranslational control of core 2 GlcNAc-T activity (28,45). Furthermore, a number of studies have shown (46) that TNF-α mediates a number of endothelial cell responses via PKC activation. It is conceivable that glucose (11,14) and TNF-α mediate higher core 2 GlcNAc-T activity through the same mechanism. We propose that glucose likely causes an increase in TNF-α levels (30–32), which then mediates the engagement of PKCβ2 and the subsequent posttranslational modification of the core 2 GlcNAc-T protein. Elevated D-glucose and TNF-α may activate PKCβ2 through intracellular production of reactive oxygen species in human leukocytes (47).

In summary, our results suggest that TNF-α present in the plasma of diabetic patients leads to upregulated levels of the Golgi enzyme core 2 GlcNAc-T via posttranslational modification of the protein following PKC phosphorylation. Because TNF-α is thought to be involved in the progression of diabetic retinopathy and ocular inflammation (15–20), our finding that TNF-α raises the activity of core 2 GlcNAc-T suggests the physiological relevance of O-linked oligosaccharides in the development and/or progression of this pathological condition. In this respect, it is possible that leukocyte-endothelial cell adhesion in the retina is mediated by P-selectin glycoprotein ligand-1, a predominant ligand for selectins, which requires decoration with core 2 branched O-glycans for proper function (48–50). This would be consistent with the altered O-glycosylation pattern on P-selectin glycoprotein ligand-1 that was previously shown in U937 cells upon elevation of core 2 GlcNAc-T activity (11). Finally, our results suggest a novel therapeutic strategy to prevent retinopathy, based on the use of bioavailable, specific core 2 GlcNAc-T inhibitors. These could be screened and further evaluated

TABLE 2
Plasma from diabetic patients and TNF-α activate total PKC activity in leukocytes

Treatment	Total PKC activity (pmol · h^{-1} · mg $protein^{-1}$)
Nondiabetic plasma	4.29 ± 0.51 (n = 3)
Diabetic plasma	9.29 ± 0.70 (n = 3)*
Diabetic plasma + 50 nmol/l LY379196	3.79 ± 0.20 (n = 3)†
Control medium	3.01 ± 1.22 (n = 4)
TNF-α (8 pg/ml)	15.50 ± 3.34 (n = 4)‡
TNF-α (8 pg/ml) + 50 nmol/l LY379196	3.93 ± 0.97 (n =)§

Data are means ± SE. Human leukocytes (U937) were exposed to 5% plasma from nondiabetic healthy control subjects (n = 3), diabetic patients in the presence (n = 3) and absence (n = 3) of 50 nmol/l LY379196. After 24 h of incubation, PKC activity was measured. Cells were also exposed to RPMI growth medium containing 8 pg/ml TNF-α in the presence and absence of 50 nmol/l LY379196. After 24 h of incubation, total PKC activity was measured. *P = 0.0.0045 vs. nondiabetic plasma, †P = 0.019 vs. diabetic plasma; ‡P = 0.012 vs. control medium; §P = 0.016 vs. TNF-α treatment.

via our U937 cell-based system that may be readily adapted to high-throughput screening platforms.

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