

# Induction of Endothelin-1 Expression by Glucose

## An Effect of Protein Kinase C Activation

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Enhanced actions or levels of endothelin-1 (ET-1), a potent vasoconstrictor, have been associated with decreased blood flow in the retina and peripheral nerves of diabetic animals and may be related to the development of pathologies in these tissues. Hyperglycemia has been postulated to increase ET-1 secretion in endothelial cells. We have characterized the mechanism by which elevation of glucose is increasing ET-1 mRNA expression in capillary bovine retinal endothelial cells (BREC) and bovine retinal pericytes (BRPC). Elevation of glucose, but not mannitol, from 5.5 to 25 mmol/l for 3 days increased membranous protein kinase C (PKC) activities and ET-1 mRNA in parallel levels by 2-fold in BREC and BRPC. These effects were reversed by decreasing glucose levels to 5.5 mmol/l for an additional 2 days. Glucose-induced ET-1 overexpression was inhibited by a general PKC inhibitor, GF109203X, and a mitogen-activated protein kinase kinase inhibitor, PD98059, but not by wortmannin, a phosphatidylinositol 3-kinase inhibitor. By immunoblot analysis, PKC- $\beta$ 2 and - $\delta$  isoforms in BREC were significantly increased relative to other isoforms in the membranous fractions when glucose level was increased. Overexpression of PKC- $\beta$ 1 and - $\delta$  isoforms but not PKC- $\zeta$  isoform by adenovirus vectors containing the respective cDNA enhanced in parallel PKC activities, proteins, and basal and glucose-induced ET-1 mRNA expression by at least 2-fold. These results showed that enhanced ET-1 expression induced by hyperglycemia in diabetes is partly due to activation of PKC- $\beta$  and - $\delta$  isoforms, suggesting that inhibition of these PKC isoforms may prevent early changes in diabetic retinopathy and neuropathy. *Diabetes* 49:1239–1248, 2000

**A**bnormal hemodynamic changes such as blood flow and contractility have been observed in many organs of diabetic animals and patients, especially in the retina and microvessels of peripheral nerves (1). Decreased blood flow to the retina and peripheral nerves has been reported in early diabetic retinopathy (2–4) and neuropathy (5,6) and could be related to protein kinase C (PKC) activation in vascular cells. Intravitreal injection of phobol esters and PKC agonists can induce PKC activation and decrease retinal blood flow in the retina (7). In addition, PKC inhibitors may normalize retinal and nerve blood flow abnormalities in diabetic animals (6–8), supporting the hypothesis that PKC activation may play an important role in the pathogenesis of hemodynamic abnormalities in the retina and peripheral nerves.

One possible consequence of PKC activation induced by diabetes, which can lead to decreased blood flow in the retina and peripheral nerves, is an increase of endothelin (ET)-1 expression and activities. ET-1, an endothelium-derived vasotropic cytokine that can bind to 3 distinct types of endothelin receptors (termed ET-A, ET-B, and ET-C), has been studied in detail (9–12). In the diabetic state, multiple reports have suggested that ET-1 expression or action may be altered. Previously, we have reported that intravitreal application of ET-1 caused sustained retinal vasoconstriction and decreased blood flow in nondiabetic rats (13). In addition, it has been reported that ET-1 gene expression is increased in diabetic rat retina, which may be responsible for the decrease in retinal blood flow in diabetic animals because the ET-A receptor antagonist BQ-123 prevented the hemodynamic changes (14,15). Neurovascular dysfunctions in diabetic rats have also been reported to be prevented by the ET-A antagonist (16).

The levels of preproET-1 mRNA can be regulated by various chemical and mechanical stimuli such as thrombin, angiotensin II, vasopressin, transforming growth factor- $\beta$ ,  $Ca^{2+}$  ionophore, insulin, tumor necrosis factor- $\alpha$ , hypoxia, and hemodynamical shear stress (17). PKC activation is involved in basal and agonist-induced ET-1 synthesis or secretion in different cell types (18–22). Yamada and Yokoda (19) have reported that PKC activation by phobol 12-myristate 13-acetate increased and PKC inhibition decreased ET-1 release from human aortic and pulmonary endothelial cells. Emori et al. (20) showed that the PKC inhibitors H-7 and staurosporin decreased angiotensin- and vasopressin-induced ET-1 release from bovine endothelial cells.

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AP-1, activated protein 1; BREC, bovine retinal endothelial cells; BRPC, bovine retinal pericytes; BSA, bovine serum albumin; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; EBM, endothelial cell basal medium; EIA, endothelin enzyme immunoassay; ET, endothelin; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PKC, protein kinase C.

In diabetes, hyperglycemia is the most important risk factor for the development of diabetic microvascular complications. Intensive glycemic control as reported in the Diabetes Control and Complications Trial (23) and U.K. Prospective Diabetes Study (24,25) has been shown to delay the onset and progression of diabetic complications, including retinopathy and neuropathy. We have previously reported (7,8,26–29) that diabetes and hyperglycemia can activate different PKC isoforms depending on the source of vascular tissues. PKC- $\beta$  and possibly PKC- $\delta$  isoform activation have been associated with multiple biochemical and functional changes in the vascular cells and tissues in diabetic animals (1,28). Furthermore, a PKC- $\beta$  isoform-specific inhibitor (LY333531) prevented vascular dysfunctions, including glomerular hyperfiltration, increased albumin excretion rate, and decreased retinal blood flow, in diabetic rats (8). In cultured vascular cells, increasing glucose levels have been shown to increase the secretion of ET-1 protein (30).

Thus, ET-1 clearly has potent vascular effects in the retina, and its action and expression are increased in the diabetic state. In this study, we have characterized the mechanism by which glucose increases ET-1 expression in the retinal vascular cells.

## RESEARCH DESIGN AND METHODS

**Materials.** Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and calf serum were purchased from Gibco BRL (Grand Island, NY). Endothelial cell basal medium (EBM) was purchased from Clonetics (San Diego, CA). Tri-Reagent was purchased from Molecular Research Center (Cincinnati, OH). The endothelin enzyme immunoassay (EIA) kit was purchased from Biomedica (Biomedica Gruppe, Vienna, Austria). Anti-PKC- $\alpha$ , - $\beta$ 1, - $\beta$ 2, - $\delta$ , - $\epsilon$ , and - $\zeta$  antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [ $\alpha$ - $^{32}$ P]dCTP and [ $\gamma$ - $^{32}$ P]ATP were purchased from Du Pont-NEN (Boston, MA). The following items were also purchased: polyvinylidene difluoride membrane from Novex (San Diego, CA), nylon membrane from ICN Biomedicals (Aurora, OH), multiprime DNA labeling system and enhanced chemiluminescence kit from Amersham Life Sciences (Arlington Heights, IL), bisindolylmaleimide I (GF109203X) and 2'-amino-3'-methoxyflavone (PD98059) from Calbiochem-Novabiochem (La Jolla, CA), protein A-Sepharose 6MB from Pharmacia Biotech AB (Uppsala, Sweden), protein assay kit from Bio-Rad (Hercules, CA), phosphocellulose squares (P-81) from Whatman Institute (Maidstone, U.K.), and plasmid maxi kit from Qiagen (Valencia, CA). PKC- $\beta$  isoform inhibitor, (S)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1H,13H-dibenzo[e,k]pyrrolo[3,4-h][1,4,3]oxadiazacyclohexadecene-1,3(2H)-dione (LY333531) was provided by Lilly Research Laboratories (Indianapolis, IN) (8). All other materials were from Fisher Scientific (Pittsburgh, PA) and Sigma (St. Louis, MO).

**Cell culture.** Primary cultures of bovine retinal endothelial cells (BREC) and bovine retinal pericytes (BRPC) were isolated from fresh bovine eyes by homogenization and a series of filtration steps as described previously. The purities of BREC and BRPC were determined to be >95% by immunostaining with the von Willebrand factor and 3G5 antibody (31,32). BREC were subsequently cultured with EBM supplemented with 10% plasma-derived horse serum, heparin (50 mg/l), and 50  $\mu$ g/ml endothelial cell growth factor. BRPC were cultured in DMEM with 5.5 mmol/l glucose and 20% fetal bovine serum. The cells were used within 10 passages for the following studies. All cells were cultured at 37°C in 5% CO<sub>2</sub>, 95% air, and media were changed every 2–3 days. Subconfluent BREC or BRPC were exposed to normal growth media containing indicated concentrations of glucose or mannitol. After incubating for various periods of time, the cells were incubated in DMEM containing 1% calf serum with the same concentration of glucose for another 24 h and then used in the assay.

**RNA extraction and Northern blot analysis of ET-1 mRNA expression.** Total RNA was extracted from the cells by the guanidium thiocyanate method with phenol-chloroform using Tri-Reagent (33). Total RNA (30  $\mu$ g for BRPC, 20  $\mu$ g for BREC) was loaded on a 1% agarose gel with 9% formaldehyde, which was separated in MOPS buffer (20 mmol/l MOPS, 5 mmol/l Na acetate, and 0.5 mmol/l EDTA, pH 7.0). The RNA was transferred onto a nylon membrane. After ultraviolet cross-linking, the membranes were prehybridized and hybridized to a  $^{32}$ P-labeled bovine preproET-1 cDNA probe prepared by the

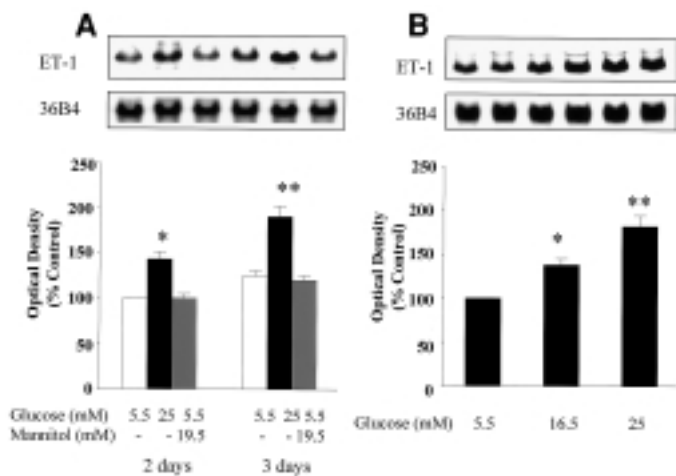
multiprime DNA labeling system in 0.1 mol/l Pipes, 0.2 mol/l NaPO<sub>4</sub>, 0.1 mol/l NaCl, 1 mmol/l EDTA, 5% SDS, and 30  $\mu$ g/ml salmon sperm DNA at 65°C. Stringent washing was done in 50% of 1 $\times$  sodium chloride and sodium citrate, 5% SDS at 65°C for over 1 h. The expression of mRNA was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and normalized using 36B4 as the standard cDNA probe (34).

**In situ PKC activity assay.** PKC activity was measured using a modified *in situ* method described previously (35). Briefly, subconfluent BREC or BRPC in 12-well culture plates were exposed to 5.5 or 25 mmol/l glucose for indicated time periods. The cells were washed rapidly 3 times with 2 ml DMEM containing 20 mmol/l HEPES (pH 7.4) and then were replaced with a salt solution consisting of 137 mmol/l NaCl, 5.4 mmol/l KCl, 0.3 mmol/l Na<sub>2</sub>HPO<sub>4</sub>, 0.4 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 5.5 mmol/l glucose, 10 mmol/l MgCl<sub>2</sub>, 25 mmol/l  $\beta$ -glycerophosphate, 5 mmol/l EGTA, 2.5 mmol/l CaCl<sub>2</sub>, 20 mmol/l HEPES, 50 mg/ml digitonin, 100  $\mu$ mol/l ATP mixed with [ $\gamma$ - $^{32}$ P]ATP (<1,500 cpm/pmol), and 100  $\mu$ mol/l PKC-specific peptide substrate (RKRTLRL) (36) for 15 min at room temperature. The kinase reaction was terminated with 5% trichloroacetic acid (wt/vol; final concentration), spotted on 2  $\times$  2-cm phosphocellulose squares of Whatman P-81 and washed 3 times with 0.75% phosphoric acid. Before being quantitated as described previously (4,6), the ratios of total to peptide negative control activities were usually >5- to 10-fold, depending on the stimulant used.

**Measurement of intracellular distribution of PKC activity and isoforms.** After exposure to various glucose levels, BREC and BRPC were washed 3 times with ice-cold phosphate-buffered saline and once with a buffer consisting of 20 mmol/l Tris-HCl, 2 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l dithiothreitol, 300 mmol/l sucrose, and 25 mg/ml leupeptin and fractionated into cytosolic and membrane fractions according to the method described previously (27). PKC in the membranous and cytosolic fractions were partially purified using diethylaminoethyl cellulose (7,26), and then, protein concentrations were determined by the method of Bradford (37). PKC activities in both fractions were determined by measuring phosphatidylserine/diacylglycerol-dependent phosphorylation of an exogenous PKC-specific peptide substrate (RKRTLRL) in the presence of Ca<sup>2+</sup>. The kinase reaction was terminated after 5 min at 30°C. Specific PKC activity was calculated by subtracting the nonspecific kinase activities from those obtained in the presence of phosphatidylserine/diacylglycerol and Ca<sup>2+</sup>. For determining the distribution of PKC isoforms in BREC and BRPC, aliquots of equivalent amounts of protein extracted from both cytosolic and membrane fractions were analyzed as described previously (38). Partially purified PKC fractions were separated by 8% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked overnight with phosphate-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin (BSA). The membranes were incubated with several types of PKC isoform-specific antibodies for 1 h at room temperature. The blots were washed and then incubated with peroxidase-conjugated anti-rabbit immunoglobulins. Sites of antibody binding were visualized using an enhanced chemiluminescence Western blotting detection system and quantified using an image densitometer.

**Construction of replication-deficient adenoviruses containing PKC- $\alpha$ , - $\beta$ 1, and - $\delta$  cDNA.** Construction of replication-deficient recombinant adenoviruses containing full-length cDNA of PKC- $\alpha$ , - $\beta$ 1, - $\delta$ , and - $\zeta$  isoforms was carried out as described previously (39,40). Essentially, full-length mouse PKC- $\beta$ 1 cDNA obtained from P. Ashendal (Purdue University, Indianapolis, IN) and full-length human PKC- $\alpha$  and - $\delta$  cDNA obtained from Dr. Kirk Ways (Lilly Laboratory, Indianapolis, IN) were ligated into the adenoviral shuttle vector pAC-CMV-pLpA downstream of the cytomegalovirus (CMV) promoter and upstream of an SV40 polyA tail signal. This PKC- $\alpha$ , - $\beta$ 1, and - $\delta$ -containing shuttle vector was then cotransfected with the E1A transcription factor-deficient adenoviral genomic vector JM17 into 293 E1A-transformed cells by calcium phosphate precipitation (39,40). Subsequent generation of recombinant adenovirus expressing PKC- $\alpha$ , - $\beta$ 1, and - $\delta$  (Adv-CMV-PKC- $\alpha$ , - $\beta$ 1, and - $\delta$ ) in 293 cell lysates was confirmed by Southern blot analysis. As a control, recombinant  $\beta$ -galactosidase adenovirus (Adv-CMV- $\beta$ gal) was similarly generated (39,40). Recombinant adenoviruses were purified by cesium chloride gradient centrifugation, desalted, and then assayed for effectiveness of infection in a 293-cell plaque assay. Purified adenovirus stocks (10<sup>12</sup> pfu/ml) were stored in 20% glycerol at -80°C. Adenoviral-mediated gene transfer to confluent BREC or BRPC was performed by a 1-h adenoviral infection of 10<sup>8</sup> pfu/ml at 37°C in DMEM without serum as described previously (39,40). Expression of  $\beta$ -galactosidase was detected in >95% of BREC and BRPC infected with 10<sup>8</sup> pfu/ml of Adv-CMV- $\beta$ gal, PKC- $\alpha$ , PKC- $\beta$ 1, and PKC- $\delta$  isoforms.

**ET-1 enzyme immunoassay.** EIA was performed using an EIA kit from Biomedica (Biomedica Gruppe). Subconfluent BREC in 100-mm dishes infected with or without adenovirus as described above were exposed to EBM containing indicated concentrations of glucose for 24 h, and then the cells were



**FIG. 1. A:** Effect of elevated glucose and mannitol levels on ET-1 mRNA expression in BREC. After 48 or 72 h of exposure to 5.5 and 25 mmol/l glucose or 5.5 mmol/l glucose and 19.5 mmol/l mannitol, the cells were lysed, and ET-1 mRNA levels were measured by Northern blot analysis and quantitated using phosphoimages of the blots probed with a whole-length bovine preproET-1 cDNA probe or reprobed with 36B4 as a loading control, as described in RESEARCH DESIGN AND METHODS. The results were derived from 3 separate experiments, with duplicates performed in each experiment. Each bar represents the mean  $\pm$  SE in A and B. \* $P < 0.05$ , \*\* $P < 0.01$  vs. 5.5 mmol/l glucose. **B:** Dose-response effects of glucose on ET-1 mRNA expression in BREC. After 72 h of incubation with 5.5, 16.5, or 25 mmol/l glucose, the cells were lysed, and ET-1 mRNA levels were quantitated as described. The results were derived from 3 separate experiments, with duplicates performed in each experiment. \* $P < 0.05$ , \*\* $P < 0.01$  vs. 5.5 mmol/l glucose.

incubated in DMEM containing 0.1% BSA with the same concentration of glucose for another 24 h. Collected media were centrifuged, and the 100  $\mu$ l of resulting supernatants were diluted 100 times with DMEM containing 0.1% BSA. The EIA was performed according to the manufacturer's instructions.

**Statistical analysis.** Results are expressed as means  $\pm$  SE. Statistical significance was estimated by 1-way analysis of variance and a Student-Newman-Keuls test for comparison of several groups. The differences were considered significant at  $P < 0.05$ .

## RESULTS

**Effect of glucose on ET-1 mRNA expression and ET-1 secretion.** The effects of increasing glucose levels in the media from 5.5 to 25 mmol/l were studied. After 48 and 72 h of exposure to 25 mmol/l glucose, ET-1 mRNA levels increased by  $43 \pm 8$  and  $90 \pm 12\%$  in BREC (Fig. 1A), respectively, compared with 5.5 mmol/l glucose. After 48 and 72 h

of exposure to 25 mmol/l glucose, ET-1 mRNA levels increased by  $24 \pm 5\%$  and  $45 \pm 9\%$  in BRPC, respectively, compared with 5.5 mmol/l glucose. In contrast, the addition of 19.5 mmol/l mannitol, an osmotic control, to 5.5 mmol/l glucose did not have any effect on ET-1 mRNA expression compared with 5.5 mmol/l glucose alone (Fig. 1A). The effect of glucose was concentration-dependent, since ET-1 mRNA increased by  $38 \pm 7\%$  and  $85 \pm 12\%$  when glucose levels were increased from 5.5 to 16.5 and 25 mmol/l, respectively (Fig. 1B). To determine whether elevated glucose levels can increase ET-1 secretion as well as mRNA expression, immunoreactive ET-1 protein was measured in the media from BREC. After 48 h of exposure to 25 mmol/l glucose, ET-1 protein levels were significantly increased by  $81 \pm 5\%$  in BREC (Table 1) compared with 5.5 mmol/l glucose. These results showed that increasing glucose levels to 16.5 mmol/l or higher increased ET-1 mRNA expression and secretion via metabolic effect.

**Effect of PKC on ET-1 mRNA expression induced by glucose.** To characterize the possible roles of PKC activation and other intracellular signaling pathways in glucose-induced ET-1 expression, we examined the effects of GF109203X, a general PKC-specific inhibitor; PD98059, a mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor; and wortmannin, an inhibitor of phosphatidylinositol 3-kinase on ET-1 mRNA expression (1,7,8). Elevating glucose levels from 5.5 to 25 mmol/l for 3 days increased ET-1 mRNA expression to  $85 \pm 12\%$  of the control. The increase in ET-1 mRNA was inhibited by 5  $\mu$ mol/l GF109203X in both BREC (Fig. 2A) and BRPC (Fig. 2B). The addition of PD98059 also inhibited glucose-induced ET-1 expression by  $95 \pm 6\%$  in BRPC (Fig. 2B) and by  $52 \pm 4\%$  in BREC (Fig. 2A), but wortmannin (100 nmol/l) did not have any effect on glucose-induced ET-1 expression (Fig. 2B). These data suggest that both PKC and MAPK activation are partly responsible for glucose-induced ET-1 mRNA expression.

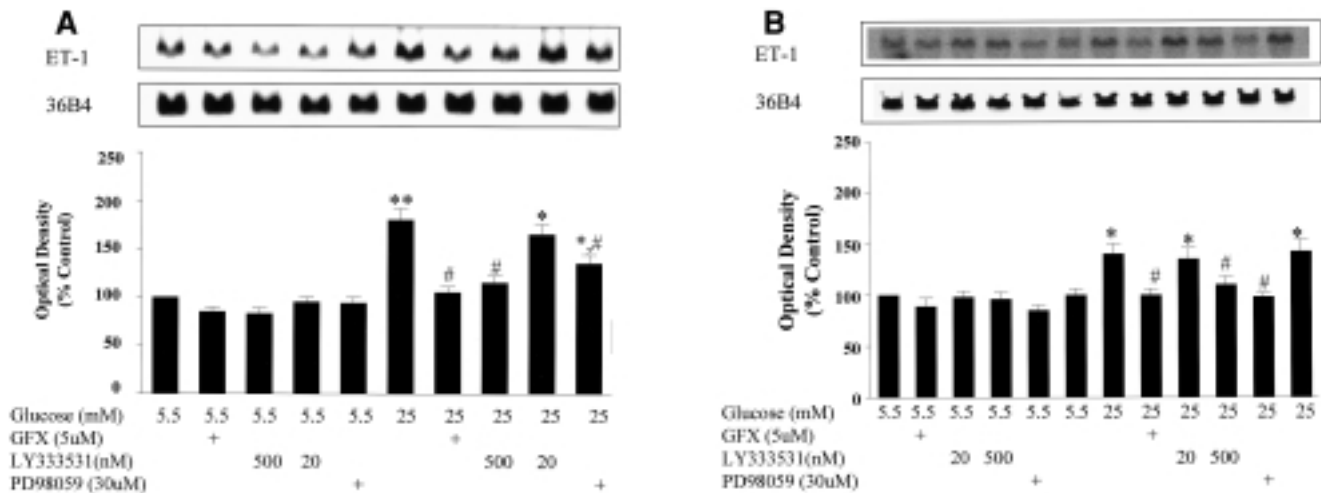
To determine directly whether the PKC pathway was activated by increasing glucose levels from 5.5 to 25 mmol/l, the time course of in situ PKC activities was measured in BREC and BRPC. PKC activities were increased by  $25 \pm 5$ ,  $31 \pm 4$ , and  $45 \pm 5\%$  in BREC (Fig. 3A) and  $24 \pm 4$ ,  $30 \pm 5$ , and  $42 \pm 5\%$  in BRPC (Fig. 3B) after 24, 48, and 72 h of incubation, respectively, at 25 mmol/l glucose compared with 5.5 mmol/l glucose. PKC activation in the retinal capillary cells was also assessed by measuring changes in the cytosolic and membranous fractions. Increasing the glucose concentration in the media from 5.5 to 25 mmol/l for 3 days increased PKC-specific activities

TABLE 1

Effects of glucose levels and overexpression of various PKC isoforms on immunoreactive ET-1 protein in the media from cultured bovine retinal endothelial cells

Glucose (mmol/l)	ET-1 pmol $\cdot$ ml <sup>-2</sup> $\cdot$ 24 h <sup>-1</sup> $\cdot$ 10 <sup>-6</sup> cells				
	Uninfected	$\beta$ -Gal	PKC- $\alpha$	PKC- $\beta$ 1	PKC- $\delta$
5.5	1.02 $\pm$ 0.05	1.16 $\pm$ 0.06	1.41 $\pm$ 0.05†	2.19 $\pm$ 0.09‡	1.46 $\pm$ 0.06†
25.0	1.85 $\pm$ 0.05*	—	—	—	—

Data are means  $\pm$  SE of 4 experiments. Cells were infected with or without adenovirus containing  $\beta$ -gal PKC- $\alpha$ , - $\beta$ 1, and - $\delta$ cDNA and then exposed to 5.5 or 25 mmol/l glucose for 2 days. \* $P < 0.01$  vs. 5.5 mmol/l glucose; † $P < 0.05$  vs.  $\beta$ -gal control; ‡ $P < 0.01$  vs.  $\beta$ -gal control;  $\beta$ -gal,  $\beta$ -galactosidase.



**FIG. 2.** *A:* Effect of GF109203X, LY333531, and PD98059 on ET-1 mRNA expression in BREC. After 72 h of exposure to 25 mmol/l glucose, the cells were treated or not treated with a PKC-specific inhibitor, GF109203X (GFX, 5 μmol/l), or an MEK inhibitor, PD98059 (30 μmol/l), for 6 h, or a PKC-β-specific inhibitor, LY333531 (20 nmol/l), for 12 h; then, the cells were lysed, and ET-1 mRNA levels were measured by Northern blot analysis and quantitated using phosphorimages of the blots probed with full-length bovine preproET-1 cDNA probe or reprobbed with 36B4 as a loading control, as described in RESEARCH DESIGN AND METHODS. The results were derived from 3 separate experiments, with duplicates performed in each experiment. Each bar represents the mean ± SE in *A* and *B*. \**P* < 0.05, \*\**P* < 0.01 vs. 5.5 mmol/l glucose; #*P* < 0.05 vs. 25 mmol/l glucose. *B:* Effect of GF109203X, LY333531, PD98059, and wortmannin on ET-1 mRNA expression in BRPC. Cells were treated as described above. The results were derived from 3 separate experiments, with duplicates performed in each experiment. Each bar represents the mean ± SE in *A* and *B*. \**P* < 0.05 vs. 5.5 mmol/l glucose; #*P* < 0.05 vs. 25 mmol/l glucose.

in the membranous fraction of BREC (Fig. 3C) and BRPC (Fig. 3D) by  $93 \pm 15$  and  $49 \pm 13\%$ , respectively.

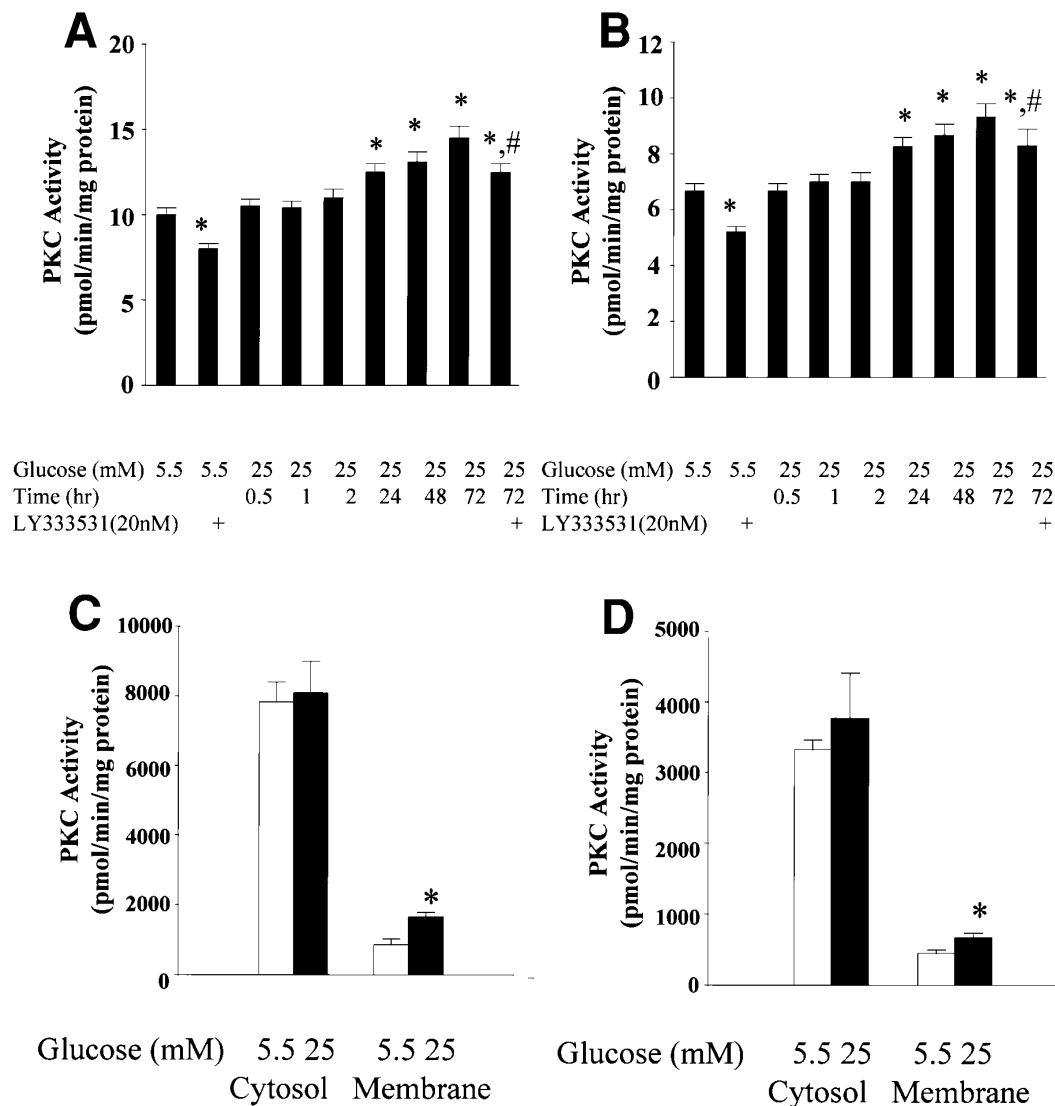
We examined the reversibility of PKC activation induced by 25 mmol/l glucose by returning glucose levels to 5.5 mmol/l as well as correlating PKC inactivation with the changes of ET-1 expression. PKC activation induced by 25 mmol/l glucose was decreased by 60% after returning the concentration of glucose to 5.5 mmol/l for 24 h and returned to normal after 2 days (Fig. 4A). Paralleling PKC activity, ET-1 expression induced by glucose was also restored to a normal level after normalization of glucose for 2 days (Fig. 4B).

**Effects of glucose levels on PKC isoform expression.** We and others (7,8,23,26–29,35,41) have reported previously that elevation of glucose levels in vivo or in vitro increased or activated PKC, particularly the β and δ isoforms, in the vascular cells. Thus, the effect of the PKC-β isoform selective inhibitor LY333531 (42) on glucose-induced PKC activation and ET-1 mRNA expression was determined in BREC and BRPC cultured with 5.5–25 mmol/l of glucose. The addition of 20 nmol/l LY333531, which we had previously reported to inhibit the PKC-β isoform specifically (8), reduced basal PKC activities by ~25% and glucose-induced PKC activation by  $44 \pm 4$  and  $49 \pm 4\%$  in BREC and BRPC, respectively (Fig. 3A and B), but did not significantly inhibit ET-1 expression (Fig. 2A and B). Glucose-induced PKC activation is defined as the difference between cells incubated with 5.5 mmol/l for 72 h and 25 mmol/l for 72 h. However, LY333531 at 500 nmol/l, which can inhibit multiple PKC isoforms (41), prevented the glucose-induced ET-1 mRNA expression, similar to GF109203X (Fig. 2A and B). Results from the studies using the PKC-β isoform-specific inhibitor LY333531 suggested that the activation of PKC isoforms other than β isoforms could be involved in glucose-induced ET-1 mRNA expression (8).

To determine the specific isoforms that are translocated to the membrane or activated by high glucose, we examined the effects of high glucose levels on the expression of various PKC isoforms in cytosolic and membrane fractions of BREC by immunoblot analysis.

The expression of PKC-α, -β1, -β2, and -δ isoforms in BREC was significantly increased to  $65 \pm 40$ ,  $59 \pm 47$ ,  $240 \pm 69$ , and  $254 \pm 65\%$ , respectively, by exposure to 25 mmol/l glucose compared with 5.5 mmol/l glucose for 72 h in the membranous fractions (Fig. 5A–D). No significant changes were observed in the cytosolic fractions for PKC-α, -β1, -β2, and -δ isoforms (Fig. 5A–D) and in the cytosolic and membranous fractions for PKC-ε and -ζ isoforms (Fig. 5D and 5E).

**Effects of various PKC isoforms on ET-1 expression and secretion.** The results of immunoblot analysis of BREC incubated with 25 mmol/l glucose suggested that PKC-α, -β1, -β2, and -δ isoforms were increased in the membranous fraction. To determine which of these PKC isoforms were mediating the effects of glucose on ET-1 expression, we overexpressed PKC-α, -β1, -δ, and -ζ isoforms in the BREC and BRPC by using replication-deficient adenoviral vectors or β-galactosidase separately. BRPC infected with adenovirus containing PKC-α, -β1, and -δ cDNA exhibited 16-, 23-, and 17-fold increases in PKC-α, -β1, and -δ protein levels and 10.5-, 11-, and 5.6-fold increases in PKC activities compared with cells infected with β-galactosidase control (Fig. 6A and B). Interestingly, PKC-α overexpressed cells also exhibited 2-fold increases in PKC-β1 expression. Compared with β-galactosidase control, BRPC overexpressed with PKC-α, -β1, and -δ increased basal ET-1 mRNA expression by 40%, 2.5-fold, and 2-fold, respectively. To determine whether PKC overexpression can also increase ET-1 secretion, we measured ET-1 protein levels in the culture media from BREC



**FIG. 3. A:** Time course of glucose effects and the effects of LY333531 on in situ PKC activities in BREC. After the indicated time of incubation with 25 mmol/l glucose, the cells were untreated or treated with a PKC- $\beta$ -specific inhibitor, LY333531 (20 nmol/l), for 12 h, and PKC activities were measured by in situ PKC assay, as described in RESEARCH DESIGN AND METHODS. LY333531 was solubilized with DMSO, with final concentrations of DMSO of 0.1%. \* $P < 0.05$  vs. 0 h; # $P < 0.05$  vs. 72 h. The results were derived from 3 separate experiments. Each bar represents the mean  $\pm$  SE in A, B, C, and D. Cells were all incubated for 72 h in 1% calf serum and DMEM. LY222531 was added 12 h before the end of the experiments and total time of incubation at either 5.5 or 25 mmol/l glucose was 72 h. **B:** Time course of glucose effects and the effects of LY333531 on in situ PKC activities in BRPC. Cells were prepared as described above. PKC activities were measured by in situ PKC assay, as already described. \* $P < 0.05$  vs. 0 h; # $P < 0.05$  vs. 72 h. **C:** The effects of glucose on PKC translocation in BREC. After incubation with 25 mmol/l glucose for 3 days, total PKC-specific activities were measured in both cytosolic and membrane fractions from cultured BREC, as described in RESEARCH DESIGN AND METHODS. \* $P < 0.05$  vs. 5.5 mmol/l glucose. **D:** The effects of glucose on PKC translocation in BRPC. \* $P < 0.05$  vs. 5.5 mmol/l glucose.

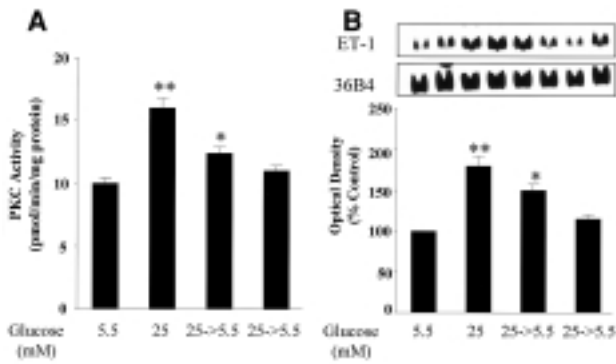
overexpressed with PKC- $\alpha$ , - $\beta$ 1, - $\delta$ , or - $\zeta$  isoforms. BREC infected with adenovirus containing PKC- $\alpha$ , - $\beta$ 1, and - $\delta$  cDNA increased ET-1 protein levels by 30%, 2.2-fold, and 43%, respectively, compared with cells infected with  $\beta$ -galactosidase control (Table 1). These changes are comparable to the effect of increasing glucose levels from 5.5 to 25 mmol/l (which increased ET-1 production by 85%). Overexpression of BREC with adenovirus containing PKC- $\zeta$  isoform increased  $\zeta$  protein levels by approximately 10-fold but did not enhance the expression of ET-1 mRNA or protein levels in the media (data not shown in figures).

To identify the effects of PKC isoforms on the change of PKC activities and ET-1 expression by high glucose, we measured in situ PKC activity and ET-1 mRNA expression in PKC- $\alpha$ ,

- $\beta$ 1, and - $\delta$  overexpressed BREC cultured with 5.5 and 25 mmol/l glucose. PKC- $\alpha$ , - $\beta$ 1, and - $\delta$  overexpression increased basal PKC activities by 9-, 9.5-, and 4.7-fold (Fig. 6C) and ET-1 expression by 30%, 2.2-fold, and 2.0-fold, respectively (Fig. 7A). In PKC- $\alpha$ , - $\beta$ 1, and - $\delta$  overexpressed cells, exposure to 25 mmol/l glucose increased PKC activities by 11.5-, 13-, and 7.8-fold (Fig. 6C) and ET-1 mRNA expression by 2.8-, 4.2-, and 4.1-fold compared with  $\beta$ -galactosidase control, respectively (Fig. 7A), and was greater than PKC activities and ET-1 expression in BREC exposed to 5.5 mmol/l glucose.

**DISCUSSION**

Abnormal blood flow to the retina and the peripheral nerves are observed in early phases of diabetic retinopathy and neu-

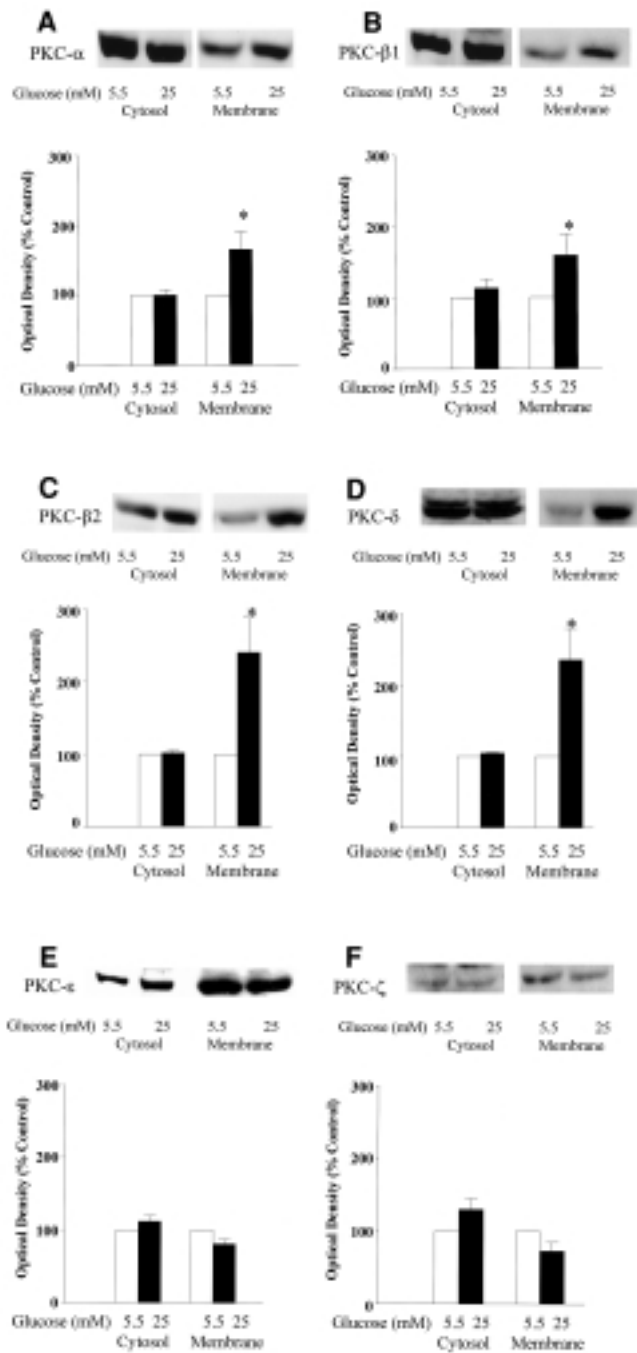


**FIG. 4. A:** Reversible effects of glucose on in situ PKC activities in BREC. After 72 h of incubation with 25 mmol/l glucose, the high-glucose medium was changed to 5.5 mmol/l glucose for 24 or 48 h. PKC activities were measured by in situ PKC assay, as described in RESEARCH DESIGN AND METHODS. The results were derived from 3 separate experiments. Each bar represents the mean  $\pm$  SE in A and B. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. 5.5 mmol/l of glucose. **B:** Reversible effects of glucose on ET-1 mRNA expression in BREC. Cells were prepared as described above. The cells were lysed, and ET-1 mRNA levels were measured by Northern blot analysis and quantitated using phosphoimages as described. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. 5.5 mmol/l of glucose.

ropathy (1–5) and may contribute to the establishment of pathologies in these tissues. Among the various vasotropic cytokines, increases in the levels or actions of ET-1 have been postulated to have a prominent role for multiple reasons, especially in the retina. Increased levels of ET-1 have been reported in the retina and plasma of diabetic animals and patients (14,15,43,44). Competitive inhibition of ET-1 to its receptors can prevent the decreases of blood flow in the retina and peripheral nerves (14–16).

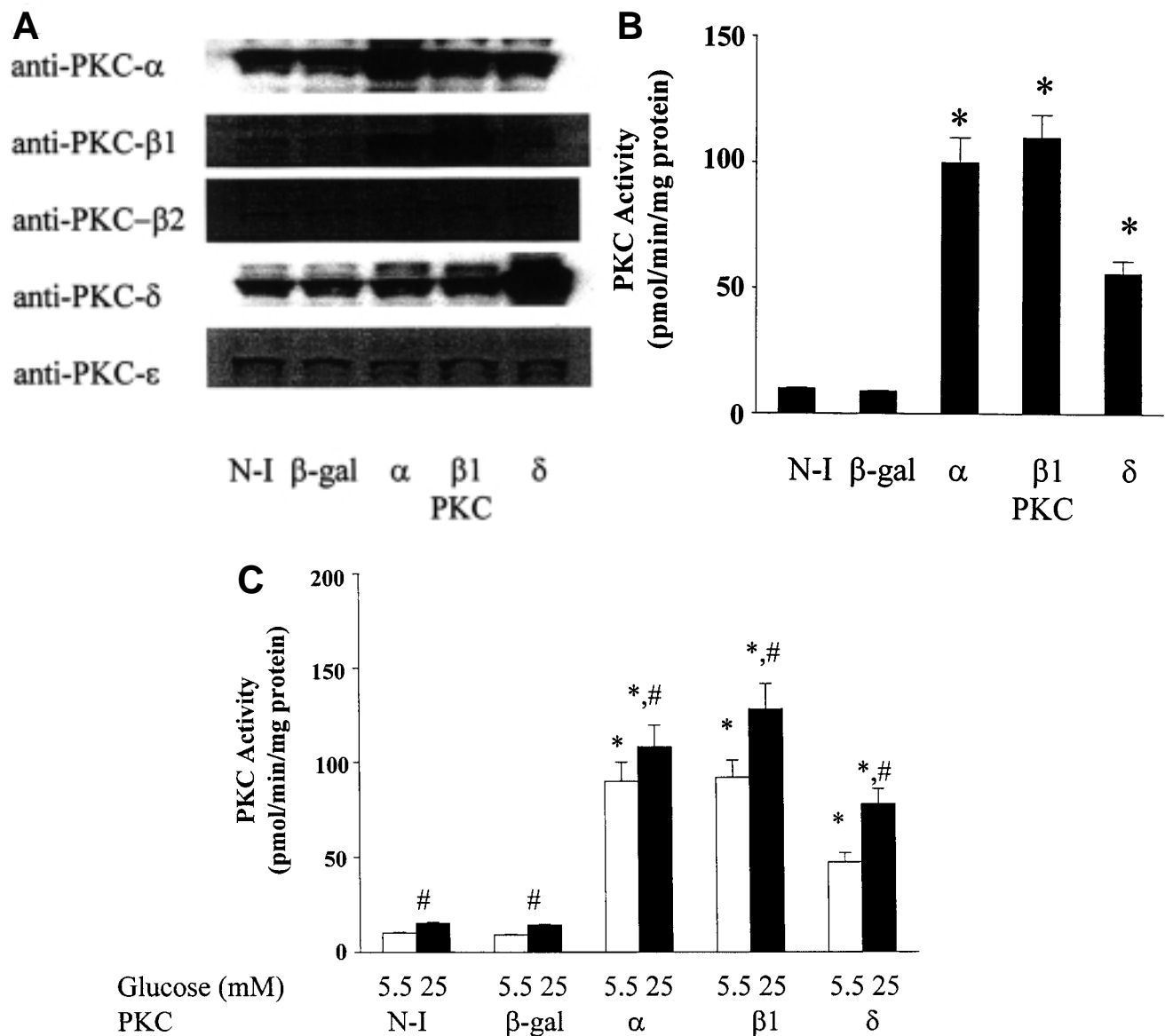
Hyperglycemia is the most likely metabolic change inducing the increases in ET-1 levels, although hyperinsulinemia may also have an effect when present. Yamauchi et. al. (30) have reported that aortic endothelial cells exposed to increased levels of glucose enhanced the production of ET-1 protein. This change was not induced by similar levels of mannitol, suggesting that the change caused by glucose was not due to osmotic effect. Our results have extended these findings by showing glucose induction of ET-1 expression can be increased both in retinal capillary endothelial cells and pericytes, even at a glucose concentration of 16.5 mmol/l, which is commonly encountered in diabetic patients. The effect of glucose on ET-1 expression appeared to be reversible within 1–2 days. This time frame is surprising because the reversal of PKC activation in large arteries and retinal blood flow in diabetic rats required several weeks of euglycemia induced by insulin before normalization was observed (45). However, in aortic smooth muscle cells, Williams and Schrier (46) reported that PKC activation induced by hyperglycemia can be reversed within 1 h after reducing glucose levels in the media. Thus, reversibility of PKC activation and its consequences appears to be rapid in cultured cells but rather slow in vivo, requiring days and weeks. This result suggests that the mechanism for glucose-induced PKC activation may be different in vivo from in vitro.

The increases in ET-1 expression in the retinal vascular cells included both mRNA and protein levels, suggesting that the changes in ET-1 expression may be functionally important.



**FIG. 5. Effect of glucose on PKC isoforms expression in BREC (A–F).** After 72 h of exposure to 5.5 or 25 mmol/l glucose, the cells were lysed, and PKC proteins in both membranous and cytosolic fractions were partially purified. The proteins were separated by 8% SDS-PAGE, and immunoblot analysis was performed using various types of antibodies on PKC isoforms, as described in RESEARCH DESIGN AND METHODS. The results are derived from 4 separate experiments. Each bar represents the mean  $\pm$  SE. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. control cells at 5.5 mmol/l glucose.

This suggestion is supported by our previous report (14) that inhibition using ET-A receptor antagonist BQ-123 prevented or reversed hemodynamic changes such as decreased blood flow in diabetic rats. In addition, other groups (47,48) have shown by immunohistological study that ET-1 expression may also be increased in certain retinal cells in the diabetic state.



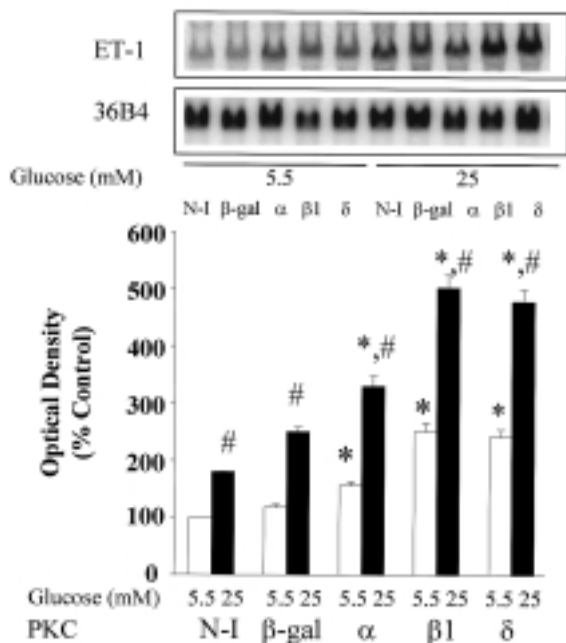
**FIG. 6. A and B:** Effect of PKC- $\alpha$ , - $\beta$ 1, and - $\delta$  overexpression in BRPC. **A:** Immunoblot analysis. Total cell lysate was isolated from noninfected (N-I) control cells or cells infected with Adv-CMV- $\beta$ gal ( $\beta$ -gal) or Adv-CMV-PKC- $\alpha$ , - $\beta$ 1, or - $\delta$  (PKC- $\alpha$ , - $\beta$ 1, or - $\delta$ ). PKC proteins were detected by immunoblot analysis. **B:** In situ PKC activity. Three days after infection with adenovirus, PKC activities were measured by in situ PKC assay, as described in RESEARCH DESIGN AND METHODS. \* $P < 0.01$  vs. N-I. The results were derived from 3 separate experiments. **C:** Effects of overexpressing PKC isoforms on glucose-induced in situ PKC activities. After adenovirus infection, BRPC were exposed to 5.5 or 25 mmol/l glucose for 2 days. PKC activities were measured by in situ PKC assay as already described. \* $P < 0.01$  vs.  $\beta$ -gal; # $P < 0.05$  vs. 5.5 mmol/l glucose. The results were derived from 3 separate experiments.

The mechanisms of glucose effects could be multiple. The present study suggests that both PKC and MAPK activation, but not phosphatidylinositol 3-kinase, may be involved. There is a great deal of evidence to support the concept that elevation of glucose levels can activate PKC and MAPK pathways (1). Our results showing that general PKC inhibitors GF109203X and LY333531 at 500 nmol/l completely prevented all the effects of glucose on ET-1 expression strongly indicate that PKC activation is mediating most of the glucose effect. In contrast, PD98059, an MEK inhibitor, was only partially effective, suggesting the ras-MAPK could also play a role. A similar mechanism appears to be activated in both retinal endothelial cells and pericytes, although the level of ET-1 expression by the endothelial cells appears to be 100 times higher than that of per-

icytes, suggesting that the endothelial cell is the main contributor of ET-1 production in the retinal vasculature.

The present study also directly demonstrates for the first time that elevation of glucose levels activates PKC in pericytes. Similar to previous reports, the effects of glucose on PKC activation were not immediate but were delayed for 24–48 h (35). These findings indicate that glucose effects on PKC are not solely due to an increase in flux of glucose metabolites but also that changes in the expression of regulatory enzymes may be occurring.

The results from the use of general PKC inhibitor GF109203X and PKC- $\beta$  isoform specific inhibitor LY333531 suggest that PKC isoforms other than the  $\beta$  isoform are also activated by hyperglycemia to increase ET-1 expression. This



**FIG. 7.** Effects of various PKC isoforms on basal and glucose-induced ET-1 mRNA expression in BREC. After adenovirus infection, BREC were exposed to 5.5 or 25 mmol/l glucose for 2 days. The cells were lysed, and ET-1 mRNA levels were measured and quantitated as described. \* $P < 0.01$ , \*\* $P < 0.01$  vs.  $\beta$ -gal; # $P < 0.05$  vs. 5.5 mmol/l glucose. The results were derived from 3 separate experiments.  $\beta$ -gal, Adv-CMV- $\beta$ gal; N-I, noninfected.

possibility was substantiated by the analysis of different PKC isoforms that showed that PKC- $\alpha$ , - $\beta$ 1, - $\beta$ 2, and - $\delta$  were all increased or activated by glucose. These findings are not surprising because we and others (7,26–29,49–52) have reported that PKC- $\alpha$ , - $\beta$ , and - $\delta$  isoforms can be activated in the vascular tissues of diabetic animals. However, in a previous report (7), we did not observe activated PKC- $\delta$  in the retina of diabetic rats, but PKC- $\alpha$ ,  $\beta$ 1, and  $\beta$ 2 were observed to be activated there. The most likely explanation for this difference is that PKC- $\delta$  activation may occur only in the vasculature, which represents only a small pool of PKC in the whole retinal protein lysates. But recent results from our laboratory and others (28,52) have reported that PKC- $\delta$  isoform was activated in aortic smooth muscle cells exposed to increased glucose concentration or in the aorta and kidney of diabetic rats (28,52).

The results derived from the infection of vascular cells by adenovirus containing the various PKC isoforms provided strong support that activation of PKC- $\alpha$ , - $\beta$ 1, or - $\delta$  can increase ET-1 expression. However, quantitatively, the effect of PKC- $\beta$  and - $\delta$  isoforms are much greater than PKC- $\alpha$  isoform in inducing ET-1 expression. Recently, we have overexpressed PKC- $\beta$ 1 and - $\beta$ 2 isoforms in the endothelial cells. Protein levels of PKC- $\beta$ 1 and - $\beta$ 2 were increased by 10-fold. ET-1 mRNA expression was similar in endothelial cells overexpressing PKC- $\beta$ 1 or - $\beta$ 2. In addition, it is not clear from the present study whether PKC- $\alpha$  is increasing ET-1 expression directly or indirectly, since enhancing PKC- $\alpha$  isoform also enhanced PKC- $\beta$  isoform. Further studies using the selective PKC- $\beta$  isoform inhibitor need to be performed to test these possibilities. The availability of the adenoviral-infected cells has also provided vascular cell models that contain large

amounts of PKC, which facilitates the detection of PKC activation and its cellular targets. The ability of PKC activation to enhance ET-1 expression is limited mainly to PKC- $\beta$  and - $\delta$  isoforms, since the PKC- $\zeta$  isoform did not have any effect. This is clearly demonstrated by the activation of these different PKC isoforms in the adenoviral-infected cells when they are exposed to a glucose level increase from 5.5 to 25 mmol/l.

Several mechanisms have been suggested by which PKC activation can enhance ET-1 expression. One possible explanation is that the transcription of ET-1 is enhanced via the activated protein 1 (AP-1) site. Inoue et al. (53) have shown that the 5'-flanking region of the human PPET-1 gene contains octanucleotide sequences for phorbol ester-responsive elements, also known as the binding elements for the fos jun complex (AP-1). Lee et al. (54) have reported that cotransfection of c-fos and c-jun plasmid markedly increased the transcription rate of ET-1 gene in bovine endothelial cells. Soh et al. (55) have recently reported that transient transfection of PKC- $\alpha$  and - $\epsilon$  in NIH 3T3 cells activated a serum response element in the c-fos promoter. These data suggest that the human PPET-1 gene can be directly regulated by intracellular signaling mediated by PKC via the *trans*-acting transcription factors fos and jun. In addition, it is possible that PKC activation can in turn activate MAPK, which then can induce c-fos, c-jun, and ET-1 transcription rates. However, in preliminary data using adenoviruses to overexpress PKC- $\alpha$ , - $\beta$ 1, and - $\delta$ , MAPK activity was significantly increased at the basal state but not in stimulated levels. Further study will be needed to identify the various mechanisms of increased ET-1 gene expression by PKC activation induced by glucose. It is interesting to note that glucose induced an increase in PKC activity by only 45% but increased ET-1 expression by 85%. In contrast, overexpression of PKC- $\beta$ 1 and - $\delta$  individually increased PKC activities by 5- to 10-fold but only increased ET-1 expression 2-fold and 43%, respectively. These findings show the importance of measuring the activation of each PKC isoform because glucose induced 2-fold increases in PKC- $\beta$ 2 and - $\delta$  isoforms, whereas glucose increased total activity by 45%. However, the apparent inability of PKC activation, even when increased by 10-fold, to enhance ET-1 expression by >2- to 4-fold is not clear. One explanation is that the PKC-dependent pathway of enhancing the ET-1 transcription rate is not rate-limiting after a 2-fold increase in PKC activity. In addition, glucose may induce other pathways, such as the MAPK pathway, to increase ET-1 expression. Further studies will be needed to clarify these possibilities.

In summary, these results have provided a mechanistic explanation of glucose's inducing effects on ET-1 expression in the retinal vascular cells. For the first time, we have characterized the different PKC isoforms that are activated in the retinal microvascular cells and identified PKC- $\beta$  and - $\delta$  isoforms as the main mediators of glucose effect on ET-1 expression. Further studies using inhibitors of PKC- $\beta$  and - $\delta$  isoforms are needed to delineate the role of ET-1 expression in the development of diabetic retinopathy and other complications.

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## REFERENCES

- Koya D, King GL: Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859–866, 1998
- Feke GT, Buzney SM, Ogasawara H, Fujio N, Goger DG, Spack NP, Gabbay KH: Retinal circulatory abnormalities in type 1 diabetes. *Invest Ophthalmol Vis Sci* 35:2968–2975, 1994
- Bursell SE, Clermont AC, Kinsley BT, Simonson DC, Aiello LM, Wolpert HA: Retinal blood flow changes in patients with insulin-dependent diabetes mellitus and no diabetic retinopathy. *Invest Ophthalmol Vis Sci* 37:886–897, 1996
- Small KW, Stefansson E, Hatchell D: Retinal blood flow in normal and diabetic dogs. *Invest Ophthalmol Vis Sci* 28:672–675, 1987
- Tesfaye S, Malik R, Ward JD: Vascular factors in diabetic neuropathy. *Diabetologia* 37:847–851, 1994
- Cameron NE, Cotter MA, Lai K, Hohman TC: Effects of protein kinase C inhibition on nerve function, blood flow and Na<sup>+</sup>, K<sup>+</sup> ATPase defects in diabetic rats (Abstract). *Diabetes* 46 (Suppl. 1):31A, 1997
- Shiba T, Inoguchi T, Sportsman R, Heath WF, Bursell SE, King GL: Correlation of diacylglycerol level and protein kinase C activity in rat retina to retinal circulation. *Am J Physiol* 265:E783–E793, 1993
- Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, Bursell S-E, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL: Amelioration of vascular dysfunction in diabetic rats by an oral PKC  $\beta$  inhibitor. *Science* 272:728–731, 1996
- Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332:411–415, 1988
- Rai H, Hori S, Aramori I, Ohkubo H, Nakanishi S: Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 348:730–732, 1990
- Sakurai T, Yanagisawa M, Takawa Y, Miyazaki H, Kimura S, Goto K, Masaki T: Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 348:732–735, 1990
- Karne S, Jayawickreme CK, Lerner MR: Cloning and characterization of an endothelin-3 specific receptor (ET<sub>C</sub> receptor) from *Xenopus laevis* dermal melanophores. *J Biol Chem* 25:19126–19133, 1993
- Bursell SE, Clermont AC, Oren D, King GL: The in vivo effect of endothelin on retinal circulation in non-diabetic and diabetic rats. *Invest Ophthalmol Vis Sci* 36:596–607, 1995
- Takaki C, Bursell S-E, Lin Y-W, Takaki H, Duh E, Jiang Z, Clermont AC, King GL: Regulation of retinal hemodynamics in diabetic rats by increased expression and action of endothelin-1. *Invest Ophthalmol Vis Sci* 37:2054–2058, 1996
- Takei K, Sato T, Nonoyama T, Hommura S, Miyauchi T, Goto K: Analysis of vasocontractile responses to endothelin-1 in rabbit retinal vessels using an ET<sub>A</sub> receptor antagonist and an ET<sub>B</sub> receptor agonist. *Life Sci* 53:PL111–PL115, 1993
- Cameron NE, Cotter MA: Effects of a non-peptide endothelin-1 ET<sub>A</sub> antagonist on neurovascular function in diabetic rats: interaction with the renin-angiotensin system. *J Pharmacol Exp Ther* 278:1262–1268, 1996
- Benatti L, Bonocchi L, Liviana C, Sarmientos P: Two endothelin-1 mRNAs transcribed by alternative promoters. *J Clin Invest* 91:1149–1156, 1993
- Luscher TF, Boulanger CM, Dohi Y, Yang Z: Endothelin-derived contracting factors. *Hypertension* 19:117–130, 1992
- Yamada Y, Yokoda M: Effects of protein kinase C activation and inhibition on endothelin-1 release from human aortic and pulmonary artery endothelial cells: comparison with effects on bovine endothelin-1 and human prostaglandin 12 release. *Am J Hypertens* 10:32–42, 1997
- Emori T, Hirata Y, Ohta K, Kanno K, Eguchi S, Imai T, Shichiri M, Marumo F: Cellular mechanism of endothelin-1 release by angiotensin and vasopressin. *Hypertension* 18:165–170, 1991
- Hattori Y, Kasai K, Banba N, Hattori S, Nakamura T, Shimoda S: Effects of a phorbol ester on immunoreactive endothelin-1 release from cultured porcine aortic endothelial cells. *Endocrinol Jpn* 39:341–345, 1992
- Baumgartner-Parzer SM, Wagner L, Reining G, Sexl V, Nowotny P, Muller M, Brunner M, Waldhausl W: Increase by tri-iodothyronine of endothelin-1, fibronectin and von Willebrand factor in cultured endothelial cells. *J Endocrinol* 154:231–239, 1997
- The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993
- U.K. Prospective Diabetes Study (UKPDS) Group: Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 352:837–853, 1998
- U.K. Prospective Diabetes Study (UKPDS) Group: Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet* 352:854–865, 1998
- Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL: Preferential elevation of protein kinase C isoform  $\beta$  II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycaemic control by islet cell transplantation. *Proc Natl Acad Sci U S A* 89:11059–11063, 1992
- Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL: Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes* 43:1122–1129, 1994
- Babazono T, Kapor-Drezgic J, Dlugosz JA, Whiteside C: Altered expression and subcellular localization of diacylglycerol-sensitive protein kinase C isoforms in diabetic rat glomerular cells. *Diabetes* 47:668–676, 1998
- Kunisaki M, Bursell SE, Umeda F, Nawata H, King GL: Normalization of diacylglycerol-protein kinase C activation by vitamin E in aorta of diabetic rats and cultured rat smooth muscle cells exposed to elevated glucose levels. *Diabetes* 43:1372–1377, 1995
- Yamauchi T, Ohnaka K, Takayanagi R, Umeda F, Nawata H: Enhanced secretion of endothelin-1 by elevated glucose levels from cultured bovine aortic endothelial cells. *FEBS Lett* 267:16–18, 1990
- King GL, Goodman AD, Buzney S, Moses A, Kahn CR: Receptors and growth-promoting effects of insulin and insulin-like growth factors on cells from bovine retinal capillaries and aorta. *J Clin Invest* 75:1028–1036, 1985
- Nayak RC, Berman AB, George KL, Eisenbarth GS, King GL: A monoclonal antibody (3G5)-defined ganglioside antigen is expressed on the cell surface of microvascular pericytes. *J Exp Med* 167:1003–1015, 1988
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
- Aiello LP, Robinson GS, Lin YW, Nishio Y, King GL: Identification of multiple genes in bovine retinal pericytes altered by exposure to elevated levels of glucose by using mRNA differential display. *Proc Natl Acad Sci U S A* 91:6231–6235, 1994
- Xia P, Kramer RM, King GL: Identification of the mechanism for the inhibition of Na<sup>+</sup>, K<sup>+</sup>-adenosine triphosphate by hyperglycemia involving activation of protein kinase C and cytosolic phospholipase A<sub>2</sub>. *J Clin Invest* 96:733–740, 1995
- Yasuda I, Kishimoto A, Tanaka S, Tominaga M, Sakurai A, Nishizuka Y: A synthetic peptide substrate for selectivity assay of protein kinase C. *Biochem Biophys Res Commun* 166:1220–1227, 1990
- Bradford MM: A rapid and sensitive method of the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
- Inoguchi T, Xia P, Kunisaki M, Higashi S, Feener EP, King GL: Insulin's effect on protein kinase C and diacylglycerol induced by diabetes and glucose in vascular tissues. *Am J Physiol* 267:E369–E379, 1994
- Becker TC, BeltrandelRio H, Noel RJ, Johnson JH, Newgard CB: Overexpression of hexokinase I in isolated islet of Langerhans via recombinant adenovirus: enhancement of glucose metabolism and insulin secretion at basal but not stimulatory glucose levels. *J Biol Chem* 269:21234–21238, 1994
- Becker TC, Noel RJ, Coats WS, Gomez-Foix AM, Alam T, Gerard RD, Newgard CB: Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol* 43:161–189, 1994
- Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL: Characterization of protein kinase C  $\beta$  isoform activation on the gene expression of transforming growth factor- $\beta$ , extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest* 100:115–126, 1997
- 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, Winerowski LL, Faul MM: (S)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16, 21-dimetheno-1H, 13H-dibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecene-1,3(2H)-dione (LY333531) and related analogues: isozyme selective inhibitors of protein kinase C  $\beta$ . *J Med Chem* 39:2664–2671, 1996
- Makino A, Kamata K: Elevated plasma endothelin-1 level in streptozotocin induced diabetic rats and responsiveness of the mesenteric arterial bed to endothelin-1. *Br J Pharmacol* 123:1065–1072, 1998
- Haak T, Jungmann E, Felber A, Hillmann U, Usadel KH: Increased plasma levels of endothelin in diabetic patients with hypertension. *Am J Hypertens* 5:161–166, 1992
- Clermont AC, Britts M, Shiba T, McGovern T, King GL, Bursell S-E: Normalization of retinal blood flow in diabetic rats with primary intervention using insulin pumps. *Invest Ophthalmol Vis Sci* 35:981–990, 1994
- Williams B, Schreier RW: Characterization of glucose-induced *in situ* protein kinase C activity in cultured vascular smooth muscle cells. *Diabetes* 41:1464–1472, 1992
- Chakrabarti S, Sima AA: Endothelin-1 and endothelin-3-like immunoreactivity in the eyes of diabetic and non-diabetic BB/W rats. *Diabetes Res Clin Pract* 37:109–120, 1997
- Chakrabarty U, Hayes RG, Stitt AW, Douglas A: Endothelin expression in ocu-

- lar tissues of diabetic and insulin-treated rats. *Invest Ophthalmol Vis Sci* 38:2144–2155, 1997
49. Ayo SH, Radnik R, Garoni JA, Troyer DA, Kreisberg JI: High glucose increases diacylglycerol mass and activates protein kinase C in mesangial cell cultures. *Am J Physiol* 261:F571–F577, 1991
50. Haller H, Baur E, Quass P, Behrend M, Lindschau C, Distler A, Luft FC: High glucose concentrations and protein kinase C isoforms in vascular smooth muscle cells. *Kidney Int* 47:1057–1067, 1995
51. Berti L, Mosthaf L, Kroder G, Kellerer M, Tippmer S, Mushack J, Seffer E, Klaus Seedorf, Haring H: Glucose-induced translocation of protein kinase C isoforms in Rat-1 fibroblasts is paralleled by inhibition of the insulin receptor tyrosine kinase. *J Biol Chem* 269:3381–3386, 1994
52. Igarashi M, Wakasaki H, Takahara N, Ishii H, Jiang Z-Y, 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
53. Inoue A, Yanagisawa M, Takuwa Y, Mitsui Y, Kobayashi M, Masaki T: The human preproendothelin-1 gene: complete nucleotide sequence and regulation of expression. *J Biol Chem* 264:14954–14959, 1989
54. Lee M-E, Dhady MS, Temizer DH, Clifford JA, Yoshizumi M, Quertermous T: Regulation of endothelin-1 gene expression by fos and jun. *J Biol Chem* 265:19034–19039, 1991
55. Soh JW, Lee EH, Prywes R, Weinstein IB: Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. *Mol Cell Biol* 19:1313–1324, 1999