Role of Protein Kinase C on the Expression of Platelet-Derived Growth Factor and Endothelin-1 in the Retina of Diabetic Rats and Cultured Retinal Capillary Pericytes

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Increased expression of endothelin-1 (ET-1) is associated with diabetic retinopathy and vasculopathy, although the molecular explanation has not been defined. The effects of high glucose and protein kinase C (PKC) activation on platelet-derived growth factor (PDGF)-BB and of ET-1 expression in the retina of streptozotocin (STZ)-induced diabetic rats and bovine retinal pericytes (BRPC) were examined. In 4-week diabetic rats, PDGF-B and prepro-ET-1 (ppET-1) mRNA levels increased significantly by 2.8- and 1.9-fold, respectively, as quantified by RT-PCR. Treatment with PKC-β isoform-specific inhibitor (LY333531) or insulin normalized retinal ET-1 and PDGF-B expression. In BRPC, high glucose levels increased ppET-1 and PDGF-B mRNA expression by 1.7- and 1.9-fold, respectively. The addition of PDGF-BB but not PDGF-AA increased expression of ppET-1 and vascular endothelial growth factor mRNA by 1.6- and 2.1-fold, respectively, with both inhibited by AG1296, a selective PDGF receptor kinase inhibitor. A general PKC inhibitor, GF109203X, suppressed PDGF-BB's induction of ET-1 mRNA. Thus, increased ET-1 expression in diabetic retina could be due to increased expression of PDGF-BB, mediated via PDGF-β receptors in part by PKC activation. The novel demonstration of elevated expression of PDGF-B and its induction by PKC activation identifies a potential new molecular step in the pathogenesis of diabetic retinopathy. Diabetes 52:838–845, 2003

Hyperglycemia has been identified as the major risk factor for the development of diabetic microvascular complications, including retinopathy (1,2). There is a great deal of evidence to support the conclusion that hyperglycemia is responsible for many early retinal capillary dysfunctions and lesions such as micro-aneurysms, basement membrane thickening, increases in permeability, and alteration of retinal blood flow (1,2). Elucidating the molecular and biochemical pathways by which hyperglycemia mediates its multiple effects to induce these retinal hemodynamic changes and pathologies has been difficult, although several hypotheses have been proposed (3,4).

Previously, we and others have reported that increases in the expression of cytokines such as endothelin-1 (ET-1) and vascular endothelial growth factor (VEGF) are observed after a short duration of diabetes, preceding the development of functional and histological abnormalities in the retinal microvessels (5–8). Many of these retinal changes in diabetes are associated with changes in ET-1 and VEGF levels. For example, increases in VEGF expression have been correlated with an increase in the retinal capillary permeability and micro-anerysm formation (9,10). Elevated levels of ET-1 expression have been reported to decrease retinal blood flow and loss of autoregulation in response to oxygen tension in the retina (11,12).

The biochemical mechanisms that are responsible for the increased expression of ET-1 and VEGF in the retina are not clear, although it is very likely that they are initiated by hyperglycemia or glucose metabolites. Among the various metabolites that are derived from hyperglycemia, increased levels of glycation products and oxidants and the activation of signaling pathways such as protein kinase C (PKC) have been reported to increase the expression of VEGF and ET-1 in cultured vascular cells (13–15). However, the various biochemical steps by which glucose or its metabolites are specifically inducing ET-1 and VEGF expression are largely unknown.

In this study, the expression of platelet-derived growth factor (PDGF) and its potential role in increasing the expression of ET-1 and VEGF in the retina of diabetic rats and cultured retinal capillary pericytes have been characterized. We chose to study the effect of PDGF expression and action because previous studies have reported that PDGF-BB can increase the expression of ET-1 and VEGF mRNA in cultured vascular smooth muscle and mesangial cells (16–18). However, a change in PDGF expression has not been reported in the retina of diabetic states. We also investigated the possibility that diabetes and hyperglycemia can activate PKC signaling pathways and regulate the
expression of PDGF-BB, which can lead to increases in ET-1 and VEGF expression in the retina.

RESEARCH DESIGN AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and calf serum were purchased from Gibco (Grand Island, NY). TRI-REAGENT was from Molecular Research Center (Cincinnati, OH). Human recombinant PDGF-AA and PDGF-BB were obtained from Sigma Chemical (St. Louis, MO). α,β-32P dCTP was purchased from Du Pont NEN Research Products (Boston, MA). The following items were purchased: nylon membrane from ICN (Aurora, OH); Multiprime DNA labeling system from Amersham Life Sciences (Arlington Heights, IL); and 6,7-dimethoxy-3-phenylquinoxaline (AG1296), bisphenol A dimethacrylate (GF109203X), 2-amino-3-methoxyflavone (PD98059), and BQ-123 sodium salt from Calbiochem-Novabiochem (La Jolla, CA).

Animals. Male Sprague-Dawley rats (200 g; Taconic Farms, Germantown, NY) were divided into four groups: control, 4 weeks of diabetes, 4 weeks of diabetes treated with insulin implant (one implant/200 g body wt; Linshin Canada, Scarborough, ON, Canada), and 4 weeks of diabetes treated with PKC-β selective inhibitor LY333531 (0.062% wt/wt) diet as described previously (19). Male Lewis rats (200 g; Taconic Farms) were divided into two groups: control and 8 weeks of diabetes. The control group received 1 ml/kg body wt of sterile 20 mmol/l citrate buffer (pH 4.5) by intraperitoneal injection. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (60 mg/kg body wt; Sigma Chemical) in citrate buffer. The diabetic state was confirmed 24 h after STZ injections by blood glucose levels exceeding 250 mg/dl. Blood glucose concentration was determined weekly in all animals. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Isolation of bovine retinal pericytes. Primary cultures of bovine retinal pericytes (BRPC) were isolated from fresh bovine eyes by homogenization and a series of filtration steps as described previously (20). BRPC were cultured in DMEM with 5.5 mmol/l glucose and 20% FBS. Cells up to subconfluent were used for the various assays. The expression of PDGF-B chain mRNA was also evaluated in the retinal diencephalons for 30 min after the addition of PDGF. After addition of PDGF-AA or -BB for 4 h, the cells were used for the various assays.

RNA extraction. Total RNA was extracted from retinas of experimental animals and BRPC isolated by the guanidinium thiocyanate method with phenol-chloroform using TRI-REAGENT (19).

Northern blot analysis. Northern blot analysis was performed on 20 μg of total RNA/lane in 1% agarose gel with 1.8% formaldehyde gel electrophoresis, which was separated in MOPS buffer (20 mmol/l MOPS, 5 mmol/l Na acetate, 0.5 mmol/l EDTA [pH 7.0]). Total RNA was transferred onto a nylon membrane. After ultraviolet cross-linking, the membrane was prehybridized and hybridized to 32P-labeled human ET-type DNA probe (0.7 kb), human VEGF cDNA clone (1.9 kb), and human PDGF-B DNA probe (0.8 kb), prepared by the Multiprime DNA labeling system in PERFECTHYB PLUS (Sigma) at 65°C. Washing was performed in 2× SSC, 0.1% SDS at room temperature for 5 min, and two times in 0.1× SSC, 0.1% SDS at 65°C for 20 min. The expression of mRNA was quantified with a Phosphoimager (Molecular Dynamics, Sunnyvale, CA) and normalized using 36B4 as the standard cDNA probes (19).

RESULTS

PDGF-B mRNA levels in the retina. For evaluating the possibility that diabetes may induce PDGF expression in the retina, the expression of PDGF-B chain mRNA in the retina of STZ-induced diabetic rats was evaluated in Sprague-Dawley rats (Fig. 1). PDGF-B mRNA level in the retina of diabetic Lewis rats was significantly increased by 2.8 × 0.3-fold (P < 0.01) after 4 weeks of diabetes (Fig. 1) compared with controls. Similar results were obtained when PDGF-B mRNA levels were normalized to 18 s ribosomal RNA (data not shown). Treatments using insulin implant or oral administration of PKC-β specific inhibitor (LY333531 at 0.062% wt/wt) suppressed totally the increases of PDGF-B mRNA levels in the retina by 120 ± 4% (P < 0.01) and 100 ± 5% (P < 0.01), respectively, in diabetic rats after 4 weeks of duration as compared with control rats (19,21). The expression of PDGF-B chain mRNA was also evaluated in the retina of STZ-induced diabetic Lewis rats. PDGF-B mRNA level in the retina of diabetic Lewis rats was significantly increased by 1.4 ± 0.2-fold (P < 0.05) after 8 weeks of diabetes compared with controls (data not shown).

Inhibition of ET-1 mRNA expression in the retina by PKC-β isoform–specific inhibitor or insulin in vivo. The expression of prepro-ET-1 (ppET-1) mRNA in the retina was also characterized and correlated with the changes in PDGF-B mRNA levels. To determine the role of PKC activation as a mediator of ET-1 expression, we
Inhibition of ppET-1 and PDGF-B mRNA expression by glucose levels in BRPC. To identify the mechanism that may induce ET-1 expression in the retina of diabetic rats, we investigated ET-1 and PDGF-B expression in BRPC when exposed to 5.5 vs. 25 mmol/l glucose. Figure 3A showed that media containing 25 mmol/l glucose increased the expression of ppET-1 mRNA by 1.7 ± 0.2-fold (P < 0.05) compared with cells cultured in 5.5 mmol/l glucose. Furthermore, after exposure to 25 mmol/l glucose-containing media for 72 h, ET-1 expression at basal or stimulated by the addition of PDGF-BB were increased by 3.5 ± 0.28-fold (P < 0.05) and 7.0 ± 0.59-fold (P < 0.05), respectively, compared with 5.5 mmol/l glucose. After exposure to media containing 25 mmol/l glucose for 72 h, PDGF-B mRNA level was significantly increased by 1.9 ± 0.2-fold (P < 0.05) compared with 5.5 mmol/l glucose (Fig. 3B). These results suggested that the exposure of BRPC to 25 mmol/l glucose increased ET-1 and PDGF-B mRNA expression, and PDGF-BB, via an autocrine or paracrine effect, can increase further the expression of ppET-1 mRNA.

Effect of PDGF-AA and PDGF-BB on ET-1 and VEGF expression in BRPC. The expressions of ppET-1 and VEGF mRNA were measured in BRPC that had been cultured in the presence of PDGF-AA or -BB for 4 h with or without AG1296, which is reported to be a specific inhibitor of PDGF receptor tyrosine kinase (22). The addition of PDGF-AA for 4 h did not induce any change in ET-1 and VEGF expression compared with control (Fig. 4A and B). In contrast, the addition of PDGF-BB for 4 h increased ppET-1 and VEGF mRNA expression in BRPC significantly by 1.6 ± 0.2-fold (P < 0.05) and 2.1 ± 0.1-fold (P < 0.01), respectively, compared with control, and the addition of AG1296 completely suppressed increases in ET-1 (Fig. 4C) and VEGF expression (Fig. 4D). These data suggest that PDGF-β, not PDGF-α receptor, is involved in PDGF-BB-induced expression of ET-1 and VEGF mRNA in BRPC.
Effect of GF109203X on ET-1 and VEGF expression in BRPC. To characterize the possible role of PKC activation in PDGF-BB induced ET-1 and VEGF expression, we examined the effects of GF109203X (GFX), a general PKC specific inhibitor, on their expression when stimulated by PDGF-BB (23). PDGF-BB increased ppET-1 and VEGF expression significantly by 1.7 ± 0.2-fold ($P < 0.05$) and 2.1 ± 0.1-fold ($P < 0.01$), respectively. Addition of 5 μmol/l GFX inhibited the PDGF-BB–induced ET-1 expression by 130 ± 20% ($P < 0.05$) compared with PDGF group (Fig. 5A). However, GFX only partially suppressed PDGF-induced VEGF expression by 40 ± 9% ($P < 0.05$) when compared with PDGF-BB alone group (Fig. 5B). These results suggest that PKC mediates the induction of ppET-1 expression by PDGF-BB, but the increase in VEGF expression induced by PDGF-BB is only partly mediated by PKC activation along with other unknown transduction pathways.

Effect of MEK inhibitor on ET-1 expression in BRPC. Previously, we have reported that glucose-induced ppET-1 expression in BRPC was inhibited by the addition of PD98059, a mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor (24). PDGF-BB increased ET-1 mRNA, significantly, by 1.6 ± 0.2-fold ($P < 0.05$). To characterize the role of the MAPK pathway activation in PDGF-BB–stimulated ET-1 expression, we examined the effects of PD98059 on ppET-1 mRNA expression stimulated by PDGF-BB. The addition of PD98059 completely abolished the expression of ET-1 mRNA induced by PDGF-BB to 25 ± 8% ($P < 0.05$) below the control (Fig. 6). These results suggest that both PKC and MAPK activation can regulate PDGF-BB–induced ET-1 mRNA expression in BRPC.

Effect of ET-A receptor specific antagonist on ET-1 and VEGF expression in BRPC. It has previously been reported that VEGF can also enhance the expression of...
ppET-1 mRNA in bovine aortic endothelial cells (25). However, in rat vascular smooth muscle cells, ET-1’s effect on VEGF mRNA expression was abolished by a selective ET-A receptor antagonist. To determine whether ET-A receptor could also play a role in the increase of ET-1 expression induced by PDGF-BB, we measured the effects of BQ123, a selective ET-A receptor antagonist, on PDGF-BB–stimulated ET-1 and VEGF expression in BRPC (7,11). PDGF-BB increased ppET-1 and VEGF mRNA expression significantly by 1.7 ± 0.2-fold (P < 0.05) and 2.1 ± 0.1-fold (P < 0.01), respectively. The addition of BQ123 inhibited PDGF-BB–induced ET-1 expression completely by 112 ± 4% (P < 0.05) and VEGF expression partially by 28 ± 10% (P < 0.05), compared with PDGF-BB only (Fig. 7). These data indicated that the increased expression of ET-1 is partly due to elevation of intrinsic ET-1, which then exerts its action via the ET-A receptors on BRPC. In contrast, ET-A receptor has minimal effects on PDGF-BB–induced increase in the expression of VEGF mRNA.

**DISCUSSION**

Changes in retinal capillary endothelial cells and pericytes occur years before the onset of clinical diabetic retinopathy as manifested by increases in permeability, pericytic death, basement membrane thickening, and endothelial cell proliferation (26). Hyperglycemia, the main initiating factor for the microvascular abnormalities, mediates many of its diverse effects by altering the expression of cytokines such as in the increased expression of ET-1 and VEGF (6,7,27). We and others have also reported that the expression and the actions of ET-1 and VEGF are increased not only in the retina but also in the renal glomeruli of diabetic animals (6,28,29). In the present study, we have focused the studies on the expression of ET-1 and PDGF in the pericytes and retina of diabetic animals because both of those cytokines are known to have important effects on pericyte activation, survival, and growth (30–32).

In this study, we can confirm that the expression of ppET-1 mRNA is increased in the retina even after 8 weeks of diabetes in several species of rats (7,8). Maintaining euglycemia with insulin treatments prevented the increased expression of ET-1 in the retina (33). This finding concurs with our reports that ET-1 actions on decreasing retinal blood flow in the diabetic rats can also be prevented with treatments with insulin (33). We have extended these previous findings on the regulation of ET-1 expression in the retina of diabetic animals by finding that treatment with PKC-β isoform inhibitor LY335351 orally can also prevent the increase in the expression of ET-1 mRNA even in the presence of hyperglycemia. These data provided direct evidence that PKC-β isoform activation is responsible for the elevated retinal ET-1 expression induced by diabetes. This finding supports our previous report that using this same PKC-β isoform inhibitor can normalize retinal blood flow associated with short duration of diabetes (21). This finding that the ET-1 expression in diabetic rats can be prevented or reversed by PKC inhibitor is very different from the regulation of the retinal expression of VEGF mRNA by diabetes, which we have reported that treatment with PKC-β isoform inhibitor was unable to reduce VEGF expression (6). Thus, PKC-β isoform activation is involved in the induction ET-1 ex-
expression but not VEGF expression in the retina of diabetic rats.

The causes for the increases in ET-1 expression in the retina of diabetic rats were further characterized by evaluating the changes in PDGF mRNA expression, which has been reported to regulate the expression of both ET-1 and VEGF in nonretinal vascular cells (16–18). The importance of PDGF on pericyte growth and survival is strongly suggested by the reports that PDGF-β receptor–deficient mice in utero do not have capillary pericytes, which results in death as a result of both retinal and central nervous system hemorrhages (31,32). Our studies have clearly demonstrated that PDGF-B mRNA expression is significantly increased in the retina of diabetic rats. This represents the first report of PDGF-B mRNA level being elevated in the retina of the diabetic state. Because the changes in PDGF-B mRNA level in the retina moved in concurrence with ET-1 expression after treatments with insulin and PKC-β isoform inhibitor LY333531, we suggest that the increase in retinal PDGF-B expression is induced by PKC, which is activated and partially responsible for the increased expression of ET-1 in the retina of diabetic animals (21). The finding that PDGF-B levels could be increased in the retina of diabetic rats is surprising because retinal capillary pericyte loss is a very specific and early pathological finding of diabetic states (26). The report that PDGF-β receptor–deficient mice are lacking in pericytes has led to the speculation that the loss of pericytes in diabetic retinopathy could be due to a general decrease or loss of PDGF expression or action (31,32).

Additional studies will have to be performed to confirm that the protein levels of PDGF-BB are also increased similar to PDGF-B mRNA levels. If it is confirmed, then our results would suggest that the deficiency of PDGF-BB is not the explanation of retinal pericytes loss in diabetes. However, it is still possible that hyperglycemia or diabetes has induced a resistance to PDGF-B’s actions, which could be responsible for the increases in PDGF-B levels in retinas. It is also possible that the increase in PDGF-B expression could be due to the activation of PKC-β isoform by hyperglycemia, which may induce PDGF-B expression (4,34). The loss of retinal pericytes could be due to the toxic effect of hyperglycemia by another mechanism even in the presence of elevated PDGF-B, which have an antiapoptotic action on retinal pericytes and other vascular cells (31,32,35,36).

It is likely that hyperglycemia is causing the elevation of PDGF-B and ET-1 in the retina because the exposure of pericyte to elevated glucose levels can increase the expression of both ET-1 or PDGF-B mRNA (24). We have previously reported that elevating glucose levels to hyperglycemic levels induced the expression of ET-1 mRNA from retinal capillary endothelial cells and pericytes by PKC and MAPK pathways (24). In this study, we suggest that elevated glucose levels increased PDGF-B mRNA expression in the retinal microvessels, most likely as a result of PKC-β isoform activation. PKC activation has been reported to induce PDGF-B expression by activating c-fos and c-jun sites in the promoter region (36,37). Increases in PDGF-B levels can then bind specifically to PDGF-β receptors for biological action because PDGF-AA was ineffective and the inhibition of PDGF-β receptor was completely effective in inhibiting ET-1 expression (36). Once PDGF-β receptors are activated, ET-1 expression is induced via the activation of PKC and MAPK signaling pathways, which have been reported to be activated by PDGF-BB (36). Because the PKC-β isoform–specific inhibitor (LY333531) prevented ET-1 expression both in vivo and in cultured cells, PDGF-B’s effect on increasing ET-1 expression is regulated mostly by PKC-β isoform activation, which then activates or enhances the activation of ERK-1 and -2 pathways and phospholipase C to mediate its vascular actions (36,38).

The above hypothesis is not only supported by our results but also is suggested by previous studies, which have shown that PDGF-β receptors can activate PKC-γ and MAPK (38). In addition, MAPK activation can be regulated by PKC isoforms α, β, γ, and ξ (39,40) or by PKC-independent pathway. In the present study, the results suggest that hyperglycemia and diabetes are inducing the expression of ET-1 and PDGF-β mainly via the PKC and MAPK pathways, because both inhibitors of PKC and MEK were able to prevent their expression in the retina completely.

Our results also compared the increases of ET-1 and VEGF in the retina as related to PDGF-B expression and PKC activation. Previous reports have showed that the expression of VEGF mRNAs is elevated in the retina of diabetic rats, which can be normalized by insulin treatment maintaining euglycemia but not by PKC-β isoform inhibitor (5,6). The results in the present study on retinal ET-1 expression support that diabetes and hyperglycemia...
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are regulating the expression of these two genes differently, especially with regard to PKC activation. The results in diabetic rats is supported by studies in cultured cells in which ET-1 expression induced by PDGF-BB is inhibited by general PKC inhibitors, but PDGF-BB–induced VEGF mRNA was unaffected. However, the finding that PDGF-BB can increase VEGF mRNA expression in retinal vascular cells suggests that increase in PDGF-B expression or increased activation of PDGF-β receptors may contribute to increased retinal levels of VEGF in the diabetic or hyperglycemic state (5,6). It is unlikely that insulin levels played an important role because treatment with insulin decreased both VEGF and ET-1 expression in the retina (6,33). A surprising finding was the ability of BQ123, an antagonist of ET-1 binding to the ET-A receptor, was able to inhibit PDGF-BB–induced ET-1 expression but not VEGF expression. This study was initiated because of previous reports in cell culture studies that VEGF and ET-1 may regulate the expression of each other in vascular cells (16–18). However, our results suggest that ET-A receptor is not mediating PDGF-B expression but may enhance PDGF-BB–induced ET-1 expression in an autocrine or paracrine manner. Additional studies are in progress to clarify this interesting new finding.

In summary, our study has demonstrated for the first time that diabetes and probably hyperglycemia can increase PDGF-B levels in the retina, which could potentially be the initiator for the increase in ET-1 expression in the retina. It is also likely that PKC activation induced by hyperglycemia could be regulating the increase in PDGF-B expression and its actions on ET-1 expression. The finding that retinal PDGF-B levels could be elevated in diabetes may have biological implications in addition to those related to ET-1 actions on retinal blood flow. Additional studies will be needed to determine the pathophysiological consequence of increased PDGF-B expression in the retina in the expression of adhesion molecules, leukocyte adhesion, endothelial and pericyte interactions, extracellular matrix production, and cellular survival and growth. All of these biological actions have been known to be regulated by PDGF-B and are reported to be abnormal in the retinal vasculature of diabetic state.

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