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Effects of protein kinase C inhibition and activation on proliferation and apoptosis of bovine retinal pericytes

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Abstract

Aims/hypothesis. Drop-out of capillary pericytes occurs early and selectively in diabetic retinopathy. High glucose concentrations decrease replication and increase apoptosis of cultured pericytes. Since glucose activates protein kinase C, we investigated the effects of modulating this intracellular mediator on replication, cell cycle and apoptosis of cultured bovine retinal pericytes.
Methods. Pericytes cultured in 5.6 or 28 mmol/l glucose were exposed to a protein kinase C activator (phorbol 12-myristate 13-acetate) and/or a selective inhibitor of its β2 isoform (LY379196). Cells were counted after 7 days. Proliferation by the tetrazolium to formazan assay and DNA synthesis by 5-bromo-2′-deoxyuridine incorporation were measured at day 4. Cell cycle by flow cytometry and apoptosis by ELISA were assessed at day 2.
Results. High glucose reduced pericyte replication and increased apoptosis. Protein kinase C activation increased proliferation, while inhibition of its β2 isoform decreased it. Cell cycle was accelerated by protein kinase C activation and delayed by inhibition. Apoptosis was enhanced by protein kinase C inhibition and reduced by activation.
Conclusions/interpretation. Protein kinase C inhibition amplifies the anti-proliferative and pro-apoptotic effects of high glucose on cultured pericytes, whereas stimulation reduces apoptosis and promotes proliferation both in physiological glucose and high glucose. Protein kinase C inhibition, proposed for the treatment of diabetic macular edema and proliferative retinopathy, might accelerate pericyte dropout in earlier stages when these cells are still present in retinal capillaries. [Diabetologia (2003) 46:416–419]

Keywords Diabetic retinopathy, pericytes, glucose, protein kinase C, cell replication, cell cycle, apoptosis.

Selective loss of capillary pericytes occurs early in diabetic retinopathy [1]. Previous work in vitro showed that high glucose concentrations reduce replication of bovine retinal pericytes (BRP) by non-osmotic mechanisms [2]. High glucose also stimulates synthesis of protein kinase C (PKC), via increased diacylglycerol (DAG) production from glycolytic metabolites [3]. PKC activation, in turn, could mediate basement membrane thickening, increased capillary permeability and reduction of Na^+-K^+ ATPase [3]. However, little is known on the effects of PKC on the viability of capillary pericytes.

The aim of this work was to evaluate the effects of PKC inhibition and activation on replication, apoptosis and cell cycle traversal of BRP. A specific inhibitor of the β2 isoform of PKC (LY379196) and phorbol 12-myristate 13-acetate (PMA), a non-selective PKC activator, were tested in the presence of physiological and high glucose concentrations.

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Abbreviations: BRP, bovine retinal pericytes; PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; iPKC, protein kinase C β2 inhibitor LY379196; BrdU, 5-bromo-2′-deoxyuridine; AGE, Advanced Glycosylation End-products.

Materials and methods

Cell cultures. BRP were obtained from pools of 15 to 20 bovine retinas and characterized by 3G5 fluorescence immunostaining, as described previously [4]. All experiments were repeated using a different pool every time. Pericytes were grown in DMEM with 5.6 mmol/l D-glucose (Sigma Chemical, St.Louis, Mo., USA) and 10% FCS (Sigma), until confluent. Cells were then trypsinized, seeded in 6-, 24- or 96-well plates as appropriate and cultured in media with 5.6 (physiological) or 28 mmol/l (high) D-glucose (Sigma). Dose-response curves were done with LY379196 (generously provided by Eli Lilly, Indianapolis, Ind., USA) 0, 100, 500, 1,000, 2,000, 5,000 and 10,000 nmol/l. PKC was stimulated by exposing BRP to PMA 100 or 500 nmol/l for 1 h per day.

Cell counts. Second-passage BRP (10,000 cells/cm^2) were seeded in 6-well plates in the different experimental conditions, trypsinized after 7 days and counted in a Bürker chamber, after vital colouring with Trypan blue 0.2% (Sigma).

Cell proliferation (MTT). BRP were seeded in 24-well plates (10,000 cells/cm^2). After 4 days, proliferation was measured using the Cell Proliferation Kit I, (Roche Diagnostics, Basel, Switzerland), which measures cleavage of the tetrazolium salt MTT by mitochondrial dehydrogenases in metabolically active cells. Absorbances were detected spectrophotometrically at 580 nm.

DNA synthesis. BRP were seeded in 24-well plates. After 4 days, DNA incorporation of the pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU) instead of thymidine was detected by immunoassay (Cell Proliferation ELISA, BrdU, Roche). Absorbances were read at 450 nm in an ELISA reader.

Cell cycle. BRP were seeded in 96-well plates (10,000 cells/cm^2). After 2 days, BRP were fixed and permeabilized by 70% ethanol and DNA stained with propidium iodide (20 µg/ml in PBS+0.1% BSA). Fluorescence was evaluated by flow cytometry, using FACScan equipment (Becton Dickinson Immunocytometry Systems, San José, Calif., USA). Results are expressed as percentages of elements detected in the different phases of the cell cycle, namely G0/G1 (no DNA synthesis), S (active DNA synthesis), G2 (pre-mitotis) and M (mitosis). The apparatus was calibrated to test 10,000 cells from each sample.

Apoptosis. BRP were seeded in 96-well plates. Apoptosis was measured after 2 days by the Cell Death Detection ELISA^{PLUS} kit (Roche), based on a quantitative sandwich-enzyme-immunoassay principle, using monoclonal antibodies directed against DNA and histones that allow specific measurement of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates.

Statistical analysis. Data are expressed as percentages (means±SD) of results obtained with positive control conditions (BRP in 5.6 mmol/l glucose) within each experiment. Statistical comparisons among groups were done by one-way analysis of variance and two-tailed Student's *t*-test for paired data. Because of the multiple comparisons done in some experiments, a *p* value of less than 0.01 was considered significant.

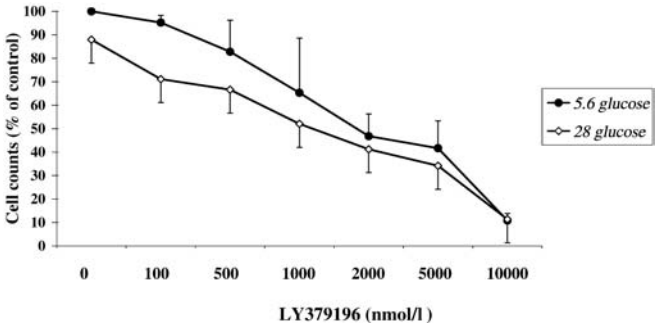

Fig. 1. Dose-response curves, cell counts of BRP cultured in 5.6 and 28 mmol/l glucose with increasing concentrations of the PKC-β2 inhibitor LY379196 (*n*=6)

Results

Dose-response curves show that BRP counts decreased with increasing concentrations of LY379196, both in physiological and high glucose (Fig. 1). A trend to reduced replication was seen already at 100 nmol/l, both in 5.6 mmol/l (100 vs 95.2±3.1, p=0.013) and 28 mmol/l glucose (87.9±10.1 vs 71.1±7.9, p=0.048). LY379196 100 nmol/l plus glucose 28 mmol/l had a combined effect, when compared to physiological glucose alone, p<0.001. Cell counts were approximately halved at LY379196 1,000–2,000 nmol/l. Consequently, the following experiments were carried out using 1,000 nmol/l LY379196.

Cell counts confirmed that BRP grown in high glucose were less than in physiological glucose (Table 1). LY379196 1000 nmol/l reduced counts in both physiological and high glucose, while PMA 100 and 500 mmol/l increased them. LY379196 1,000 nmol/l nearly halved the proliferative effect of both PMA concentrations. The cell count results were confirmed by the MTT cleavage method (Table 1).

High glucose reduced BrdU incorporation (83.2±15.8%, p=0.034 vs 5.6 glucose). LY379196 1000 nmol/l reduced BrdU incorporation in normal glucose (66.4±14.6%; p=0.004 vs 5.6 glucose) though not high glucose (70.8±18.9%). PKC stimulation by PMA 500 nmol/l increased DNA synthesis both in physiological (167.0±39.0%, p=0.007 vs 5.6 glucose) and high glucose (154.0±35.1%, p<0.001 vs 28 glucose).

Flow cytometry indicated that pericytes in phases S and G2/M decreased after PKC inhibition and increased after PMA. Conversely, pericytes in G0/G1 were more numerous after PKC inhibition and less after PMA. BRP in G0/G1 phase were 36.0±13.8% of total in physiological glucose, 31.3±15.9% in high glucose, 46.2±12.6% in glucose 5.6+LY379196 1,000 nmol/l, 43.1±14.5% in glucose 28+LY379196, 21.4±12.6% in glucose 5.6+PMA 500 mmol/l, and 20.5±14.3% in glucose 28+PMA. Cells in S phase were 41.9±16.5% in glucose 5.6, 45.1±19.7% in glu-

Table 1. Cell counts after 7 days and cell proliferation (MTT cleavage) after 4 days of the addition of LY379196 (1,000 nmol/l) and/or PMA (500 nmol/l) to BRP cultured in glucose 5.6 mmol/l and 28 mmol/l (n=6)

	5.6 mmol/l glucose				28 mmol/l glucose			
	Cell counts		MTT		Cell counts		MTT	
	Percentage	p value	Percentage	p value	Percentage	p value	Percentage	p value
Control	100	–	100	–	89.7±6.6	p=0.012 vs 5.6 glucose	85.4±7.6	p=0.008 vs 5.6 glucose
LY379196 (1,000 nmol/l)	52.3±22.8	p=0.004 vs 5.6 glucose	74.3±14.5	p=0.007 vs 5.6 glucose	44.9±11.5	p<0.001 vs 28 glucose	58.4±11.9	p=0.012 vs 28 glucose
PMA (100 nmol/l)	581.3±328	p=0.016 vs 5.6 glucose	246.0±106.4	p=0.02 vs 5.6 glucose	391.1±139.2	p=0.003 vs 28 glucose	137.1±40.8	p=0.026 vs 28 glucose
PMA (100 nmol/l) + LY379196 (1,000 nmol/l)	264.7±90.6	p=0.026 vs 5.6 glucose + PMA 100	120.4±48.0	p=0.002 vs 5.6 glucose + PMA 100	112.2±54.8	p=0.004 vs 28 glucose + PMA 100	89.9±4.7	p=0.041 vs 28 glucose + PMA 100
PMA (500 nmol/l)	696.9±276.9	p=0.003 vs 5.6 glucose	422.9±121.5	p=0.001 vs 5.6 glucose	566.8±272.7	p=0.007 vs 28 glucose	308.1±106.6	p=0.005 vs 28 glucose
PMA (500 nmol/l) + LY379196 (1,000 nmol/l)	310.9±100	p=0.003 vs 5.6 glucose + PMA 500	152.8±54.8	p=0.004 vs 5.6 glucose + PMA 500	145.2±36.2	p=0.008 vs 28 glucose + PMA 500	144.1±59.6	p=0.043 vs 28 glucose + PMA 500

cose 28, 36.2±15.2% in glucose 5.6+LY379196, 37.2±15.2% in glucose 28+LY379196, 48.9±15.7% in glucose 5.6+PMA, 50.1±16.8% in glucose 28+PMA. Cells in G2/M were 16.1±3.6% in glucose 5.6, 15.5±4.5% in glucose 28, 13.7±2.8% in glucose 5.6+LY379196, 14.6±3.0% in glucose 28 + LY379196, 21.1±2.9% in glucose 5.6+PMA, and 19.7±3.0% in glucose 28+PMA.

Apoptosis rate was higher in BRP kept in high glucose (122.8±14.3%, p=0.011) than in 5.6 mmol/l. LY379196 increased apoptosis (138.5±24.7%, p=0.012), and PMA decreased it (72.0±23.8%, p=0.035) in 5.6 glucose. This pro-apoptotic effect of LY379196 further increased that of high glucose (146.7±34.7%, p=0.049 vs glucose 28 alone), while PMA abolished it (68.8±27.9%, p=0.02 vs glucose 28).

Discussion

High glucose concentrations can accelerate glycolysis, with increased production of glycerol-3-phosphate, which either directly stimulates the synthesis of DAG or is transformed into lysophosphatidic acid, phosphatidic acid and again into DAG [3, 5]. Increased cytoplasmic DAG enhances PKC affinity for calcium, causing its translocation to the cell membrane and consequent activation [6].

Excess PKC activation was implicated in tissue damage caused by chronic hyperglycaemia, including increased capillary permeability and basement membrane thickening, reduced Na^+-K^+-ATPase activity, enhanced monocyte adhesion to the vessel wall, and impaired smooth muscle contractility [3]. PKC also regulates cell proliferation, differentiation and survival. Although the specific roles of its different isoforms have not been fully clarified, the α and β isoforms seem to be specifically implicated in cell proliferation [7]. PKC-β2, in particular, mediates the angiogenic and permeability-enhancing effects of vascular endothelial growth factor-vascular permeability factor (VEGF-VPF), involved in sight-threatening diabetic retinopathy [8]. Evidence for a central role of PKC in diabetic macular oedema and proliferative retinopathy led to develop specific β isoform inhibitors for therapeutic purposes. However, the possibility that PKC inhibition affects capillary cell replication at earlier stages of retinopathy was not investigated in vitro or in experimental diabetes. Mural pericytes, in particular, are lost early in the natural history of this complication and their absence could facilitate subsequent events altering capillary permeability, autoregulation and patency [1].

We show that selective inhibition of the PKC β2 isoform results in dose-dependent reduction of BRP replication in normal and high glucose. Conversely, PKC stimulation by PMA increases cell replication five- to sixfold, an effect inhibited by LY379196, con-

firming that PMA action was mediated by PKC. LY379196 increased the percentage of resting cells (G0/G1), whereas PKC stimulation markedly increased actively replicating ones (S and G2/M), confirming a major role for PKC in pericyte progression along the cell cycle.

Apoptosis could play a role in early pericyte dropout of diabetic retinopathy [9]. Mechanisms proposed to account for this phenomenon include rapid fluctuations of blood glucose, increased Bax expression during hyperglycaemia, aldose-reductase hyperactivity, increased AGE production and nuclear factor-kappa B activation [9, 10]. Our results confirm that high glucose increases pericyte apoptosis and suggest that PKC inhibition could further amplify it. The potential role of other in vivo determinants, like hypoxia and/or altered haemodynamic forces, in modifying pericyte survival in the retinal microcirculation remains to be established.

In contrast with what has been suggested for the sight-threatening stages of retinopathy, when most pericytes are presumably lost and endothelial cell dysfunction and/or proliferation prevail, activation of the DAG-PKC pathway in earlier phases might have a role in contrasting the effects of hyperglycaemia on pericyte proliferation and apoptosis. We tested LY379196 at 1000 nmol/l, presumably higher than plasma concentrations reached after administration of LY333531, a similar drug currently undergoing clinical evaluation. However, LY379196 100 nmol/l was synergistic with high glucose in causing a statistically significant, albeit small, reduction of BRP counts. Thus, the possibility that pharmacological inhibition of PKC β-isoforms might accelerate pericyte loss in pre-clinical and/or mild retinopathy should be carefully evaluated in animal models.

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