Impaired membrane translocation of thrombin stimulated PKC ϵ by high glucose

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Abstract

Hyperglycaemia is known to cause endothelial dysfunction and to promote diabetic angiopathy. Therefore, this study was designed to evaluate the effect of long term incubation (16 ± 1 days) in 30 mM vs 5 mM glucose on ligand induced translocation of protein kinase C (PKC) in paired cultures of individual isolates of human umbilical vein endothelial cells (HUVECs). Cells were stimulated with increasing concentrations of thrombin (0.01, 0.1, 1, 10 and 100 nM) for 30 seconds in the presence of 5 mM and 30 mM glucose, respectively, and analyzed by immunoblotting for PKC-isoforms α and ϵ . Stimulation by thrombin of confluent cultures displayed a concentration dependent rise in membrane bound PKC α and ϵ .

Translocation of PKC α by thrombin remained unaffected by high versus normal ambient glucose, whereas translocation of PKC ϵ in cells grown in 30mM glucose was reduced at maximal thrombin concentrations (area under the curve, AUC: $90.4 \pm 7\%$ of control cells; $p < 0.008$; $n=6$) versus control cultures kept in 5mM glucose. In the identical isolates translocation of PKC ϵ was not reduced by 30 mM mannitol used as osmotic control. No change was induced by long term incubation of resting cells with 30 mM vs 5 mM glucose as to total and membrane bound PKC α or PKC ϵ . The obtained data suggest modulation by 30 mM glucose of ligand induced PKC translocation in an isoform specific manner, whereas subcellular distribution of PKC isoforms in the absence of thrombin stimulation remains unaffected by 30 mM glucose.

Introduction

Diabetic vascular complications are related to the prevailing metabolic milieu with hyperglycaemia being the main causal factor. In that context, activation of protein kinase C (PKC) in response to elevated glucose concentrations via increased synthesis of diacylglycerol (DAG) (Inoguchi *et al.* 1992) has been shown in a variety of cells from vascular tissue (Lee *et al.* 1989; Xia *et al.* 1994). Protein kinase C consists of several distinct isoforms, differing in molecular structure, function, as well as localisation within the cell and is known to modulate besides proliferation and signal transduction also endothelial barrier function (Lynch *et al.* 1990, Hempel *et al.* 1997). To better understand the role of the PKC pathway in hyperglycaemia exposed human vascular endothelium we evaluated the effect of 30 mM vs 5 mM glucose longterm incubation in human umbilical vein endothelial cells (HUVECs) on basal PKC subcellular distribution and on ligand induced translocation of

PKC using thrombin, which binds to a G-protein coupled receptor (Obberghen-Schilling & Pouyssegur 1993).

Materials & Methods

Endothelial cells were derived from human umbilical cord veins and isolated by collagenase treatment and cultured in Medium 199 (Sigma St. Louis, MO) supplemented with 17% fetal calf serum (Gibco-BRL, Grand Island, NY), 0.08 mg/ml endothelial cell growth factor (Technoclone, Vienna, Austria), 83 IU/ml penicillin-streptomycin (Gibco, BRL, Paisley, Scotland), 0.4 μ g/ml fungizone (Gibco, BRL, Paisley, Scotland) and 4 U/ml heparin (Biochrom, Berlin, Germany) as described previously (Baumgartner-Parzer *et al.* 1994). The cultures were maintained at 37°C under 5% CO₂ / 95% O₂ and subcultures were performed using 0.02% EDTA in PBS. Endothelial cells were identified by typical phase contrast „cobblestone" morphology.

PKC epsilon

Figure 1 Translocation of PKC ϵ from the cytosolic to the membrane fraction after stimulation with 0.01, 0.1, 1, 10 and 100 nM thrombin for 30 seconds. Equal amounts of protein of the membrane fraction and the cytosolic fraction respectively were separated on SDS-PAGE transferred to nitrocellulose and probed with isoform specific antibodies against PKC ϵ .

Incubation conditions

From each umbilical cord vein, cells were split up for longterm incubation with either 5 mM or 30 mM glucose or 30 mM mannitol for osmotic control, respectively. For all experiments, endothelial cells were used as individual isolates in first subculture. Cells determined for incubation in 5 mM glucose were plated at 2.8×10^5 cells per 100 mm dish, whereas companion cells (from the same individual isolate) for incubation in 30 mM glucose were plated with 25% higher density. Differential plating density was implemented to study experimental and control cells at identical final density (Baumgartner-Parzer *et al.* 1995). Confluent cultures of the first passage were starved for 12 h with 0.4% fetal calf serum in Medium 199 and harvested for preparation of whole cell lysates or were stimulated with increasing concentrations of thrombin (0.01, 0.1, 1, 10 and 100 nM; Sigma, St. Louis, MO) for 30 seconds with subsequent preparation of membrane fraction.

Preparation of the membrane fraction

After extensive washing with cold PBS, the cells were quickly scraped in 1 ml of buffer A containing 8.5 mM HEPES, pH 7.6, 4.25 mM EDTA, 0.425 mM EGTA, 0.1 mg/ml aprotinin, 0.1 mg/ml leupeptin, 1.0 mM phenylmethylsulfonylfluoride, 0 mM β -mercaptoethanol, 240 mM sucrose and 10% (v/v) glycerol. After two freeze and thaw cycles, cytosolic and membrane fractions were separated by ultracentrifugation ($100,000 \times g$ 1 h $4^\circ C$). The pellet

was resuspended in 100 μ l of buffer B (10 mM HEPES, pH 7.6, 10 mM EDTA, 5 mM EGTA, 0.1 mg/ml aprotinin, 0.1 mg/ml leupeptin, 1.0 mM phenylmethylsulfonyl-fluoride, 10% (v/v) glycerol and 1% (v/v) Triton X-100), incubated for 30 minutes at $4^\circ C$ and centrifugated at $100,000 \times g$ for 30 minutes. The supernatant was collected and represents the Triton extractable component of the membrane fraction.

Whole cell lysate

Cells were washed with cold PBS and homogenized in buffer B supplemented with 0.1% (v/v) SDS.

Western blotting

Equal amounts of protein (6 μ g / lane; determined by Micro-BCA Kit, Pierce, Rockford, IL) were separated on SDS-PAGE (8%), transferred to nitrocellulose membranes (Schleicher&Schuell, Inc, Keen, NH) and analyzed by immunoblotting using polyclonal antibodies (Santa Cruz Biotechnology, S. Cruz, CA) against PKC isoforms alpha (PKC α), and epsilon (PKC ϵ). After incubation with donkey anti rabbit antibody (Amersham Corp, Arlington Heights, IL) the respective bands were visualized using the ECL system (Amersham Corp, Arlington Heights, IL). A cell lysate from rat brain was used as a positive control, for negative control experiments the primary antibody was omitted. Quantification of the intensity of isoform specific bands was performed by scanning

Figure 2 Dose dependent increase in membrane bound PKC upon stimulation with thrombin. Human umbilical vein endothelial cells incubated in 30 mM glucose (O) as well as control cells (■) from the same isolate kept in 5 mM glucose were stimulated with 0.01, 0.1, 1, 10 and 100 nM thrombin for 30 seconds. Equal amounts of protein of the membrane fraction were separated on SDS-PAGE, transferred to nitrocellulose and probed with isoform specific antibodies against PKC α and ϵ . The results obtained by scanning densitometry of the isoformspecific bands are given as the means ($\pm$ SEM) of 6 individual HUVEC- cultures (* $p < 0.01$; ** $p < 0.001$; ¶ $p = n. s.$).

densitometry (Hirschmann Elscript 400 densitometer, Munich, Germany).

Statistical analysis

Data are expressed as means $\pm$ SD unless indicated otherwise and were analyzed by Student's paired t-test.

Results

Paired HUVEC cultures obtained from 6 individual human umbilical cord veins were exposed to 5 mM and 30 mM glucose as well as to 30 mM mannitol

and reached confluence in first subculture after 16 ± 1 days. Cell numbers determined after starvation with 0.4 % FCS in Medium 199 for 12 hours did not differ between groups (5 mM glucose: $2.19 \pm 0.4 \times 10^6$, 30 mM glucose: $2.24 \pm 0.5 \times 10^6$ and 30 mM mannitol: $2.21 \pm 0.4 \times 10^6$).

No change was induced by longterm (16 ± 1 days) exposure to 30 mM glucose vs cells kept in 5 mM glucose (100%) in total amount of PKC α ($88.4 \pm 17.4\%$; $p = n. s.$) and PKC ϵ ($80.7 \pm 26\%$; $p = n. s.$; $n = 6$ each) using western blot analysis.

The amount of PKC bound to the membrane fraction in resting cells displayed no difference after exposure to 5 mM and 30 mM glucose, respectively, neither for PKC ϵ ($95.2 \pm 8\%$ of control cells; $p = n.s.$; $n = 6$) nor for PKC α ($119 \pm 15\%$ of control cells $p = n.s.$; $n = 6$).

Stimulation of HUVECs with thrombin (0.01, 0.1, 1, 10 and 100 nM) for 30 seconds resulted in a dose-dependent increase in membrane bound PKC ϵ and PKC α (Fig. 1 and 2). Ligand mediated stimulation of PKC α was unaffected by 30 mM glucose (Area Under the Curve / AUC: $111.9 \pm 14\%$ of control, $p < 0.089$; $n = 6$). The amount of PKC ϵ translocated to the membrane fraction in cells grown in 30 mM glucose was significantly reduced at concentrations of 1, 10 and 100 nM thrombin (AUC: $90.4 \pm 7\%$ of control cells, $p < 0.008$, $n = 6$) versus control cultures kept in 5 mM glucose (Fig. 2). Cells cultured in 30 mM mannitol did not show reduced translocation of PKC ϵ .

Discussion

Endothelial cells are subject to damage by hyperglycaemia finally resulting in diabetic angiopathy. In this context, particular attention has been paid to the diacylglycerol (DAG) - protein kinase C (PKC) pathway. PKC β II, one of its isoforms, has been shown to be activated by elevated glucose concentrations in vascular cells (Inoguchi *et al.* 1992). Human umbilical vein endothelial cells are a common model for the study of vascular complications in diabetes mellitus, but so far the influence of high ambient glucose on PKC isoforms in HUVECs has not yet been evaluated. Investigating the impact of longterm (16 ± 1 days) 30 mM glucose exposure on PKC α , a classical PKC isoform (cPKC), as well as PKC ϵ , one of the new PKCs (nPKC), neither changes of protein expression nor of the subcellular distribution of PKC has been observed in this study which is in concordance with reports by others (Cagliero *et al.* 1991).

Besides its role as an important clotting factor, thrombin modulates the function of endothelial cells via binding to its receptor, thrombomodulin, and subsequent translocation of PKC from the cytosol to the membrane fraction as demonstrated in bovine pulmonary artery endothelial cells (Stasek & Garcia 1992). In this study, thrombin is shown to induce translocation of PKC isoforms alpha and epsilon in HUVECs in a dose dependent manner. Previous studies have demonstrated that PKC translocation of distinct PKC isoforms may be different (Crabos *et al.* 1992) and also depends on the stimulus (Ha & Exton 1993).

Long term exposure of HUVECs to elevated glucose concentration decreased the amount of translocated PKC ϵ but not of PKC α upon stimulation with maximal concentrations of thrombin compared to control cells. Such impairment by longterm exposure to high glucose concentration of thrombin induced translocation of PKC ϵ may indicate a pathway for detrimental glucose action on endothelial function. From this it appears that high glucose is able to interfere with the thrombin signaling system leading to translocation of PKC at a postreceptor level, even beyond G-proteins (Mancusi *et al.* 1996) possibly by altering the recruitment of a single isoform. That finding seems to be specific for glucose as maximal thrombin stimulated PKC ϵ translocation was not reduced by 30 mM mannitol. The obtained data thus suggest that part of endothelium directed glucose toxicity could be mediated via impaired translocation of PKC isoforms.

To which extent this observation also relates to other PKC isoforms and / or plays a role for the development of vascular complications in patients with diabetes mellitus remains to be elucidated.

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