Translocation of protein kinase C α and ζ in rat glomerular mesangial cells cultured under high glucose conditions

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Summary The activities and expression of protein kinase C isoenzymes were examined in glomerular mesangial cells cultured under high glucose conditions. Exposure of cells to high glucose concentrations (27.8 mmol/l) for more than 3 days resulted in a significant elevation of protein kinase C activities in the membrane fraction. Of the protein kinase C isoenzymes, the levels of protein kinase C α significantly increased in the membrane fraction after 3 days of exposure to glucose, and protein kinase C ζ

increased after 5 days of exposure. Levels of protein kinase C δ and ε remained unchanged and protein kinase C β and γ were not detected. These results indicate that protein kinase C α and ζ are translocated under high glucose conditions possibly through different mechanisms. [Diabetologia (1994) 37: 838–841]

Key words Diabetic nephropathy, glomerular mesangial cells, protein kinase C, isoenzymes, high glucose.

Protein kinase C (PKC) is involved in the intracellular signal transduction system and is generally hypothesized to be essential in agonist-induced cellular functions [1]. Vasoactive substances, such as angiotensin II, activate PKC through stimulation of phospholipase C and induce sustained contraction, synthesis of prostanoids, and proliferation of glomerular mesangial cells. PKC is composed of a family of related isoenzymes [1] the functions of which may differ, making it important to determine the isoenzyme(s) in both physiologic and pathologic states.

Recently, an increase in the basal activity of PKC has been reported in diabetic glomeruli [2] and in glomerular mesangial cells cultured under high glucose condi-

tions [3]. The de novo synthesis of DAG from excessive glucose is proposed to be responsible for the activation of PKC [2, 3]. However, little is known about the PKC isoenzymes activated under high glucose conditions. We previously have shown that agonist-induced cellular responses such as contraction and proliferation are impaired in glomerular mesangial cells of diabetic animals [4] or when cultured under high glucose conditions [5, 6]. This impairment might be due to an activation of PKC because treatment of mesangial cells with TPA desensitizes angiotensin II-induced phosphoinositide hydrolysis and this effect is considered to be mediated by the activation of PKC α [7]. Thus, the present study was performed to identify the PKC isoenzymes activated in glomerular mesangial cells cultured under high glucose conditions.

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Abbreviations: PKC, Protein kinase C; DAG, diacylglycerol; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-O-tetradecanoylphorbol 13-acetate; DEAE cellulose, diethylaminoethyl cellulose; kDa, kilodalton.

Materials and methods

Materials. Fetal bovine serum was obtained from Gibco (Grand Island, N. Y., USA). The protein kinase C assay system (RPN 77) was purchased from Amersham International plc (Amersham, Bucks., UK). Monoclonal antibodies against PKC α , β , and γ were obtained from Seikagaku Kogyo (Tokyo, Japan) and polyclonal antibodies against PKC δ , ε , and ζ were obtained from

Fig. 1. Detection of PKC isoenzymes in mesangial cells cultured under high glucose conditions. Confluent cells were cultured in the presence of 5.6 mmol/l (lane A, B, and C, cultured in different dishes) or 27.8 mmol/l (lane D, E, and F, cultured in different dishes) glucose for 5 days. Immunoblot analysis was performed on both membrane and cytosol fractions using antibodies specific to each PKC isoenzyme. Approximate molecular weight of PKC α , δ , ϵ , and ζ was 80 kDa, 80 kDa, 90 kDa, and 70 kDa, respectively. Specificity of the band of PKC δ , ϵ , and ζ was also confirmed by blocking with isoenzyme-specific peptide (supplied from Gibco)

Gibco. All other reagents were of chemical grade and purchased from standard suppliers.

Mesangial cell culture. Glomeruli were isolated from male Sprague-Dawley rats weighing 100–150 g by sieving with stainless steel and nylon meshes under sterile conditions as previously described [5, 6]. Isolated glomeruli were cultured in RPMI 1640 medium (glucose 11.1 mmol/l) containing 20% fetal bovine serum and antibiotics. Cultured cells were identified as mesangial cells as previously described [5, 6]. Confluent cells from the third to eighth passages were exposed to the experimental RPMI 1640 medium (glucose free) containing 0.4% fetal bovine serum and antibiotics supplemented with 5.6 mmol/l glucose (control group) or 27.8 mmol/l glucose (high glucose group). The medium was changed daily and the cells were harvested after 5 days unless otherwise indicated.

Measurement of PKC activity. Mesangial cells were grown on 150-mm culture dishes. PKC was partially purified at 4°C. In brief, cells were washed once with Ca^{2+} and Mg^{2+} free phosphate buffered saline, twice with a buffer containing 50 mmol/l Tris-HCl, pH 7.5, 2 mmol/l EDTA, 10 mmol/l EGTA, 10 mmol/l benzamide, and 250 mmol/l sucrose, and once with the same buffer containing 20 $\mu\text{g/ml}$ leupeptin and 50 $\mu\text{g/ml}$ PMSF (buffer A). The cells were scraped off the dishes with buffer A and centrifuged at $500 \times g$ for 3 min. The cell pellet was re-suspended in 3 ml of buffer A, sonicated, and centrifuged at $100,000 \times g$ for 60 min. The supernatant fraction was used as the cytosol fraction. The pellet was solubilized with 3 ml of buffer A with 0.5% Triton X-100. After a 30-min incubation at 4°C, the solubilized fraction was centrifuged at $100,000 \times g$ for 60 min, and the supernatant fraction was used as the membrane fraction. Both the cytosol and membrane fractions were passed through DEAE cellulose columns (bed volume 1.0 ml). The column was washed with buffer B (50 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA, 1 mmol/l EGTA, and 50 mmol/l mercaptoethanol) and eluted with 2 ml of buffer B containing 100 mmol/l NaCl. Protein kinase C activity was determined using a protein kinase C assay system, RPN 77. In summary, a 25- μl aliquot of the membranous or cytosolic fraction was added to 25 μl of a reaction mixture consisting of 50 mmol/l Tris-HCl, pH 7.5, 1 mmol/l calcium acetate, 75 $\mu\text{mol/l}$ synthetic peptide, and 2.5 mmol/l dithiothreitol with or without 0.67 mole% La phosphatidyl-L-serine and 2 $\mu\text{g/ml}$ TPA. The re-

action was started by adding 25 μl of a solution containing 50 $\mu\text{mol/l}$ γ -[^{32}P]ATP (0.2 $\mu\text{Ci/tube}$) and 15 mmol/l magnesium acetate in 50 mmol/l Tris-HCl, pH 7.5. Reactions were incubated at 25°C for 15 min and then stopped by adding 100 μl of stop reagent. The reaction mixture was then passed through a filter, and the filter was then washed with 20 ml of 5% acetic acid. Radioactivity that remained on the filter was quantitated in a liquid scintillation counter. PKC activities were calculated by subtracting the activities obtained in a mixture lacking Ca^{2+} and phosphatidylserine.

Immunoblot analysis of PKC isoenzymes. Membrane and cytosol fractions of mesangial cells were prepared as described above. Samples (75 to 100 μg protein) were electrophoresed on an SDS-PAGE (7.5% acrylamide gel) and transferred to a polyvinylidene difluoride filter (Immobilon, Millipore, Bedford, MA, USA) for 1 h at 200 mA using Multiphor II (Pharmacia LKB, Uppsala, Sweden). The filter was blocked with 2% (for PKC α , β , or γ) or 5% (for PKC δ , ϵ , or ζ) non-fat dry milk in buffer C (10 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl, 0.05% Tween 20) at 4°C overnight and then incubated with specific antibodies in buffer C at room temperature for 1 h. Filters were then washed three times with buffer C and incubated with alkaline phosphatase-conjugated anti-mouse (for PKC α , β , and γ) or anti-rabbit (for PKC δ , ϵ , and ζ) IgG at room temperature for 1 h. Colour development was performed with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. Intensities of bands were quantitated by densitometry on a Shimadzu CS-910 scanning densitometer.

Statistical analysis

Results are expressed as mean $\pm$ SD. Comparisons between two groups were analysed by Student's unpaired *t*-test.

Results

After mesangial cells were exposed to high concentrations (27.8 mmol/l) of glucose for 5 days, PKC activity increased significantly in the membrane fraction

Fig. 2. Kinetics of PKC α and ζ expression and activity in mesangial cells cultured under high glucose conditions. Confluent cells were exposed to 5.6 mmol/l or 27.8 mmol/l glucose for various times as indicated. PKC activity as well as PKC α and PKC ζ levels were analysed in cells exposed to 27.8 mmol/l glucose (●; membrane fraction, ○; cytosol fraction) and are expressed as percent of the values obtained in cells exposed to 5.6 mmol/l glucose (control) for the same period. Intensities of bands of PKC α and ζ were quantitated by densitometry on a Shimazu CS-910 scanning densitometer. Mean $\pm$ SD, $n = 6$. * $p < 0.01$ vs control

(23.8 ± 2.3 pmol $\cdot$ mg protein $^{-1} \cdot$ min $^{-1}$ in high glucose culture vs 12.5 ± 4.2 in normal glucose culture, $p < 0.01$) but not in the cytosolic fraction (75.7 ± 16.1 in high glucose culture vs 79.7 ± 13.0 in normal glucose culture). After a 5-day exposure to high glucose concentrations, the levels of PKC isoenzymes were assayed. PKC α and ζ significantly increased in the membrane fraction (%)

of control; 201.4 ± 21.1 %, mean $\pm$ SD, $n = 6$, $p < 0.01$, and 437.5 ± 59.2 %, $n = 6$, $p < 0.01$, respectively), while levels of PKC δ and ϵ remained unchanged (Fig. 1). Neither PKC β nor γ was detected under either condition. Levels of PKC α and ζ in the cytosolic fraction tended to decrease after exposure to high glucose conditions (% of control; 85.6 ± 6.0 %, $n = 6$, and 70.9 ± 11.0 %, $n = 6$, $p < 0.05$, respectively). PKC activity and levels of PKC α increased significantly in the membrane fraction of cells cultured under high glucose conditions for more than 3 days as compared with control cells (Fig. 2). A significant increase in PKC ζ levels in the membrane fraction was observed only after exposure of cells to high glucose concentrations for 5 days (Fig. 2). As previously described [7, 8], PKC α was translocated to the membrane fraction within 15 min after TPA treatment and decreased after 6 h of treatment, while the localization of PKC ζ did not change in response to TPA (data not shown).

Discussion

The present study indicates that PKC α and PKC ζ isoenzymes are translocated in glomerular mesangial cells cultured under high glucose conditions possibly through different mechanisms.

PKC isoenzymes are currently classified into three groups on the basis of their structural similarities [1]. Classic or conventional PKC (cPKC) is activated by DAG and Ca^{2+} , novel PKC (nPKC) is activated by DAG but not by Ca^{2+} , and atypical PKC (aPKC) is not affected by DAG or Ca^{2+} [1]. The PKC α , β , γ (cPKC), δ , ϵ (nPKC), and ζ (aPKC) isoenzymes were examined in this study. In agreement with the results of Huwiler et al. [7], the only cPKC we detected in cultured mesangial cells was PKC α . However, Saxena et al. [9] have detected PKC α , β_1 , and γ isoenzymes in cultured mesangial cells. Although the antibodies used by ourselves and Huwiler et al. [7] are different from those used by Saxena et al. [9], the reason for this discrepancy is not clear. Culture of mesangial cells under high glucose conditions resulted in parallel and equivalent increases in PKC activities and translocation of PKC α to the membrane fraction. This similarity of the increases in PKC activity and PKC α translocation indicates that PKC β_1 or γ might not play an important role under these conditions even if present.

In contrast to cPKC, PKC δ and ϵ (nPKC) and PKC ζ (aPKC) were all present in cultured mesangial cells. However, the response of these isoenzymes to high glucose differed. By exposing cells to high concentrations of glucose, PKC ζ was translocated to the membrane fractions, while PKC δ and ϵ remained unchanged. The response of PKC α and PKC ζ to TPA differed as previously reported by Huwiler et al. [7, 8]. Treatment of cells with TPA resulted in the translocation and eventual depletion of PKC α , while PKC ζ was insensitive to

TPA. The different responses of these isoenzymes to TPA might be explained by the responsiveness of each isoenzyme to DAG. The translocation of PKC α in high glucose conditions, an isoenzyme activated by DAG, might be due to an increase in cellular DAG mass, because de novo synthesis of DAG from glucose is enhanced under such conditions [2, 3]. In contrast, PKC ζ that is not affected by DAG, increased only after 5 days of high glucose conditions and was not responsive to TPA, indicating that its translocation might be caused by a different mechanism. Although phosphatidylinositol 3,4,5-trisphosphate has recently been reported to stimulate PKC ζ [10], the precise mechanisms or factors that activate PKC ζ have not yet been identified. High glucose is the first pathologic condition found to translocate PKC ζ .

In summary, the present results indicate that PKC α is activated in glomerular mesangial cells cultured under high glucose conditions. Our data also suggest that an atypical PKC, PKC ζ is translocated under high glucose conditions through a different pathway.

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