Characterization of Protein Kinase C Expression in Human Kidney

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FUKUZAKI, A., KANETO, H., IKEDA, S. and ORIKASA, S. *Characterization of Protein Kinase C Expression in Human Kidney*. Tohoku J. Exp. Med., 1996, **178** (3), 263-269 — Protein kinase C (PKC), the calcium and phospholipid-dependent kinase, is a key enzyme for regulation of growth and differentiation of various cells and tissues. Abnormalities of PKC modulation may cause diseases such as neoplasia. The present study was designed to demonstrate localization of PKC in normal human nephron segments. Surgically extirpated specimens for renal tumor were used. PKC was determined by immunoblotting and immunohistochemical methods using specific antibody against PKC that recognizes α and β subtypes of this enzyme. Immunoblotting study demonstrated PKC in the cytosolic fractions of homogenized cortical tubules and medullary tubules prepared from morphologically normal part of the kidney. PKC was faintly detected in glomerular supernatants. Immunohistochemical study revealed that PKC was present throughout the nephron and especially concentrated in the cytoplasm of the proximal tubules and the medullary collecting ducts. The results indicate that PKC appears to play a role in regulation of normal nephron function in human kidney. — protein kinase C; human; kidney

Protein kinase C (PKC) is a key enzyme for regulation of growth and differentiation of various cells and tissues. Activity of PKC is modulated by hormones and growth factors through receptor-mediated activation of phospholipids metabolism (Nishizuka 1984a, b, 1986, 1992; Dong et al. 1991). Agonist-stimulated phospholipid turnover can generate diacylglycerol (DAG), an intracellular second messenger that activates PKC (Nishizuka 1984a, b, 1986, 1992; Lee and Severson 1994).

PKC plays a central role in the regulation of a variety of differentiated renal functions. The abilities of hormones to regulate nutrient transport, salt and water retention, and transepithelial permeability are linked to activation of PKC (Dong et al. 1991). Molecular analyses indicate that at least three PKC isozymes, α -, β -, and ξ -PKC, are expressed in the kidney (Kosaka et al. 1988; Ono et al.

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1988). However, little has been known about the roles of PKC in human kidney. PKC in contribution to the diseases of the kidney has not been clarified. As a first step, we investigated the expression and the distribution of PKC in normal human nephron segments.

MATERIALS AND METHODS

Sample preparations

Samples used in this study were derived from kidneys surgically extirpated from twelve patients (6 men and 6 women) for small sized renal cell carcinoma. The ages of the patients ranged from 36 to 74 years (mean age 59 years). Morphologically normal part of the kidney was separated from the tumor. Samples were immediately transferred in cold Hanks' balanced salt solution (HBSS) and divided into cortices and medullae. We dissected and minced each sample and prepared glomeruli (GL), cortical tubules (CT) and medullary tubules (MT) by the sieving technique as previously reported from our laboratory (Ishidoya et al. 1994). We captured GL from 150 μm -sized meshes. CT and MT were obtained from 250 μm -sized meshes. The isolated preparations were washed twice with cold HBSS by centrifugation at 1,500 rpm for 5 min, then washed again with Ca^{2+} , Mg^{2+} free HBSS by centrifugation at 1,500 rpm for 5 min. Samples were suspended and homogenized in an appropriate volume (200 to 500 μl) of ice-cold homogenate buffer (25 mM Tris-HCl, pH 7.5; containing 1 mM EDTA, 1 mM dithiothreitol, and 100 KI Units/ml of Aprotinine) with 10 strokes in a Teflon-glass homogenizer. Then, they were centrifuged at $100,000 \times g$ for 60 min. The supernatants were stored at -70°C as cytosolic fractions. The protein content of each preparation was assayed by the method of Lowry et al. (1951). Part of the samples was prepared for immunohistochemical study.

Immunoblotting analysis

Samples were prepared to separate proteins by means of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described by Laemmli (1970). The gels containing 13.5% acrylamide were used to separate proteins. Each gel electrophoresis ran with prestained molecular weight markers (Bio-Rad, Richmond, CA, USA) to optimize the separation. Separated proteins were transferred from the polyacrylamide gels to nitrocellulose membrane by the method of Towbin et al. (1979). The membranes were washed with 20 mM Tris buffer (pH 7.5, containing 500 mM NaCl), then exposed to the specific mouse monoclonal antibody against PKC that recognizes α and β forms of the enzyme (Amersham, Buckinghamshire, UK), and diluted in 1 : 100 with the same buffer containing 1% gelatin and 0.02% NaN_3 at 37°C for 60 min. The membranes were washed again with 20 mM Tris buffer containing 0.05% tween 20. Rabbit anti-mouse IgG (whole molecule) conjugated with alkaline phosphatase (Sigma Immunochemicals, St. Louis, MO, USA) diluted 1 : 1,000 with the same buffer containing

1% gelatin was used as a second antibody. The membranes were incubated with the second antibody at 37°C for 60 min. Incubations were terminated by washing the membrane with 20 mM Tris buffer, and the membranes were stained with nitroblue tetrazolium/5-bromo-4-chloro-3-indoxyl phosphate (NBT/BCIP). Determining the level of PKC expression was carried out by measuring the bands on each membrane with video densitometer (Model GS670; Bio-Rad) and analyzed by a computer.

Immunohistochemical analysis

Samples were immersed in periodate-lysine-paraformaldehyde (PLP) fixative overnight at 4°C. They were washed subsequently with phosphate buffered saline (PBS), 10% sucrose/PBS, 20% sucrose/PBS, and embedded in OCT compound (Tissue Tek, Elkhart, IN, USA) with liquid nitrogen. Frozen sections, 4 μ m in thickness, were preincubated with blocking solution (10% sheep serum in PBS) for 30 min, then incubated with mouse monoclonal anti-PKC antibody diluted 1:100 in PBS. We used mouse IgG_{2a} from normal non-immunized mouse (Dako Japan, Kyoto) diluted in 1:100 as a primary antibody for negative controls. Sections were immersed in 3% H₂O₂/methanol for 15 min, then washed in PBS and incubated with sheep anti-mouse IgG/F(ab')₂ peroxidase conjugated antibody (Amersham) diluted in 1:100 at room temperature for one hr. The reaction was terminated by washing the section with PBS. Finally, the sections were stained with 0.03% 3, 3'-diaminobenzidine (DAB) in 0.05 M Tris-HCl, pH 7.6 containing 0.005% H₂O₂. We counterstained the sections with methylgreen at room temperature for 60 min. The sections were washed, dehydrated, mounted and inspected microscopically.

Fig. 1. Immunoblot of samples prepared from various parts of human kidney segment incubated with anti-PKC antibody. In each lane, 30 μ g of proteins are applied from cytosolic fractions of glomeruli (GL), cortical tubules (CT) and medullary tubules (MT). Numerals represent molecular weights determined from pre-stained molecular weight standard (STD).

Fig. 2.

RESULTS

Immunoblot analysis

Immunoblotting study identified α and β forms of PKC as an approximately 79 kDa protein. In normal human kidney, PKC was apparently detected in cytosolic fractions derived from medullary tubules (MT) or cortical tubules (CT). PKC was faintly demonstrated in some samples prepared from glomeruli (GL) (Fig. 1).

Immunohistochemical analysis

Immunohistochemical staining of normal human kidney with anti-PKC antibodies demonstrated that PKC was present throughout the nephron and was especially concentrated in cytoplasm of cortical tubules and outer medullary collecting ducts. Staining was weak in glomeruli. Background staining or nonspecific staining was unremarkable in negative controls (Fig. 2).

DISCUSSION

Protein kinase C (PKC) was first found as the enzyme that is responsible for the phosphorylation of the endogenous platelet protein (Nishizuka 1984a, b). Later, it was shown that PKC takes part in cellular responses to various agonists including hormones, neurotransmitters, and some growth factors (Nishizuka 1984a, b, 1992). PKC is a family of distinct proteins, and at least ten subspecies have now been identified. The classical PKCs consist of α , β I, β II, and γ subspecies that are activated by Ca^{2+} , phosphatidylserine, and DAG. The new PKCs consist of δ , ϵ , η , and θ subspecies that do not require Ca^{2+} but are activated by phosphatidylserine and DAG. The atypical PKCs consist of ξ and ι subspecies that are dependent on phosphatidylserine but are not affected by Ca^{2+} or DAG (Nishizuka 1992; Lee and Severson 1994). Several initial studies indicate that there are identifiable differences among the isozymes that may eventually be related to isozyme-specific function. Tissue distributions of the isozymes differ substantially. Whereas α -PKC is found in all cells and tissues, β -PKC is found only in selected cells and tissues, and γ -PKC is found only in the central nervous system (Dong et al. 1991). PKC can be subdivided into two domains: a kinase (catalytic) domain and a regulatory domain that contains the binding sites for Ca^{2+} and lipids and an autoinhibitory sequence (Lee and Severson 1994). PKC

Fig. 2. Immunohistochemical photomicrographs of morphologically normal human kidney sections incubated with anti-PKC antibody. a, Cortical view shows faint glomerular staining and remarkable staining of the cortical tubules ($\times 100$). b, View of cortex demonstrates staining in cytoplasm of proximal tubules ($\times 200$). c, Outer medullary view shows intense staining of outer medullary portion of proximal tubules and collecting ducts ($\times 100$). d, Background staining or nonspecific staining is unremarkable in negative control ($\times 100$).

activity in intact cells can be stimulated by tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA), which presumably binds to the same site in the regulatory domain as DAG (Lee and Severson 1994).

PKC is widely distributed in tissues and organs of mammals and other organisms (Nishizuka 1984a). In most tissues other than brain, this enzyme was recovered mainly from the soluble fraction as an inactive form (Nishizuka 1986). Although it has been shown that PKC is a family of distinct proteins consisting with subspecies (Ono et al. 1988; Lee and Severson 1994), immunoabsorption assays demonstrated that 34% of the total rat kidney PKC was α -PKC (Dong et al. 1991). It appears reasonable to anticipate the existence of this PKC subtype in the human nephron segments.

In the immunohistochemical study of rat kidney, Dong et al. (1991) demonstrated that α -PKC was present throughout the nephron and was especially concentrated in proximal tubules and papillary collecting ducts. They noted intense staining in the S3 segment of the proximal tubules comparing with the S1-S2 segments. Only a few cells in the glomeruli were stained. The results of our present study on the localization of PKC in the human nephron segments were similar to those of rat kidney.

In summary, we demonstrated the presence of PKC in human kidney, using immunoblotting and immunohistochemical methods. The present study revealed that PKC presents throughout the nephron and especially localizes in the cytoplasm of the outer medullary tubules. Further evidence is necessary to clarify the role of this enzyme in the modulation of renal function.

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