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Release of Renal Dipeptidase from Rabbit Renal Proximal Tubules and Its Inhibition by Gentamicin

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Effects of several drugs on rabbit renal proximal tubules were examined for the applicability of renal dipeptidase (RDPase, EC 3. 4. 13. 11) release as a model system to study nephrotoxicity. The proximal tubule prepared by the method of Taub (1990) released RDPase spontaneously in the control experiment which was confirmed by Western blotting. RDPase was also released from cisplatin, lipopolysaccharide (LPS), and indomethacin-treated tubules. Gentamicin inhibited RDPase release in a concentration-dependent manner. This RDPase release system may not be a general model to screen nephrotoxicity but could be a useful source of RDPase purification in a simple and inexpensive way.

Key words : Proximal tubule, Glycosylphosphatidylinositol (GPI)-protein, Renal dipeptidase (RDPase), Phosphatidylinositol specific phospholipase C (PI-PLC), Gentamicin

INTRODUCTION

The primary site of renal injury by various drugs is often the proximal tubule which may be attributable to the drug-accumulating capacity of this cells (Baylis *et al.*, 1977; Kaloyanides and Pastoriza-Munoz, 1980; Olsen, 1989; Park *et al.*, 1992; Kuhlmann *et al.*, 1997). A large number of amphiphilic drugs including aminoglycosides were concentrated within the lysosomes of proximal tubule cells and induced the formation of myeloid bodies containing undegraded remnants of cytomembranes. It is presumably caused by the inhibition of the lysosomal hydrolases, thus resulting in nephrotoxicity (Kaloyanides and Pastoriza-Munoz, 1980; Olier *et al.*, 1986). Focal degeneration of brush border following gentamicin treatment is common features (Baylis *et al.*, 1977; Olsen, 1989). The morphological damage by cisplatin, one of the most widely used chemotherapeutic agent of cancer, was also focused in the proximal tubules (Safirstein *et al.*, 1987; Kuhlmann *et al.*, 1997).

Renal dipeptidase (RDPase, EC 3. 4. 13. 11) is a well known glycosylated phosphatidylinositol (GPI)-anchored protein of microvilli of renal proximal tubules. It was released into the urine of healthy and renal patients, and was termed as urinary dipeptidase (Udpase; Park *et al.*, 1992). RDPase and Udpase were reported to be an identical enzyme (We *et al.*, 1997). When cephaloridine was administered to the rabbit, maximal release of Udpase with concomitant necrosis of proximal tubules was obvious 2 days post in-jection (Park *et al.*, 1992). Similar result was observed with aminoglycoside treated LLC-PK1 cells. Alkaline phosphatase and aminopeptidase, the marker enzymes of the apical membranes, were released into the culture medium in increased amount after aminoglycoside treat-ment (Hori *et al.*, 1984). These observations suggested that various lesions at the same locus could be probed by RDPase release assay of proximal tubules caused by nephrotoxicity by various drugs. The nephrotoxicity caused by cephaloridine, gentamicin and cisplatin takes 5 to 10 days to manifest the effects *in vivo* (Park *et al.*, 1992; Olsen, 1989).

In this study, we wished to examine the rabbit proximal tubule in relation to RDPase as a fast and general model system for the evaluation of renal toxicity of several drugs.

MATERIALS AND METHODS

New Zealand white rabbits (1.5 kg) were provided by a local animal farm. The 83 and 253 μm nylon meshes from Tetko, Inc., Kansas City, MO were soaked in 70% ethanol before each use. Dulbecco's modified Eagle's medium (DMEM) from Sigma, St. Louis, MO was filter-sterilized. Western blotting kit was purchased from Clon-tech, Palo Alto, CA. The purification of RDPase and its antibody

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production were described previously (Park *et al.*, 1992). Glycyldehydrophenylalanine (Gdp), a substrate of RDPase, was synthesized according to the method of Campbell *et al.* (1963). Solutions, surgical tools, glass wares and other necessary equipments were autoclaved. Experimental procedures were carried out at 4°C, otherwise stated.

Proximal tubule preparation

New Zealand white rabbits were sacrificed by cervical dislocation and the proximal tubules were prepared by the method of Taub (1990). Antibiotics were not added in the tubule preparation since the effect of drugs were studied compounds. Fresh kidneys were perfused through renal artery over a sterile 20-gage needle three times with 40 ml of phosphate buffered saline (PBS) followed by iron oxide (0.5%) perfusion twice (Cook and Pickering, 1958). The perfused cortex was cut into small pieces and homogenized with 3 volume of DMEM-44 mM sodium bicarbonate buffer, pH 7.5 using a Dounce tissue homogenizer and was passed through the 253, then 83 μm nylon mesh. The tubules and glomeruli on 83 μm mesh were resuspended in small volume of DMEM-bicarbonate buffer and the iron oxide-entrapped glomeruli were removed with magnetic bar. The remaining tubules were incubated for 2 min with soybean trypsin inhibitor (0.025%) and centrifuged (100 × g, 10 min). The pellet was resuspended in DMEM-bicarbonate buffer, and referred to as proximal tubule'.

Effect of incubation time, temperature and pH

Proximal tubules (2 mg/ml) were incubated at 37°C with shaking (100 rpm) as a function of time up to 10 hours. After centrifugation (18,300 × g, 15 min) the supernatants were assayed for the released RDPase. Maximum release of RDPase was achieved with 6 hour incubation. The effect of incubation temperature on RDPase release was examined with 6 hour incubation while pH was determined after with 2 hour incubation.

Effect of drugs on the proximal tubules

Total volume of 250 μl reaction mixture contained proximal tubules (approximately 2 mg/ml) and different concentration of drug compounds; gentamicin, cisplatin, LPS or indomethacin in Eppendorf tubes. They were incubated for 2 and 6 hours. After centrifugation (18,300 × g, 15 min) the effect of various drugs on the release of RDPase was examined. The RDPase released in the control tube, which does not contain any drug, was taken as 100%.

Enzyme assay

The released RDPase was determined by Gdp ($E_{cm}^{275\ nm} = 1.56 \times 10^4$) hydrolysis according to the method of Campbell *et al.* (1963). The enzyme activity, unit (U), was defined as μmole Gdp hydrolyzed per min. The protein concentration was determined by the method of Bradford (1976).

Electrophoresis and Western blotting

The electrophoresis of the native PAGE (8%) was done according to the method of Laemmli (1970). Western blotting was carried out according to the manufacturer's protocol (Clontech) with the rabbit IgG raised against the human RDPase as described previously (Park *et al.*, 1992).

RESULTS

The control experiment, incubation of proximal tubules in the absence of drug compound, was carried out as a function of time. There was no detectable RDPase release up to 2 hours, but thereafter the enzyme release was increased reaching the maximum at 6 hour followed by a rapid decrease (Fig. 1). At the peak point, the released RDPase was 175% of the membrane-bound form. The effect of temperature and pH on the tubule was examined. There was no detectable RDPase activity at 4 or 23°C with 6 hour incubation but the maximum release (100%) of RDPase was reached at 37°C followed by a decrease at 42°C (Table I). There was pH change from 7.5 to 6.0 with 6 hour incubation. The enzyme release was not detected after 2 hour incubation at

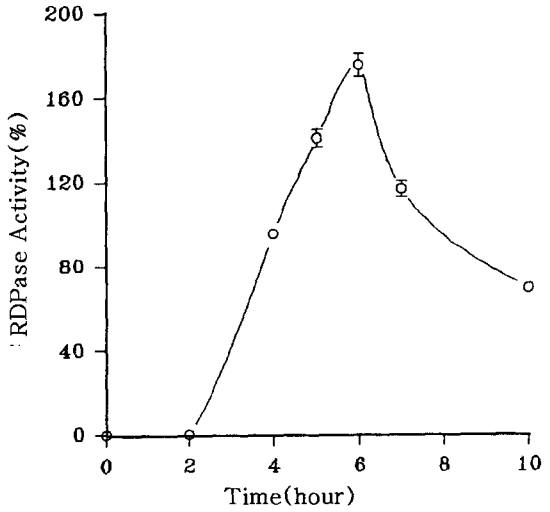

Fig. 1. Release of RDPase from rabbit proximal tubules as a function of time. Rabbit proximal tubules (250 μl, 1.6 mg/ml, 174.9 mU/ml) were incubated at 37°C for 10 hours. After centrifugation (18,300 × g, 15 min), the supernatant was assayed for the released RDPase as described in the 'Materials and Methods'. The RDPase activity in the proximal tubule before incubation was taken as 100%. Each data point represents the average of triplicates.

Table I. Effect of incubation temperature on RDPase release from rabbit proximal tubules

Incubation (°C)	RDPase release (%)[a]
4	ND[b]
23	ND
37	100
42	58.6

[a]RDPase at maximal release was taken as 100 %.
[b]ND, not detected.

37°C at any pH (pH 6.0, 7.0 and 7.5).

The release of RDPase from the proximal tubule with 6 hour incubation in the control was confirmed with Western blotting with 8% native polyacrylamide gell (Fig. 2). The rabbit RDPase immunologically cross-reacted with polyclonal IgG raised against human RDPase. The human RDPase in lane 1 exhibited two bands. Most of the physico-chemical characteristics of the major band was elucidated but the fast moving minor band was not, except that it was a glycoprotein with Gdp-hydrolyzing activity (Park *et al.*, 1992; Park *et al.*, 1994). The released rabbit RDPase in lane 2 exhibited the same mobility with the minor band of human RDPase.

The effect of several drugs on RDPase release of proximal tubules was examined after 2-and 6-hour incubation (Table II). The maximum release of RDPase observed with 6 hour incubation in the control tube was taken as 100%. Various drugs including cisplatin (5×10^{-6} and 5×10^{-5} M), indomethacin (1×10^{-5} and 1×10^{-4} M) and LPS (1 and 10 μg/ml) did not affect the release of RDPase significantly. However, gentamicin blocked the RDPase release almost completely with 6 hour incubation in a concentration-dependent manner. With 8×10^{-8} M gentamicin, 70% of RDPase release was demonstrated but it became negligible with higher drug concentration (8×10^{-5} M and 8×10^{-6} M). There was no detectable RDPase release with 2 hour incubation in the drug-treated samples as well as the control tube.

Fig. 2. Western blotting of released RDPase. The native polyacrylamide gel (8 %) was used for the analysis of purified human RDPase (l0 ng, lane 1), and released rabbit RDPase (26 μg) in the supernatant of the control tube with 6 hour incubation (Lane 2). The purification of human RDPase and its antibody production was described previously (Park *et al.*, 1992). The blotting was carried out according to the protocol of manufacturer (Clontech).

Table II. Effect of drugs on RDPase release from the rabbit proximal tubules

Incubation time (hour)	Released RDPae activity (%)[a]	
	2	6
Control	ND[b]	100
Nephrotoxic drug		
Gentamicin (8×10^{-8} M)	ND	70.0±11.2
Gentamicin (8×10^{-6} M)	ND	2.6±0.8
Gentamicin (8×10^{-5} M)	ND	3.4±0.1
Cisplatin (5×10^{-6} M)	ND	100.5±0.8
Cisplatin (5×10^{-5} M)	0.1±0.1	100.1±0.1
Inflammation factor		
LPS (1 μg/ml)	ND	101.3±1.6
LPS (10 μg/ml)	ND	100.2±6.0
Anti-inflammation factor		
Indomethacin (1×10^{-5} M)	ND	102.1±2.9
Indomethacin (1×10^{-4} M)	ND	106.0±6.0

[a]Released RDPase of the control experiment was taken as 100 %.
[b]ND, not detected.

DISCUSSION

When rabbits were treated with nephrotoxic cephaloridine, necrosis of proximal tubules was observed with a dose-dependent release of RDPase into urine (Udpase) suggesting its use as a nephrotoxic index (Park *et al.*, 1992). We hoped that the drugs tested in this study could give us better understanding of nephrotoxicity and the applicability of this model system to screen the nephrotoxicity.

Gentamicin and cisplatin are well established nephrotoxic agents with different effects, although their mechanisms are not clear, yet. Gentamicin was suggested to inhibit PI-PLC of lysosomal and of brush border membrane resulting in abnormal degradation of phospholipid, thus forming myeloid bodies (Lipsky and Lietman 1982; Ramsammy *et al.*, 1989). Schwertz *et al.* (1984) demonstrated an uncompetitive inhibition of gentamicin on brush border membrane-associated PI-PLC of rabbit proximal tubules. The central event of cisplatin toxicity in proximal tubule was pointed as the mitochondrial injury (Singh, 1989; Brady *et al.*, 1990). Bacterial LPS is an inflammatory factor and LPS-treated mice developed a proliferative glomerulonephritis associated with renal in-

sufficiency and proteinuria (Cavallo and Granholm, 1990). Indomethacin probably inhibited prostaglandin synthesis and exerted its effect as a nonsteroidal anti-inflammatory agent. According our results, neither LPS nor indomethacin affected RDPase release from the proximal tubules. We did not observe any difference of RDPase release by cisplatin, LPS and indomethacin compared with the control experiment. Only gentamicin showed significant difference; the inhibition of RDPase release. Thus, the proposed model system of proximal tubule in relation of RDPase release may not be used with for general purpose of screening nephrotoxicity, but it clearly distinguished the effect of two well known nephrotoxic drugs, gentamicin and cisplatin. However, it can't be interpreted as the difference of the nephrotoxic mechanism of two drugs because we do not understand the spontaneous release mechanism of RDPase from the proximal tubule, yet. This relatively fast RDPase release system using isolated proximal tubules was expected to be useful for screening drug-associated nephrotoxicity but it was not accredited for its general use.

The accompanied pH change from 7.5 to 6.0 hour incubation does not appear to be the primary cause of the RDPase release, since an incubation of the tubule for 2 hours did not release detectable RDPase in pH 6.0. Traditionally the solubilization of RDPase was achieved by detergent (Triton X-100, octyl-β-D-glucoside) or organic solvent (n-butanol) followed by extensive dialysis or other measures to remove the solubilizing agents (Hitchcock *et al.*, 1987; Adachi *et al.*, 1989; Hooper and Turner, 1989; Campbell *et al.*, 1988; Park *et al.*, 1993), and bacterial PI-PLC (Hooper and Turner, 1989; Littlewood *et al.*, 1989). We did not add solubilizing agents in our control sample but the released RDPase was 175% of the starting proximal tubules (Fig. 1). Such activity increase was reported by a number of investigators (We, 1997; Campbell *et al.*, 1990; Brewis *et al.*, 1994) for bacterial PI-PLC. Campbell *et al.* (1990) interpreted this as a result of altered accessibility to the active site. Brewis *et al.* (1994) proposed the result as the conformational change of the RDPase. Thus, our spontaneous release of RDPase from the proximal tubules is simpler and easier than the methods using solubilizing agents and less expensive than the PI-PLC method.

The very fact that the membrane bound RDPase was released into the supernatant indicated the presence of a hydrolase acting on GPI-proteins of the proximal tubule. It was supported by the temperature effect on RDPase release which is indicative of a heat-labile enzyme-catalyzed reaction (Table I). We temporarily named it hydrolase-X. Currently we are working on the purification of hydrolase-X from the proximal tubules in control experiment. The characterization of hydrolase-X will give us better understanding of this selective inhibition of RDPase release by gentamicin and the mechanism of spon-taneous release of RDPase from the proximal tubules.

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REFERENCES CTED

Adachi, H., Kubota, I., Okamura, N., Iwata, H., Tsujimoto, M., Nakazato, H., Nishihara, T. and Noguchi, T., Purification and characterization of human microsomal dipeptidase. *J. Biochem.*, 105, 957-961 (1989).

Baylis, C., Rennke, H. R. and Brenner, B. M., Mechanisms of the defect in glomerular ultrafiltration associated with gentamicin administration. *Kidney Int.*, 12, 344-353 (1977).

Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72, 248-254 (1976).

Brady, H. R., Kone, B. C., Stromski, M. E., Zeidel, M. L., Giebisch, G. and Gullans, S. R., Mitochondrial injury: an early event in cisplatin toxicity to renal proximal tubules. *Am. J. Physiol.* 258:F1181-F1187 (1990).

Brewis, I. A., Ferguson, M. A. J., Mehlert, A., Turner, A. J. and Hooper, N. M., Structures of the glycosylphosphatidylinositol anchors of porcine and human renal membrane dipeptidase *J. Biol. Chem.*, 270, 22946-22956 (1995).

Brewis, I. A., Turner, A. J. and Hooper, N. M., Activation of the glycosylphosphatidylinositol-anchored membrane dipeptidase upon release from pig kidney membranes by phospholipase C. *Biochem. J.*, 303, 633-638 (1994).

Campbell, B. J., Baker, S. F., Shukla, S. D., Forrester, L. J. and Zahler, W. L., Bioconversion of leukotriene D_4 by lung dipeptidase. *Biochim. Biophys. Acta*, 1042, 107–112 (1990).

Campbell, B. J., Lin, Y. C. and Bird, M. E., Renal aminopeptidase and copper-activated peptide hydrolysis. *J. Biol. Chem.*, 238, 3632-3640 (1963).

Campbell, B. J., Shih, Y. D., Forrester, L. J. and Zahler, W. L., Specificity and inhibition studies of human renal dipeptidase. *Biochim. Biophys. Acta*, 956, 110-118 (1988).

Cavallo, T. and Granholm, N. A., Bacterial lipopolysaccharide transforms mesangial into proliferative lupus nephritis without interfering with processing of pathogenic immune complexes in NZB/W mice. *Am. J. Pathol.*, 137, 971-978 (1990).

Clontech Lab: Affinity Purified Biotinylated Goat Anti-Rabbit IgG. Palo Alto, Clontech Lab, cat No. K1005a.

Cook, W. F. and Pickering, G. W., A rapid method for

separating glomeruli from rabbit kidney. *Nature*, 182, 1103-1104 (1958).

Hitchcock, M. J., Farrell, C. A., Huybensz, S., Luh B. Y. and Phelps, D. J., Affinity purification of renal dipeptidase solubilized with detergent. *Anal. Biochem.* 163, 219-223 (1987).

Hooper, N. M. and Turner, A. J., Ectoenzymes of the kidney microvillar membrane : Isolation and characterization of the amphipathic form of renal dipeptidase and hydrolysis of its glycosyl-phosphatidylinositol anchor by an activity in plasma. *Biochem., J.* 261, 811-818 (1989).

Hori, R., Yamamoto, K., Saito, H., Kohno, M. and Inui, K., Effect of aminoglycoside antibiotics on cellular functions of kidney epithelial cell line (LLC-PK1): a model system for aminoglycoside nephrotoxicity. *J. Pharmacol. Exp. Ther.*, 230, 724-728 (1984).

Kaloyanides, G. J. and Pastoriza-Munoz, E., Aminoglycoside nephrotoxicity. *Kidney. Int.*, 18, 571-582 (1980).

Kuhlmann M. K., Burkhardt, G. and Kohler, H., Insights into potential cellular mechanisms of cisplatin nephrotoxicity and their clinical application, *Nephrol. Dial. Transplant.* 12, 2478-2480 (1997).

Laemmli, U. K., Cleavage of structural proteins during the assembly of bacteriophage T_4. *Nature*, 227, 680-685 (1970).

Lipsky, J. J. and Lietman, P. S., Aminoglycoside inhibition of a renal phosphatidylinositol phospholipase C. *J. Pharmacol. Exp. Ther.*, 220, 287-292 (1982).

Littlewood, G. M., Hooper, N. M. and Turner, A. J., Ectoenzymes of the kidney microvillar membrane. Affinity purification, characterization and localization of the phospholipase C-solubilized form of renal dipeptidase. *Biochem., J.* 257, 361-367 (1989).

Olier, B., Borsa, F., Morin, J. P., Humbert, G. and Fillastre, J. P., Urinary excretion of phospholipids: index of aminoglycoside nephrotoxicity. *Pathol. Biol. (Paris)*, 34, 577-581 (1986).

Olsen, S., Acute tubular necrosis and toxic renal injury, In Tisher C. C. and Brenner, B. M. (eds.). *Renal Pathology with Clinical and Functional Correlation.*. Philadelphia, Lippincott, vol. 1, pp. 656-699, 1989.

Park, H. S., Kim, D. H., Kwark, H. S. E., Park, S. K., Kang, S. K., Chung, B. H. and Yoo, G. S., Human renal dipeptidase from kidneys of renal stone patients: Partial purification. *Arch. Pharm. Res.*, 16, 295-299 (1993).

Park, H. S., Kim, D. H., Kwark, H. S. E., Park, S. K. and Kang, S. K., Human renal dipeptidase from kidneys of renal stone patients: Partial characterization. *Arch. Pharm. Res.*, 17, 21-25 (1994).

Park, H. S., Kim, D. H., Park, S. K., Kang, S. K., Burks, M., Mullins, J. M. and Campbell, B. J., Tissue origin of urinary dipeptidase. *Korean Biochem. J.*, 25, 329-336 (1992).

Ramsammy, L. S., Josepovitz, C., Lane, B. and Kaloyanides, G. J., Effect of gentamicin on phospholipid metabolism in cultured rabbit proximal tubular cells. *Am. J. Physiol.* 256, C204-C213 (1989).

Safirstein, R., Winston, J., Moel, D., Dikman, S. and Guttenplan, J., Cisplatin nephrotoxicity: insights into mechanism. *Int. J. Androl.*, 10, 325-346 (1987).

Schwertz, D. W., Kreisberg, J. I. and Venkatachalam, M. A., Effects of aminoglycosides on proximal tubule brush border membrane phosphatidylinositol-specific phospholipase C. *J. Pharmacol. Exp. Ther.*, 231, 48-55 (1984).

Singh, G., A possible cellular mechanism of cisplatin-induced nephrotoxicity, *Toxicology*, 58, 71-80 (1989).

Taub, M., Primary kidney cells, In Pollard, J. W. and Walker, J. M. (Eds.). *Methods in Molecular Biology. vol. 5, Animal cell culture.* Humana Press, Clifton, N. J., pp. 189-196, 1990.

We, J. S., Kang, B. Y., Lee, J. C., Lee, H. B. and Park, H. S., Identification of urinary dipeptidase as the released from of renal dipeptidase. *Kidney Blood Press. Res.* 20, 411-415 (1997).