Korean J Biol Sci 7: 309-315, 2003

Chitosan Increases the Release of Renal Dipeptidase from Porcine Renal Proximal Tubule Cells

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Key Words:

Renal dipeptidase
Chitosan
$HgCl_2$
Nitric oxide

Renal dipeptidase (RDPase, membrane dipeptidase, dehydropeptidase 1, EC 3.4.13.19) has been widely studied since it was first purified from porcine kidney brush border membrane. It was reported that RDPase activity in urine samples of acute and chronic renal failure patients decreases. Nitric oxide (NO) is a highly reactive free radical involved in a number of physiological and pathological processes. NO is able to act in a dual mode, leading either to induction of apoptosis or to blunted execution of programmed cell death. NO inhibited the RDPase release from porcine renal proximal tubules, which could be blocked by L-NAME. Chitosan, the linear polymer of D-glucosamine in β(1→4) linkage, not only reversed the decreased RDPase release by NO but also increased NO production in the proximal tubule cells. The stimulatory effect of NO on RDPase release from proximal tubules in the presence of chitosan must be different from the previously proposed mechanism of RDPase release via NO signaling pathway. Chitosan stimulated the RDPase release in the proximal tubules and increased RDPase activity to 220% and 250% at 0.1% and 1%, respectively. RDPase release was decreased to about 40% in the injured proximal tubules and was recovered in proportion to the increase of chitosan. Chitosan may be useful in recovery of renal function from $HgCl_2$ injury.

Renal dipeptidase (RDPase, membrane dipeptidase, dehydropeptidase 1, EC 3.4.13.19) has been widely studied since it was first purified from porcine kidney brush border membrane (Campbell et al., 1966). RDPase is a zinc-metalloenzyme principally localized in the brush-border of the renal proximal tubule (Welch and Campbell, 1980), but is also found with high activity in other tissues such as the lung, pancreas, testis and intestinal mucosa (Campbell et al., 1990; Kera et al., 1999). Dipeptidase activity was also detected in the urine of human, where it was named as urinary dipeptidase (Udpase), and was subsequently purified from human urine (Park et al., 1992). RDPase and Udpase were identified as the same enzyme on the basis of various physicochemical characteristics such as substrate specificity, immunoreactivity and amino acid sequence identity (We et al., 1997). Various physiological role of this enzyme has been suggested such as hydrolysis of dipeptides for reabsorption in the kidney (Welch and Campbell, 1980), and metabolism of glutathione and leukotriene D_4 (Campbell et al., 1990). It was reported that RDPase activity in urine samples of acute and chronic renal failure patients decreased (Fukumura et al., 1999). RDPase is the only known mammalian enzyme to exhibit β-lactamase activity that hydrolyzes carbapenem antibiotics such as thienamycin, imipenem and meropenem and reduces their antibacterial activity (Kropp et al., 1982). RDPase is a well-characterized mammalian glycosylphosphatidylinositol (GPI)-anchored ectoenzyme and subsequently the complete structure of its GPI anchor was determined with core glycostructure and diacylglycerol (DAG), predominantly distearoyl phosphatidylinositol (PI), as opposed to the more common alkylacylglycerol structure present in other mammalian GPI-anchors (Brewis et al., 1995). The GPI anchor on both human and porcine RDPase has been extensively characterized in terms of its hydrolysis by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) and a serum phospholipase D (PLD) (Brewis et al., 1994).

Chitosan, the linear polymer of D-glucosamine in β(1→4) linkage, has been recommended as a suitable

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functional material because of its biocompatibility, biodegradability, non-toxicity and adsorption properties (Kumar, 2000). It was reported that chitosan could regulate cell biology such as differentiation, proliferation, and cytokine production (Pae et al., 2001). Recently it was reported that chitinous materials inhibit nitric oxide (NO) production by activated macrophages (Hwang et al., 2000) but not by the resting macrophages which have different metabolic pathways (Carina et al., 2003).

NO is a highly reactive free radical involved in a number of physiological and pathological processes (Moncada et al., 1991). In numerous mammalian cells and tissues, the oxidation of terminal guanidino nitrogen of L-arginine (Arg) (Schmidt et al., 1989), yielding NO and citrulline, is catalyzed by different NO synthases (NOS) (Schmidt and Murad, 1991), which are either Ca^{2+}-dependent epithelial and neuronal NOS (eNOS and nNOS, respectively) or Ca^{2+}-independent inducible NOS (iNOS). The role of NO is well established in relaxation of vascular smooth muscle (Hassid et al., 1994), inhibition of mitogenesis and growth of glomerular mesangial cells (Rupprecht et al., 2000), and macrophage toxicity (Lakics and Vogel, 1998). NO acts immunologically as a cytotoxic agent on invading microorganisms in macrophages or on tumor cells (Stuehr and Nathan, 1989). NO also mediates apoptosis in neuronal PC12 cells after stimulation with tumor necrosis factor-α (TNF-α) and bacterial LPS (Heneka et al., 1998), a cell wall component that causes inflammation via activation of macrophage. Interferon-γ has also been shown to produce NO by iNOS synergistically with TNF-α in activated macrophages resulting in apoptosis. Olsson et al. (1998) demonstrated that intraperitoneally injected LPS induced iNOS and elevation of NO content in kidney and urinary bladder. LPS has also been known to induce endotoxemia and ARF. Lipid A, the biologically active component of LPS, also induces NO generation by renal proximal tubules and leads to NO-dependent cytotoxicity of tubular cells (Traylor et al., 1996). The abnormal increase of iNOS, cGMP and NO with some inflammatory cytokines (TNF-α, IL-6 and IL-8) in urine was reported to be accompanied by the pathological symptoms such as urinary tract infections (Smith et al., 1996) and interstitial cystitis (Wheeler et al., 1997). These imply the possibility that NO may play an important role in inflammation and apoptosis of epithelial cells through certain signal transduction mechanism. It was reported that NO did not suppress PLC activation or IP_3 generation in response to angiotensin II (Wang et al., 2000) and that activation of NOS and increased formation of NO result in increased activity of PLC (Llansola et al., 2000).

In this work, experiments were carried out to investigate the relation between RDPase release and NO production of chitosan.

Materials and Methods

Animal sample

Porcine kidneys were supplied by the Samho Slaughterhouse (Gwangju, Korea) and were transferred to laboratory immediately after death.

Chemicals

Chitosan was purchased from the ECOBIO INC. (Kwangju, Korea). Ala-Ala, Ala dehydrogenase, β-NAD^+, L-Arg, diaphorase and resazurin were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Sodium nitroprusside (SNP) was purchased from Abott (Chicago, IL, USA) and the bicinchoninic acid (BCA) protein assay kit from Pierce (Rockford, IL, USA). Mercuric chloride was purchased from the Junsei Chemical Company (Tokyo, Japan). All procedures were carried out at 4°C, unless otherwise stated.

Preparation of porcine proximal tubules

Proximal tubules were prepared fresh for each experiment with a slight modification of the protocol of Taub et al. (1990). Briefly, the kidneys were perfused with cold phosphate-buffered-saline (PBS) to completely remove the blood via a polyethylene catheter, which was inserted above the bifurcation of the renal artery. The renal outer sack was removed; the cortex was minced into small pieces and homogenized by one stroke each with a polytron tissue homogenizer and a teflon-glass homogenizer (GlasCol, Terre Haute, IN, USA) set on low speed (25-30 rpm). The crude homogenate was passed through a 253 nm nylon mesh and then an 83 nm mesh (Tetko, Kansas City, MO, USA). The proximal tubules retained on the 83 nm mesh were washed with 10 volumes of cold PBS and then with an equal volume of Krebs-Ringer-Hepes (KRH) buffer consisting of 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose and 25 mM Hepes (pH 8.0). The scraped proximal tubules were then resuspended in 2 volumes of KRH buffer and centrifuged (600 g, 5 min). The pellet was resuspended in 50 volumes of KRH buffer and was referred to as the 'proximal tubules'.

Spectrofluorometric assay for RDPase

The enzymatic activity of the released RDPase was assayed as described previously according to the fluorometric method of Ito et al. (1984). A typical 2.5 ml reaction mixture consisted of 1.5 M L-Ala-L-Ala, 0.4 U L-Ala dehydrogenase, 1.5 M β-NAD^+, 0.06 U diaphorase and 0.1 M resazurin sodium in 12.5 mM Na_2CO_3 (pH 9.0) buffer. The incubation supernatant (20 μl), containing the released RDPase, was added last to the pre-incubated

reaction mixture and the reaction was carried out for 2 min at 25°C. The concentration of resorufin, the fluorescent product of the coupled reactions, was measured photometrically for 2 min using its excitation and emission maxima of 568 nm and 589 nm, respectively, using a JASCO spectrofluorimeter (JASCO Co., Hachioji City, Tokyo, Japan).

Measurement of protein

Samples (25 μl) were transferred to a 96-well microplate and then 200 μl of bicincchoninic acid (BCA) reagent that consists of reagent A and reagent B (50:1, respectively) was added. Bovine serum albumin was used as standard. After 1 h incubation at 37°C, protein concentrations were determined from the absorbance at 540 nm using microplate reader (Molecular Devices, USA).

Treatment of proximal tubules with various agents

Aliquots of proximal tubules (500 μl) were treated with various agents such as chitosan, L-Arg (NO synthase substrate), SNP (direct donor of NO) and mercuric chloride alone or in combination at various concentrations. In all experiments, otherwise stated, samples were incubated for 10 min at 37°C and were centrifuged at 18,000g for 3 min using a tabletop centrifuge or for 5 min using a high speed refrigerated centrifuge (MEGA17R, Hanil Co. Seoul, Korea). The supernatant was used as the source of released RDPase and NO.

Measurement of nitrite

Aliquot of the incubation supernatant (100 μl) was transferred to 96 well plates and nitrite was determined spectrophotometrically by mixing a 100 μl of Griess reagent (0.8% sulfanilamide, 0.75% N-(naphthyethylene) diamine in 0.5 N HCl) (Traylor et al., 1996). After 15-min incubation at room temperature, the nitrite concentrations were measured at 540 nm using a microplate reader. Sodium nitrate (0.5 to 100 μM) was used as nitrite standards and nitrite was linear over this concentration range.

Statistic analysis

For *in vitro* data analysis, Students t-test was performed by Sigmaplot computerized program and a p value less than 0.05 was used as the significance criterion. The comparison was carried out with control or with reagent-treated data.

Results

We used L-NAME, a NOS inhibitor, to confirm that L-Arg acts on RDPase release via NO generated by NOS (Fig. 1). NOS was inhibited by pretreatment with 5 mM L-NAME for 5 min and then the proximal tubules were reacted with 10 mM L-Arg for 30 min. NO production was increased by L-Arg and was approximately 3 fold (1.96±0.19 nM/mg protein) at 10 mM L-Arg compared with control (0.75±0.12 nM/mg protein) (Fig. 1A). L-NAME diminished nitrite concentrations to 1.30±0.0, 1.01±0.02, and 0.66±0.02 nM/mg protein with increasing concentrations (1, 5, and 10 mM), respectively. In Fig. 1B, RDPase release was decreased to 48.7±5.0% by L-Arg (10 mM) and then restored to 80.9±6.8% at 10 mM of L-NAME. These results demonstrate that NO produced by NOS from L-Arg sequentially inhibits the GPI-hydrolase leading to a reduction of RDPase release.

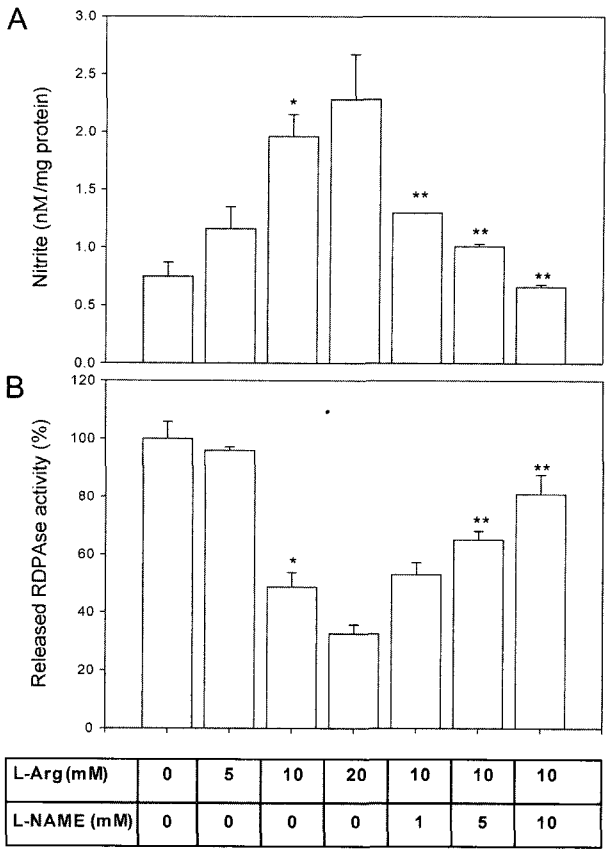


Fig. 1. Effect of L-NAME on L-Arg-induced NO generation and inhibition of RDPase release. Proximal tubules were diluted by 20-fold KRH buffer and treated for 5 min at 37°C with increasing concentrations of L-NAME (0, 1, 5 and 10 mM) and further incubated for 30 min with or without L-Arg (10 mM), and treated with L-Arg (5, 10, and 20 mM). A, Nitrite concentration in the incubation supernatants was determined with Griess reagent using 96-well microplate reader at 540 nm (*p<0.05 vs. control and **p<0.05 vs. L-Arg). B, The released RDPase activity was measured by the spectrofluorometric method described in the "Methods" and expressed as percentage compared with control. The bars illustrated show the results as mean±S.D. of 3 determinants (*p<0.05 vs. control and **p<0.05 vs. L-Arg).

When proximal tubules were treated with chitosan as

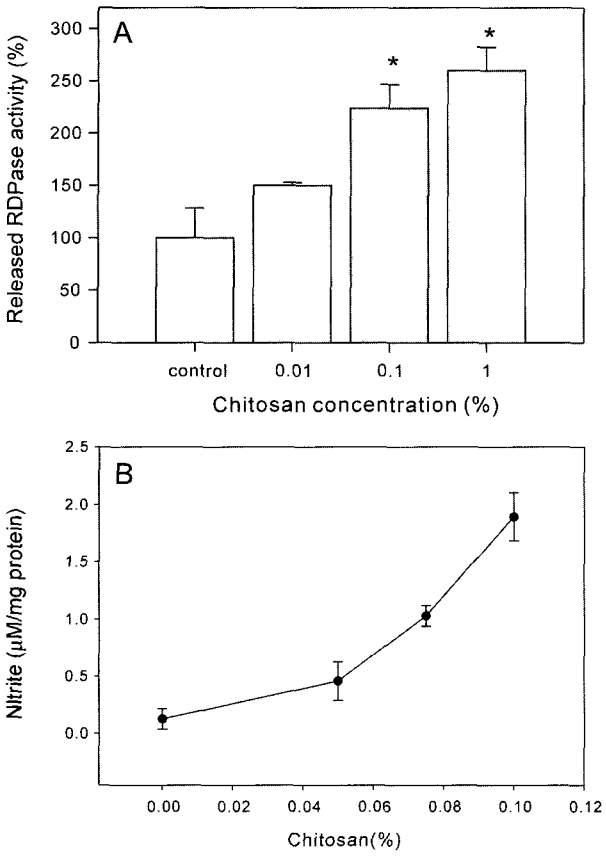


Fig. 2. Effect of chitosan on RDPase release and NO production from renal proximal tubules. A, Proximal tubules were diluted 50-fold by KRH buffer and incubated with chitosan (pH 7.0) for 10 min at 37°C at various concentrations. The released RDPase activity was expressed as percentage compared with control (100%) (n=3-6, mean±S.D., $^{*}P<0.05$ vs. control). B, Proximal tubules were diluted 50-fold by KRH buffer and incubated for 10 min at 37°C with chitosan (0, 0.05, 0.75 and 0.1%) and nitrite was assayed as described in the "Methods".

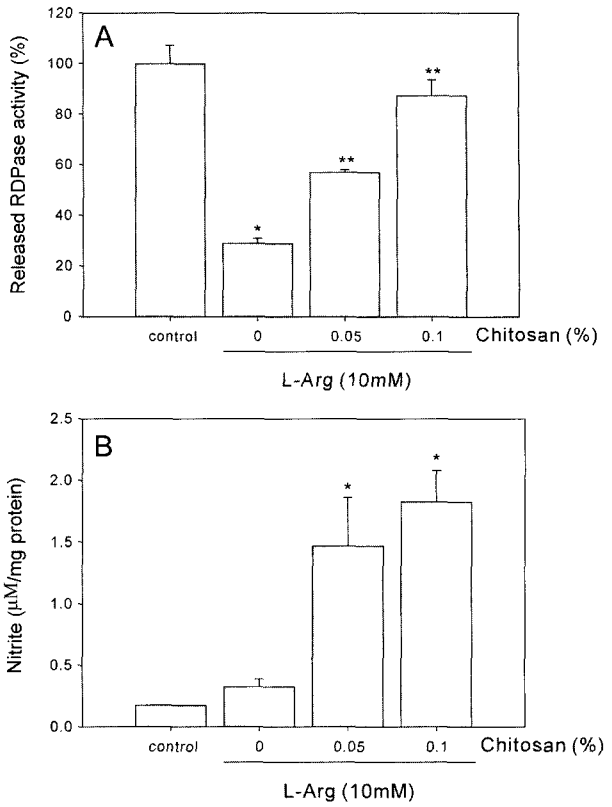


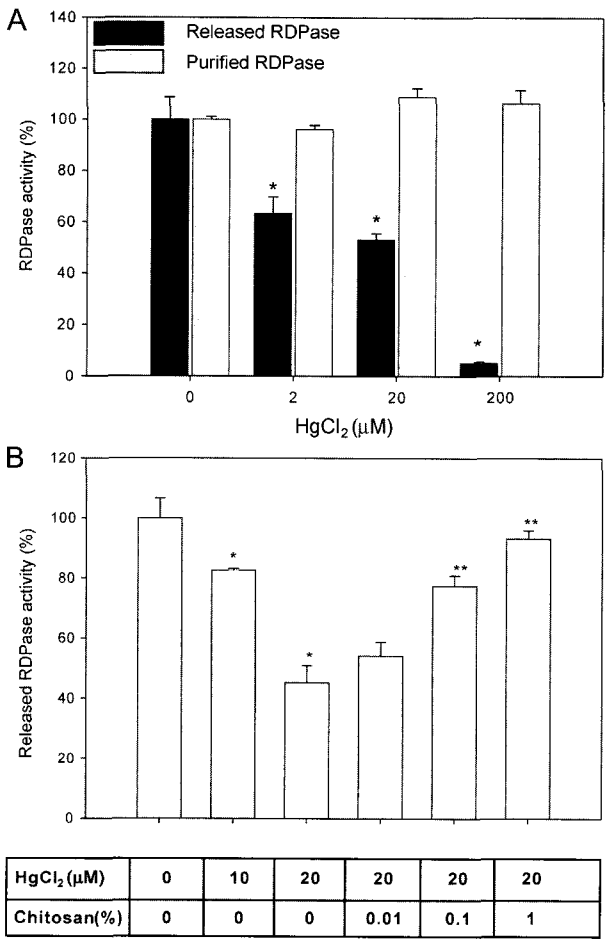
Fig. 3. Effect of chitosan on RDPase release and NO production in the presence of L-Arg. Proximal tubules were diluted 50-fold by KRH buffer and incubated for 10 min at 37°C with chitosan (0, 0.05 and 0.1%) in the presence of L-Arg (10 mM). A, The released RDPase activity was measured by spectrofluorometric method as described in the "Methods" and expressed as percentage compared with control (100%). (n=3-6, mean±S.D., $^{*}P<0.05$ vs. control and $^{**}P<0.05$ vs. 0 mM L-Arg). B, Nitrite was measured as described in the "Methods" in the incubation supernatant. Nitrite concentrations were expressed as μmol/mg of protein (n=3-6, mean±S.D., $^{*}P<0.05$ vs. control).

shown in Fig. 2A, RDPase release was increased to 150%, 220%, and 250% at 0.01%, 0.1%, and 1% chitosan, respectively. In Fig. 2B, NO production was increased by chitosan treatment by approximately 14.5 fold (1.89±0.21 nM/mg protein) compared with non-treatment (0.13±0.09 nM/mg protein).

To examine whether the chitosan treatment could reverse the inhibitory effect of NO on RDPase release, the proximal tubules were treated with chitosan at various concentrations in the presence of 10 mM L-Arg. As shown in Fig. 3A, L-Arg decreased the RDPase release below 30% levels compared with control, but chitosan with L-Arg recovered the RDPase release in proportion to chitosan concentration (56% in 0.05% chitosan and 88% in 0.1% chitosan). Treatment with chitosan in the presence of SNP also showed similar results in the recovery tendency (data not shown). Additionally, in Fig. 3B, NO production with chitosan in the presence of L-Arg was far more prominent than NO production by L-Arg alone.

In Fig. 1, we showed that RDPase release was decreased with increased NO. However, not only the RDPase release (Figs. 2A, 3A) but also NO production (Figs. 2B, 3B) were increased by chitosan in a concentration-dependent manner.

To examine whether this RDPase release was decreased in injured proximal tubules and chitosan could recover the decreased RDPase release, the proximal tubules were treated with $HgCl_2$ to induce acute renal damage. The proximal tubules were treated with $HgCl_2$ (20 μM) and chitosan at various concentrations was examined and the RDPase release. As shown in Fig. 4A, the release of RDPase was significantly decreased with increasing concentrations of $HgCl_2$ (2, 20, and 200 μM) without any change in purified RDPase activity. In Fig. 4B, we observed that the RDPase release was decreased to about 40% in the $HgCl_2$-injured proximal tubules with 20 μM $HgCl_2$ and was recovered in



$HgCl_2$ (μM)	0	10	20	20	20	20
Chitosan(%)	0	0	0	0.01	0.1	1

Fig. 4. Effect of chitosan on RDPase release in the presence of $HgCl_2$ from renal proximal tubules. A, Proximal tubules were diluted 50-fold by KRH buffer and incubated for 20 min at 37°C without $HgCl_2$ (control) or supplemented with increasing concentrations of $HgCl_2$ (2, 20, and 200 μM). The released RDPase was assayed as described in the "Methods". Alternatively, $HgCl_2$ was reacted with partially purified RDPase at the same conditions. The partially purified RDPase activity was measured by a UV-spectrophotometric method using Gdp (glycyl-dehydrophenylalanine) as a substrate as described in the "Methods". The bars show the results of 3 determinants (mean±S.D.) expressed as percentage compared with control activity (*p<0.01 vs. control). B, Proximal tubules were diluted 50-fold by KRH buffer and incubated with 10 and 20 μM $HgCl_2$, and chitosan (pH 7.0) for 10 min at 37°C at various concentrations in the presence of 20 μM $HgCl_2$. The released RDPase activity was expressed as percentage compared with control (100%) (n=3-6, mean±S.D., *p<0.005 vs. control and **p<0.05 vs. $HgCl_2$).

proportion to the concentration of chitosan.

Discussion

In the this study, we evaluated the effects of chitosan on the RDPase release in the proximal tubules. The induction of iNOS protein by cytokines such as TNFα is known to relate to cell and/or tissue injury via the cytotoxic effect of the free radical, NO, produced in large quantities by various NOS, including NOS, iNOS and eNOS (Pinsky et al., 1995). In host defense system, activated macrophages by inflammatory agents such as interferon-γ (INF-γ) and bacterial LPS are known to produce a large quantity of NO as the major cytotoxic mediator and inhibit the growth of invading microorganisms and tumor cells (Moncada et al., 1993). Chitinous materials inhibit NO production by activated macrophages (Hwang et al., 2000) but the resting macrophages have different metabolic pathways (Carina et al., 2003). NO inhibited the shedding of the RDPase from porcine renal proximal tubules (Park et al., 2002). This result was also confirmed from our experiments (Fig. 1). However, subsequent study, as shown in Fig. 2 and 3, demonstrated different results. We confirmed that chitosan not only increased RDPase release and recovered the NO-induced decrease of RDPase release (Figs. 2A, 3A), but also increased the NO production in the proximal tubule cells (Figs. 2B, 3B). These data seem to suggest that there is a direct relationship between chitosan and RDPase release.

Insulin has been known to stimulate mammalian glycosylphosphatidyl-inositol (GPI) proteins, including heparan sulfate proteoglycan from rat hepatocyte (Ishihara et al., 1987), alkaline phosphatase from the cell surface of BC3H1 myocytes (Romero et al., 1988), 5'-nucleotidase (Klip et al., 1988), and membrane dipeptidase (RDPase) in 3T3-L1 adipocytes (Movahedi et al., 2000). The increase and recovery of RDPase activity were suggested as an indication of strengthening of renal function and improvement of renal failure when the RDPase activity in urine samples of acute and chronic renal failure patients decreases (Fukumura et al., 1999). $HgCl_2$ is known to cause acute renal failure by severely damaging proximal tubule cells. The onset of $HgCl_2$-induced ARF results from tubular obstruction and backleak due to acute tubular necrosis (Kreisberg et al., 1983). $HgCl_2$ inhibits the RDPase release without change of RDPase activity in the proximal tubule cells. The purified RDPase activity was not decreased by $HgCl_2$ up to the tested range, 200 μM. The RDPase release was decreased with increasing concentrations in the injured proximal tubules and was recovered in proportion to the increase of chitosan. We showed that chitosan is able to increase and recover the RDPase release in the proximal tubules. This result may imply that chitosan could be used for strengthening of renal function and improvement from renal failure.

This chitosan result demonstrated two pathways leading to RDPase release; one reciprocally controlled by NO and another proportionally controlled by NO in the presence of chitosan.

Acknowledgement

This study was financially supported by grants awarded to Dr. H. S. Park from Chonnam National University, Korea, in the program 2002.

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[Received June 31, 2003; accepted September 7, 2003]