

Mechanism of Cd-induced inhibition of Na-glucose cotransporter in rabbit proximal tubule cells: Roles of luminal pH and membrane-bound carbonic anhydrase

Shuichi Tsuruoka, Erik R. Swenson, Akio Fujimura, and Masashi Imai

Department of Pharmacology, Jichi Medical University, Shimotsuke, Tochigi, Japan (S.T., A.F., M.I.); Department of Nephrology, University of Tsukuba, Tsukuba, Ibaraki, Japan (S.T.) and Medical Service, Veterans Affairs, Puget Sound Health Care System, University of Washington, Seattle, Washington, USA (E.R.S.)

RH: luminal pH and carbonic anhydrase in Cd-nephropathy

Address correspondence to: Shuichi Tsuruoka, MD, Department of Nephrology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan. Tel: +81-29-853-3202; fax: +81-29-853-3202. E-mail: tsuru@md.tsukuba.ac.jp

List of abbreviations: Cd, cadmium; MT, metallothionein; CA, carbonic anhydrase; pFB-ABZ, p-fluorobenzyl-aminobenzolamide; ACZ, acetazolamide.

ABSTRACT

Background/Aims. We have previously reported that a complex of cadmium-metallothionein (Cd-MT) directly affects the apical Na-glucose cotransporter on the luminal side in proximal tubules, suggesting that Cd-MT is more toxic than CdCl₂ in causing tubulopathy. To find potential mechanisms, we evaluated the effect of luminal pH alteration and carbonic anhydrase(CA) inhibition on Cd-MT-induced reduction of glucose-dependent transmural voltage in rabbit S2 segments perfused in vitro.

Methods. Before and after the addition of Cd-MT(1μgCd/ml) to the lumen, the deflections of transmural voltage upon the elimination of glucose from the perfusate($\Delta V_{t_{glu}}$) were measured as a parameter of activity of the Na-glucose cotransporter.

Results. During perfusion with a control solution of pH7.4, the $\Delta V_{t_{glu}}$ significantly decreased after addition of Cd-MT for 10min. A reduction of pH to 6.8 significantly shortened the time needed to reduce the $\Delta V_{t_{glu}}$ to less than 5min, whereas an increase of pH to 7.7 significantly prolonged the time to more than 20min. Furthermore, simultaneous addition of acetazolamide with control perfusate prevented the reduction. p-Fluorobenzyl-aminobenzolamide(pFB-ABZ), a membrane-impermeable CA inhibitor, added to the lumen also completely prevented the reduction of $\Delta V_{t_{glu}}$. In rabbits with chronic Cd exposure, acetazolamide prevented the glucosuria.

Conclusion. Cd-MT-induced inhibition of Na-glucose cotransporter activity in S2 segment strongly depends on luminal pH, and that an increase of pH by inhibition of luminal membrane-bound CA is useful for preventing renal Cd-toxicity.

Introduction

Chronic exposure of cadmium (Cd) causes kidney injury [1]. The earliest manifestation of Cd nephrotoxicity is proximal tubular dysfunction such as glucosuria and aminoaciduria. Although the mechanisms involved in Cd-induced renal dysfunction have been extensively studied, detailed cellular mechanisms remain to be established. Cd in the tissue is mainly bound to metallothionein (MT). We have previously reported, by using an *in vitro* microperfusion technique, that Cd-MT impairs absorption of glucose from the luminal side at a very early phase (within 10 min after exposure) in rabbit S2 segments [2]. In that article, we found a rapid and possibly direct effect (within 1 min) of Cd-MT on impairment of Na-glucose cotransport activity as well as a late (>5 min) and more prominent effect on inhibition of Na-K-ATPase activity subsequent to endocytosis of Cd-MT [2]. Since the conformation of the proteins is sometimes altered by extrinsic pH, such a change might occur in Cd-MT by alteration of extracellular pH. If this is true, the Cd-induced renal toxicity might be dependent on the extracellular pH. On the other hand, Cd-MT complex dissociates at very low pH into Cd²⁺ and holo-metallothionein [3]. The purpose of this study was to evaluate pH dependence of the Cd-MT-induced impairment of glucose absorption in isolated rabbit proximal tubules perfused *in vitro*. We also evaluated the effect of carbonic anhydrase inhibitors, which increase luminal pH by reduction of H⁺ secretion *in vitro* and *in vivo*.

Methods

In Vitro Microperfusion. In vitro microperfusion of isolated renal tubules, developed by Burg et al. [4] , was used as modified previously [2,5-7]. Female Japanese white rabbits weighing 1.5–2.3 kg were maintained on regular laboratory chow and allowed free access to distilled water. On the day of the experiments, animals were anesthetized with sodium pentobarbital (35 mg/kg, i.v.). The left kidney was removed, and coronal slices were made. Slices were transferred to a chilled dish containing modified Collins' solution of the following composition: 14 mM KH_2PO_4 , 44 mM K_2HPO_4 , 15 mM KCl, 9 mM NaHCO_3 , and 160 mM sucrose, with pH maintained at 7.4. Segments of proximal tubules (S2), with length ranging from 0.8 to 1.2 mm, were isolated from the medullary ray of renal cortex by fine forceps under a stereomicroscope. Isolated tubules were transferred to the perfusion chamber mounted on an inverted microscope (IX-71, Olympus, Tokyo, Japan) and perfused in vitro at 37°C. Tubules were hooked up to the holding pipette, and a single-barreled perfusion pipette was inserted into the tubular lumen. Triple-barreled polyethylene tubing was inserted into the perfusion pipette to allow rapid exchange of the perfusion fluid. The perfusion rate was controlled at 10–20 nl/min by adjusting the height of the fluid reservoir, which was connected to the back end of the perfusion pipette. A system of a flow-through bath was used to permit rapid exchange of the bathing fluid. The bathing fluid was maintained at 37°C by using a warm water jacket. The flow rate of the bathing fluid ranged from 3 to 5 ml/min, allowing the bath fluid to be exchanged within 2 sec. The composition of the basal solution used in this study was as follows: 110 mM NaCl, 5 mM KCl, 25 mM NaHCO_3 , 0.8 mM Na_2HPO_4 , 0.2 mM NaH_2PO_4 , 10 mM sodium acetate, 1.8 mM CaCl_2 , 1.0 mM MgCl_2 , 8.3 mM glucose, and 5 mM alanine. The pH of the solution was maintained at 37°C by bubbling with 95% O_2 , 5% CO_2 gas. In some experiments, bicarbonate-free HEPES buffer solution with the following composition was used: 110 mM NaCl,

5 mM KCl, 25 mM Na-cyclamate, 10 mM HEPES, 5 mM Tris, 0.8 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, 10 mM sodium acetate, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 8.3 mM glucose, and 5 mM alanine.

Electrophysiological Studies. Transmural voltage (V_t) was measured by connecting a 1-M KCl agar bridge to a saturated KCl reservoir, where a calomel half-cell electrode was placed. The electrode was connected to a dual-channel electrometer (Duo 773, W.P. Instruments, New Haven, CT), and measured voltage was recorded on a two-pen recorder (R301, Rikadenki, Tokyo, Japan). A flowing-boundary 1M KCl agar bridge connected to a calomel half-cell was placed at the outflow of the bath and served as a ground. The deflection of apical membrane voltage on abrupt elimination of glucose from the luminal fluid reflects activity of Na⁺-dependent glucose cotransporter in the apical membrane [8,9]. Because we have previously confirmed that deflection of V_t on abrupt elimination of glucose from the luminal fluid ($\Delta V_{t_{glu}}$) moves in parallel with the deflection of apical membrane voltage on abrupt elimination of glucose from the luminal fluid by simultaneous recording [2], we used $\Delta V_{t_{glu}}$ as a parameter of the Na-glucose cotransporter activity, instead of the apical membrane voltage on abrupt elimination of glucose from the luminal fluid, in this study. The $\Delta V_{t_{glu}}$ was measured before and, 10 and 20 min. after addition of Cd-MT (10^{-6} g Cd/ml) to lumen. This dose is comparable to that of chronic Cd exposure in rabbit plasma [12].

Net Bicarbonate Transport. The concentrations of inulin and total CO₂ (assumed to be equal to that of HCO₃) in perfusate and collected fluid were measured in a continuous-flow microfluorometer (Nanoflo, WPI, Sarasota, FL) [10]. Three 47-nl collections were made per period and stored under water-saturated mineral oil. Aliquots (15 nl) of each collection were analyzed for total CO₂ on the day of the experiment and for inulin on the following day, using procedures specified by the manufacturer [11]. Samples of perfusate were processed similarly.

The perfusion rate was generally 5–8 nl/min. The HCO_3 transport rate (JHCO_3) was calculated as

$$\text{JHCO}_3 = (V_i [\text{HCO}_3]_i - V_o [\text{HCO}_3]_o)/L,$$

where V_i and V_o are perfusion rate and collection rate, respectively, $[\text{HCO}_3]_i$ and $[\text{HCO}_3]_o$ are HCO_3 concentrations in the perfusate and collected fluid, respectively, and L is tubular length in millimeters. Positive values of JHCO_3 indicate net HCO_3 absorption.

In Vivo Study. Female Japanese white rabbits weighing 1.5–2.2 kg ($n = 23$) were used. They had free access to standard chow (CR-3, Nippon Crea containing 0.06 μg Cd/g, 7 μg Cu/g, 31 μg Zn/g, 392 μg Fe/g, and 20.3 mg Ca/g) and distilled water until the end of the study. They were kept in the standard animal room in our medical school, with a 12-h light/dark cycle. The temperature and humidity were maintained automatically. CdCl_2 (0.3 mg Cd /Kg, s.c. once every day) [12] and/or acetazolamide (ACZ, 2 mg/kg for low dose and 20 mg/kg for high dose, i.p. twice daily) were given for 8 weeks to the rabbits. The rabbits consisted of the following four groups: Group 1 ($n = 5$): vehicle; Group 2 ($n = 6$): CdCl_2 ; Group 3 ($n = 6$): $\text{CdCl}_2 + \text{ACZ}$ (2 mg/kg); Group 4 ($n = 6$): $\text{CdCl}_2 + \text{ACZ}$ (20 mg/kg).

Blood and 1-day urine samples were obtained before and 4 and 8 weeks after the treatment. The urine was collected by using a metabolic cage. Plasma and urine creatinine (Cr) were measured by modified Jaffe's reaction with autoanalyzer. Urine glucose concentration was measured by Glucose FA test (Wako Pure Chemicals, Osaka, Japan) with autoanalyzer (Abbott VP, Abbott Park, Illinois, USA) and expressed as the ratio to creatinine concentration [12]. The Cd-MT in the urine specimen was fractionized by high-performance gel-filtration column (Toyo soda, HLC-803A, Tokyo, Japan) and Cd concentration was measured by flameless atomic absorption spectrophotometer (Hitachi Z 9000, Tokyo, Japan) after dilution with nitric acid [12].

Chemicals. All solutions containing bicarbonate were bubbled with 95% O₂, 5% CO₂ gas to adjusted pH of 7.4. The pH of HEPES solution was adjusted by addition of Tris. All reagents, except p-fluorobenzyl-aminobenzolamide, were purchased from Sigma (St. Louis,, USA). p-Fluorobenzyl-aminobenzolamide (pFB-ABZ), a carbonic anhydrase inhibitor impermeable to the cell membrane [11,13], was synthesized by the method of Tewson et al. [13]. Concentration of Cd-MT was expressed as grams of Cd per milliliter to represent Cd concentration contained in the molecule.

Ethical aspect. All of the experiments shown in this study were performed according to the *Guidelines of Animal Research* of our medical school, which thoroughly incorporated the National Guideline of Animal Research of Japan.

Statistics. All data were expressed as means \pm SE. Statistical comparison was performed using Student's *t*-test and ANOVA, where appropriate; *p* values less than 0.05 were regarded as significant.

Results

pH Dependence of Acute Effect of Luminal Cd-MT on $\Delta V_{t_{glu}}$. First, we evaluated the effect of luminal pH on $\Delta V_{t_{glu}}$ after luminal Cd-MT using HEPES buffer solution. As shown in Fig. 1, the $\Delta V_{t_{glu}}$ significantly decreased within 10 min after addition of Cd-MT (10^{-6} g Cd/ml) when the luminal pH was 7.4 (mean $\Delta V_{t_{glu}}$ before and 10 min after addition of Cd-MT = 2.0 ± 0.1 mV and 0.5 ± 0.1 mV, respectively; $n = 17$). The time needed to cause the change was slightly but significantly slower than in our previous report, in which we used bicarbonate buffer solution with the same pH [2]. When the pH of the perfusate was decreased to 6.8, the reduction of $\Delta V_{t_{glu}}$ was significantly accelerated, whereas it was prolonged when the pH was changed to 7.7 (Fig. 1). The $\Delta V_{t_{glu}}$ was maintained at a similar level for at least 20 min when Cd-MT was not added in the pH 6.8 and 7.7 solutions: $\Delta V_{t_{glu}}$ before and 20 min after the experiments was 1.9 ± 0.2 mV and 1.8 ± 0.2 mV at pH6.8, and 2.0 ± 0.2 mV and 1.9 ± 0.2 mV at pH7.7, respectively; $n = 4$ in each.

Effect of Carbonic Anhydrase Inhibitors on Cd-MT-Induced $\Delta V_{t_{glu}}$. Because the effect of luminal Cd-MT on $\Delta V_{t_{glu}}$ was dependent on luminal pH, we next examined the effect of carbonic anhydrase (CA) inhibitors, which alter luminal pH in a bicarbonate buffer solution. As shown in Fig. 2A, luminal Cd-MT (10^{-6} g Cd/ml) almost completely suppressed $\Delta V_{t_{glu}}$ within 10 min after addition, whereas the vehicle did not show any effects (Fig 2B), confirming our previous observation [2]. We also found that comparable concentration of native MT itself did not affect to $\Delta V_{t_{glu}}$ ($\Delta V_{t_{glu}}$ before and 20 min after addition of MT was 2.0 ± 0.3 mV and 1.8 ± 0.3 mV at pH7.4, $P > 0.05$). When 10^{-5} M ACZ was added to the lumen, luminal administration of Cd-MT did not cause any inhibition of $\Delta V_{t_{glu}}$, and subsequent elimination of ACZ from the lumen caused marked inhibition of $\Delta V_{t_{glu}}$ (Fig. 3A). On the other hand, administration of ACZ to the bath did not prevent the inhibitory effect of Cd-MT on $\Delta V_{t_{glu}}$ (Fig. 3B).

To confirm that the observed effect of ACZ is due to inhibition of membrane-bound CA, rather than intracellular CA, we tested effects of pFB-ABZ, an impermeable inhibitor specifically restricted in its binding to membrane-bound CA [11,13]. Luminal pFB-ABZ (10^{-5} M) completely prevented the decrease of Cd-MT-induced $\Delta V_{t_{glu}}$, and this effect was reversed by elimination of the drug from the lumen (Fig. 4A). Once Cd-MT decreased $\Delta V_{t_{glu}}$, it was not recovered by addition of pFB-ABZ (Fig. 4B). Furthermore, addition of CA (2 mg/dl) in combination with pFB-ABZ reversed the preventive effect of pFB-ABZ against Cd-MT (Fig. 5A). CA alone did not affect the $\Delta V_{t_{glu}}$ (Fig. 5B). When pFB-ABZ was added to the bath, it did not prevent the change of $\Delta V_{t_{glu}}$ by luminal Cd-MT (Fig. 6).

Effect of Luminal Carbonic Anhydrase Inhibitors on Bicarbonate Transport. JHCO_3 decreased from 68.2 ± 3.4 to 33.4 ± 3.7 pmol/min/mm by addition of pFB-ABZ to lumen ($n = 4$, $P < 0.01$), which is consistent with our previous study [11]. Luminal ACZ also reduced bicarbonate absorption from 70.8 ± 3.1 to 37.0 ± 4.7 pmol/min/mm ($n = 4$, $P < 0.01$). These results indicated that pFB-ABZ and ACZ to lumen prevented absorption of bicarbonate and consequently prevented reduction of luminal pH in vitro.

Effect of Modulator of Cytoskeleton on Cd-MT-Induced Dysfunction of the Proximal Tubule. Since the pH-dependent effect of Cd-MT was time-dependent rather than instantaneous (Fig. 1), the effect might not be a direct one on Na^+ -glucose cotransporter. It is possible that Cd-MT exerts the inhibitory effect after intracellular uptake of the complex. To evaluate this possibility, we examined the effect of cytoskeleton modulators. Pretreatment with colchicine (10^{-5} M) from lumen prevented the reduction of Cd-MT-induced reduction of $\Delta V_{t_{glu}}$, (Fig 7A), whereas lumicolchicine, a biologically inactive colchicine analogue, did not prevent it (Fig. 7B). Cytochalasin D (10^{-6} M) (Fig. 8A) and Y-27632 (10^{-4} M) (Fig. 8B), an inhibitor of rho-kinase [14], also prevented the Cd-MT induced reduction.

In Vivo Effect of ACZ in Preventing Cd-Induced Nephrotoxicity. Repeated administration of Cd caused nephrotoxicity as revealed by increased serum creatinine (Cr) concentration and urinary excretion of glucose. The renal Cd toxicity represented by these data was prevented by administration of ACZ in a dose-dependent manner (Fig. 9A, B). Urinary Cd-MT content at the end of the study was significantly increased by co-administration with high-dose of acetazolamide, but not by low dose (Fig. 9C). Body weights did not decrease in either group (data not shown).

Discussion

The present study demonstrates in rabbit proximal tubule cells that the inhibitory effect of Cd-MT on $\Delta V_{t_{glu}}$ is pH-dependent: an increase of luminal pH prevented, and a decrease of the pH worsened, the impairment of sodium-dependent glucose transporter activity following acute luminal exposure of Cd-MT. This is the first study showing that the impairment of sodium-dependent glucose transporter activity by luminal Cd-MT is dependent on luminal pH. We observed that the reduction of bicarbonate absorption was prevented by luminal pFB-ABZ and ACZ in this study. Because luminal pH decreased along the proximal tubule by reabsorption of bicarbonate in the filtered fluid, the reduction of bicarbonate absorption by pFB-ABZ and ACZ to lumen inhibited the reduction of luminal pH. Therefore a preventive effect of pFB-ABZ and ACZ on Cd-MT-induced toxicity was mediated by an increase of luminal pH. The luminal pH in the normal kidney can be changed from about 6.8 to 7.7 to adapt to the systemic acid-base status [15]. Thus it is important to note that the prevention of the toxic effect of Cd-MT is attainable within physiological range of the luminal pH in the proximal tubule.

Inhibition of carbonic anhydrase in the cytosol and/or apical membrane of proximal tubule cells prevented the reduction of luminal pH and decreased urinary acid excretion [15]. Therefore the prevention of the Cd-MT-induced damage by acetazolamide and pFB-ABZ in this study must be due to alteration of luminal pH by these drugs. We also found that the effects of luminal ACZ and pFB-ABZ, a CA inhibitor impermeable to cell membrane but able to affect HCO_3^- absorption in proximal tubule cells [11], were comparable, while basolateral addition of ACZ was not effective. These results suggest that inhibition of luminal membrane-bound CA is important for preventing the Cd-MT-induced damage. Among many isoforms of CA, CA IV, XII, XIV, and XV are membrane-associated and reported to be located in kidney, while CA XIV is not detected in the rabbit, while it is detected in mouse kidney [16,17].

It is uncertain how alteration of luminal pH affects the toxicity of Cd-MT. It is generally believed that Cd-MT enters the cell as Cd-MT and dissociates into Cd^{2+} in the cytoplasm. Alteration of luminal pH will mainly affect the phenomenon in the lumen. Therefore conformational change of Cd-MT in the luminal space by an increase of pH reduces the transport of luminal Cd-MT into the cell. It is also possible that carrier protein of Cd-MT in the apical membrane might be reduced in its activity by an increase of luminal pH. It is reported that endocytosis of Cd-MT is mediated by megalin [18,19] and that the expression of megalin is reduced by Cd-MT exposure [20]. However, the influence of intracellular pH on activity of megalin was not reported. Another possibility is that the increase of luminal pH directly enhances glucose transporter activity. We think that this may not be true because we have some preliminary data that $\Delta V_{t_{\text{glu}}}$ was maintained at a similar level for at least 20 min when Cd-MT was not added in pH 6.8 and 7.7 solutions.

We found that modifiers or modulators of cytoskeleton (such as colchicine, cytochalasin D) and rho-kinase inhibitor Y-27632 prevented Cd-MT-induced functional damage. This may be compatible with the finding that endocytosis was reduced in renal tubular cells from Cd-treated animals [21]. It was reported that inhibition of acidic endocytotic compartments by chloroquine, or LY294002, prevented Cd-MT-induced apoptosis [22]. Alkalinization of luminal fluid may secondarily alter intracellular pH, which subsequently affects the endosomal uptake of Cd/Cd-MT.

We also found that the time needed to reduce $\Delta V_{t_{\text{glu}}}$ after addition of luminal Cd-MT was slightly but significantly increased in HEPES buffer solution than in a bicarbonate buffer solution of similar pH. The reason for this difference is uncertain at the present time. HEPES is not membrane-permeable and is less likely to affect intracellular pH than bicarbonate, which may affect the results. It may suggest the existence of other factor(s) beside pH contributing to

the Cd-MT-induced damage.

Based on the *in vitro* data as discussed above, we propose that the inhibition of CA would be a therapeutic option for the prevention of Cd-induced nephropathy. This is another important feature of this study. The *in vivo* experiments were designed to support this issue. Although we did not examine detailed kidney functions such as clearance studies, both plasma creatinine levels and glucosuria are reasonable parameters to represent Cd nephrotoxicity. Under chronic experimental conditions in the *in vivo* study, we think that glucosuria is one of the parameters of nephrotoxicity rather than simple alteration of the cotransporter. The data clearly support the notion that administration of ACZ prevents Cd-induced nephrotoxicity in a dose dependent manner. Our *in vivo* data is compatible with the findings of a previous article by Nomiya et al [12], although their interpretation of this effect was somewhat different. CA inhibitors are mainly used as diuretics, but not commonly used for prevention of nephrotoxicity. From this point of view, the present data are unique. We also found that urinary excretion of Cd-MT increased by high dose of acetazolamide, which supported for the relationship between *in vivo* and *in vitro* data. Low dose of acetazolamide did not increase the Cd-MT excretion. We think that this model of chronic intoxication excreted very large amount of Cd-MT into urine, and the low dose of acetazolamide may not be enough for *in vivo* treatment. We have previously reported that alkalinization of systemic pH by addition of citrate prevented cyclosporine-A-induced distal renal tubular acidosis in rats [23]. Acetazolamide might also be used for prevention of the cyclosporine-A-induced damage.

Nomiya et al [12] previously reported in the rabbit that nephrotoxicity by systemic Cd exposure was also prevented by the addition of glycyrrhizic acid, the active pharmacological ingredient of licorice. They speculated that glycyrrhizic acid prevented the release of Cd-MT from the liver into systemic circulation because of its protective effect on hepatic functions. The

glycyrrhizic acid, however, was also reported to cause pseudo-aldosteronism, which subsequently causes metabolic alkalosis [24]. Therefore it is necessary to examine whether effect of glycyrrhizic acid was due to an increase of systemic pH by the drug.

Recently, it was reported that expression of NHE3 protein decreased while CA IV was unchanged in brush border membrane vesicles from renal proximal tubule cells in Cd-intoxicated rats. These results indicate that urinary alkalization will occur in Cd intoxication [25]. We did not evaluate Na-H exchanger activity in this study. Because our finding indicates that alkalization of luminal fluid prevented the Cd-MT-induced toxicity, the reduction of NHE3 protein may be an adaptive response of the kidney to prevent the damage of proximal tubule cells.

In summary, we found for the first time that the impairment of sodium-dependent glucose transporter activity caused by luminal Cd-MT is influenced by luminal pH and that an increase in pH prevented the impairment in isolated perfused rabbit proximal tubules. We also found that inhibition of luminal CA activity, which increases the luminal pH, also prevented the impairment. Addition of cytoskeleton modifiers also prevented the toxicity. Furthermore, administration of acetazolamide in vivo also prevented the nephrotoxicity of Cd exposure. These results may point to new therapy options in preventing the nephrotoxicity of systemic Cd exposure.

Acknowledgement

This work was partly supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Cronin R, Henrich W: Toxic nephropathy. in Brenner B (ed): Brenner and Rector's the Kidney. Philadelphia: Saunders, 1996, 1680-1711.
2. Tsuruoka S, Sugimoto K, Muto S, Nomiya K, Fujimura A, Imai M: Acute effect of cadmium-metallothionein on glucose and amino acid transport across the apical membrane of the rabbit proximal tubule perfused in vitro. *J Pharmacol Exp Ther* 2000;292:769-777.
3. Nomiya K, Nomiya H: Cadmium-induced renal dysfunction: New mechanism, treatment and prevention. *J Trace Elem Exp Med* 1998;11:275-288.
4. Burg M, Grantham J, Abramow M, Orloff J: Preparation and study of fragments of single rabbit nephron. *Am J Physiol* 1966;210:1293-1298.
5. Tsuruoka S, Takeda M, Yoshitomi K, Imai M: Cellular heterogeneity of ammonium ion transport across the basolateral membrane of the hamster medullary thick ascending limb of Henle's loop. *J Clin Invest* 1993;92:1881-1888.
6. Tsuruoka S, Koseki C, Muto S, Tabei K, Imai M: Axial heterogeneity of potassium transport across hamster thick ascending limb of Henle's loop. *Am J Physiol* 1994;267:F121-129.
7. Tsuruoka S, Schwartz GJ, Wakaumi M, Nishiki K, Yamamoto H, Purkerson JM, Fujimura A: Nitric oxide production modulates Cyclosporin A-induced distal renal tubular acidosis in the rat. *J Pharmacol Exp Ther* 2003;305:845-850.
8. Maruyama T, Hoshi T: The effect of D-glucose on the electrical potential profile across the proximal tubule of newt kidney. *Biochim Biophys Acta* 1972;282:214-225.
9. Fromter E: Electrophysiological analysis of rat renal sugar and amino acid transport. I. Basic phenomena. *Pflugers Arch* 1982;393:179-189.

10. Zhelyaskov V, Liu S, Broderick M: Analysis of nanoliter samples of electrolytes using a flow-through microfluorometer. *Kidney Int.* 2000;57:1764-1769.
11. Tsuruoka S, Swenson ER, Petrovic S, Fujimura A, Schwartz GJ: Role of basolateral carbonic anhydrase in proximal tubular fluid and bicarbonate absorption. *Am J Physiol Renal Physiol* 2001;280:F146-154.
12. Nomiya K, Nomiya H, Kameda N: Plasma cadmium-metlothionein, a biological exposure index for cadmium-induced renal dysfunction, based on the mechanism of its action. *Toxicology* 1998;129:157-168.
13. Tewson T, Sttekhova S, Swenson E: The synthesis and preliminary evaluation of [F-18]-N-p-fluorobenzyl-aminobenzamide, an inhibitor of carbonic anhydrase IV. *J Labelled Cpd Radiopharm* 1999;Suppl 1:42-43.
14. Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M, Narumiya S: Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol Pharmacol* 2000;57:976-983.
15. Rose D, Post T: Regulation of acid-base balance. in *Clinical Physiology of acid-base and electrolyte disorders.* New York: McGraw-Hill, 2001, 325-371.
16. Purkerson J, Schwartz G: Expression of membrane-associated carbonic anhydrase isoforms IV, IX, XII, and XIV in the rabbit: induction of CA IV and IX during maturation. *Am J Physiol Regul Integr Comp Physiol.* 2005;288:R1256-R1263.
17. Kaunisto K, Parkkila S, Rajaniemi H, Waheed A, Grubb J, Sly W: Carbonic anhydrase XIV: luminal expression suggests key role in renal acidification. *Kidney Int.* 2002;61:2111-2118.
18. Klassen R, Crenshaw K, Kozyraki R, Verroust P, Tio L, Atrian S, Allen P, Hammond T: Megalin mediates renal uptake of heavy metal metallothionein complexes. *Am J Physiol*

- Renal Physiol 2004;287:F393-F403.
19. Wolff N, Abouhamed M, Verroust P, Thévenod F: Megalin-dependent internalization of cadmium-metallothionein and cytotoxicity in cultured renal proximal tubule cells. *J Pharmacol Exp Ther* 2006;318:782-791.
 20. Sabolic I, Ljubojevic M, Herak-Kramberger C, Brown D: Cd-MT causes endocytosis of brush-border transporters in rat renal proximal tubules. *Am J Physiol Renal Physiol* 2002;283:F1389-1402.
 21. Herak-Kramberger C, Brown D, Sabolic I: Cadmium inhibits vacuolar H⁺-ATPase and endocytosis in rat kidney cortex. *Kidney Int.* 1998;53:1713-1726.
 22. Erfurt C, Roussa E, Thevenod F: Apoptosis by Cd²⁺ or CdMT in proximal tubule cells: different uptake routes and permissive role of endo/lysosomal CdMT uptake. *Am J Physiol Cell Physiol.* 2003;285:C1367-1376.
 23. Tsuruoka S, Schwartz GJ, Ioka T, Yamamoto H, Ando H, Fujimura A: Citrate reverses cyclosporin A-induced metabolic acidosis and bone resorption in rats. *Am J Nephrol* 2005;25:233-239.
 24. Monder C, Stewart P, Lakshmi V, Valentino R, Burt D, Edwards C: Licorice inhibits corticosteroid 11 beta-dehydrogenase of rat kidney and liver: in vivo and in vitro studies. *Endocrinology* 1989;125.
 25. Ahn D, Chung J, Kim J, Kim K, Park Y: Inhibition of renal Na⁺/H⁺ exchange in cadmium-intoxicated rats. *Toxicol Appl Pharmacol.* 2005;204:91-98.

Figure legends

Fig. 1. pH-dependent effect of luminal Cd-MT on glucose-induced Vt deflection. Values are given as Mean \pm SE. * $p < 0.05$ vs. pH 7.4 by repeated ANOVA; $n = 4-17$ in each. Abbreviation: Pre ; pretreatment period/ The same are applied for all other figures.

Fig. 2. Comparison of the effects of luminal Cd-MT (A) and vehicle (B) at pH7.4 on glucose-induced Vt deflection. Time courses of glucose-induced Vt deflection are shown. Individual results and mean \pm SE are also shown. * $p < 0.05$ vs. pre.

Fig. 3. Comparison of effect of ACZ added to the lumen (A) and to the bath (B). Luminal addition of ACZ for 10 min prevented the suppression of the glucose-induced Vt deflection caused by luminal Ca-MT, and removal of ACZ reversed the effect (A). Administration of ACZ to the bath was without any effect (B). Individual results and mean \pm SE are also shown. * $p < 0.05$ vs. Pre.

Fig. 4. Effect of luminal p-fluorobenzyl-aminobenzolamide (pFB-ABZ) for 10 min on Cd-MT-induced reduction of Vt deflection upon glucose removal: (A) pFB-ABZ added from zero to 10 min. (B) pFB-ABZ added from 10 to 20 min. Individual results and mean \pm SE are also shown. * $p < 0.05$ vs. Pre.

Fig. 5. (A) carbonic anhydrase reverses the protective effect of pFB-ABZ on Cd-MT inhibition of glucose-dependent Vt deflection. (B) carbonic anhydrase alone had no effect. Individual results and mean \pm SE are also shown. * $p < 0.05$ vs. Pre.

Fig. 6. No effect of basolateral p-fluoro benzolamide (pFB-ABZ) on Cd-MT-induced reduction of Vt deflection upon glucose removal. Individual results and mean \pm SE are also shown. $*p < 0.05$ vs. pre.

Fig. 7. (A) Effect of colchicine on Cd-MT-induced reduction of Vt deflection upon glucose removal. (B) lumicolchicine had no effect. Individual results and mean \pm SE are also shown. $*p < 0.05$ vs. pre.

Fig. 8. Effects of cytochalasin D (A) and Y-27632 (B) on Cd-MT-induced reduction of Vt deflection upon glucose removal. Individual results and mean \pm SE are also shown.

Fig. 9. Effect of ACZ on CdCl₂-induced renal dysfunction in vivo. Mean \pm SE. $*p < 0.05$ vs. CdCl₂. Nephrotoxicity of CdCl₂ was represented by plasma creatinine (A), glucosuria (B) concentrations. Urinary Cd-MT (C) excretion at eight week after the study was increased by high dose of acetazolamide than CdCl₂ group.

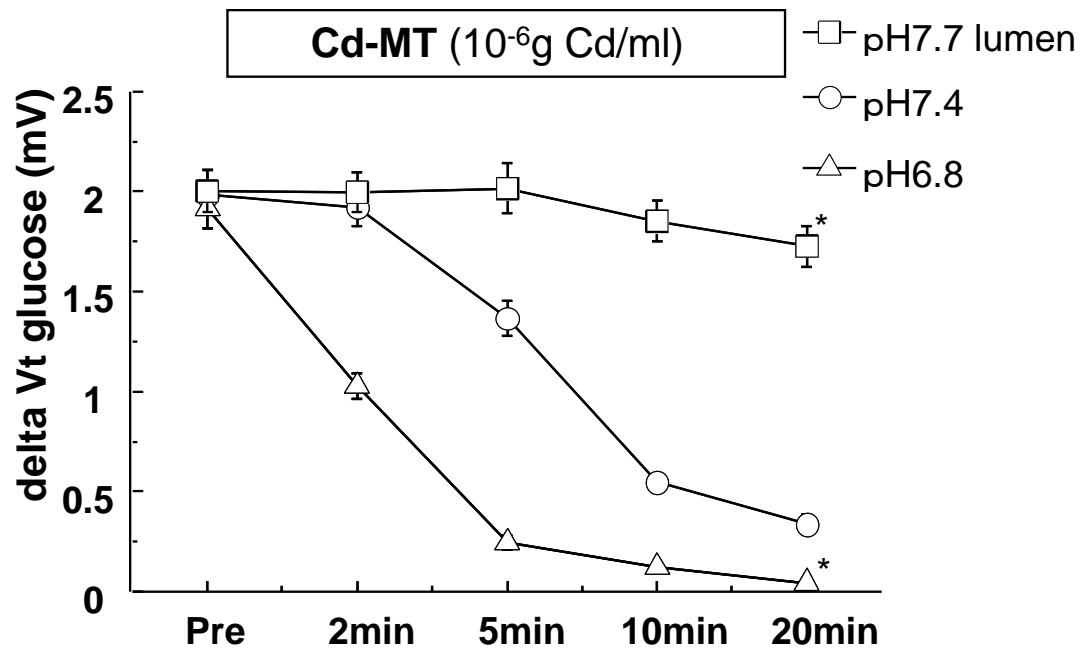


Fig.1

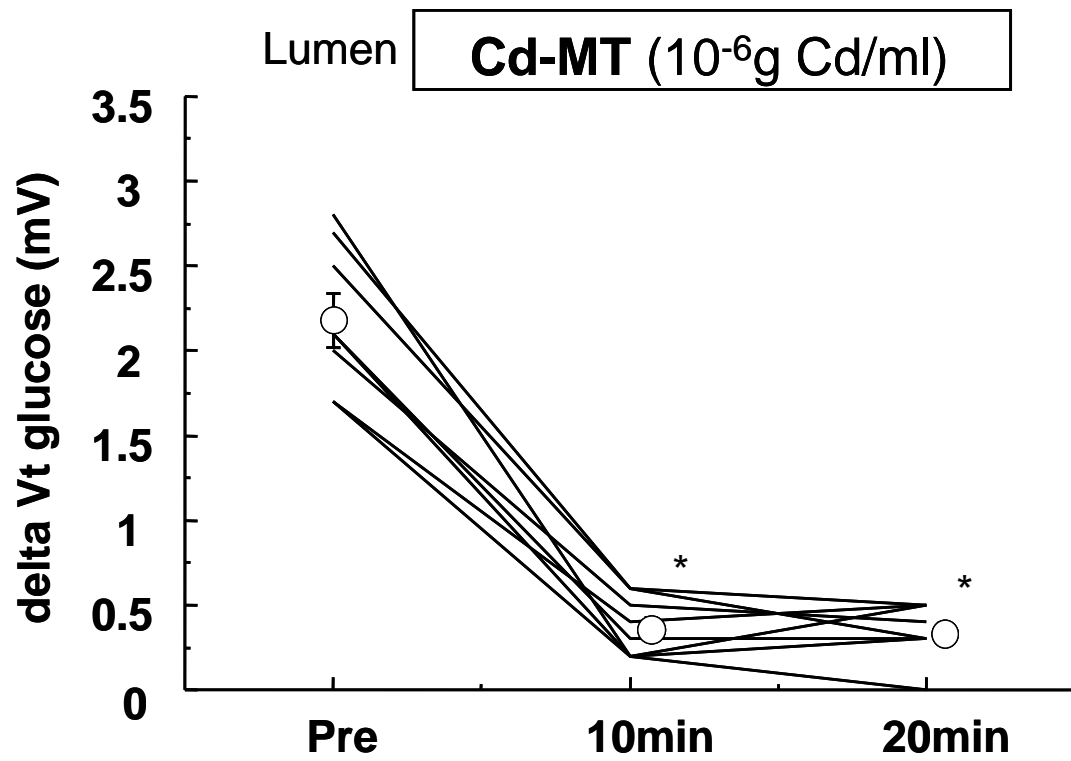


Fig.2A

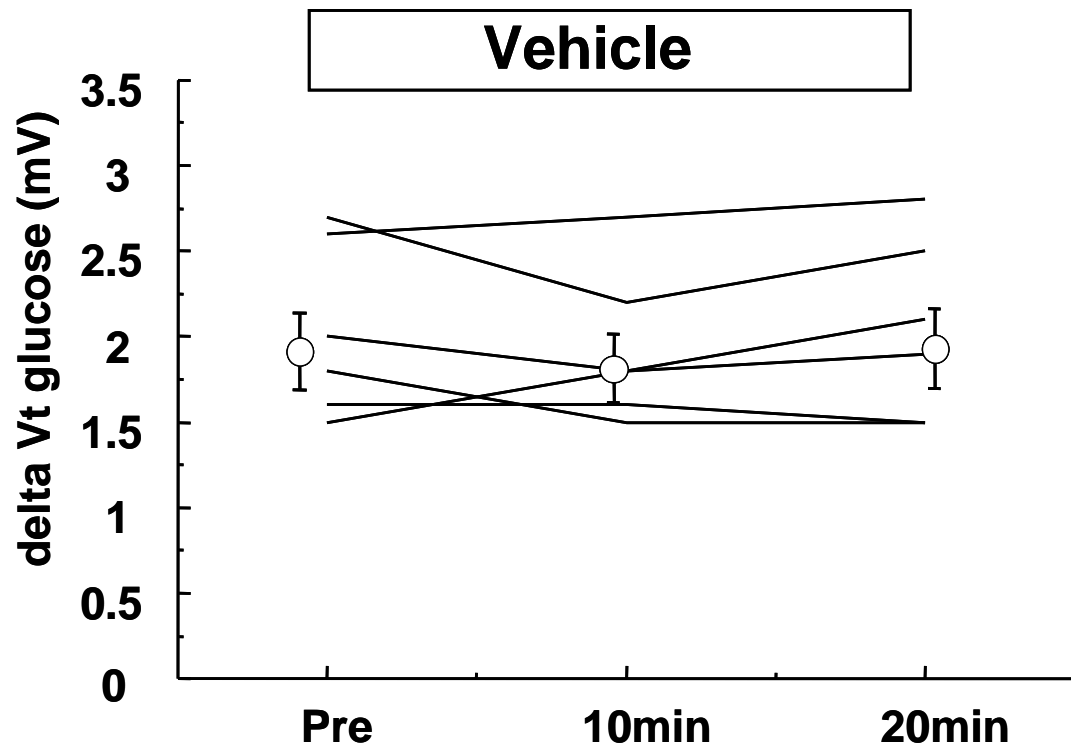


Fig.2B

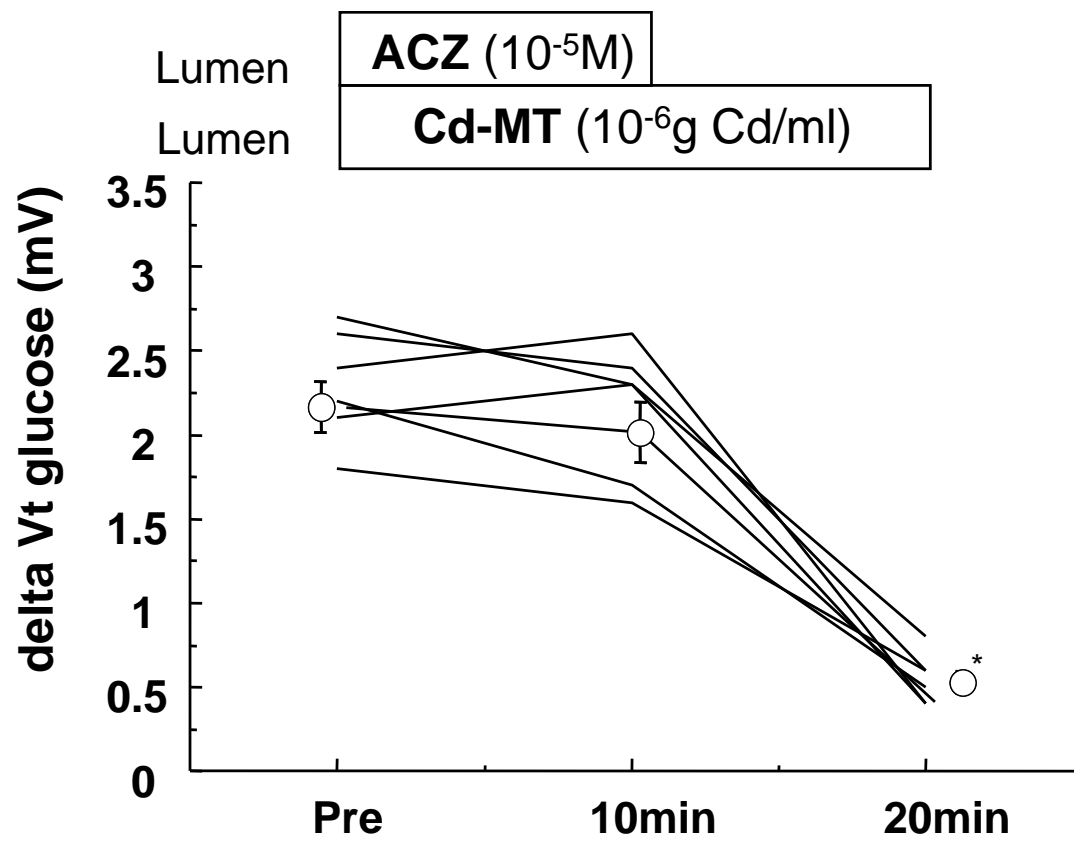


Fig.3A

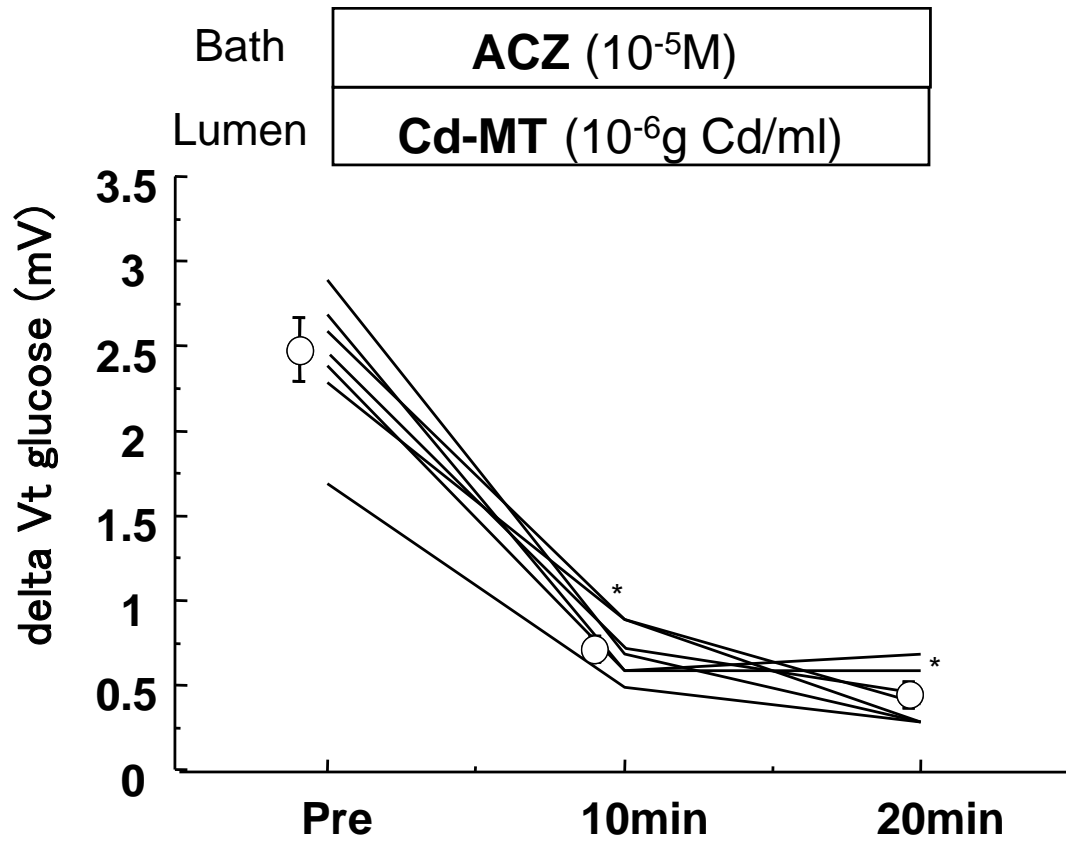


Fig.3B

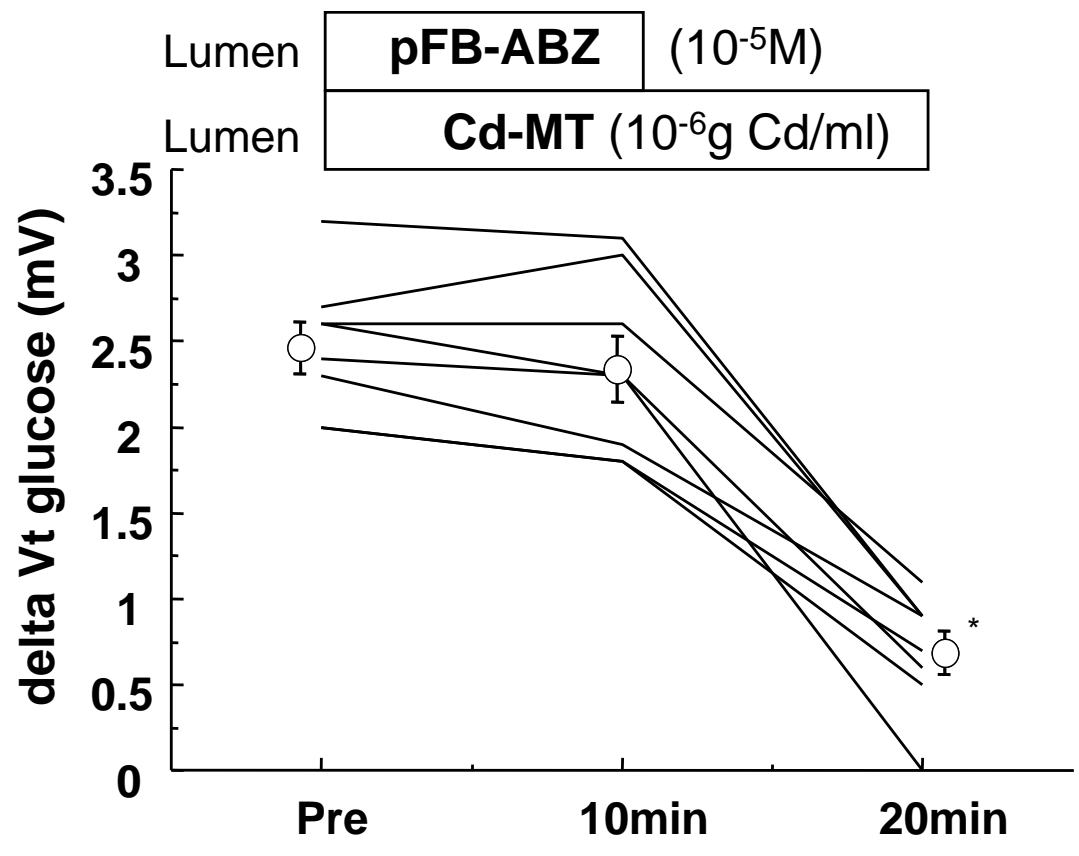


Fig.4A

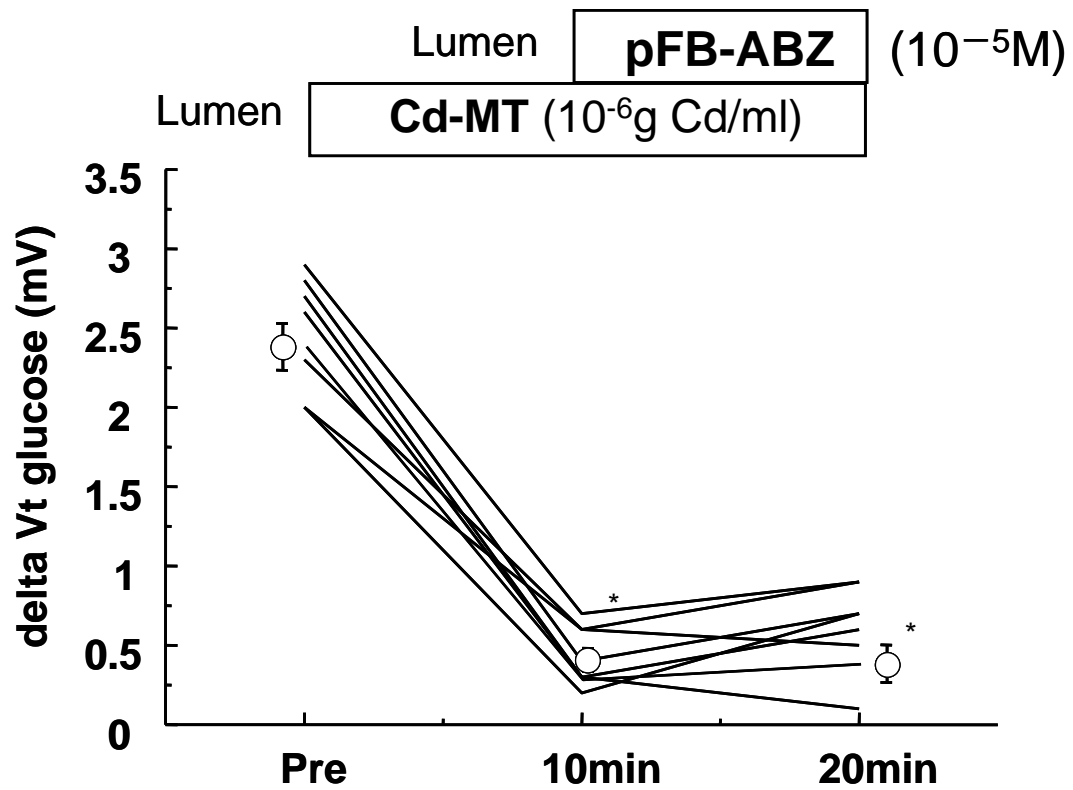


Fig.4B

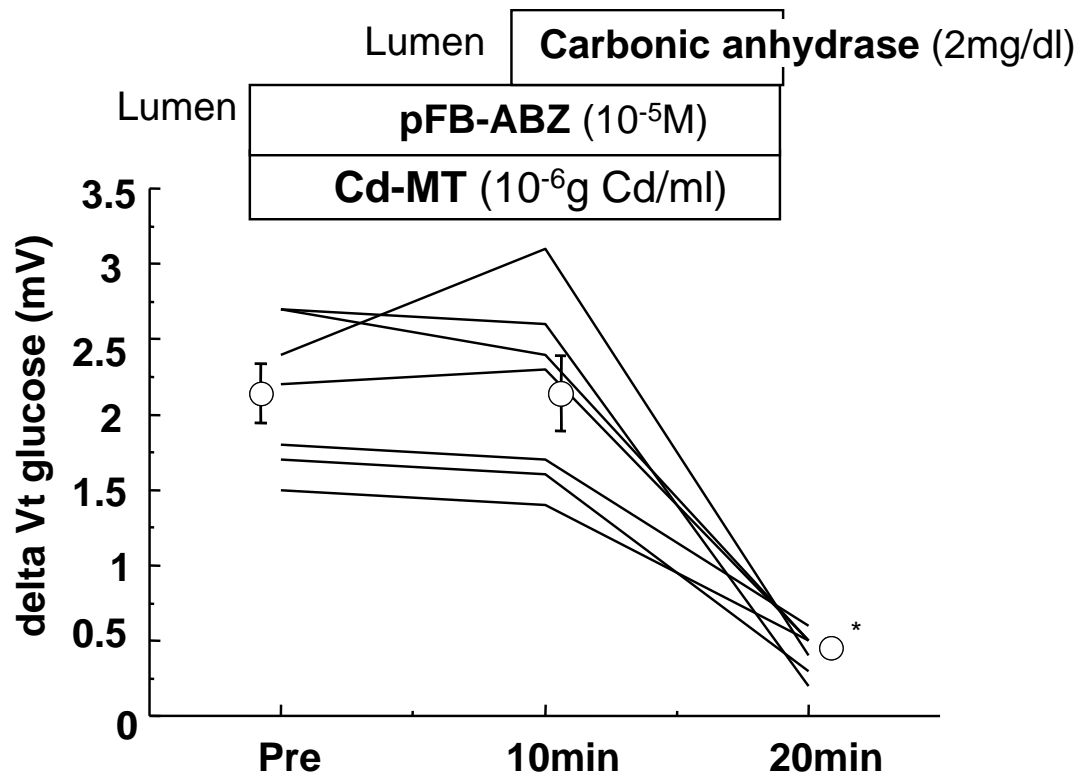


Fig.5A

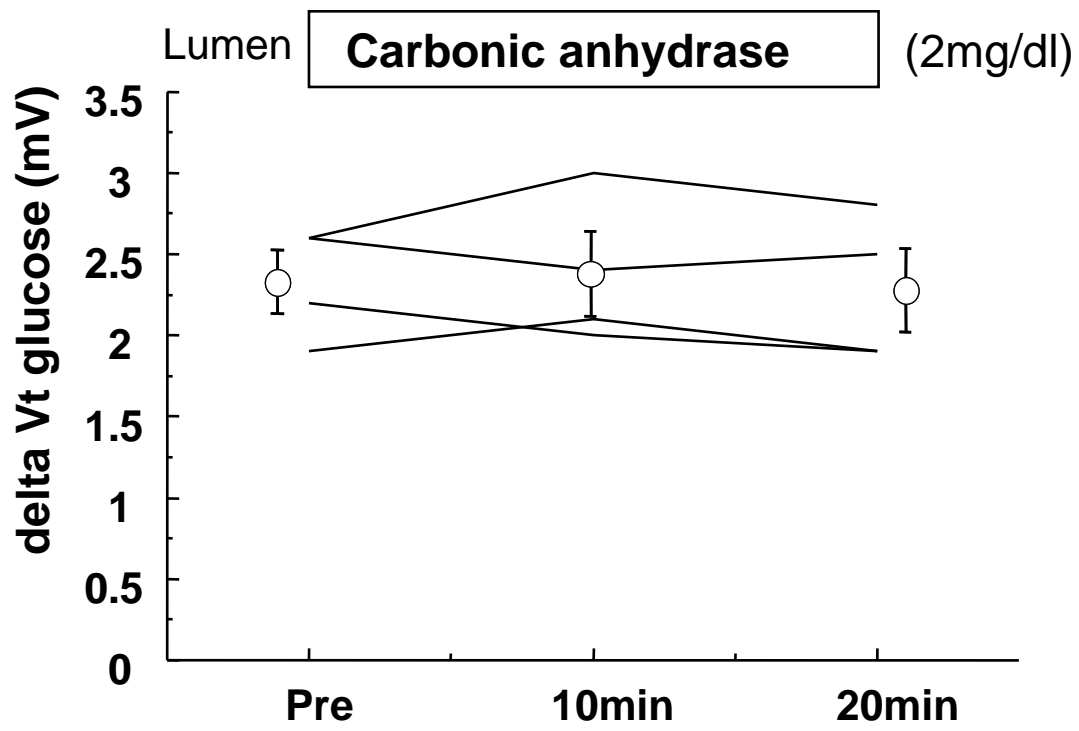


Fig.5B

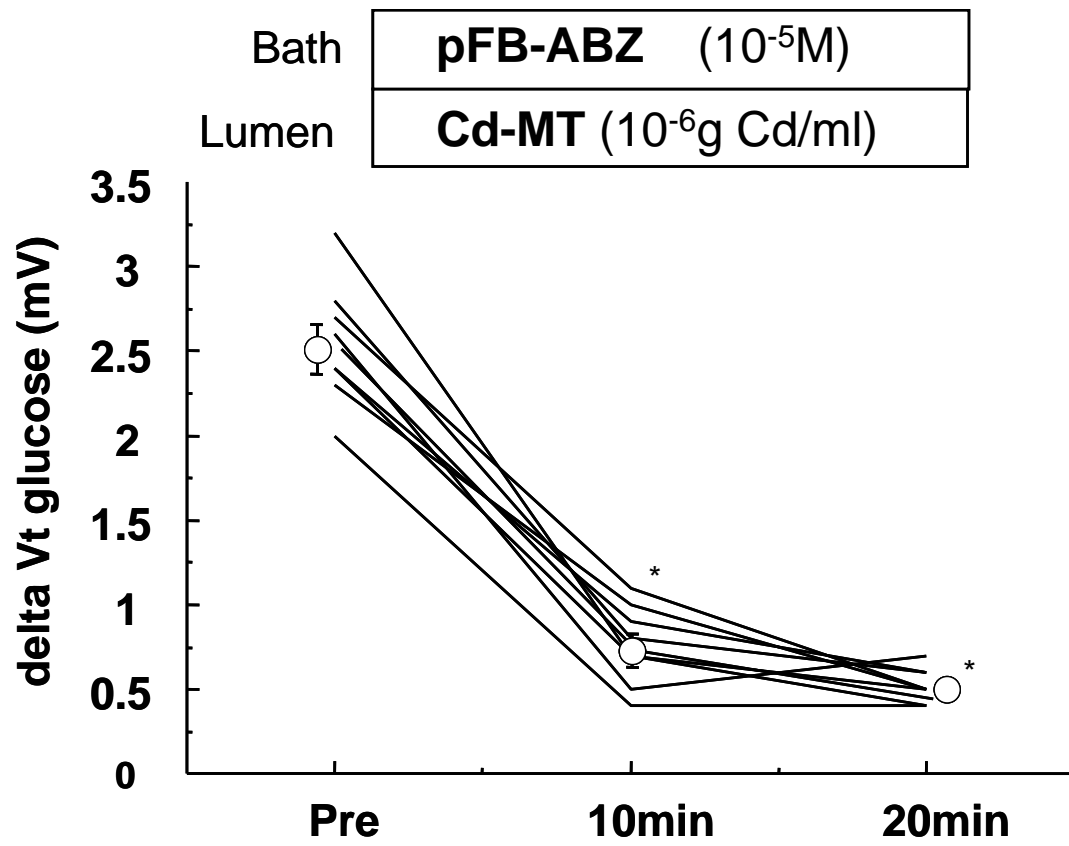


Fig.6

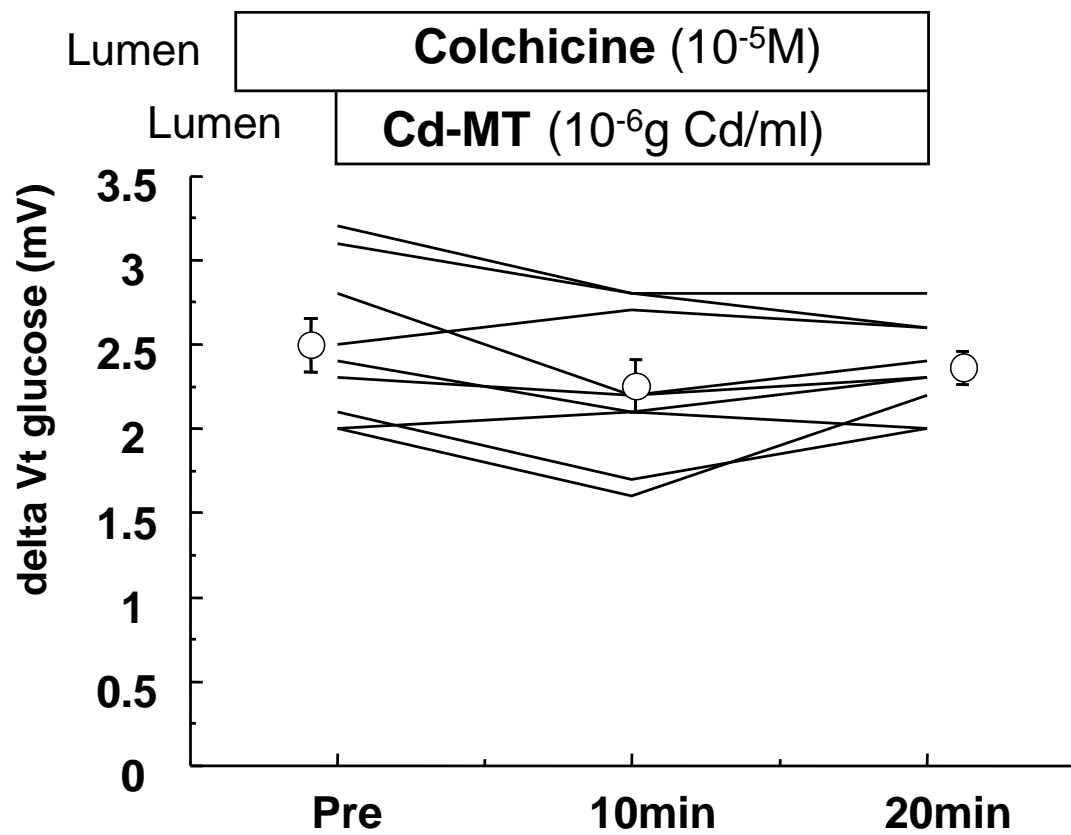


Fig.7A

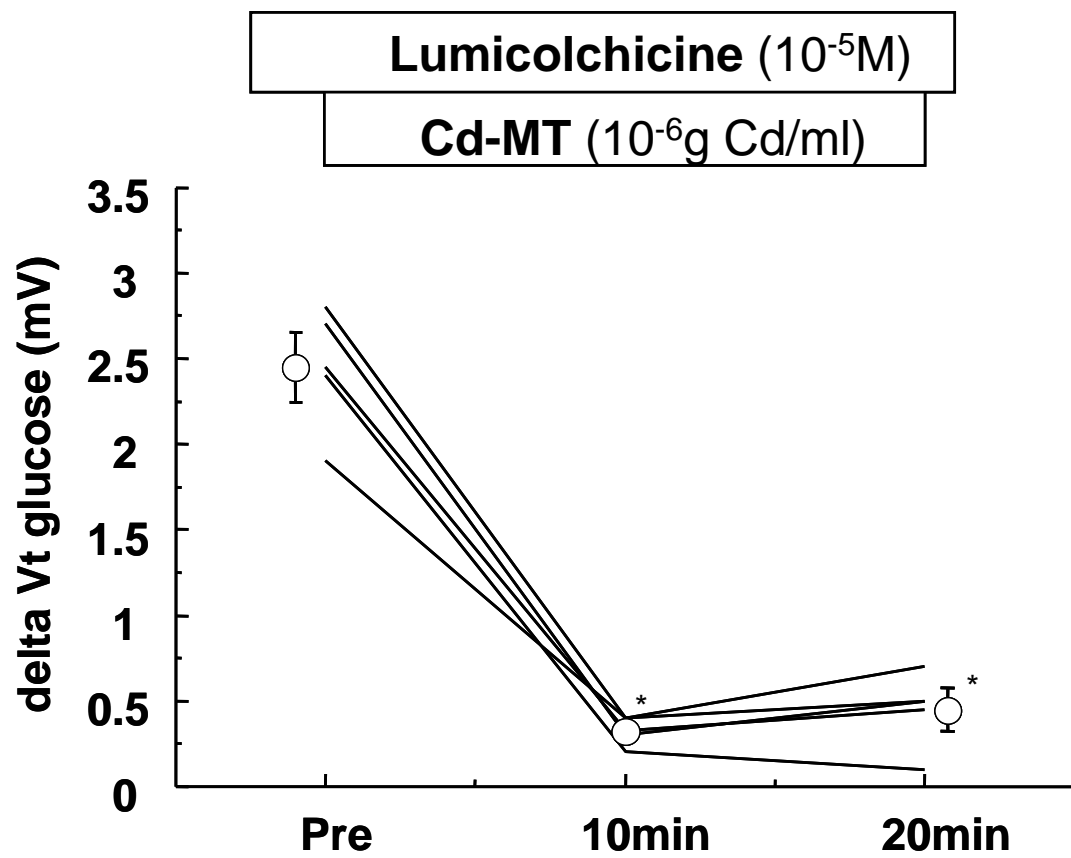


Fig.7B

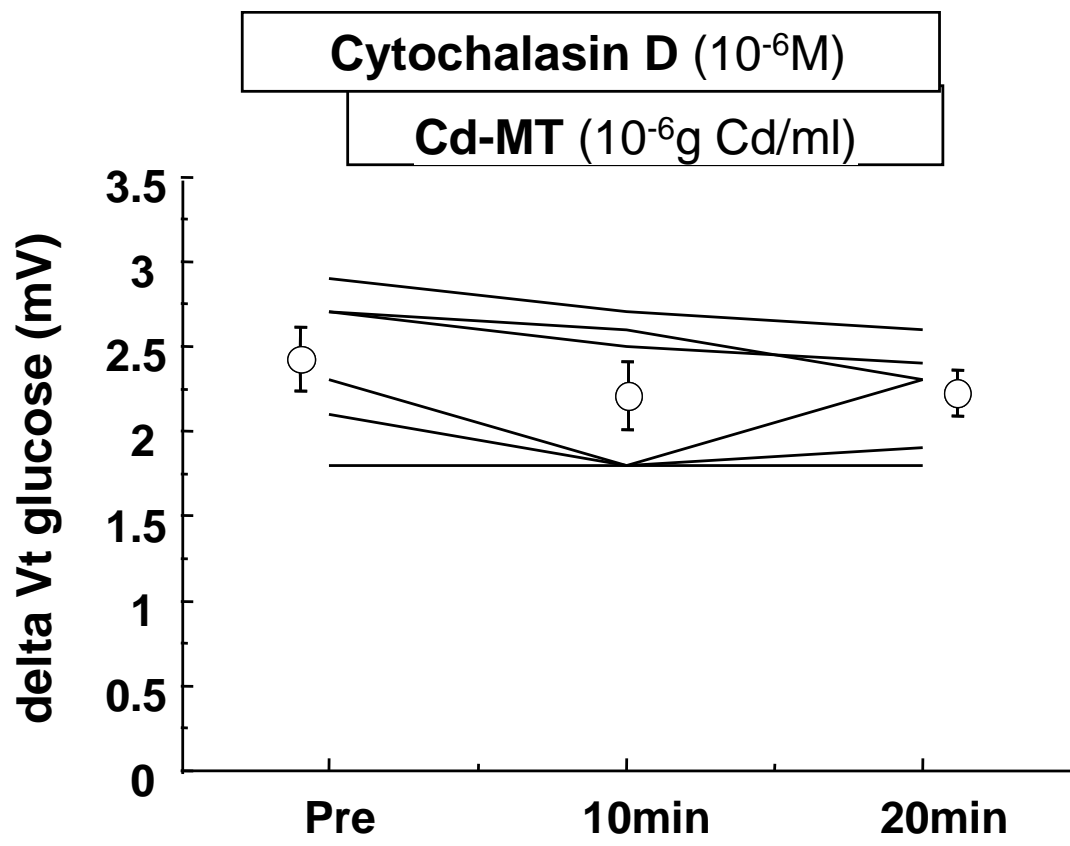


Fig.8A

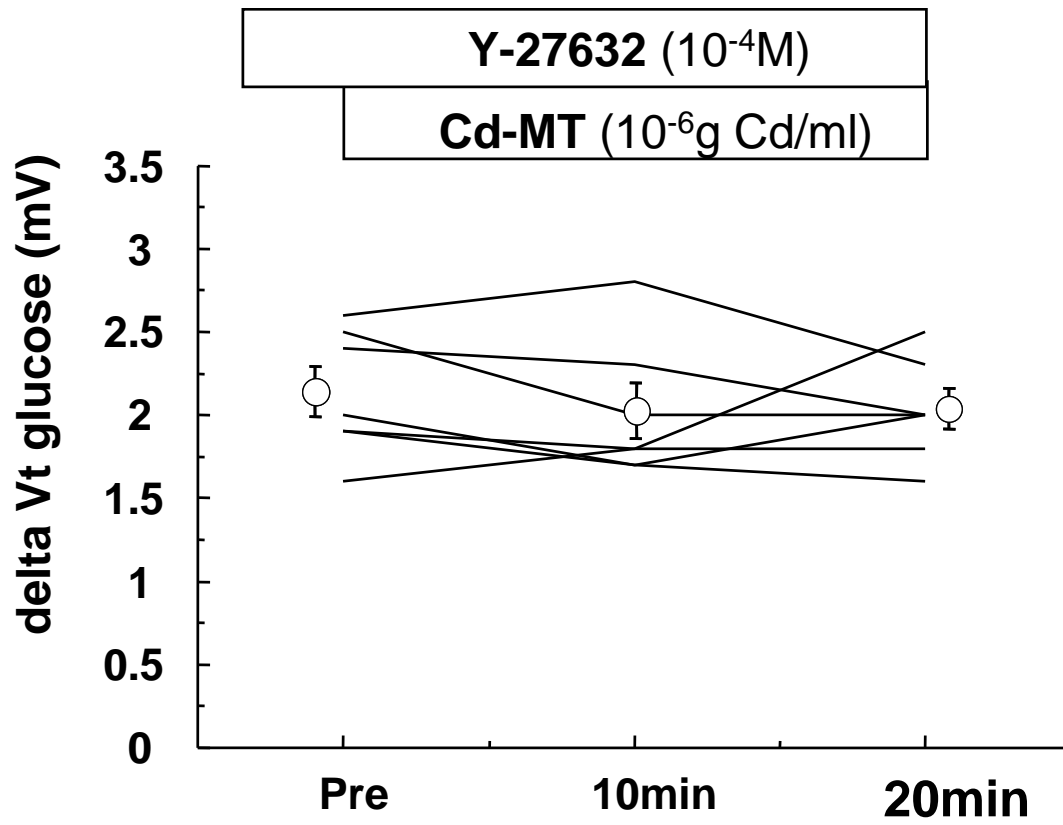


Fig.8B

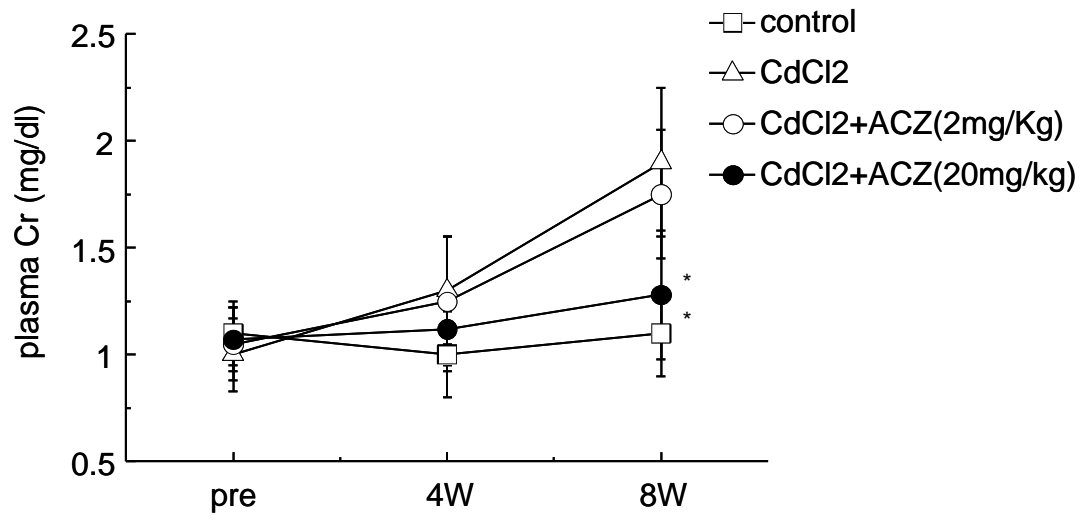


Fig.9A

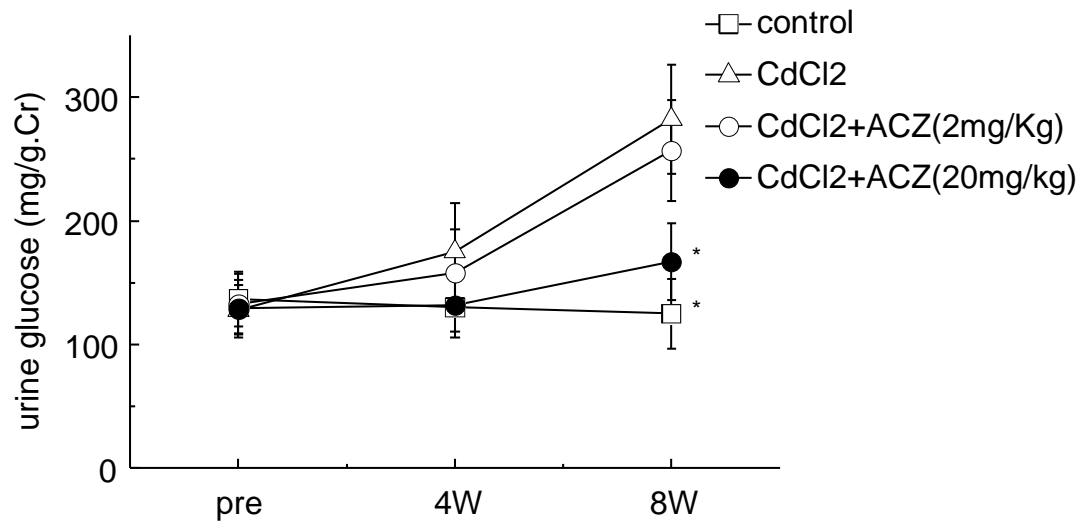


Fig.9B

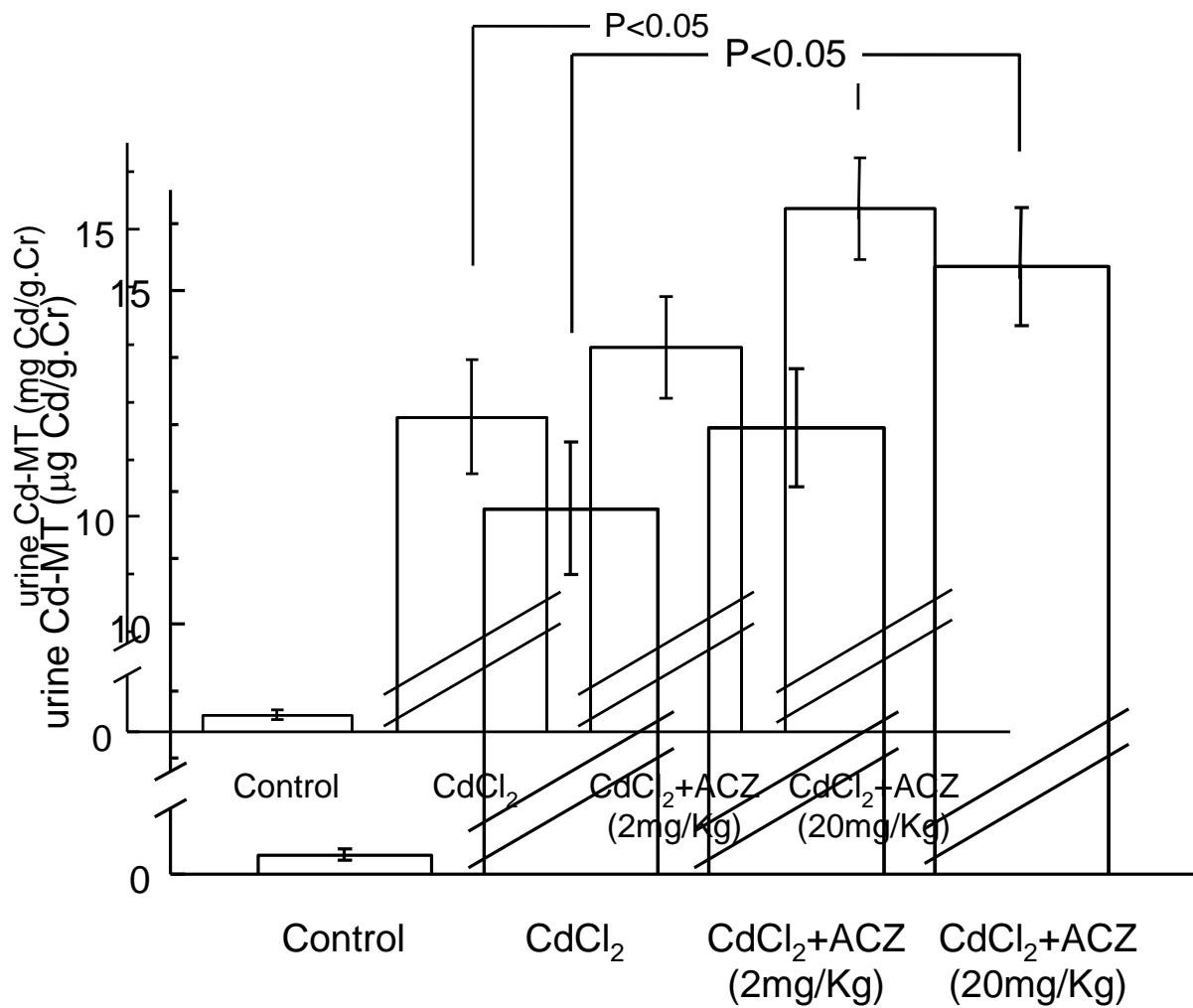


Fig. 9C