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Expressions of Uroplakins in the Mouse Urinary Bladder with Cyclophosphamide-Induced Cystitis

Even though uroplakins (UPs) are believed to serve a strong protective barrier against toxic materials, cyclophosphamide (CP) causes extensive cystitis. We investigated the expression of UPs in the urothelium in CP induced mouse cystitis. A total of 27 ICR female mice received a single intraperitoneal injection of 200 mg CP/kg. Nine CP-treated mice and 6 controls were sequentially killed at 12, 24, and 72 hr post injection. Extensive cystitis and an increased vesical weight were seen. These all peaked within 12 hr post injection and they tended to decrease thereafter. The level of all the UPs mRNA, the protein expressions of UP II and III on immunoblotting study, and the expression of UP III on immunolocalization study were maximally suppressed within 12 hr; this partially recovered at 24 hr, and this completely recovered at 72 hr post CP injection. In conclusion, CP reduced the expression of UPs. The reduction of the UPs mRNA and protein was time dependent, and this peaked within 12 hr after CP injection. However, the damage was rapidly repaired within 24 hr. This study demonstrates a dynamic process, an extensive reduction and rapid recovery, for the UPs expression of the mouse urinary bladder after CP injection.

Key Words : *Cyclophosphamide; Cystitis; Uroplakin*

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INTRODUCTION

Cyclophosphamide (CP) is an extensively used as an anticancer and immunosuppressive agent (1, 2). However, the urologic side effects of CP have been reported to range from asymptomatic microhematuria to life-threatening hemorrhagic cystitis (3, 4). CP also induces chronic bladder inflammation in experimental animal models that resemble the clinical syndrome of interstitial cystitis (5).

CP causes mucosal ulceration, transmural edema and epithelial necrosis that are associated with acute hemorrhage (6, 7). Vigorous diuresis and agents that can detoxify CP, such as 2-mercaptoethene sulfate (MESNA), have been used to decrease the urotoxicity (8). However, despite the positive results after the prophylactic use of MESNA, bladder protection is not always achieved (8, 9). Hemorrhagic cystitis still occurs after CP administration. It is important to investigate the basic mechanisms involved in the destruction of the uro-epithelial barrier in CP induced cystitis. However, it is not clear which mechanisms are actively involved in incurring mucosal damage.

The mammalian bladder maintains high electrochemical gradients between the urine and blood (10). The apical membrane in the bladder mucosa contains a group of four related transmembrane proteins; the uroplakins (UPs), which together with tight junctions form a specialized membrane compartment that represents one of the tightest and most impermeable barriers in the body (11-13). We formed a hypothesis that when the UPs are damaged by toxic materials, urine leaks into the underlying bladder layers and this induced inflammatory reactions in the bladder wall.

Studies have recently been done concerning the mechanisms involved in the pathogenesis of experimental alkylating agent-induced urologic complications (14, 15). Although much has been learned about the molecular changes, as well as some aspects of their physiological significance in the uro-epithelium, relatively little is known about the UPs' function when they are exposed to noxious chemicals.

In the current studies we investigated whether UPs were actively involved in the pathogenesis of CP-induced urothelial toxicity in mice. For this purpose we analyzed the timely expression of uroplakin Ia, Ib, II, and III in CP induced mouse cystitis.

MATERIALS AND METHODS

The present animal study protocol was approved by the IACUC of Dankook University College of Medicine. A total of 27 female ICR mice received an intramuscular injection

of ketamine (15 mg/kg) and xylazine (5 mg/kg), and then they received an intraperitoneal injection of 200 mg CP/kg (Sigma Chemicals, St. Louis, MO, U.S.A.) dissolved in distilled water (7). Eighteen mice (control group) received sterile water only. Nine CP-treated mice and 6 controls were sacrificed at 12, 24, and 72 hr post injection, respectively. The mice' bladders were removed and weighed. At each time, three treated mice bladders and two control bladders were fixed with 4% paraformaldehyde for immunohistochemistry, whereas six treated mice bladders and four control bladders were stored within a deep freezer.

Reverse transcription polymerase chain reaction (RT-PCR)

The total RNA was extracted from the whole bladder specimen with using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) and chloroform. We designed the PCR primers for all the uroplakins that spanned at least one intron of the corresponding genes. The primers synthesized for β-actin were 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′ according to the known cDNA sequence (GenBank code: NM007393). The primers for uroplakin Ia were 5′-TGT CGT CGT CGG TAC ATG AT-3′ and 5′-CAG AGT CGG GTT AGC TCC TG-3′ (GenBank code: NM081091). The primers for uroplakin Ib were 5′-CCT CTT CTG CTT GTC CGT TC-3′ and 5′-CTG CTT CAG GAA GAG GTT GG-3′ (GenBank code: NM178924). The primers for uroplakin II were 5′-AGA GCC AAC GAC AGC AAA GT-3′ and 5′-GGT GTA GCC AGA CCC ACT GT-3′ (GenBank code: NM009476). The primers for uroplakin III were 5′-CTG ACC CCT TGT GGT GAC TT-3′ and 5′-GGA CGT GAT GAC AAT CAT GC-3′ (GenBank code: AF222750). We used the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, U.S.A.) to prepare cDNA according to the manufacture's introduction. PCR was performed with 25 μL of the following reaction mixture: 12.5 μL of 2x GoTaq Green Master mixture (Promega), 10 pM of each primer and 1 μL of the DNA template. The amplification conditions were as follows: each step consisted of denaturation at 95℃ for 30 sec, annealing at 53℃ for 30 sec and extension at 72℃ for 30 sec. Thirty cycles for amplification were performed. The amplified products were resolved by electrophoresis through 2% agarose gels and then they were stained with ethidium bromide; the images were digitally captured using LAS 3000 software (Fujifilm, Tokyo, Japan).

Western blotting

We prepared the whole bladder lysate in lysis solution (50 mM Tris-Cl [pH7.5], 250 mM NaCl, 0.5% Triton x-100, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 10% glycerol and 2 μg/mL each of aprotinin, leupeptin and antipain). The amount of loaded proteins for uroplakin II and III were 30 μg each. The proteins were separated by 15% SDS-polyacrylamide gel electrophoresis, and they were transferred to nitrocellulose membranes. After incubation with 5% skim milk in TBST (10 mM Tris-Cl [pH7.5], 100 mM NaCl, 0.1% Tween20) for 1 hr at room temperature, the membrane was incubated with goat polyclonal primary antibody (uroplakin II, III. 1:1,000 dilutions) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in 5% skim milk in TBST solution for overnight at 4℃. After washing with TBST solution, the membranes were incubated with the secondary antibody (donkey anti-goat IgG-HRP polyclonal antibody, 1:2,500 dilutions) (Santa Cruz) for 1 hr at room temperature. After extensively washing, the membranes were enhanced with western blotting liminol reagent (Santa Cruz) for 1 min, and they were exposed from 30 sec to 30 min and the pictures were taken using a LAS-3000 image reader (Fujifilm).

Immunohistochemistry

The paraffin blocks were deparaffinized in xylene and alcohol. The antigens were retrieved by heating the specimens for 10 min at 121℃ in TE buffer (10 mMTris-HCl, 1 mM EDTA, pH 7.8), and the endogenous avidin binding sites in the urothelium were blocked with using 10% skim milk in TBST solution for 1 hr. Immunolabeling with anti-uroplakin III polyclonal antibody (Santa Cruz) at a 1:100 dilution was performed in 10% skim milk in TBST solution. The rabbit anti-goat immunoglobins HRP (Zymed, South San Francisco, CA, U.S.A.) at a 1:500 dilution was used for the secondary antibody. The peroxidase activity was demonstrated with 3,3′-diaminobenzidine. The sections were counterstained with Mayer haematoxylin, and then they were dehydrated and mounted.

RESULTS

A single dose of CP caused mucosal injury in the urothelium, and this was followed by a regenerative process. Significant increases in bladder wet weight were noted within 12 hr post injection, and this tended to decrease thereafter. When compared with normal bladder (Fig. 1A), the bladder revealed extensive cystitis that was characterized by acute inflammation with vascular congestion, edema and hemorrhage at 12 hr post injection (Fig. 1B). However, the submucosal edema was reduced at 24 hr (Fig. 1C) and the urothelium almost completely restored at 72 hr (Fig. 1D). A strong uroplakin III expression appeared along the bladder epithelium in the control bladder (Fig. 2A). At 12 hr after CP injection, there was a significantly decrease or loss of the uroplakin III expression in the intact bladder mucosa (Fig. 2B). However, this expression was weakly restored at 24 hr (Fig. 2C) and it was completely recovered at 72 hr (Fig. 2D). The messenger RNA expressions of all the uroplakins were signifi-

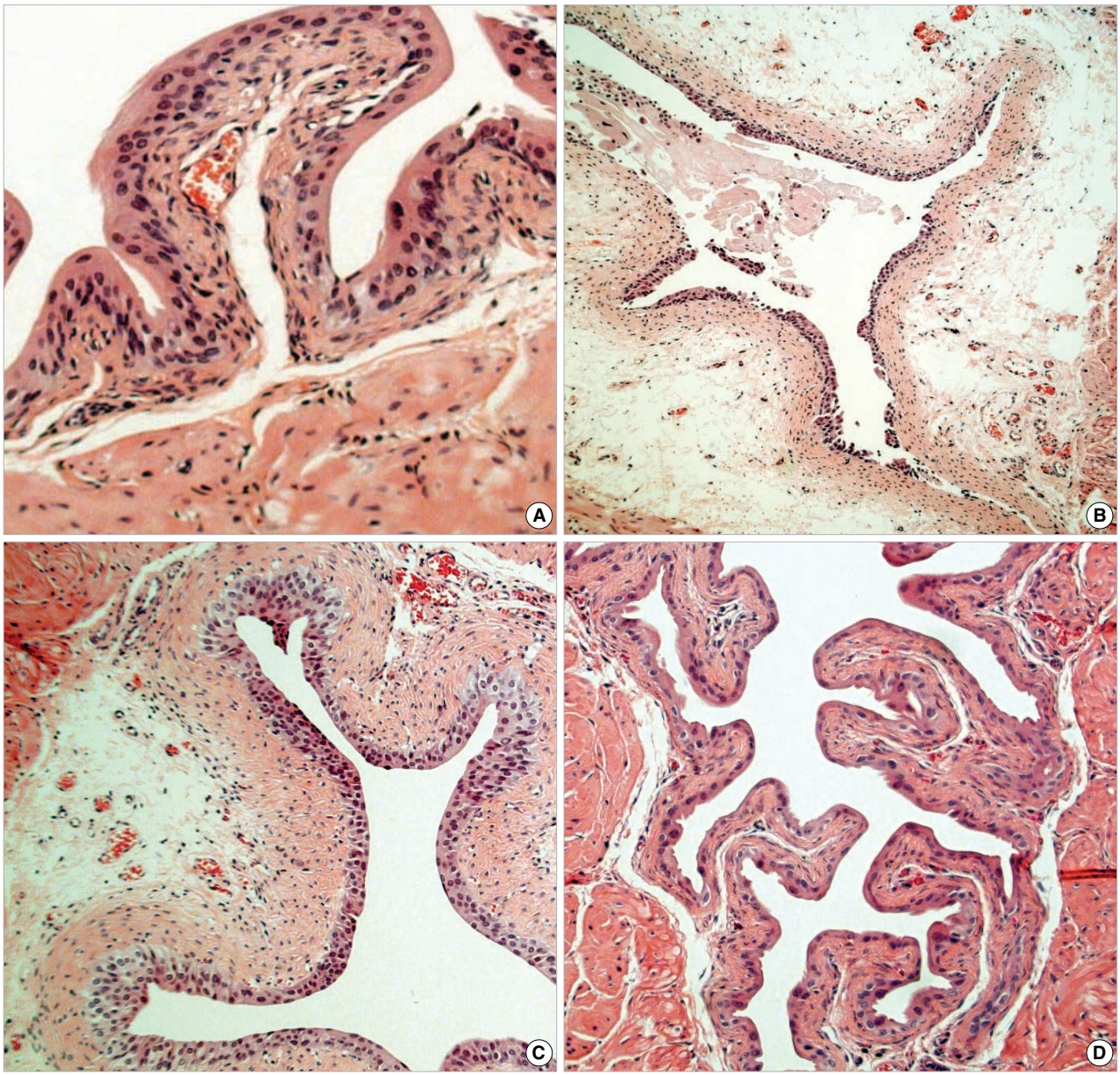

Fig. 1. Cyclophosphamide (CP) induced bladder histological alterations. (A) Normal epithelium projected to bladder lumen in controls. (B) Histological section of the urinary bladder of mice showed epithelial ulceration, hemorrhage, and submucosal edema at 12 hr post CP injection. (C, D) Cystitis improved at 24 and 72 hr, respectively. Hematoxylin and eosinstaining. Original magnifications (A) ×40 and (B-D) ×10.

cantly decreased after 12 hr and they completely recovered at 24 hr post CP injection. The protein expressions of uroplakin II and III were significantly decreased after 12 hr and they were restored 24 hr after CP injection (Fig. 3, 4).

DISCUSSION

It is well known that acrolein, which is one of the metabolites of CP in urine, contacts the urothelial mucosa and detaches the superficial cells. This may increase the bladder's permeability, permitting increased access of the constituents in the urine to the neurons and inflammatory cells in the mucosa or submucosa (6, 16). However, there have been very few studies that have focused on explaining the interaction between the toxic material and the bladder's barrier. Moreover, previous animal studies with using CP have mainly focused on the neurochemical and electrophysiological properties of the bladder detrusor muscle or the afferent neurons in the bladder wall (5, 17, 18).

The most characteristic feature of the terminally differentiated superficial cells of the bladder mucosa is their unique

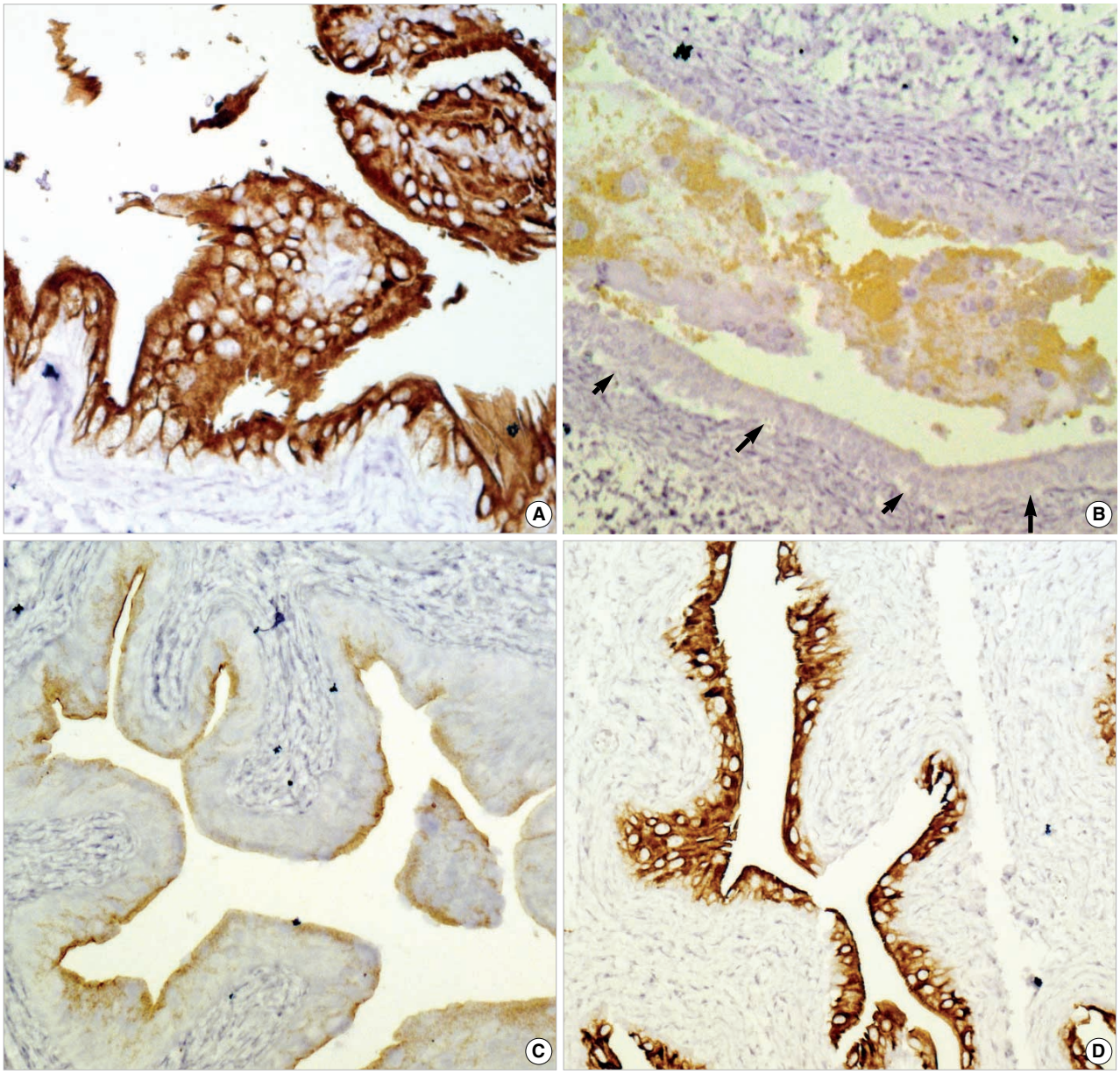

Fig. 2. Immunohistochemical reaction with anti-uroplakin III antibody. (A) A strong uroplakin III expression appeared along the bladder epithelium in control bladder. (B) At 12 hr after cyclophosphamide injection, there was a significantly decrease or loss of uroplakin III expression in intact bladder mucosa (arrows). (C, D) However, the expression was weakly restored at 24 hr and completely recovered at 72 hr. All original magnifications were x10.

apical plasma membrane, which is covered with rigid-looking UPs. Four UPs (UP Ia, UP Ib, UP II, and UP III) have been isolated and characterized, and they are considered to be biochemical markers of urothelial differentiation and they are biochemically unique (11, 12). Because these membranes are unusually stable in a number of harsh conditions including 2% NP-40, 2% sodium sarcosine, 25 mM NaOH, 9 M urea and 6 M guanidium chloride, these membranes are believed to serve as an exceptionally effective permeability barrier (11-13). As a result, we had a concern about the changes of the molecular expression of the UPs, which are an ideal urothelial protector, when they are insulted by CP.

Two mechanisms may be possible. First, the toxic metabolites contact to the uroepithelium and they mechanically destroy the epithelium. Toxic materials and water freely pass through the defect and accumulate into the submucosa. Second, CP directly suppresses the expression of UPs in the uroepithelium by unknown mechanisms, and this diminishes or weakens the barrier function of the urothelium and there is passive accumulation of water into the submucosal area.

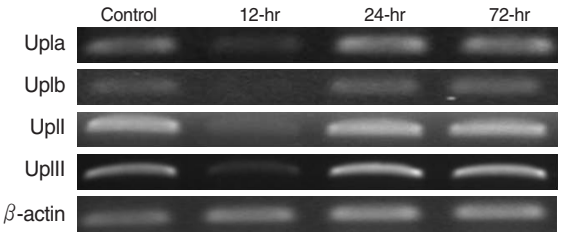

Fig. 3. Cyclophosphamide affected the mRNA expression of all uroplakins (UP). Note the mRNA expressions of all uroplakins were maximally decreased at 12 hr post injection, and restored after that. β-actin was used as an internal standard.

Fig. 4. Immunoblot analyses of uroplakin II and III. Cyclophosphamide reduced the expression of uroplakin II and III. Note the protein labelings of uroplakins were maximally decreased at 12 hr post CP injection, and tended to increase thereafter. As an internal standard, β-actin (Cell Signaling Technology, Danvers, MA, U.S.A.) was used.

Our finding was a little different from a previous study for which the maximal damage in the bladder mucosa was noted at 48 hr, and normal morphological restoration was noted within 7-10 days after CP injection (19). We think these differences may have been caused by using a different model or a different CP dosage.

Terminal differentiation in the apical plasma membrane takes place within the superficial layer of cells that are characterized by the presence of UPs. Since urothelial plaques and their subunits, i.e. the UPs, are synthesized in large quantities by only the terminally differentiated urothelial cells, the UPs can be regarded as a major urothelial differentiation product (12, 20). Fig. 2A shows that the expression of uroplakin III is uniquely distributed in a dense layer that's concentrated on the luminal surface of the umbrella cells in the urothelium. Interestingly, the protein expression of UP III was markedly reduced in the apical membrane within 12 hr after CP injection. However, the loss of UP III was time limited. It was partially recovered within 24 hr after CP treatment and it completely recovered at 72 hr. This finding matched with Veranic et al.'s study (20). They revealed that a generalized denuded urothelium was found after CP injection. However, focal intact microvilli were negative to anti-UP antibodies on their apical surfaces at day 1 after CP injection. We also can found the same finding on Fig. 2B. Even though a large area of urothelium was gone with CP injection, some focal intact uroepithelium showed a significantly loss of uroplakin III expression. With above findings we can confirm the hypothesis that CP may actively depress the expression of UPs in uroepithelium when are damaged by CP.

From days 1-4 days, epithelial cells with weak anti-UP labeling were seen and this phenomenon was not limited to a single UP, but all UPs were simultaneously reduced on the electron microscope images (20). Even though we used only one antibody, UP III, we believe that immunohistochemical staining with UP Ia, Ib, and II antibodies may reveal the same findings. The morphologic and immunohistochemical studies were also supported by the mRNA expression study and the western blotting study. The mRNA expressions of all the UPs were maximally reduced at 12 hr after CP injection, and these expressions were recovered at 24 hr. We tried using all the UPs antibodies. It was very difficult to get good bands from using the anti-UP Ia and Ib antibodies. We estimated this finding may be due to a structural uniqueness, the tetraspanin family, in UP Ia and Ib. The protein levels of UP II and III were maximally decreased at 12 hr after CP injection, and they were restored thereafter.

The ablation of UP III led to abnormal synthesis and processing of UP Ib, i.e., the level of UP Ib mRNA was greatly increased, whereas the amount of UP Ib protein was reduced. Because UP III and UP Ib are known to interact, these UP Ib changes were caused by the removal of its partner, UP III (13, 21). The UP II ablation led to an up-regulation of the UP Ib mRNA level (21). However, our results showed that the expressions of all the UPs were simultaneously reduced at the mRNA and protein levels, which mean a different mechanism may be involved.

A number of conditions lead to disruption of the bladder permeability barrier, with leakage of urine constituents into the underlying cell layers. These include bacterial infection, exposure to noxious chemicals and the dysplasia of tumor growth (22, 23). In addition, interstitial cystitis is a chronic painful condition of an unknown cause, and it is associated with disruption of the permeability barrier in cats (24). Our result may be helpful for understanding the injury and repair mechanisms of the apical membrane. This may lead to important insights for the care of patients with interstitial cystitis, and this may also shed light on developing new drugs for ameliorating the urotoxicity after cyclophosphamide-based chemotherapy.

In conclusion, CP induces actively down regulation of all the UP genes, and this damages the urothelial protective barrier in the early injured phase. However, the damaged urothelium is rapidly recovered within 24 hr post injection. That means rapid resealing of injuries to the bladder permeability barrier is of major physiological importance to restore the bladder's protection.

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