Fucoidan elevates surface organic cation transporter 2 expression via upregulation of protein kinase A in uric acid nephropathy

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Abstract. Uric acid nephropathy (UAN) is caused by excessive uric acid, and is a key risk factor for uric acid nephrolithiasis, gouty arthritis, renal diseases and cardiovascular diseases. The present study aimed to evaluate the protective effect of fucoidan, a sulfated polysaccharide component of brown algae, on UAN and to elucidate the underlying molecular mechanism. A rat model of UAN was induced by adenine treatment, and rats were then randomly assigned to control, model or fucoidan treatment groups. Hematoxylin and eosin staining of the kidney tissues of rats with UAN was subjected to conventional morphological evaluation. Cellular infiltrate in the tubules, atrophic glomeruli, tubular ectasia, granuloma hyperplasia focal fibrosis and accumulated urate crystals in the tubules of UAN rat renal tissues were observed. These symptoms of kidney damage were reduced in the fucoidan treatment group. Periodic acid methenamine silver-Masson staining was performed and the results indicated that renal interstitial fibrosis was reduced among renal tissues from the fucoidan treatment group compared with the model group. Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling staining revealed a lower proportion of apoptotic nuclei in the kidneys of the fucoidan treatment group compared with the model group. Protein kinase A (PKA) 2β and phosphorylated PKA 2β protein levels were significantly elevated in renal tissues of the fucoidan treatment group compared with the model group ($P<0.05$ and $P<0.01$, respectively), suggesting that PKA expression was upregulated by fucoidan. Immunohistochemistry staining of PKA in rat renal tissues demonstrated increased expression of PKA. The surface organic cation transporter 2 (OCT2) level was significantly increased by fucoidan treatment compared with the model group ($P<0.01$), with no significant change in total OCT2 level. COS-7 cells ectopically expressing OCT2 were established. It was indicated that fucoidan was able to activate PKA and upregulate surface OCT2 in OCT2-expressing COS-7 cells. This further demonstrated that upregulation of surface OCT2 expression in OCT2-expressing cells was induced by PKA upregulation. In conclusion, fucoidan upregulated surface OCT2 expression in renal tissues to alleviate the symptoms of UAN via upregulated expression of PKA.

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Introduction

Fucoidan is a sulfated polysaccharide extracted from marine brown algae and some echinoderms that has various beneficial health effects (1-3). There is a growing interest among producers and consumers in using new functional ingredients, such as fucoidan, in the diet (1). Fucoidan has been extensively studied due to it having multiple biological activities and potential applications, including anticancer, antiviral, anti-metastasis, anti-lymphangiogenesis, immune modulation, blood anticoagulant, protection from radiation damage, tissue engineering and pathogen inhibition effects (2-6).

Fucoidan may also have beneficial effects on kidney function. Previous studies have reported that fucoidan may reduce the metabolic abnormalities of diabetic rats and delay the progression of diabetic renal complications in a rat model of streptozoin-induced diabetes (7). Fucoidan has also been demonstrated to have a renoprotective role in oxalate-mediated peroxidative injury (8). Furthermore, previous studies have reported that fucoidan may alleviate the symptoms of

chronic kidney disease in a model of subtotal nephrectomy chronic kidney disease and have a renoprotective effect on active Heymann nephritis (9,10).

Although fucoidan has a protective effect against uric acid nephropathy (UAN) (11), little is known about the underlying molecular mechanism of this. In the present study, a rat model of UAN was established using adenine in order to further investigate the renoprotective role of fucoidan and its molecular mechanism. Using hematoxylin and eosin (H&E) and periodic acid-methenamine silver (PAM)-Masson staining, it was indicated that fucoidan was able to alleviate symptoms of UAN in a rat model.

Organic cation transporter 2 (OCT2) is expressed predominantly in the kidney, at the basolateral membrane of S2 and S3 segments of the proximal tubule (12,13). OCT2 is a major transporter of various organic cations from the blood stream into renal epithelial cells and is important for the cellular uptake of cationic drugs across the basolateral membrane of proximal tubule cells, including metformin, lamivudine and cimetidine (14). OCT2 is regulated by protein kinase A (PKA), phosphoionositide 3-kinase, p56lck tyrosine kinase and mitogen-activated protein kinase kinase 1 and 2 (15). To gain insight into the possible roles of PKA and OCT2 in the protective effect of fucoidan, PKA 2β, phosphorylated (p)-PKA 2β, surface OCT2 and total OCT2 protein levels were evaluated in renal tissues of a rat model of UAN in the present study. The results indicated that PKA 2β and p-PKA 2β levels were significantly elevated in renal tissues of the fucoidan treatment group compared with the UAN model group, suggesting that PKA was activated in the kidney tissue of fucoidan treatment group rats. Notably, the surface OCT2 level was markedly increased by fucoidan treatment with no change in total OCT2 level.

To further ascertain the role of fucoidan in regulating PKA and surface OCT2 expression, COS-7 cells ectopically expressing OCT2 were established. The level of PKA 2β and p-PKA 2β and surface OCT2 was significantly increased by treatment with fucoidan compared with the control in OCT2-expressing COS-7 cells. These observations indicated that fucoidan may activate PKA and upregulate surface OCT2 in OCT2-expressing COS-7 cells. Furthermore, it was confirmed that activation of PKA with 8-bromoadenosine 3',5'-cyclic monophosphate (8-bromo-cAMP) upregulated surface OCT2 in OCT2-expressing COS-7 cells. In summary, fucoidan upregulated surface OCT2 via upregulating the expression of PKA in order to alleviate the symptoms of UAN.

Materials and methods

Rat model of UAN and experimental procedures. A total of 30 male Sprague Dawley rats (weight, 167.2±1.9 g; age, 6-8 weeks), were randomly assigned into three experimental groups. The rats were housed in temperature-(22-25˚C) and humidity-(40-70%) controlled conditions with a 12-h light/dark cycle and *ad libitum* access to food and water. All rats were treated by intragastric administration. Group 2 and 3 rats were administered with 30 mg/kg adenine once a day for 18 days to establish a rat model of UAN. Group 1 was allocated as the normal control group and rats werefed an equivalent amount of carboxymethylcellulose sodium (CMC-Na; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Rats in Group 2 were then fed with an equivalent amount of saline buffer (30 mg/kg body weight) for 18 days to establish the disease model. Rats in Group 3 were fed with a single dose of fucoidan polysaccharide sulfate (400 mg/kg body weight) to establish the fucoidan treatment group.

Rats were obtained from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). The research was carried out in accordance with the Guidelines for Human Treatment of Animals set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Sun Yat-sen University. The study was approved by the Committee of Biomedical Ethics of Sun Yat-sen University [SYXK (yue): 2007-0081].

Chemicals and reagents. Fucoidan polysaccharide sulfate was diluted with 10% dimethyl sulfoxide in ethanol to a final concentration of 50 mg/ml. Fucoidan was purchased from South Product Co., Ltd. (Uruma, Japan). Adenine tablets were purchased from Amresco, LLC (Solon, OH, USA) and diluted with 0.15% CMC-Nato a final concentration of 3%. Cell Surface Protein Isolation kit (K295) was purchased from BioVision, Inc., (Milpitas, CA, USA). Sulfo-NHS-SS-biotin and Avidin Agarose beads were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). COS-7 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin and 100 U/ml streptomycin (Amresco, LLC). The cells were cultured in a 5% CO_2 atmosphere at 37˚C. 8-bromo-cAMP (Sigma-Aldrich; Merck KGaA) was used asaPKA activator. OCT2-expressing COS-7 cells were treated with PKA activator 8-bromo-cAMP (1 μmol/l) or phosphate-buffered saline (PBS) for 12 h at 37˚C.

Tissue surface protein extraction/cell surface biotinylation and western blot analysis. After treatment for 18 days, rats were euthanized by CO_2 inhalation, and the renal tissues from rats in different groups were frozen and stored at -20˚C immediately after dissection. Renal tissues were mechanically dissociated and homogenized according to the manufacturer's instructions for the surface protein extraction kit. For COS-7 cell surface biotinylation, after treatment with fucoidan (500 μg/ml) or control salinefor 24 h, cells were washed and incubated with PBS supplemented with 0.5 mg/ml sulfo-NHS-SS biotin for 40 min at 4˚C, and excess biotin was quenched with 50 mM Tris-PBS buffer for 20 min at 4˚C. Subsequently, cells were collected, lysed in radioimmunoprecipitation buffer from BioVision, Inc., and subjected to streptavidin-agarose beads at 4˚C for a further 3 h. The protein samples (30 μg) obtained were boiled, subjected to 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered saline-Tween-20 containing 2% bovine serum albumin (Sigma-Aldrich; Merck KGaA) at room temperature for 2 h, and the membranes were then incubated with specific antibodies against OCT2 (sc-233; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:1,500), PKA 2β (sc-365615; Santa Cruz Biotechnology, Inc; 1:1,500), p-PKA 2β (sc-293036; Santa Cruz Biotechnology, Inc.;

1:1,000), GAPDH (ab8245; Abcam, Cambridge, UK; 1:8,000). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (20162; ProMab Biotechnologies, Inc., Richmond, CA, USA; 1:1,000) were used. For incubation, all antibodies were incubated at 4˚C for 12 h. The membranes were washed inTBST (Sigma-Aldrich; Merck KGaA) at room temperature four times between the incubations with the primary and secondary antibodies. Protein bands were detected using an enhanced chemiluminescence reaction (Biological Industries, Kibbutz Beit Haemek, Israel). The intensity of each band was quantified using Quantity One software v4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were repeated three times.

H&E staining of renal tissues. Renal tissues of rats were removed, fixed with 4% paraformaldehyde in PBS at room temperature for 2 h, decalcified for 10 days at room temperature with EDTA and embedded in paraffin for histological analysis. Kidney sections (5 μm thick) were stained with hematoxylin for 2 min, then eosin for 30 sec. Images of the kidney from multiple sections in each rat were observed under a light microscope with identical parameters (magnification, resolution and light intensity).

PAM-Masson staining. In brief, rat kidneys were harvested, cut along the short axis at the maximum area of the whole kidney, fixed in Carnoy's solution (Beyotime Institute of Biotechnology, Haimen, China) at 37˚C for 2 h, embedded in paraffin, sectioned (1 μm thick), and stained with PAM. Images of the kidney from multiple sections in each rat were observed under a light microscope with identical parameters (magnification, resolution and light intensity).

Immunohistochemistry assay for PKA. Immunohistochemistry was performed by National Engineering Center for Biochip at Shanghai (China), as described previously (16,17). Immunohistochemistry was carried out with antibodies specific for PKA using anti-rat PKA (sc-365615; Santa Cruz Biotechnology, Inc.; 1:300) in blocking buffer and incubated overnight at 4˚C. Negative control sections were incubated with PBS instead of primary antibodies.

OCT2-expressing plasmid construction and transfection. Green fluorescent protein (GFP)-OCT2 and GFP-C (control) vector plasmids were purchased from OriGene Technologies, Inc., (Rockville, MD, USA) and transfected into COS-7 cells using a Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) reagent, according to the manufacturer's protocol.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) assay. Frozen renal tissue sections (3 μm thick) were cut and mounted on 3-aminopropyltriethoxysilane-treated glass slides. The TUNEL protocol was performed using the *In Situ* Cell Death Detection kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer's instructions. Sections were dried overnight, fixed in 1% formal dehydeat room temperature for 2 h, washed with PBS four times for 5 min each, permeabilized with 0.2% Triton X-100 at 4˚C for 10 min, and re-washed with PBS before application of TUNEL reagents. Images were observed under a light microscope with identical parameters (magnification, resolution and light intensity).

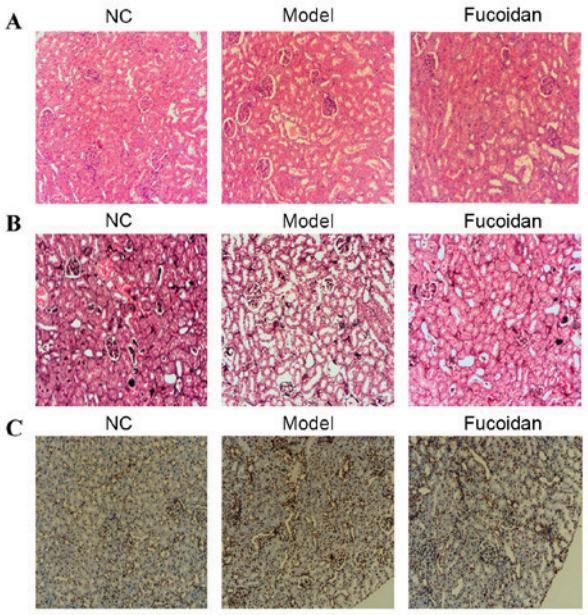

Figure 1. Fucoidan alleviates symptoms of UAN. (A) Hematoxylin and eosin staining of renal tissue from NC rats and rats with UAN. Magnification, x400. (B) Periodic acid methenamine silver-Masson staining of renal tissue from NC rats and rats with UAN. Magnification, x400. (C) Apoptotic cells in renal tissues from NC rats and rats with UAN, visualized using a TUNEL assay. NC, normal control; UAN, uric acid nephropathy. Magnification, x200.

Statistical analysis. All data were presented as the mean ± standard error of the mean of at least three independent experiments. Student's t-test was used to evaluate differences between two groups with GraphPad Prism v5.01 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant result.

Results

Fucoidan alleviates the symptoms of UAN in a rat model of UAN. An experimental model of UAN was induced using uricopoiesis promoter adenine. Histopathological analysis of renal tissues in each group was performed. H&E staining of kidney tissue indicated that the control group maintained normal glomerular size, integrity and demonstrated no notable inflammatory response (Fig. 1A). In the UAN model group, cellular infiltrate in the tubules, atrophic glomeruli, tubular ectasia, granuloma hyperplasia focal fibrosis and accumulated urate crystals were observed. However, treatment with fucoidan decreased the deposition of urate crystals and inflammatory cellular influx in the tubule compared with the model group. Furthermore, tubular ectasia and interstitial fibrosis were improved by fucoidan treatment, suggesting that kidney damage was attenuated by fucoidan. The renal tissues were also processed and stained with PAM-Masson for examination under a light microscope. The PAM-Masson results revealed that renal interstitial fibrosis was less prominent among renal tissues from the fucoidan treatment group compared with the model group (Fig. 1B). In addition, the fucoidan treatment group demonstrated a lower proportion

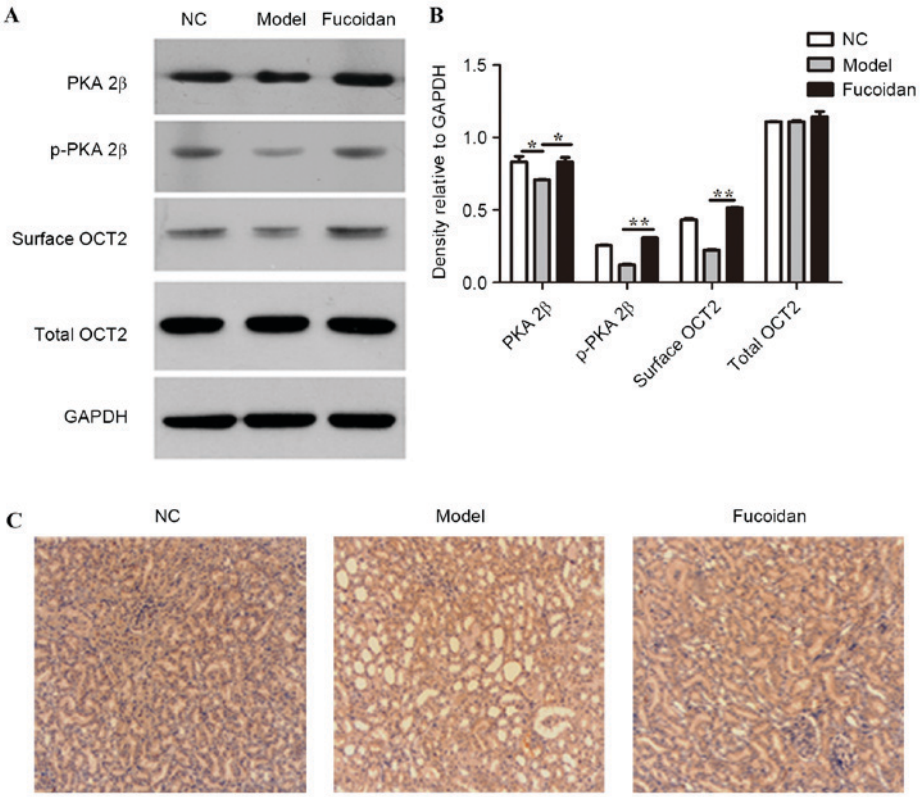

Figure 2. Fucoidan activates PKA and upregulates surface OCT2 in the renal tissue of a rat model of UAN. (A) Western blot analysis of the PKA 2β, p-PKA 2β, surface OCT2 and total OCT2 protein level in renal tissues. GAPDH was used as a loading control. (B) Densitometry plot of results from western blot analysis. Relative expression levels were normalized to GAPDH. Data are presented as the mean ± standard error of the mean (n=3). (C) Immunohistochemistry analysis of PKA expression in kidney tissues of rats with UAN. Magnification, x400. *P<0.05; **P<0.01. PKA, protein kinase A; NC, normal control; UAN, uric acid nephropathy; OCT2, organic cation transporter 2; p, phosphorylated.

of apoptotic nuclei in the kidney compared with the model group, as assessed by TUNEL staining (Fig. 1C). These results suggested that fucoidan was able to alleviate the symptoms of UAN in a rat model.

Fucoidan activates PKA and upregulates surface OCT2 in renal tissues of a rat model of UAN. Given that fucoidan alleviated the symptoms of UAN, further study to investigate the specific molecular mechanism was required. Thus, the protein levels of PKA 2β, p-PKA 2β, surface OCT2 and total OCT2 were evaluated in renal tissues of the different groups. As demonstrated in Fig. 2A and B, PKA 2β and p-PKA 2β protein expression levels were significantly elevated in the renal tissues of the fucoidan treatment group compared with the model group (P<0.05 and P<0.01, respectively), suggesting that increased expression of PKA occurred in kidney tissue of fucoidan treatment group rats. The upregulation of PKA protein expression was further demonstrated by immunohistochemistry staining of PKA in rat renal tissues (Fig. 2C). The level of surface OCT2 protein was significantly increased by fucoidan treatment compared with the model group (P<0.01), with no significant change in total OCT2 level (Fig. 2A and B).

Surface OCT2 is upregulated with PKA upregulation by fucoidan in OCT2-expressing COS-7 cells. To further ascertain the role of fucoidan in regulating PKA and surface OCT2 expression, COS-7 cells ectopically expressing OCT2 were established. COS-7 cells ectopically expressing OCT2 were treated with fucoidan (500 μg/ml) or PBS for 24 h. A significant increase in the proportion of surface OCT2 protein was induced by fucoidan treatment compared with the control (P<0.05; Fig. 3), with no significant difference in total OCT2 level between the two groups. A significantly elevated PKA 2β and p-PKA 2β level was observed in OCT2-expressing COS-7 cells treated with fucoidan compared with the control (both P<0.05; Fig. 3). These results demonstrated that fucoidan may increase the expression of PKA and upregulate surface OCT2 in OCT2-expressing COS-7 cells.

PKA upregulates surface OCT2 in OCT-2 expressing COS-7 cells. Previous studies have reported that PKA regulates OCT2-mediated organic cation transport (18,19). The increased expression of PKA by forskolin leads to stimulation of 4-[4-(dimethylamino) styryl]-*N*-methylpyridinium uptake in IHKE-1 cells (20). To study the relationship between PKA expression and surface OCT2 upregulation in COS-7 cells, surface, intracellular and total OCT2 protein levels were evaluated in OCT2-expressing COS-7 cells treated with PKA activator 8-bromo-cAMP. OCT2-expressing COS-7 cells were treated with PKA activator, 8-bromo-cAMP (1 μmol/l) or PBS. As demonstrated in Fig. 4, in the 8-bromo-cAMP-treated COS-7 cells, the surface OCT2 protein level was increased significantly compared with the control (P<0.05), while intracellular OCT2 level decreased significantly (P<0.01), with no significant change in total OCT2. The findings presented in the present study indicate that fucoidan may upregulate surface OCT2 via PKA to alleviate the symptoms of UAN.

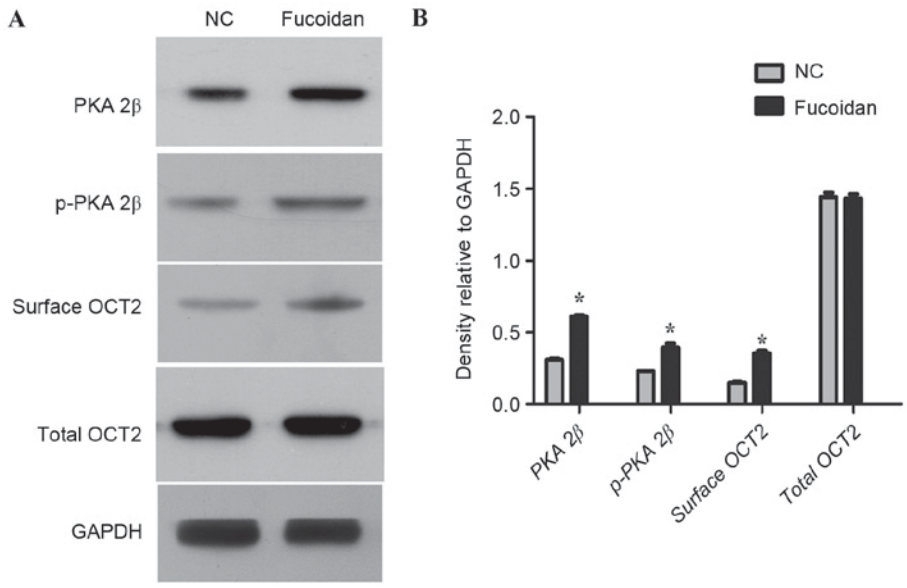

Figure 3. Surface OCT2 is upregulated by fucoidan in OCT2-expressing COS-7 cells. OCT2-expressing COS-7 cells were treated with fucoidan (500 μg/ml) or phosphate-buffered saline (NC) for 24 h. (A) Western blot analysis was performed to evaluate the protein expression of PKA 2β, p-PKA 2β, total OCT2 and surface OCT2. (B) Densitometry plot of results from western blot analysis. Relative expression levels were normalized to GAPDH. Data are expressed as the mean ± standard error of the mean (n=3). *P<0.05 vs. NC. PKA, protein kinase A; NC, normal control; OCT2, organic cation transporter 2; p, phosphorylated.

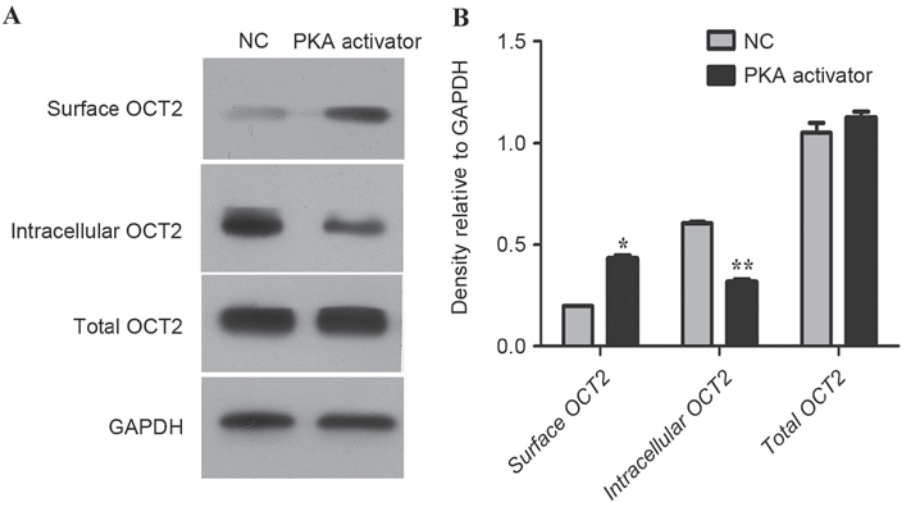

Figure 4. PKA upregulates surface OCT2 in OCT2-expressing COS-7 cells. OCT2-expressing COS-7 cells were treated with PKA activator, 8-bromoadenosine 3',5'-cyclic monophosphate (1 μmol/l) or phosphate-buffered saline (NC) for 12 h. (A) Western blot analysis was performed to evaluate the protein expression of surface OCT2, total OCT2 and intracellular OCT2. (B) Densitometry plot of results from western blot analysis. Relative expression levels were normalized to GAPDH. Data are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01 vs. NC. PKA, protein kinase A; NC, normal control; OCT2, organic cation transporter 2.

Discussion

UAN is one of the most common and extensive metabolic diseases worldwide, and is a key risk factor for the development of uric acid nephrolithiasis, gouty arthritis, renal diseases and cardiovascular diseases, particularly hypertension (21-23). The typical characteristic of UAN is high serum uric acid level, resulting in the accumulation of urate crystals in the joints and kidneys (24,25). Due to the fact that acid is the insoluble, circulating end product of purine nucleotide metabolism in humans, the urate crystals deposit in the collecting duct (23,26). This induces acute kidney injury, resulting in decreased filtering capacity of the glomerulus and renal blood flow, toxic or obstructive injury to the renal tubule, edema and tubular interstitial inflammation (26).

The multiple biological activities of fucoidan have been widely studied. Its anticancer properties have been indicated by its ability to induce apoptosis and macrophage-induced tumor cell death, block the interactions between cancer cells and the basement membrane and inhibit angiogenesis by interfering with the binding of vascular endothelial growth factor and basic fibroblast growth factor (6,27). The anticoagulant activity of fucoidan from *Fucus evanescens* was revealed *in vitro* and *in vivo* (5). In addition, fucoidan has been indicated to have a potent antiviral effect against herpes simplex virus types 1 and 2, as well as human cytomegalovirus (28). The gastroprotective

effect of fucoidan against aspirin-inducedulceration in rats demonstrated the immunomodulatory effect of fucoidan (29). Fucoidan also suppresses hypoxia-induced lymphangiogenesis and lymphatic metastasis in murine hepatocarcinoma by suppressing hypoxia-inducible factor-1α/vascular endothelial growth factor C (30).

Consistent with previous studies, the results of the present study indicated that fucoidan was able to alleviate the symptoms of UAN in a rat model. H&E staining revealed cellular infiltrate in the tubules, atrophic glomeruli, tubular ectasia, granuloma hyperplasia focal fibrosis and accumulated urate crystals in the tubules of renal tissues from rats with UAN, suggesting that the rat model of UAN was established successfully. The deposition of urate crystals and inflammatory cellular influx in the tubules were decreased by fucoidan administration. The proportion of kidney apoptosis was decreased by fucoidan treatment compared with the model group, as evaluated by a TUNEL staining assay. The protein levels of PKA 2β, p-PKA 2β, surface OCT2 and total OCT2 were evaluated in renal tissues. It was indicated that both the PKA 2β and p-PKA 2β level were significantly elevated in renal tissues of the fucoidan treatment group compared with the UAN model group. These results suggested that increased expression of PKA occurred in the kidney tissue of fucoidan treatment group rats. The upregulated protein level of PKA was further demonstrated by immunohistochemical staining of PKA in rat renal tissues. Furthermore, the surface OCT2 level was significantly increased by fucoidan treatment compared with the model group, with no significant change in total OCT2 level.

OCT2 is critical for organic cation transport, absorption and excretion of endogenous and exogenous cationic substances and urinary excretion (31-33). COS-7 cells ectopically expressing OCT2 were established in the present study to further evaluate the role of fucoidan in regulating PKA expression and surface OCT2 expression. It was indicated that fucoidan was able to increase the expression of PKA and upregulate surface OCT2 in OCT2-expressing COS-7 cells. The relationship between PKA and surface OCT2 upregulation in COS-7 cells was also investigated. The increased expression of PKA induced by 8-bromo-cAMP increased surface OCT2 and decreased intracellular OCT2 protein levels compared with the control, with no significant change in total OCT2. Consistent with previous studies, the upregulation of PKA stimulated OCT2 activity (18,20).

Previous studies have reported that PKA regulates OCT2 activity in both a heterologous cell system and intact renal proximal tubules (18-20). The activation of PKA by forskolin stimulated rabbit OCT2 activity, while inhibition of PKA (by H-89) reduced transport activity of rabbit OCT2 in CHO-K1 cells (20). However, human OCT2 was reported to be inhibited by PKA activation and activated by a calmodulin-dependent signaling pathway in human embryonic kidney HEK-293 cells (34).

In conclusion, the present study identified that fucoidan upregulated surface OCT2 expression in renal tissues. This suggests that fucoidan has a protective effect against UAN may have a potential use in treating UAN. Further in-depth study of the mechanism by which fucoidan regulates human OCT2 expression and PKA activation is required.

Acknowledgements

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