Water Intake Restriction Induces Primary Cilia Shortening and Concentrated Urine Formation via Hdac6 Activation in Mice

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Research

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Abstract

**Background:** The primary cilium, a microtubule-based cellular organelle, acts as a mechano-sensor for monitoring the fluid flow in cells. In kidneys, the primary cilia protrude into the tubular lumen from the tubular cells and therefore, directly contact pro-urine flow and components. However, it remains to be defined how the cilia are associated with kidney function and diseases. Here, we investigated whether water access restriction affects the cilia length in the renal tubular cells, whether cilia length changes are associated with kidney functions, and how cilia lengths are regulated.

**Methods:** C57BL/6 mice were provided free access to water (control), but water supply was stopped for some mice for 24 to 48 h (water restriction). Among each group, some mice were administered with tubastatin A (10 mg/kg BW), a specific inhibitor of histone deacetylase 6 (HDAC6), daily from 2 days before water restriction. Cultured tubular epithelial cells were treated with either 10 or 20 mM NaCl or 20 mM mannitol with or without tubastatin A. Primary cilia were determined by immunofluorescence staining using acetylated-α-tubulin antibody or scanning electron microscope.

**Results:** Water restriction shortened the primary cilia of kidney tubular epithelial cells along with increasing urine osmolality. Water restriction increased the activity of HDAC6 with increased the deacetylation of α-tubulin, a substrate of HDAC6 and a major comprising protein of microtubule of primary cilia. HDAC6 inhibitor blocked water restriction-induced primary cilia shortening along with the inhibition of α-tubulin deacetylation. In addition, HDAC6 inhibitor blocked the increase in water restriction-induced urine osmolality. Increases of NaCl or mannitol concentration in the medium for Madin-Darby canine kidney tubule cell culture shortened the cilia length and increased HDAC6 activity and α-tubulin deacetylation. HDAC6 inhibitor blocked those NaCl and mannitol-induced effects.

**Conclusions:** Our data have demonstrated that water restriction shortened the primary cilia of kidney tubular cells via HDAC6 activation and α-tubulin deacetylation along with increasing urine osmolality, suggesting that the alteration of primary cilia length is an adaptive response to the water intake to maintain body water balance and that the primary cilia length regulation may be a therapeutic strategy of kidney diseases related to the body water and electrolyte imbalances.

**Introduction**

Primary cilium is a non-motile antenna-like cellular organelle that acts as a mechano- and chemo-sensing organelle that maintains cell functions. The core of the primary cilium consists of microtubules with 9 + 0 configuration that is anchored to the basal body. The length of the primary cilium dynamically alters under physiological and pathological conditions. This alteration includes both elongation and shortening, based on the assembly and disassembly of tubulins that comprise microtubules [1], and proteins docking and incorporating to the ciliary membranes. Recent findings have demonstrated that alteration in the primary cilium length is linked to the function of cells and that the aberrant function and structure of
primary cilia are associated with several diseases, such as obesity, hypertension, diabetes, and kidney cystic diseases [2–4].

In the kidneys, the primary cilia project to the tubular lumens [5, 6]. Therefore, the primary cilia directly contact to the kidney pro-urine flow, electrolytes, and osmolality, all of which change considerably under physiological and pathophysiological conditions [7]. Recent reports have shown the localization of transient receptor potential melastatin-3, an osmosensitive transient receptor potential channel, or transient receptor potential vaniloid 4, an osmosensitive Ca$^{2+}$ channel, on the primary ciliary membrane of kidney tubular epithelial cells and bile duct cholangiocytes [7] and that the defect of primary cilia in cells causes the loss of flow sensing ability and osmotic stress response [7]. We have also recently reported that in mice, the alterations in the primary cilia length occurred in the high-burdened contralateral kidneys after unilateral ureteral obstruction and unilateral nephrectomy, in injured kidneys after kidney ischemia/reperfusion and hepatic ischemia/reperfusion, and in fibrotic kidneys after recovery from damage [3, 4, 8]. In humans, Vergesse et al. reported that the primary cilia lengths of tubular epithelial cells in allografted kidneys are dynamically changed in correlation with graft kidney functional changes [2]. In a similar manner, studies have demonstrated that the lengths of the primary cilia can be diversely modified during kidney diseases that induce the perturbation of the regulatory ability of pro-urine flow and osmolality. Thus, the primary cilia may play important roles in the maintenance of kidney function, and abnormal function of the primary cilia due to aberrant structure and abnormal cilia-related signaling are associated with the pathogenesis of various kidney diseases. However, the role of the primary cilia on kidney functions and how primary cilia respond to intra-kidney environments in terms of filtrated fluid flow, osmolality, and components, remain largely unknown. Therefore, we investigated whether water restriction in mice and the medium osmolality change in established kidney tubular epithelial cells affect the primary cilia length; and if so, what are their underlying mechanisms and the role of primary cilia length change in the kidney function. Here, we report that water restriction shortens the length of the primary cilia, this shortening is linked to the urine-concentrating function of the kidneys, and that histone deacetylase 6 (HDAC6) activation is associated with this shortening.

Results

Water restriction shortens the primary cilia of the kidney tubule cells. To investigate how primary cilia respond to intra-kidney environments in terms of filtrated fluid flow, osmolality, and components, first, we determined the primary cilia lengths of kidney tubular epithelial cells in mice which were restricted to access to drinking water for 24 or 48 h. Water restriction dramatically shortened the lengths of the primary cilia in the kidney tubular epithelial cells; after water restriction, the primary cilia lengths decreased in all uriniferous tubular epithelial cells, with an exception in the intercalated cells that do not possess primary cilium; these primary cilia lengths were greater in order in PT, DT, and CD cells (Fig. 1) [9, 10]. These water restriction-induced shortenings of the primary cilia were augmented, depending on the period of water restriction (Fig. 1). When the primary cilia were observed under scanning electron
microscopy (SEM), in consistence with IF-staining data, water restriction reduced the primary cilia lengths in the distal tubular cells and the principal cells of the collecting ducts (Fig. 2).

Next, to evaluate kidney functions such as urine concentration, we determined the urine and plasma osmolality. Water restriction gradually increased urine osmolality overtime, without changing the plasma osmolality and hematocrit (Table 1). These results indicate that water restriction shortens the length of the primary cilia of the kidney tubular epithelial cells, showing a positive correlation with increased urine osmolality.

**Water restriction-induced shortening of primary cilia is not due to cell proliferation and primary cilia disruption.** Shortening of the primary cilia occurs via both resorption into the cell body by cell cycle entry and deciliation [11, 12, 4, 13]. Therefore, we determined that water restriction-induced shortenings of the primary cilia are associated with cell proliferation or deciliation. When cell proliferations were evaluated by BrdU-incorporated cell numbers and proliferative cell nuclear antigen (PCNA) and p21 (a cyclin-dependent kinase inhibitor 1) expression levels [13, 14]. Water restriction did not increase the number of BrdU-positive cells (Fig. 3A and B) and PCNA expressions in the kidneys as compared to that in the water-supplied kidneys (Fig. 3C and D), whereas it increased p21 expression (Fig. 3C and E).

Since detached or fragmented primary cilia from the tubular cells directly releases into urine through tubular lumens [13], to define that primary cilia shortening is associated with deciliation, we determined a primary ciliary protein, acetylated-α-tubulin (ac-α-tubulin, a major component of microtubules which are the core structures of primary cilia) in the urine. Water restriction did not increase the level of ac-α-tubulin in the urine; the last lane in Fig. 3F represents positive control urine collected from the kidneys with ischemia/reperfusion injury (Fig. 3F). These results indicate that water restriction-induced shortening of the primary cilia is not associated with, at least in part, cell mitosis and deciliation, suggesting that the shortening of primary cilia is associated with cell differentiating processes that are induced in response to water restriction.

**Water restriction deacetylates α-tubulin along with the increase of HDAC6 activity in the kidney.** The disassembly of the microtubule is associated with the shortening of the primary cilium; this microtubule disassembly is regulated by the deacetylation of α-tubulins [15, 16]. Therefore, we determined the levels of ac-α-tubulin. First, we determined the levels of α-tubulin in the kidneys. Water restriction significantly reduced the levels of ac-α-tubulin in the kidney, without causing significant changes in α-tubulin (Fig. 4A-D). These results indicate that water restriction causes α-tubulin deacetylation. Next, we determined the activity and expression of HDAC6 in the kidneys; α-tubulin is a major cytosolic substrate of HDAC6 [14, 17]. HDAC6 activity was significantly increased in the kidneys by water restriction (Fig. 4E). HDAC6 mRNA expression was also significantly higher in the kidneys by water restriction when compared with control (Fig. 4F). These results indicate that water restriction-induced primary cilia shortening may be associated with increased HDAC6 activity, leading to α-tubulin deacetylation and subsequent disassembly of the microtubule.
**Tubastatin A, an inhibitor of HDAC6, blocks water restriction-induced primary cilia shortening, HDAC6 activation, and urine osmolality decrease.** To test whether HDAC6 inhibition blocks the shortening of the primary cilia, tubastatin A, a specific inhibitor of HDAC6, was administered to the mice 2 d before water restriction daily. As shown in Fig. 5, HDAC6 inhibitor almost completely blocked primary cilia shortening induced by water restriction in all the kidney tubulars cells (Fig. 5A-D). Tubastatin A treatment blocked the water restriction-induced decrease of acetylated α-tubulin expression in kidneys without any changes in α-tubulin expression (Fig. 6A-D). Moreover, tubastatin A prevented the increases in the HDAC6 activity and HDAC6 mRNA expression induced by water restriction (Fig. 6E and F).

Next, we investigated whether tubastatin A administration affects urine osmolality. Tubastatin A administration almost completely blocked the urine osmolality increases induced by water restriction (Table 2). Tubastatin A administration in water-supplied mice slightly, but not significantly, reduced urine osmolality (Table 2). These results indicate that water restriction-induced shortening of the primary cilia is associated with increased HDAC6 activity, and this shortening is associated with kidney urine-concentration.

**NaCl and mannitol treatment shortens the primary cilia length in the kidney epithelial cells and activates HDAC6.** To test whether primary cilia shortening is associated with pro-urine components, such as electrolytes concentrations and pro-urine osmolality, we treated 10 and 20 mM of NaCl or 20 mM mannitol in the culture medium of Madin-Darby canine kidney cells (MDCK). Mannitol was treated to evaluate the effect of medium osmolality because mannitol does not enter the cells [18]. Treatment of NaCl or mannitol shortened the primary cilia of MDCK cells in a concentration-dependent manner (Fig. 7A-C). Tubastatin A treatment blocked the NaCl and mannitol-induced shortening of the primary cilia (Fig. 7D and E). NaCl and mannitol treatment decreased ac-α-tubulin expressions without causing any significant changes in the α-tubulin expression (Fig. 8A-D). These NaCl and mannitol-induced decreases in the ac-α-tubulin expression were blocked by tubastatin A treatments (Fig. 8A-D). To test whether the shortening of primary cilia induced by NaCl or mannitol treatment is associated with the deciliation of primary cilia, we determined the ac-α-tubulin levels in the culture mediums of MDCK cell culture system. The levels of ac-α-tubulin expressions in the cell culture medium after NaCl and mannitol treatments remained unchanged as compared to those of vehicle treatments (Fig. 8E). Next, we determined the activity and HDAC6 mRNA expression. NaCl and mannitol treatment significantly increased the activities of HDAC6 and the increases were prevented by tubastatin A treatment (Fig. 8F). In consistency, HDAC6 mRNA expressions were same pattern with HDAC6 activities (Fig. 8G). These results indicate that increases in the electrolyte concentration and osmolality induce the shortening of the primary cilia via HDAC6 activation, subsequent α-tubulin deacetylation, and microtubule disassembly.

**Discussion**

To our knowledge, this is the first study to demonstrate that 1) water restriction in mice shortens the primary cilia lengths of kidney tubular cells, 2) this shortening is blocked by HDAC6 inhibitor, tubastatin A, treatment along with prevention of water restriction-induced HDAC6 activation and α-tubulin
deacetylation, and 3) HDAC6 inhibitor treatment in the water-restricted mice impairs the urine-concentrating ability of the kidneys. In addition, NaCl and mannitol treatments in the culture medium for established kidney tubular epithelial cells shorten the primary cilia, and these shortenings are blocked by HDAC6 inhibitor treatment along with the inhibition of α-tubulin deacetylation. These data indicate that the shortening of the primary cilia may be an adaptive response for the preservation of water and for electrolyte homeostasis in the body under water-restricted condition; further, this adaptive response is associated with the HDAC6 pathway. Our data answer the following questions: why the primary cilia lengths dynamically alter under various physiological conditions and how cells regulate the primary cilia, suggesting that the development of primary cilia length regulatory tools warrants the development of therapeutics of kidney diseases related to body water and electrolyte imbalances. In addition, our experimental in vivo and in vitro models, water restriction, as well as NaCl and mannitol treatment, could be broadly used for future studies in this field.

Primary cilia are localized on the surfaces of kidney tubular epithelial cells and visceral epithelial cells in the Bowman's capsule with the exception of intercalated cells. Therefore, they primarily contact and sense the ultrafiltrate flow, osmolality, and electrolytes [19]. Studies have demonstrated that the primary cilia sense the flow, and this sensing ability is positively correlated with their lengths [20–22]. Nag and Resnick reported that the primary cilium length is functionally involved in flow sensing via the enabling the setting of the bending strain of cilium [19]. In this study, we found that water restriction shortens the primary cilia in all uriniferous tubular epithelial cells along with urine volume reduction and increased urine osmolality. These data indicate that the reduction in the kidney flow shortens the primary cilia length. Water restriction results in the reduction of glomerular filtration rate (GFR), resulting in tubular fluid flow decrease [23]; in the inner medulla, the blood flow decreases by 34% at the end of 48 h of water restriction [24]. Urine flow blockage due to ureteral obstruction induces the shortening of the primary cilia of tubular cells [3, 13], while the increase in the urine flow in the remaining kidney after nephrectomy of one kidney, the fluid flow of which is increased, elongates the primary cilia [8]. Therefore, we consider that decreased fluid flow rate may affect the shortening of primary cilia length in water deprivation mice.

Along with the reduction in the urine flow and volume, water restriction results in increased urine electrolyte concentrations, leading to increased urine osmolality. This indicates that the primary cilia of tubular epithelial cells are exposed to a hypertonic solution with a high electrolyte concentration and reduced fluid flow. Furthermore, the primary cilia membrane contains osmolality sensing-associated proteins [7]. In present study, the supply of NaCl and mannitol into the culture medium induced the shortening of the primary cilia. These data indicate that increased fluid osmolality and/or electrolyte concentrations along with reduction in fluid flow causes the shortening of the primary cilia in the kidney tubular epithelial cells. Therefore, we speculate that the change in the primary cilia length is an adaptive response of the kidney epithelial cells for the maintenance of body water balance resulting from the reduced fluid flow and the increased fluid-electrolyte concentration. Paretorius et al. reported that primary cilia are required for the response to osmotic stress in the kidney tubular epithelial cells [7]. Furthermore,
damaged kidneys that present reductions in the glomerular filtrate rate (GFR) and fractional excretion of Na⁺ possess short primary cilia [25, 26].

Shortening in the primary cilia is associated with resorption of primary cilia during cell cycle entry and deciliation [11, 27]. In the present study, when we determined the cell cycle entry by BrdU incorporation assay and PCNA and p21 expressions, we found no significant changes in the BrdU-incorporated cell numbers and PCNA and p21 expressions in the water-restricted kidneys than in the water-supplied kidneys. In addition, we found no significant changes in the primary cilia proteins in the urine of water-restricted kidneys. These data indicate that water restriction-induced shortening of primary cilia is not caused by cell proliferation and deciliation (deciliated primary cilia in the kidneys are excreted in the urine) [11, 28], suggesting that reduction in fluid flow activates primary cilia shortening.

The shortening of the primary cilia is regulated by the disassembly of the microtubule and the release of primary cilia proteins. The disassembly of microtubule is dependent on the deacetylation of α-tubulins that are the main components of microtubule and major substrate of HDAC6 among cytosolic proteins [29, 17]. Pufacheva et al. reported that that deacetylation of α-tubulin by HDAC6 induces cilia disassembly [17]. In the present study, we found that water restriction dramatically decreased ac-α-tubulin expression, without changing the total α-tubulin expression in the kidneys along with increased HDAC6 activity and mRNA levels. Thus, we speculate that the shortening of the primary cilia is mediated by microtubule disassembly through the deacetylation of α-tubulin. To confirm this, we treated mice with tubastatin A, a well-known inhibitor of HDAC6, before water depletion. The injection of tubastatin A blocked the water restriction-induced deacetylation of α-tubulin. Along with this decrease in the ac-α-tubulin level, tubastatin A treatment almost completely blocked water restriction-induced shortening of the primary cilia. In the cultured tubular epithelial cells, tubastatin A treatment prevented NaCl and mannitol treatment-induced α-tubulin deacetylation and shortening of the primary cilia. These results indicate that water restriction-induced primary cilia shortening is regulated by increased HDAC6 activation, deacetylation of α-tubulin, and disassembly of microtubules, suggesting that HDAC6 signal pathway is a major primary cilia length-regulating factor. Supporting this, recent several studies have demonstrated that HDAC6 plays an important role in the regulation of primary cilia length; Smith et al. reported that HDAC6 activation induces the deacetylation of α-tubulin and subsequently shortens the primary cilia length [30]; Ehnert S. reported that HDAC6 induces the shortening of primary cilia in human osteoblasts [31]; Thompson CL et al. reported that primary disassembly down-regulates mechanosensitive hedgehog signaling in chondrocytes [32]; Li reported that the HDAC6-specific inhibitor, tubacin, stabilizes cilia [33]; Kamemura et al. also reported that HDAC6 knockdown in lung cells increases the acetylation of α-tubulin [34].

In the present study, water restriction induced significant increase in the urine osmolality and reduction in urine volume along with primary cilia shortening, without any significant change in plasma osmolality. However, the blockage of water restriction-induced HDAC6 activation blocked the increase in the urine osmolality and reduction in the urine volume, along with the prevention of water restriction-induced primary cilia shortening. Although we do not know the exact mechanism of how water restriction reduces
the primary cilia length, our data have clearly demonstrated that the change in the primary cilia length is an adaptive response to maintain water and electrolyte homeostasis in the body extracellular fluids and that the blockage of primary cilia length change causes loss of kidney urine-concentrating function that is required for the preservation of body fluid in water-restricted condition. Our data suggest that since aberrant renal tubular flow sensing is highly associated with the pathogenesis of various kidney diseases, the development of primary cilia length regulatory tools warrant the development of therapeutics of various diseases related with the loss of body water balance including polycystic diseases.

**Conclusion**

Our data have demonstrated that the shortening of primary cilia lengths in the kidney tubular epithelial cells occurs due to water restriction and this shortening is regulated by the activation of HDAC6 which induces the deacetylation of α-tubulin, a major core component of primary cilia. This suggests that shortening of the cilia is an adaptive response to water restriction for the preservation of body water and electrolytes balance and that primary cilia length regulatory tools may warrant the development of therapeutics of kidney diseases related to the body water and electrolyte imbalances.

**Methods**

**Animal preparations.** Ten-week-old C57BL/6 male mice (Koatech, Gyounggido, Korea) were used. All experiments were approved and performed in accordance with the approved guidelines of the Institutional Animal Care and Use Committee of Kyungpook National University, Republic of Korea. The mice were provided free access to water, but water supply was stopped for some mice for 24 to 48 h. Tubastatin A (10 mg/kg BW; Selleckchem, Houston, TX, USA) or 2% DMSO/saline (vehicle) were intraperitoneal injected daily from 2 days before water restriction until sacrifice. Five mice were included in each group. The mice were euthanized by an over-dose of pentobarbital sodium (65 mg/kg body wt; Sigma-Aldrich, St. Louis, MO). To perform biochemical and histological experiments, the kidneys were either frozen in liquid nitrogen or perfusion-fixed with periodate-lysine-paraformaldehyde (PLP) (4% paraformaldehyde, 75 mM l-lysine, 10 mM sodium periodate; Sigma-Aldrich, St. Louis, MO, USA) immediately after retrieval.

Blood was withdrawn from the heart of mice using a heparinized syringe. Urine was collected with abdominal massage or by housing the mice individually in metabolic cage. Urine and plasma osmolalities were measured using cryoscopic osmometer (Osmomat 030-D, Gonotec, Berlin, Germany).

**Cell culture.** Madin-Darby canine kidney cells (MDCK, CCL-34™, ATCC Inc., Manassas, VA, USA) were cultured in MEM (Corning, NY, USA) with 5% FBS (Mediatech Inc., Herndon, VA, USA) with 100 units/mL of streptomycin/penicillin (S/P) (WelGENE Inc., Daegu, Korea). Four days after confluent growing, 10 µM of tubastatin A were treated to the cells 4 days after confluent-grown. Four h thereafter, the cells were treated with either 10 or 20 mM NaCl or 20 mM mannitol. At 24 h after these treatments, the cells were either
fixed with 4% paraformaldehyde for immunofluorescence or lysed using lysis buffer for western blot analyses.

**Immunofluorescence staining.** Kidney paraffin-sections were stained with anti-acetylated-α-tubulin (ac-α-tubulin, 1:200; Sigma-Aldrich), anti-Na/K-ATPase (1:100 dilution; Santa Cruz, Santa Cruz, CA, USA), anti-aquaporin-1 (anti-AQP1; 1:200 dilution; Alomone Laboratories, Jerusalem, Israel), and anti-AQP2 (1:200 dilution, Alomone Laboratories) antibodies. The fixed cells were stained with anti-ac-α-tubulin for the measurement of primary cilia length. To detect the cell nuclei, DAPI was applied to the samples. Images were captured using Leica microscope (DM2500, Wetzlar, Germany).

**Measurement of primary cilia length.** Images were captured using a microscope. Primary cilia length was measured blindly. We randomly captured (×400) 5–10 fields in the cortex, outer medulla, and inner medulla. More than 50 cells from each group were used to measure the length of the primary cilia using I-Solution software (IMT I-Solution, Rochester, NY, USA).

**BrdU incorporation assay.** 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg body weight; Sigma) incorporation assay was performed to determine cell proliferations. BrdU was administered to mice beginning on 1 day before cisplatin injection, every other day until sacrifice. Kidney sections were subjected to immunohistochemical staining using anti-BrdU (1:200 dilution; Serotec, Oxford, UK) antibody. Photomicrographs were obtained using a Leica microscope.

**Scanning electron microscopy.** Kidneys were perfusion-fixed with a fixing agent (0.5% glutaraldehyde and 0.5% paraformaldehyde) and then immersed in the fixing agent for 12 h. Kidneys were post-fixed with 1% osmium tetroxide for 1 h at 4 °C. After rinsing with 0.1-M phosphate buffer, the kidneys were immersed serially in 25% and 50% DMSO for 30 min each. The kidneys were rapidly frozen on a metal plate by chilling with liquid nitrogen and then cracked using a scalpel and hammer. The cracked kidneys were thawed with 50% DMSO, washed thrice with 0.1-M phosphate buffer, and placed in 1% osmium tetroxide for 1 h at 4 °C. Kidneys were transferred to 25% tannic acid for 2 h at room temperature and placed in 1% osmium tetroxide for 1 h at 4 °C. The kidneys were dehydrated using an ethanol series and isoamyl acetate. The dehydrated kidneys were subjected to critical point dry and mounted. Pictures were obtained using a scanning electron microscope (H-2500, Hitachi, Japan).

**Western blot analyses.** Western blot analyses were performed using the anti-ac-a-tubulin (Sigma-Aldrich), -α-tubulin (Sigma-Aldrich), -PCNA (DAKO, Carpinteria, CA, USA), -p21 (Santa Cruz), -GAPDH (NOVUS, Littleton, CO, USA), and -β-actin (Sigma-Aldrich).

**Quantitative real time RT-PCR (qRT-PCR) analysis.** RNA was extracted using PureHelix RNA extraction solution (Nanohelix, Seoul, Korea). For cDNA synthesis, 1 µg of RNA was used with the DiaStar RT Kit (SolGent, Daejeon, Korea). qRT-PCR was performed with AmpiGene qPCR Green Mix Hi-ROX (Enzo Life Science, Farmingdale, NY, USA) and AriaMx Real-Time PCR machine (Agilent Technology, Santa Clara, CA, USA). Mouse qRT-PCR primer sequences were 5′- AAC CCT GAG ACA AGA GTG CCA GTT-3′ and 5′- TCA GTT GCT CTC TGA TGG CAT GGA-3′ for HDAC6, sense and antisense, respectively.
**Measurement of HDAC6 activity.** Measurement of HDAC6 activity was based on deacetylase activity of HDAC6 towards a synthetic acetylated-peptide substrate resulting in the release of an AFC fluorophore. Activity level was determined using an HDAC6 activity assay kit (Biovision Incorporated, Milpitas, CA, USA). Briefly, kidney samples were homogenized in HDAC6 lysis buffer and incubated on ice for 5 min. After centrifugation of the homogenate at 16,000 g, 4°C for 10 min, the supernatant was used for determination. In 96-well plate, samples were transferred and HDAC6 substrate mix was added and incubated at 37°C for 30 min. To stop the reaction, developers were added to each well and incubated at 37°C for 10 min. Then, the fluorescence intensity was determined using a fluorescence spectrometer (Molecular Devices, California, USA) for 30 min at 380/490 nm at 37°C.

**Statistical analyses.** Using GraphPad Prism 6 software (San Diego, CA, USA), all data were analyzed. Results are expressed as mean ± standard deviation. Statistical differences among the groups were assessed using Student’s *t*-test and two-way ANOVA with repeated measures followed by post hoc Bonferroni’s multiple comparisons test. Differences were considered statistically significant at a p value < 0.05.

**Abbreviations**

- HDAC6: histone deacetylase 6
- WR: water restriction
- PT: proximal tubule
- DT: distal tubule
- CD: collecting duct
- MDCK: Madin-Darby canine kidney cell
- SEM: scanning electron microscopy
- PCNA: proliferative cell nuclear antigen
- BrdU: bromodeoxyuridine
- GFR: glomerular filtration rate
- PLP: periodate-lysine-paraformaldehyde
Declarations

Ethics approval and consent to participate

All experiments were approved and performed in accordance with the approved guidelines of the Institutional Animal Care and Use Committee of Kyungpook National University, Republic of Korea.

Consent for publication

Written informed consent for publication was obtained from all participants

Availability of data and material

All data are fully available without restriction

Competing interests

The authors declare that they have no competing interests.

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Contributions

MJK, SJH, and SYS performed the experiments and analyzed data. MJK, JIK, HKH and KMP conceived the idea, designed the experiments, analyzed the data, and drafted the manuscript. All authors read and approved the final manuscript.

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**Tables**

**Table 1. Osmolalities of urine and plasma and hematocrit after water restriction for 24 and 48 h.**

|                      | Control (n=8) | 24 h (n=6)  | 48 h (n=10) |
|----------------------|--------------|-------------|-------------|
| **Urine (mosmol/kg H2O)** | 2250.3 ± 395.0 | 3416.67 ± 27.28* | 4085.0 ± 157.3* |
| **Plasma (mosmol/kg H2O)** | 325.50 ± 7.42 | 322.66 ± 2.10 | 333.60 ± 8.98 |
| **Hematocrit (%)** | 42.30 ± 5.30 | 45.15 ± 0.75 | 45.30 ± 3.27 |

*P < 0.01.

For 24 or 48 h, some mice were given access to water (control), while some were not (water restriction). The results are expressed as means ± SD (n=6-10). *, p < 0.05 vs. water restriction.

**Table 2. Effect of tubastatin A on urine osmolarities after water restriction.**
For 48 h, some mice were given access to water (control), while some were not (water restriction, WR). Some mice were administrated with either tubastatin A (tubastatin, 10 mg/kg BW) or saline (vehicle) intraperitoneally every alternate day, starting 48 h before water restriction until the end of the experiment. Tubastatin A or saline (vehicle) were intraperitoneally injected daily, beginning from 48 h before water restriction until sacrifice. The results are expressed as mean ± SD (n=8-10). *, p < 0.05 vs. respective control. †, p < 0.01 vs. 48 h of WR

### Figures

*Fig. 1a-j*

**Figure 1**

Lengths of the primary cilia in the tubular cells of water-restricted mice kidneys. For 24 or 48 h, some mice were given access to water (control, Con), while some were not (water restriction, WR). Kidneys were harvested, fixed with the PLP-fixative, and sectioned 5 µm thickness using a microtome. The kidney sections were stained with anti-acetylated-α-tubulin (green, ac-α-tubulin, a marker of primary cilia) antibody and anti-aquaporin-1 (AQP-1, a marker of proximal tubule cell, red), -AQP-2 (a marker of principal
cell of collecting duct, red), or -Na+-K+-ATPase (a basolateral protein, a marker of distal tubule cell, red) antibodies. DAPI (blue) was used to visualize the nucleus. Pictures were taken from the cortex (a-d), outer medulla (e, f-h), and inner medulla (i and j). The primary cilia lengths were measured in S1-2 segments of the proximal tubular cells (PT, b), distal tubular cells (DT, c), and principal cells in the collecting duct (CD, d) in the cortex (a), S3 segment of proximal tubule (S3 PT, f), medullary thick ascending limb cells (mTAL, g), and principal cells in the collecting duct (CD, h) in the outer medulla (e), and principal cells in collecting duct (CD, j) in the inner medulla (i). Results are expressed as mean ± S.E.M values (n=5). Arrowheads indicate the primary cilia. *, p < 0.05 vs. control.

**Figure 2**

Primary cilia in the tubular cells of water-restricted mice kidneys. For 48 h, some mice were given access to water (control), while some were not (water restriction). The outer medulla (OM, a) and inner medulla (IM, b) lesions of kidneys were fixed with fixatives for scanning electron microscopy (SEM) analysis. The primary cilia on the distal tubular cells in the outer medulla (a) and the principal cells in the inner medulla (b) were observed using SEM. The arrows indicate the primary cilia. Statistical analysis was performed by two-way ANOVA with repeated measures followed by post hoc Bonferroni’s multiple comparisons test.
Proliferation and deciliation of tubular cells of water-restricted mice. For 48 h, some mice were given access to water (control), while some were not (water restriction). Some mice were administrated with BrdU intraperitoneally on every alternate day, beginning on 24 h before the water restriction until the end of the experiment. Kidneys were harvested and then frozen with liquid nitrogen or fixed with the PLP-fixative. (a and b) Three 3 μm thickness of kidney sections was immunohistochemically stained using anti-BrdU antibody (brown). BrdU-positive cells (BrdU+) were counted in the cortex. (c-e) The expressions of PCNA, p21, and β-actin in the kidneys were evaluated using western blotting analysis. (d and e) Band densities were measured using Image J software. (f) Mouse urines were collected and subjected to western blotting analysis using the anti-ac-α-tubulin antibody. The last lane was the positive control urine collected from kidney ischemia/reperfusion-injured mice. Results are expressed as mean ± SD (n=4). *, p < 0.05 vs. control.
Figure 4

Acetylated α-tubulin, α-TAT protein, and HDAC6 protein and HDAC6 mRNA expressions in the kidneys after water restriction. For 48 h, some mice were given access to water (control), while some were not (water restriction, WR). Kidney samples were prepared in the cortical (cortex), outer medullary (OM), and inner medullary (IM) lesions and subjected to (a-d) western blotting analysis using anti-acetylated-α-tubulin (ac-α-tub, a cytosolic substrate of HDAC6) for the determination of HDAC6 activation, and α-tubulin, antibodies. (a-c) GAPDH was used as the loading control. Densities of the blots were determined using image J software. (e) The activity of HDAC6 was determined in the whole kidneys. (f) Whole kidneys were used for the determinations of HDAC6 mRNA expression using a RT-PCR. Results are expressed as mean ± SD (n=4). *, p < 0.05 vs. control. †, p < 0.05 vs. respective-control.
Figure 5

Blockage of water restriction-induced primary cilia shortening by tubastatin A administration. For 48 h, some mice were given access to water (control, Con), while some were not (water restriction, WR). Some mice were administrated with either tubastatin A (tubastatin, 10 mg/kg BW) or saline (vehicle) intraperitoneally every alternate day, starting 48 h before water restriction until the end of the experiment. Kidneys were harvested, fixed with the PLP-fixative, and sectioned 5 μm thickness using a microtome. (a) The kidney sections were stained with anti-ac-α-tubulin (green), -aquaporin-1 (AQP-1, a marker of proximal tubule cell, red), -AQP-2 (a marker of principal cell of collecting duct, red), and -Na+-K+-ATPase (a basolateral protein, red) antibodies. DAPI (blue) used to visualize the nucleus. Pictures were taken from the cortex. (b to d) The primary cilia lengths were measured in the proximal tubular cells of the S1-2 segments of proximal tubule (PT, b), distal tubular cells of the distal tubule (DT, c), and principal cells in the collecting duct (CD, d). Results expressed as mean ± SD (n=5). The arrowheads indicate primary cilia. *, p < 0.05 vs. respective-control. †, p < 0.05 vs. vehicle-WR.
Figure 6

Blockage of changes in water restriction-induced acetylated α-tubulin and HDAC6 expressions. For 48 h, some mice were given access to water (control, Con), while some were not (water restriction, WR). Some mice were administrated either tubastatin A (tubastatin, 10 mg/kg BW) or saline (vehicle) intraperitoneally every other day, beginning 48 h before water restriction until the end of the experiment. Kidneys were harvested 48 h after water restriction. The whole kidney samples were subjected to (a-d) western blotting analysis using anti-ac-α-tubulin, and -α-tubulin antibodies. GAPDH was used as the loading control. Densities of the blots were determined using image J software. (e) The activity of HDAC6 was determined in the whole kidneys. (f) HDAC6 mRNA expressions were determined in the whole kidneys via RT-PCR. Results expressed as mean ± SD (n=4). *, p < 0.05 vs. respective-control. †, p < 0.05 vs. respective-vehicle.
Figure 7

Shortening of the MDCK cell primary cilia by NaCl and mannitol treatment and blockages of those NaCl and mannitol-induced shortening by tubastatin A treatment. (a-e) Madin-Darby canine kidney (MDCK) cells cultured on coverslips for additional 4 d after reaching confluency and treated with 10 mM and 20 mM of NaCl and mannitol for 24 h. NaCl and mannitol were dissolved with culture medium. (d) Some cells were treated with either vehicle or 10 μM of tubastatin A (tubastatin) before 10 mM of NaCl or 20 mM of mannitol treatments. (a and d) Primary cilia and nuclei were visualized using immunofluorescence staining using anti-ac-α-tubulin antibody (green) and DAPI (blue), respectively. (b, c, and e) The average lengths of the primary cilia were determined. None groups were not treated with NaCl and mannitol (None). Statistical analysis was performed by two-way ANOVA with repeated measures followed by post hoc Bonferroni’s multiple comparisons test. Results expressed as mean ± SD (n=4-6). *, p < 0.05 vs. respective none. †, p < 0.05 vs. respective vehicle-none.
Figure 8

Blockage of changes of NaCl and mannitol-induced acetylated α-tubulin and HDAC6 expressions, activity and mRNA level of HDAC6 by tubastatin A administration. (a-e) Madin-Darby canine kidney (MDCK) cells cultured on coverslips for additional 4 days after reaching confluence and treated with 10 mM of NaCl and 20 mM of mannitol (Mt) for 24 h. NaCl and mannitol were dissolved with culture medium. Some cells were treated with either vehicle or 10 μM of tubastatin A (tubastatin) before NaCl or mannitol treatments. None groups were not treated with NaCl and mannitol (None). (a-e) Cells (a-d) and culture medium samples (e) were subjected to western blotting analysis using anti-ac-α-tubulin (ac-α-tub) and -α-tubulin antibodies. GAPDH was used as the loading control. (f and g) The HDAC6 activity (f) and mRNA level (g) were determined in the whole kidneys. Densities of the blots were determined using image J software. Results are expressed as mean ± SD (n=4). *, p < 0.05 vs. respective none. †, p < 0.05 vs. respective vehicle-none.