Hypotonic Stress-Induced Down-Regulation of Claudin-1 and -2 Mediated by Dephosphorylation and Clathrin-Dependent Endocytosis in Renal Tubular Epithelial Cells*

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Running title: Hypotonic stress decreases claudin expression

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Hypotonic stress decreased claudin-1 and -2 expression levels in renal tubular epithelial HK-2 and Madin-Darby canine kidney cells. Here, we examined the regulatory mechanism involved in this decrease. The hypotonicity-induced decrease in claudin expression was inhibited by SB202190, a p38 mitogen-activated protein kinase inhibitor, but not by U0126, a MEK inhibitor, Go6983, a protein kinase C inhibitor, or SP600125, a Jun N-terminal protein kinase inhibitor. Hypotonic stress increased transepithelial electrical resistance, which was inhibited by SB202190. The mRNA expression level of claudin-1 was decreased by hypotonic stress, but that of claudin-2 was not. Hypotonic stress decreased the protein stability of claudin-1 and -2. The hypotonicity-induced decrease in claudin expression was inhibited by chloroquine (CQ), a lysosome inhibitor, dynasore and monodansylcadaverine (MDC), clathrin-dependent endocytosis inhibitors, and siRNA against clathrin heavy chain. Claudin-1 and -2 were mainly distributed in the cytosol and tight junctions (TJs) in the CQ- and MDC-treated cells, respectively. Hypotonic stress decreased the phosphorylation levels of claudin-1 and -2, which were inhibited by the protein phosphatase inhibitors okadaic acid and cantharidin. Dephosphorylated mutants of claudin-1 and -2 were mainly...
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Claudin-2 was distributed in the cytosol, which disappeared in response to hypotonic stress. In contrast, mimicking phosphorylation mutants were distributed in the TJs, which were not decreased by hypotonic stress. We suggest that hypotonic stress induces dephosphorylation, clathrin-dependent endocytosis, and degradation of claudin-1 and -2 in lysosomes, resulting in disruption of the TJs barrier in renal tubular epithelial cells.

Epithelial and endothelial cells form tight junctions (TJs) to seal adjacent cells in a narrow band just beneath their apical surface. Freeze-fracture electron microscopy has shown that TJs appear as a continuous and anastomosing network of intramembranous particle strands (1). The TJs maintain membrane polarity by restricting the exchanges of lipids and proteins between the apical and basolateral membranes, and they function as gatekeepers by regulating the paracellular permeability of ions, solutes, and water. The TJs are composed of integral membrane proteins including claudins, occludin, tricellulin, and marvelD3, and cytoplasmic plaque proteins including zonula occludens (ZO)-1, -2, and -3 (2-5). Claudins consist of a superfamily of more than 20 homologous members, and they play a key role in determining permeability properties (6,7). The expression patterns of claudins vary among tissues (8) and most cells express more than two claudins.

In the kidney, the proximal tubule has a loose epithelium that allows both transcellular and paracellular transport of water and solutes, whereas the distal and collecting tubules have a tight epithelium that primarily facilitates transcellular transport. The localization and function of claudin-1 and -2 have been well characterized. The tight segments in rabbit express claudin-1 at the TJs, whereas the leaky proximal segment lacks claudin-1 and instead expresses claudin-2 (9). In Madin-Darby canine kidney (MDCK) cells, two strains of type I (tight) and II (leaky) exhibit a differential expression pattern of claudins. Claudin-1 is expressed in both type I and II cells, whereas the expression of claudin-2 is restricted to leaky type II cells. The knockdown of claudin-2 in type II cells increases transepithelial electrical resistance (TER), whereas that of claudin-1 does not change TER (10). Claudin-2 can form paracellular cation pores, whereas claudin-1 cannot. These claudins show different properties of cation permeability, but experiments using knockout mice showed that they play an important role in the barrier function (11,12).

Renal tubular epithelial cells are commonly exposed to variations in extracellular osmolarity. The volume of the cells increases within a few minutes, which is followed by a decrease towards the initial volume under hypotonic conditions. This process is known as regulatory volume decrease (13), which may be crucial to their survival. The cells respond to hypotonic stress by controlling ion movements across their plasma membrane. Hypotonic stress inhibits the
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Na+/H+-exchanger (14), whereas it activates cystic fibrosis transmembrane conductance regulator chloride channel (15) and TWIK-related acid-sensitive potassium channel 2 in the proximal tubule (16). This stress also activates water aquaporin 2 channel and transient receptor potential vanilloid 4 cation channel in renal cortical collecting duct cells (17). Although the involvement of various ion channels, exchangers, transporters, and pumps in response to osmolarity changes has been investigated, there are few reports focusing on the effect of hypotonic stress on cell-cell contacts in the renal tubules.

In the present study, we found that hypotonic stress decreases the expression levels of claudin-1 and -2 in human renal tubule HK-2 and MDCK II cells. The hypotonicity-induced decrease in claudin expression was inhibited by monodansylcadaverine (MDC) and dynasore, clathrin-dependent endocytosis inhibitors, and chloroquine (CQ), a lysosome inhibitor. Hypotonic stress decreased the phosphorylation levels and protein stability of claudin-1 and -2. Dephosphorylated mutants of claudin-1 and -2 were mainly distributed in the cytosol, which disappeared in response to hypotonic stress. These results indicated that hypotonic stress may change the function of TJs mediated by dephosphorylation, clathrin-dependent endocytosis, and lysosomal degradation of claudin-1 and -2 in renal tubular epithelial cells.

Results

Effect of hypotonic stress on claudin expression in renal tubule cells ---

Hypotonic stress time-dependently decreased the protein levels of claudin-1 and -2 in HK-2 and MDCK II cells (Fig. 1). Isotonic medium prepared by addition of mannitol did not affect claudin expression. Therefore, claudin expression was decreased by hypotonic stress, but not by changes in sodium or chloride concentration. So far, we reported that claudin-2 expression is decreased by activation of the MEK/ERK pathway (20) and protein kinase C (PKC) in MDCK II cells (21). The hypotonicity-induced decrease in claudin-2 expression was inhibited by neither U0126, a MEK inhibitor (22), nor Go6983, a PKC inhibitor (23) (Fig. 2A). Claudin-2 expression may be down-regulated by other mechanisms under hypotonic conditions.

Inhibition of the hypotonicity-induced decrease in claudin expression and increase in TER by p38 mitogen-activated protein kinase (MAPK) inhibitor ---

Hypotonic stress increased p-p38 MAPK levels, whereas it decreased p-c-Jun amino-terminal kinase (p-JNK) levels (Fig. 2B). Therefore, we examined the effects of SB202190, a p38 MAPK inhibitor (24), and SP600125, a JNK inhibitor, on the hypotonicity-induced decrease in claudins expression. The hypotonicity-induced decrease in claudin-1 and -2 expression levels was significantly inhibited by SB202190, but not by SP600125 (Fig. 2C). Both SB202190 and SP600125 had no effect on the expression levels of claudin-1 and -2 in the absence of hypotonic stress.
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treatment (data not shown). Hypotonic stress increased TER in a time-dependent manner, which was inhibited by SB202190 (Fig. 2D) but not by SP600125 (data not shown). Claudin-2 forms cation permeable pores (25), and the knockdown of claudin-2 by siRNA increases TER (10). The hypotonicity-induced increase in TER and the inhibition by SB202190 may be contributed to by claudin-2 expression.

Effect of hypotonic stress on the stability of claudin proteins --- Hypotonic stress decreased the expression level of claudin-1 mRNA, whereas it did not decrease that of claudin-2 mRNA within 4 h (Fig. 3). There is a possibility that hypotonic stress decreases the stability of claudin-1 and -2 proteins. Therefore, we examined the effect of hypotonic stress on the protein levels of claudin-1 and -2 in the presence of cycloheximide (CHX), a translation inhibitor. The expression levels of claudin-1 and -2 time-dependently decreased in the presence of CHX (Fig. 4). Hypotonic stress accelerated the decrease in claudin expression.

Effects of endocytosis and protein degradation inhibitors on hypotonicity-induced decrease in claudins expression --- The majority of intracellular proteins are degraded by lysosomal enzymes and proteasomes. The hypotonicity-induced decrease in claudin-1 and -2 expression levels was inhibited by CQ, a lysosome inhibitor (26), but not by lactacystin (Lac), a proteasome inhibitor (Fig. 5A and 5B). Hypotonic stress may accelerate the degradation of claudins mediated by trafficking into lysosomes. Next, we examined the effects of endocytosis inhibitors on claudin expression. The hypotonicity-induced decrease in claudin-1 and -2 expressions was inhibited by MDC and dynasore, clathrin-dependent endocytosis inhibitors (27), but not by methyl-β-cyclodextrin (MβCD), a caveolae-dependent endocytosis inhibitor (Fig. 5C-5E). Furthermore, the hypotonicity-induced decrease in claudin-1 and -2 expression levels was inhibited by siRNA against clathrin heavy chain, but not by siRNA against caveolin (Fig. 6). These results indicated that hypotonic stress enhances clathrin-dependent endocytosis and lysosomal degradation of claudin-1 and -2. Immunofluorescence measurement showed that claudin-1 and -2 were mainly distributed in the TJs concomitant with ZO-1 under control conditions (Fig. 7). Hypotonic stress decreased the expression of claudins at the TJs without affecting the expression of ZO-1. Claudin-1 and -2 were colocalized with LAMP-1, a marker of lysosomes, in the CQ-treated cells. MDC increased the localization of claudin-1 and -2 at the TJs, but MβCD did not. These results indicated that hypotonic stress causes endocytosis of claudin-1 and -2 mediated by a clathrin-dependent mechanism and degradation of claudin-1 and -2 in lysosomes similar to the results in western blotting. To clarify the specificity of hypotonic stress, we examined the effect of hypotonic stress on the intracellular localization of an Na⁺/K⁺-ATPase and Na⁺/H⁺-exchanger. Both the Na⁺/K⁺-ATPase and
Na⁺/H⁺-exchanger are distributed in the cell-cell contact area under control conditions (Fig. 8). Hypotonic stress increased the intracellular localization of the Na⁺/H⁺-exchanger, but this was inhibited by SB202190 and MDC. In contrast, the intracellular localization of the Na⁺/K⁺-ATPase was not changed by hypotonic stress. These results indicated that the activation of p38 MAPK and clathrin-dependent endocytosis caused by hypotonic stress is not specific to claudins.

**Effect of hypotonic stress on the phosphorylation and expression of claudins**

It has been reported that the tight junctional localization of claudin-1 and -2 is regulated by the phosphorylation at T191 (28,29) and S208 (30), respectively. We examined the effect of hypotonic stress on the phosphorylation of claudin-1 and -2. Claudin-1 and -2 were phosphorylated at threonine and serine residues under control conditions, respectively (Fig. 9A). Hypotonic stress decreased the phosphorylation levels and the status of phosphorylation was controlled by various protein kinases and phosphatases. The hypotonicity-induced decrease in claudin-1 and -2 expression levels was inhibited by okadaic acid (OA) and cantharidin (Fig. 9B), protein phosphatase (PP) 1 and PP2A inhibitors (31,32). These results suggested that hypotonic stress decreases the phosphorylation levels of claudins mediated by the activation of PP. The activation of PP has been reported to induce de-phosphorylation of claudin-1 and occludin in MDCK cells (33). To examine the effect of dephosphorylation on the hypotonicity-induced decrease in claudin expression, we made T191A, a dephosphorylated form, and T191E, a phosphorylated form, mutants of claudin-1. Hypotonic stress decreased the expression levels of wild-type and a T191A mutant, but it did not decrease that of T191E (Fig. 10A and 10B). Similarly, hypotonic stress decreased the expression levels of wild-type and an S208A mutant, a dephosphorylated form, but it did not decrease that of S208E mutant, a phosphorylated form, of claudin-2. The expression levels of wild-type claudin-1 and -2 were not significantly different from those of mutants in the presence of CHX (Fig. 10C and 10D), indicating that the stability of protein was same among wild-type and mutants. These results indicated that hypotonic stress decreases the phosphorylation levels of claudin-1 and -2, and enhances the degradation of dephosphorylated forms.

**Intracellular localization of mutants of claudins**

A dephosphorylated T191A mutant of claudin-1 and an S208A mutant of claudin-2 were mainly distributed in the cytosol, which was decreased by hypotonic stress (Fig. 10E and 10F). In contrast, mimicking phosphorylation mutants of T191E and S208E were distributed in the TJs, which were not decreased by hypotonic stress. These results indicated that the TJs localization of claudin-1 and -2 is regulated by the phosphorylation. Next, we examined the effect of hypotonic stress on the localization of dephosphorylated mutants. T191A and S208A mutants were mainly co-localized with Rab7, a late
endosome marker, under control conditions, whereas the co-localization of claudins with LAMP-1 was low (Fig. 11). Hypotonic stress increased the co-localization of claudins with LAMP-1, whereas it decreased that with Rab7. The effect of hypotonic stress was inhibited by SB202190. These results indicated that hypotonic stress is involved in the regulation of dephosphorylation of claudin-1 and -2, and transport of dephosphorylation forms from endosomes to lysosomes.

**Discussion**

The kidney contributes to maintaining body fluid and electrolyte balance through the concentration of urine. The tubule fluid osmolarity is isosmotic with plasma or slightly hypotonic in the proximal tubule. The tubular cells are submitted to osmotic shocks either by accumulation of active osmolytes inside their cytoplasm or by dilution of the tubular fluid. Luminal hypotonicity is generated by the elevation of glucose concentration (34). Therefore, it is suggested that high glucose concentration in poorly controlled diabetes leads to excessive reabsorption of sodium and water. The reabsorption of minerals and water is coordinately regulated by transcellular and paracellular pathways. Many investigators reported that the function of ion channels, exchangers, and transporters is changed by hypotonic stress, whereas little is known about the effect of hypotonic stress on claudins, which form the paracellular barrier and pores.

In the present study, we found that hypotonic stress decreases the expression levels of claudin-1 and -2 in HK-2 and MDCK II cells. The expression of claudin-2 was regulated by transcriptional and post-translational steps. Epidermal growth factor (EGF) and hydrogen peroxide decreased claudin-2 expression mediated by activation of the MEK/ERK pathway in MDCK II cells (35,36). EGF may block transcription of claudin-2 mRNA mediated by the activation of Stat3 (37). The EGF-induced activation of Stat3 was inhibited by PP2, a Src inhibitor, indicating that Stat3 is located downstream in the Src signaling pathway. We found that the hypotonicity-induced decrease in claudin-2 expression is not inhibited by U0126 (Fig. 2), PP2 and stattic, a Stat3 specific inhibitor (data not shown). Therefore, we suggest that hypotonic stress decreases claudin-2 expression mediated by a novel regulatory mechanism. In contrast, the regulatory mechanism of claudin-1 expression still remains to be fully understood.

Interleukin-6 increased the claudin-2 mRNA levels mediated by activation of the MEK/ERK pathway and the increase in caudal-related homeobox-2 in intestinal epithelial cells (38). U0126, a MEK inhibitor, decreased claudin-2 mRNA levels mediated by the decrease in c-Fos expression in lung adenocarcinoma cells (39). Thus, several reports have indicated that claudin-2 expression is up-regulated by activation of the MEK/ERK pathway at a transcriptional level. In contrast, we reported recently that hypertonic stress
Hypotonic stress decreases claudin expression and increases phosphorylation of PKCβ and decreases claudin-2 mRNA levels, which is independent of the MEK/ERK pathway in MDCK II cells (40). In the present study, we found that hypotonic stress decreases claudin-2 expression, which is not inhibited by MEK and PKC inhibitors (Fig. 2). Furthermore, hypotonic stress did not decrease claudin-2 mRNA levels. These results suggest that the down-regulation of claudin-2 expression by hypotonic stress differs from that by activation of the MEK/ERK pathway and hypertonic stress.

Hypotonic stress activates p38 MAPK and JNK in renal epithelial A6 cells derived from Xenopus laevis (41). Our data indicated that hypotonic stress activates p38 MAPK, whereas it inhibits JNK (Fig. 2). The hypotonicity-induced decrease in claudin-1 and -2 expression levels was inhibited by a p38 MAPK inhibitor, but not by a JNK inhibitor. Furthermore, the hypotonicity-induced increase in TER was significantly inhibited by a p38 MAPK inhibitor. Recently, it has been reported that oxidative stress decreases claudin-1 expression in human retinal pigment epithelium cells and calcium oxalate monohydrate crystal decreases claudin-2 expression in MDCK cells mediated by the activation of p38 MAPK (42). We suggest that p38 MAPK plays a key role in the stress-induced decrease in claudin expression levels.

Post-translational modification often affects the function and expression of proteins. We found that hypotonic stress accelerates the decrease in claudin-1 and -2 expression levels. Molina-Jijon et al. (43) recently reported that oxidative stress decreases claudin-2 expression mediated by the activation of PKCβ and the modification of claudin-2 by nitration, phosphorylation, and SUMOylation in the kidney of type-1 diabetic rats. These regulatory mechanisms of claudin-2 expression are similar to those in hypertonic stress, but they differ from those in hypotonic stress: 1) hypotonic stress decreased the phosphorylation of claudin-2, 2) the hypotonicity-induced decrease in claudin-2 expression was not inhibited by a PKCβ inhibitor, and 3) the hypotonicity-induced decrease in claudin-2 expression was inhibited by PP1 and PP2A inhibitors. Forskolin and prostaglandin E2 decreased claudin-2 expression mediated by the inhibition of its phosphorylation in MDCK cells (30). The localization of other claudins to the TJs is reported to be regulated by its phosphorylation and phosphorylated claudin-1, -5, and -16 are localized in the TJs (44-46). In contrast, phosphorylated claudin-3 and -4 dissociate from the TJs (47,48). Thus, the effect of phosphorylation on localization differs with each claudin, but dephosphorylated claudin-1 and -2 may be transported to the lysosomal degradation pathway in MDCK II cells in response to hypotonic stimuli.

The hypotonicity-induced decrease in claudin-1 and -2 expression levels was inhibited by the clathrin-dependent endocytosis inhibitors MDC and dynasore (Fig. 5). Both claudin-1 and -2 were localized to the TJs in cells treated with hypotonic stress and MDC. These results suggest that hypotonic stress increased the...
clathrin-dependent endocytosis of these claudins. Endocytosed materials including membrane compartments are packed into endocytic vesicles that fuse to form early endosomes (49). Then, the proteins contained in endosomes can be recycled to the plasma membrane, trans-Golgi apparatus, or lysosomes. Dephosphorylated mutants of claudin-1 and -2 were mainly distributed in the late endosome under control conditions. Of note, hypotonic stress increased the localization of dephosphorylated mutants to lysosomes, which was inhibited SB202190 (Fig. 11). We suggest that the dephosphorylation and transport of claudins from endosomes to lysosomes are regulated by p38 MAPK. The activation of p38 MAPK enhanced ligand-independent endocytosis of EGF receptor (50) and transport of the EVA-1 receptor (51) from endosomes to lysosomes. Therefore, this p38 MAPK-mediated trafficking mechanism may not be specific to claudins.

In conclusion, we found that hypotonic stress decreases the expression of claudin-1 and -2 in renal tubular epithelial cells. The decrease in claudins expression was inhibited by SB202190, CQ, MDC, dynasore, OA, cantharidin, and clathrin siRNA. The possible regulatory mechanism for the expression of claudin-1 and -2 is depicted in figure 12. The renal epithelial cells control the expression of each claudin in response to changes in extracellular osmolarity. The knockdown of claudin-2 by siRNA not only decreases paracellular permeability to cation but also increases cell migration (40). The cells injured by stress stimuli including hypotonic stress, oxidative stress, and nephrotoxins are eliminated from the tubular surface. The repair of the injured tubule is accomplished by the migration of surviving cells. The change in the combination of claudin subtype expression may be involved in the maintenance of renal morphology and function.

Experimental Procedures

Materials --- Rabbit anti-claudin-1 polyclonal (71-7800) and rabbit anti-claudin-2 polyclonal (51-6100), and rabbit anti-ZO-1 polyclonal antibodies (61-7300) were obtained from Zymed Laboratories (South San Francisco, CA). Goat anti-β-actin polyclonal (sc-1615) and mouse anti-Na+/K+-ATPase monoclonal antibodies (sc-21712) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-p-p38 MAPK monoclonal (612168), mouse anti-p38 MAPK monoclonal (612280), mouse anti-p-JNK monoclonal (612540), mouse anti-JNK monoclonal (610627), and mouse anti-LAMP-1 monoclonal antibodies (51-9002014) were from BD Biosciences (San Jose, CA). Rabbit anti-Rab7 polyclonal antibodies (9367) were from Cell Signaling Technology (Beverly, MA). U0126, MDC, and mouse anti-phosphoserine (p-Ser) monoclonal (P3430), mouse anti-phosphothreonine (p-Thr) monoclonal (P3555), and rabbit anti-Na+/H+-ATPase polyclonal antibodies (SLC9A, SAB4200016) were from Sigma-Aldrich (Saint Louis, MO).
SP600125 was from Enzo Life Sciences (Farmingdale, NY). CQ, SB202190, MβCD, and mouse anti-FLAG monoclonal antibodies (DYKDDDDK, 014-22383) were from Wako Pure Chemical Industries (Osaka, Japan). Cantharidin, Go6983, and Lac were from Cayman Chemical (Ann Arbor, MI). Dynasore was from AdooQ BioScience (Irvine, CA). OA was from LC Laboratories (Woburn, MA). Lipofectamine 2000 was from Thermo Fisher Scientific (Waltham, MA). All other reagents were of the highest grade of purity available.

Cell culture --- Human renal epithelial HK-2 and canine MDCK II cells were obtained from the American Type Culture Collection (ATCC CRL-2190, Manassas, VA) and European Collection of Cell Cultures (ECACC No. EC00062107, Salisbury, UK), respectively. Cells were grown in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 5% fetal calf serum (HyClone, Logan, UT), 0.07 mg/ml penicillin-G potassium, and 0.14 mg/ml streptomycin sulfate in a 5% CO₂ atmosphere at 37 °C. For the experiments, cells were seeded at a density of 30,000 cells/cm² on plastic dishes and cultured for 3 days. They were then incubated with normal (330 mOsm/Kg H₂O) or hypotonic (165 mOsm/Kg H₂O) medium for the indicated period. Isotonic medium was prepared by adding mannitol to the hypotonic medium. The osmolarity of the medium was measured using an Osmostat OM-6020 (Kyoto Daiichi Kagaku, Kyoto, Japan).

Plasmid DNA construction and transfection --- Human claudin-1 and -2 cDNAs containing the entire open reading frame were amplified by reverse transcriptase-polymerase chain reaction (PCR) using mRNA from human kidney. The cDNAs were subcloned into the mammalian expression vector, pCMV-Tag2 containing the FLAG epitope. The mutants of claudin-1 (T191A and T191E) and claudin-2 (S208A and S208E) were generated using a KOD-Plus-Mutagenesis kit (Toyobo Life Science, Osaka, Japan) and the primers pairs described in table 1. Clathrin heavy chain and caveolin siRNAs were produced by Sigma-Aldrich. As a control, nontargeting control siRNA (Santa Cruz) was used. Plasmid vectors and siRNAs were transfected into cells using Lipofectamine 2000 as recommended by the manufacturer.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting --- Preparation of cell lysates, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were carried out as described previously (18). The primary and secondary antibodies were used at dilution of 1:1000 and 1:5000, respectively. The blots were scanned using a C-DiGit Blot Scanner (LI-COR Biotechnology, Lincoln, NE). Band density was quantified using ImageJ software (National Institute of Health software, Bethesda, MD). β-actin was used for normalization. In each experiment, three times of similar trials were performed and the data were averaged (n = 1), and such an experiment
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was independently repeated at least three times (n ≥ 3).

RNA isolation and quantitative real-time PCR --- Total RNA was isolated from MDCK II cells as described previously (19). Reverse transcription was carried out using a ReverTraAce qPCR RT Kit (Toyobo Life Science). PCR was carried out with DNA Engine Dyad Cycler (Bio-Rad, Hercules, CA) using Go Taq DNA polymerase (Promega, Madison, WI) and primers pairs for canine claudin-1, claudin-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1). Quantitative real-time PCR was carried out using a Thermal cycler Dice Real Time PCR System (Takara, Shiga, Japan) with FastStart universal SYBR Green Master (Roche Applied Sciences, Mannheim, Germany). The threshold cycle (Ct) for each individual PCR product was calculated using the instrument’s software, and Ct values obtained for claudin were normalized by subtracting the Ct values obtained for GAPDH. The resulting ΔCt values were then used to calculate the relative change in mRNA expression as a ratio (R) of the mRNA expression of hypotonic-treated samples to the mRNA expression of control samples in accordance with the equation 

\[ R = 2^{(\Delta C_t(\text{chemical treatment}) - \Delta C_t(\text{control}))} \]

Experiments were done triplicated and repeated at least three times, independently.

Measurement of TER --- MDCK II cells were plated at confluent densities on transwells with polyester membrane inserts (Corning Incorporated-Life Sciences, Acton, MA). TER was measured as described previously (18). In each experiment, three samples were used and the data were averaged (n = 1), and such experiment was independently done four times (n = 4).

Confocal microscopy --- MDCK II cells were immunostained as described previously (20). Immunolabeled cells were visualized using an LSM 700 confocal microscope (Carl Zeiss, Germany) set with an ×100 objective and a filter appropriate for Alexa Fluor 488 (488 nm excitation, 530 nm emission) and Alexa Fluor 546 (561 nm excitation, 585-615 nm emission). The scanning range is 64 μm × 64 μm. Representative images from 3-4 independent experiments are shown. The staining area of claudins, Rab7, and LAMP-1 was marked manually and analyzed in 10 different samples using ImageJ software. Similarly, the claudins-stained area co-localizing with Rab7 or LAMP-1 was marked and analyzed. After subtraction of background, the area of co-localization per cell was represented as a percentage of the total area of claudins.

Statistics --- Results are presented as the mean ± S.E.M. Differences between groups were analyzed using one-way analysis of variance, and corrections for multiple comparison were made using Tukey’s multiple comparison test. Comparisons between two groups were made using Student’s t test. Significant differences were assumed at P < 0.05.

Conflict of interest --- The authors declare that they have no conflicts of interest with
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the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author Contributions --- N. F. and Y. M. carried out main experiments and analyzed the data. T. M., S. E., H. S., M. Y., Y. Y., and J. S. contributed to the discussion of the manuscript. A. I. contributed to supervision of the project, interpretation of the data and writing the paper. All authors reviewed the results and approved the final version of the manuscript.

The abbreviations used are: CQ, chloroquine; CHX, cycloheximide; Ct, threshold cycle; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JNK, c-Jun amino-terminal kinase; Lac, lactacystin; MAPK, mitogen-activated protein kinase; MβCD, methyl-β-cyclodextrin; MDC, monodansylcadaverine; MDCK, Madin-Darby canine kidney; OA, okadaic acid; PKC, protein kinase C; PP, protein phosphatase; p-Ser, phosphoserine, p-Thr, phosphothreonine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TER, transepithelial electrical resistance; TJs, tight junctions; ZO, zonula occludens.

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FIGURE LEGENDS

FIGURE 1. Decrease in claudin-1 and -2 expression levels by hypotonic stress. HK-2 (A) and MDCK II cells (B) were incubated with hypotonic medium for the indicated period. (C) MDCK II cells were incubated with isotonic medium prepared by the addition of mannitol to the hypotonic medium. Cell lysates were immunoblotted with anti-claudin-1, anti-claudin-2, or anti-β-actin antibodies. The band densities of claudins are expressed relative to the value at 0 h. n = 3-4 (triplicate × 3-4 times). ** P < 0.01 and NS P > 0.05 compared with 0 h.

FIGURE 2. Involvement of p38 in the down-regulation of claudins by hypotonic stress. (A) MDCK II cells were incubated with hypotonic medium in the presence and absence of 10 μM U0126 or 10 μM Go6983 for 6 h. Cell lysates were immunoblotted with anti-claudin-1, anti-claudin-2, or anti-β-actin antibodies. The band densities of claudin-1 and -2 are expressed relative to the value in the control. (B) Cells were incubated with hypotonic medium for the indicated period. The cell lysate was immunoblotted with anti-p-JNK, anti-JNK, anti-p-p38, or anti-p38 antibodies. The band densities of p-JNK and p-p38 are expressed relative to the value at 0 min. (C) Cells were incubated with hypotonic medium in the presence and absence of 10 μM SP600125 or 10 μM SB202190 for 6 h. Cell lysates were immunoblotted with anti-claudin-1, anti-claudin-2, or anti-β-actin antibodies. The band densities of claudins are expressed relative to the value in the control. (D) Cells were cultured on transwells and incubated with isotonic medium (control), hypotonic medium, or hypotonic medium plus 10 μM SB202190 (SB) for the indicated period. TER was measured using volt ohmmeter. n = 4 (triplicate × 4 times). ** P < 0.01 and NS P > 0.05 compared with control or 0 h. ## P < 0.01 compared with vehicle.

FIGURE 3. Effect of hypotonic stress on mRNA levels of claudins. MDCK II cells were incubated with hypotonic medium for the indicated period. After isolation of mRNA, semi-quantitative (upper images) and quantitative RT-PCR (lower bar graph) were performed using primer pairs for claudin-1, claudin-2, and GAPDH. The expression levels of mRNA are represented relative to the values at 0 h. n = 4 (triplicate × 4 times). ** P < 0.01 and NS P > 0.05 compared with 0 h.

FIGURE 4. Acceleration of the decrease in claudin levels by hypotonic stress. MDCK II cells were incubated with isotonic or hypotonic medium in the presence of 3 μM CHX for the indicated period. Cell lysates were immunoblotted with anti-claudin-1 (A) or anti-claudin-2 (B) antibodies. The band densities of claudins are expressed relative to the value at 0 h. n = 3-4 (triplicate × 3-4 times). * P < 0.05, ** P < 0.01 and NS P > 0.05 compared with isotonic medium.
Hypotonic stress decreases claudin expression

FIGURE 5. Inhibition of hypotonicity-induced decrease in claudin expression levels by CQ, MDC, and dynasore. MDCK II cells were incubated with hypotonic medium in the presence and absence of Lac (A), CQ (B), MβCD (C), MDC (D), or dynasore (E) for 6 h. Cell lysates were immunoblotted with anti-claudin-1, anti-claudin-2, or anti-β-actin antibodies. The band densities of claudins are expressed relative to the values in the control. n = 3-4 (triplicate × 3-4 times). ** P < 0.01 compared with control. ## P < 0.01, # P < 0.05 and NS P > 0.05 compared with hypotonic alone.

FIGURE 6. Inhibition of hypotonicity-induced decrease in claudin expression levels by clathrin siRNA. (A and B) MDCK II cells were transfected with siRNA for clathrin heavy chain (+siCla) or caveolin (+siCav). Negative siRNA was used as a control (-). At 48 h after transfection, the cells were incubated with hypotonic medium for 6 h. Cell lysates were immunoblotted with anti-claudin-1, anti-claudin-2, or anti-β-actin antibodies. The band densities of claudins are expressed relative to the values in the control. n = 4-5 (triplicate × 4-5 times). ** P < 0.01 compared with control. ## P < 0.01 and NS P > 0.05 compared with hypotonic alone.

FIGURE 7. Effect of hypotonic stress on the intracellular localization of claudins. (A) MDCK II cells were incubated with isotonic or hypotonic medium in the presence and absence of 10 μM Lac or 20 μM CQ for 6 h. Then, the cells were double stained with anti-ZO-1 (green) and anti-claudin-1 (red) or anti-claudin-2 (red) antibodies. (B) Cells were treated with hypotonic medium in the presence 20 μM CQ for 6 h. Then, the cells were double stained with anti-LAMP-1 (green) and anti-claudin-1 (red) or anti-claudin-2 (red) antibodies. (C) Cells were incubated with isotonic or hypotonic medium in the presence and absence of 10 μM MβCD or 5 μM MDC for 6 h. Then, the cells were double stained with anti-ZO-1 (green) and anti-claudin-1 (red) or anti-claudin-2 (red) antibodies. The scale bar represents 10 μm. Representative images from 3-4 independent experiments are shown.

FIGURE 8. Effect of hypotonic stress on the intracellular localization of Na⁺/K⁺-ATPase and Na⁺/H⁺-exchanger. (A) MDCK II cells were incubated with isotonic or hypotonic medium for 6 h. The cells were stained with anti-Na⁺/K⁺-ATPase antibody (green). (B) Cells were incubated with isotonic or hypotonic medium in the presence and absence of 10 μM SB202190 or 5 μM MDC for 6 h. Then, the cells were stained with anti-Na⁺/H⁺-exchanger antibody (red). The scale bar represents 10 μm. n = 4 (triplicate × 4 times).

FIGURE 9. Effect of phosphorylation on hypotonicity-induced decrease in claudin expression levels. (A) MDCK II cells were incubated with hypotonic medium for the
indicated period. Cell lysates were immunoprecipitated with anti-claudin-1 or anti-claudin-2 antibodies. The immune pellets were immunoblotted with anti-claudin-1, anti-claudin-2, anti-p-Ser, or anti-p-Thr antibodies. The phosphorylation levels are expressed relative to the value at 0 h. (B) Cells were incubated with hypotonic medium for 6 h in the presence and absence of 1 µM OA or 0.5 µM cantharidin (CAN). Cell lysates were immunoblotted with anti-claudin-1, anti-claudin-2, or anti-β-actin antibodies. The band densities of claudins are expressed relative to the values in the control. n = 4-5 (triplicate × 4-5 times). ** P < 0.01 compared with control or 0 h.

**FIGURE 10. Effect of phosphorylation on the stability and intracellular localization of claudins.** MDCK II cells were transfected with wild-type (WT), T191A, or T191E of claudin-1 and WT, S208A, or S208E of claudin-2 expression vectors. (A and B) After incubation with isotonic or hypotonic medium for 6 h, cell lysates were immunoblotted with anti-FLAG or anti-β-actin antibodies. The band densities of FLAG under hypotonic conditions are expressed relative to the value in the control. (C and D) Cells were incubated with 3 µM CHX for the indicated period. The cell lysate was immunoblotted with anti-FLAG or anti-β-actin antibodies. (E and F) The cells were double stained with anti-ZO-1 (green) and anti-FLAG (red) antibodies. The scale bar represents 10 µm. n = 3 (triplicate × three times). ** P < 0.01, * P < 0.05, and NS P > 0.05 compared with WT.

**FIGURE 11. Effect of hypotonic stress on the intracellular localization of claudin mutants.** (A and B) MDCK II cells were transfected with T191A of claudin-1 or S208A of claudin-2 vector. Cells were incubated with hypotonic media in the presence and absence of 10 µM SB202190 (SB) for 6 h, then stained with anti-FLAG (red) and anti-Rab7 (green) or anti-LAPM-1 (green) antibodies. The scale bar represents 10 µm. (C and D) The localization of claudins in endosomes (Rab7) or lysosomes (LAMP-1) is shown as percentage. n = 3 (triplicate × three times). ** P < 0.01 and * P < 0.05 compared with isotonic medium. ## P < 0.01 compared with hypotonic alone.

**FIGURE 12. A tentative scheme for the decrease in claudin-1 and -2 expression levels caused by hypotonic stress.** Hypotonic stress may activate p38, PP1, and PP2A. Dephosphorylated claudin-1 and -2 are transported from the plasma membrane to the cytosol mediated by the clathrin-dependent endocytosis pathway. Internalized claudins are degraded in lysosomes.
Table 1. Primers for PCR amplification and point mutation

| Name   | Sequence                                                                 |
|--------|--------------------------------------------------------------------------|
| Claudin-1-S | 5’-ATCTACTCTCGTACGCGGCGGCGACAAC-3’                                     |
| Claudin-1-A | 5’-AGCAGCGAGTGAAGACCTGTCAC-3’                                         |
| Claudin-2-S | 5’-GCACAGGCATAACCCAGTGT-3’                                              |
| Claudin-2-A | 5’-GACAATGCAGGCCCAACGAAG-3’                                            |
| GAPDH-S  | 5’-ACGGCACAGTCAGGCTGAG-3’                                               |
| GAPDH-A  | 5’-CAGCATCACCCCATTTGATGTTG-3’                                           |
| T191A-S  | 5’-GCCTCTTACCCCAACACCAAGGACCCTATC-3’                                   |
| T191A-A  | 5’-TGTTTTTCGCGGAACAGGAACAGCAA-3’                                       |
| T191E-S  | 5’-GAATCTTACCCCAACACCAAGGACCCTATC-3’                                   |
| T191E-A  | 5’-TGTTTTTCGCGGAACAGGAACAGCAA-3’                                       |
| S208A-S  | 5’-CCTCTTCCACAGGAGCGCTCAGGCAGCCCTGTTCAAACCTC-3’                        |
| S208A-A  | 5’-GAGGTGACCCCGCTTGCAGAGCTCCTTGTAACACCTC-3’                           |
| S208E-S  | 5’-CCTCTTCCACAGGAGCGCTCCTTGTAACACCTC-3’                               |
| S208E-A  | 5’-GAGGTGACCCCGCTTGCAGAGCTCCTTGTAACACCTC-3’                           |
Figure 1  Fujii et al.
Figure 2  Fujii et al.

A) Hypotonic treatment of Claudin-1 and Claudin-2 expression in Control, Vehicle, U0126, and Go6983 conditions.

B) Time-dependent change in p-JNK, JNK, p-p38, and p38 expression following hypotonic treatment.

C) Regulation of Claudin-1 and Claudin-2 expression by SP600125 and SB202190 in hypotonic conditions.

D) Change in TER (Ω cm²) over time under Control, Hypotonic, and Hypotonic + SB conditions.
Figure 3  Fujii et al.

Hypotonic

0 2 4 (h)

Claudin-1
Claudin-2
GAPDH

Relative expression (Claudin/GAPDH)

0 0.5 1.0 1.5 2.0 2.5 3.0 3.5

Claudin-1
Claudin-2

0 2 4 (h)

Hypotonic

NS

0 2 4 (h)

Hypotonic

NS

NS

NS

**
Figure 4  Fujii et al.

A

Isotonic

Hypotonic

CHX

0 1 2 3 (h)

Claudin-1

Relative expression

(Claudin-1/β-actin)

0 0.5 1.0 1.5

0 1 2 3 (h)

CHX

0 1 2 3 (h)

Isotonic

Hypotonic

Claudin-2

Relative expression

(Claudin-2/β-actin)

0 0.5 1.0 1.5

NS NS

Claudin-1

Claudin-2

A

B

Downloaded from http://www.jbc.org/ by guest on March 22, 2020
Figure 5 Fujii et al.

A  

- + + +  Hypotonic Lac (µM)
- - + +  Claudin-1  
- - 1 10  Claudin-2  
- -  Claudin-2  
- -  β-actin  

B  

- + + +  Hypotonic CQ (µM)
- - + +  Claudin-1  
- - 2 20  Claudin-2  
- -  Claudin-2  
- -  β-actin  

C  

- + + +  Hypotonic MβCD (µM)
- - + +  Claudin-1  
- - 1 10  Claudin-2  
- -  Claudin-2  
- -  β-actin  

D  

- + + +  Hypotonic MDC (µM)
- - + +  Claudin-1  
- - 0.5 5  Claudin-2  
- -  Claudin-2  
- -  β-actin  

E  

- + + +  Hypotonic Dynasore (µM)
- - + +  Claudin-1  
- - 50 100  Claudin-2  
- -  Claudin-2  
- -  β-actin  

Relative expression (Claudin/β-actin)

0 0.5 1.0 1.5

- + + +  Hypotonic Lac (µM)
- - + +  Claudin-1  
- - 1 10  Claudin-2  
- -  Claudin-2  
- -  β-actin  

- + + +  Hypotonic CQ (µM)
- - + +  Claudin-1  
- - 2 20  Claudin-2  
- -  Claudin-2  
- -  β-actin  

- + + +  Hypotonic MβCD (µM)
- - + +  Claudin-1  
- - 1 10  Claudin-2  
- -  Claudin-2  
- -  β-actin  

- + + +  Hypotonic MDC (µM)
- - + +  Claudin-1  
- - 0.5 5  Claudin-2  
- -  Claudin-2  
- -  β-actin  

- + + +  Hypotonic Dynasore (µM)
- - + +  Claudin-1  
- - 50 100  Claudin-2  
- -  Claudin-2  
- -  β-actin  

- + + +  Hypotonic Lac (µM)
- - + +  Claudin-1  
- - 1 10  Claudin-2  
- -  Claudin-2  
- -  β-actin  

- + + +  Hypotonic CQ (µM)
- - + +  Claudin-1  
- - 2 20  Claudin-2  
- -  Claudin-2  
- -  β-actin  

- + + +  Hypotonic MβCD (µM)
- - + +  Claudin-1  
- - 1 10  Claudin-2  
- -  Claudin-2  
- -  β-actin  

- + + +  Hypotonic MDC (µM)
- - + +  Claudin-1  
- - 0.5 5  Claudin-2  
- -  Claudin-2  
- -  β-actin  

- + + +  Hypotonic Dynasore (µM)
- - + +  Claudin-1  
- - 50 100  Claudin-2  
- -  Claudin-2  
- -  β-actin  

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Figure 6  Fujii et al.
### Figure 7 Fujii et al.

| A | ZO-1 | Claudin-1 | Merged | ZO-1 | Claudin-2 | Merged |
|---|------|----------|--------|------|----------|--------|
| Control | ![Control ZO-1](image) | ![Control Claudin-1](image) | ![Control Merged](image) | ![Control ZO-1](image) | ![Control Claudin-2](image) | ![Control Merged](image) |
| Vehicle | ![Vehicle ZO-1](image) | ![Vehicle Claudin-1](image) | ![Vehicle Merged](image) | ![Vehicle ZO-1](image) | ![Vehicle Claudin-2](image) | ![Vehicle Merged](image) |
| Hypotonic | ![Hypotonic ZO-1](image) | ![Hypotonic Claudin-1](image) | ![Hypotonic Merged](image) | ![Hypotonic ZO-1](image) | ![Hypotonic Claudin-2](image) | ![Hypotonic Merged](image) |
| Lac | ![Lac ZO-1](image) | ![Lac Claudin-1](image) | ![Lac Merged](image) | ![Lac ZO-1](image) | ![Lac Claudin-2](image) | ![Lac Merged](image) |
| CQ | ![CQ ZO-1](image) | ![CQ Claudin-1](image) | ![CQ Merged](image) | ![CQ ZO-1](image) | ![CQ Claudin-2](image) | ![CQ Merged](image) |

| B | LAMP-1 | Claudin-1 | Merged | LAMP-1 | Claudin-2 | Merged |
|---|--------|----------|--------|--------|----------|--------|
| Hypotonic | ![Hypotonic LAMP-1](image) | ![Hypotonic Claudin-1](image) | ![Hypotonic Merged](image) | ![Hypotonic LAMP-1](image) | ![Hypotonic Claudin-2](image) | ![Hypotonic Merged](image) |
| CQ | ![CQ LAMP-1](image) | ![CQ Claudin-1](image) | ![CQ Merged](image) | ![CQ LAMP-1](image) | ![CQ Claudin-2](image) | ![CQ Merged](image) |

| C | ZO-1 | Claudin-1 | Merged | ZO-1 | Claudin-2 | Merged |
|---|------|----------|--------|------|----------|--------|
| Control | ![Control ZO-1](image) | ![Control Claudin-1](image) | ![Control Merged](image) | ![Control ZO-1](image) | ![Control Claudin-2](image) | ![Control Merged](image) |
| Vehicle | ![Vehicle ZO-1](image) | ![Vehicle Claudin-1](image) | ![Vehicle Merged](image) | ![Vehicle ZO-1](image) | ![Vehicle Claudin-2](image) | ![Vehicle Merged](image) |
| Hypotonic | ![Hypotonic ZO-1](image) | ![Hypotonic Claudin-1](image) | ![Hypotonic Merged](image) | ![Hypotonic ZO-1](image) | ![Hypotonic Claudin-2](image) | ![Hypotonic Merged](image) |
| MtCD | ![MtCD ZO-1](image) | ![MtCD Claudin-1](image) | ![MtCD Merged](image) | ![MtCD ZO-1](image) | ![MtCD Claudin-2](image) | ![MtCD Merged](image) |
| MDC | ![MDC ZO-1](image) | ![MDC Claudin-1](image) | ![MDC Merged](image) | ![MDC ZO-1](image) | ![MDC Claudin-2](image) | ![MDC Merged](image) |
Figure 8 Fujii et al.
A

**Figure 9 Fujii et al.**

**Relative level**

**Relative expression**

| Hypotonic | 0 | 0.5 | 1 (h) |
|-----------|---|-----|--------|
| IP: Claudin-1 | **** | **** | **** |
| IB: P-Thr | **** | **** | **** |
| IP: Claudin-1 | **** | **** | **** |
| IB: Claudin-1 | **** | **** | **** |

B

**Figure 9 Fujii et al.**

**Relative level**

**Relative expression**

| Hypotonic | 0 | 0.5 | 1 (h) |
|-----------|---|-----|--------|
| IP: Claudin-2 | **** | **** | **** |
| IB: P-Ser | **** | **** | **** |
| IP: Claudin-2 | **** | **** | **** |
| IB: Claudin-2 | **** | **** | **** |

| OA | Hypotonic | 0 | 0.5 | 1 (h) |
|-----|-----------|---|-----|--------|
| - | + | + | - | + | + | - | + | + |

| - | - | + | Hypotonic | OA |
| - | - | + | - | + | + |

| - | - | OA | + | Hypotonic | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + |

| - | - | CAN | + | Hypotonic | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |

| - | - | CAN | + | Hypotonic | OA | 0 | 0.5 | 1 (h) |
| - | - | + | - | + | + | - | + | + |
Figure 12  Fujii et al.

Hypotonic stress

p38 → p38 → P → PP1 → PP2A

Lysosome

Endosome

Tight junction

Clathrin-dependent endocytosis

P Claudin-1

P Claudin-2

Endoplasmic reticulum

Nucleus

Golgi

Claudin-1

Claudin-2
Hypotonic Stress-Induced Down-Regulation of Claudin-1 and -2 Mediated by Dephosphorylation and Clathrin-Dependent Endocytosis in Renal Tubular Epithelial Cells
Naoko Fujii, Yukinobu Matsuo, Toshiyuki Matsunaga, Satoshi Endo, Hideki Sakai, Masahiko Yamaguchi, Yasuhiro Yamazaki, Junko Sugatani and Akira Ikari

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