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High Glucose Induced Translocation of Aquaporin8 to Chicken Hepatocyte Plasma Membrane: Involvement of cAMP, PI3K/Akt, PKC, MAPKs, and Microtubule

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Abstract Aquaporin8 (AQP8) is a transmembrane water channel that is found mainly in hepatocytes. The direct involvement of AQP8 in high glucose condition has not been established. Therefore, this study examined the effects of high glucose on AQP8 and its related signal pathways in primary cultured chicken hepatocytes. High glucose increased the movement of AQP8 from the intracellular membrane to plasma membrane in a 30 mM glucose concentration and in a time- ($\geq$10 min) dependent manner. On the other hand, 30 mM mannitol did not affect the translocation of AQP8, which suggested the absence of osmotic effect. Thirty millimolar glucose increased intracellular cyclic adenosine 3, 5-monophosphate (cAMP) level. Moreover, high glucose level induced Akt phosphorylation, protein kinase C (PKC) activation, p44/42 mitogen-activated protein kinases (MAPKs), p38 MAPK, and c-jun NH_2-terminal kinase (JNK) phosphorylation. On the other hand, inhibition of each pathway by SQ 22536 (adenylate cyclase inhibitor), LY 294002 (PI3-K phosphatidylinositol 3-kinase inhibitor), Akt inhibitor, staurosporine (PKC inhibitor), PD 98059 (MEK inhibitor), SB 203580 (p38 MAPK inhibitor), or SP 600125 (JNK inhibitor) blocked 30 mM glucose-induced AQP8 translocation, respectively. In addition, inhibition of microtubule movement with nocodazole blocked high glucose-induced AQP8 translocation. High glucose level also increased the level of kinesin light chain and dynein protein expression. In conclusion, high glucose level stimulates AQP8 via cAMP, PI3-K/Akt, PKC, and MAPKs pathways in primary cultured chicken hepatocytes. J. Cell. Biochem. 103: 1089–1100, 2008. © 2007 Wiley-Liss, Inc.

Key words: AQP8; high glucose; hepatocytes; PKA; PKC

Aquaporins (AQPs) are either expressed constitutively or regulated, often by agonist-induced trafficking from an intracellular vesicular compartment to the plasma membrane allowing for rapid changes in the membrane permeability, depending upon the physiological requirements [Marinelli et al., 2004]. However, the role of AQPs in hepatobiliary physiology is a topic of only recent interest. Recent reports demonstrated that the epithelial cells of the hepatobiliary system express multiple AQPs that are likely involved in a variety of physiological and cellular functions such as bile formation [Masyuk and LaRusso, 2006]. These observations have both physiological and pathophysiological relevance. Therefore, additional work is required to provide more insights into hepatic epithelial cell physiology and pathophysiology of AQP-mediated water transport. Recently, a high glucose concentration itself has been reported to have a variety of effects on gene expression, insulin secretion, the release of neurotransmittors, and apoptosis [Schroeder et al., 2006]. Excessive glucose levels can also be transported intracellularly and be metabolized. This will result in a change in the redox potential, an increase in the level of sorbitol production through aldose reductase, or an alteration in the signal transduction pathways, such as PI3-K, Akt, and mitogen-activated protein kinase (MAPKs) [Ho et al., 2006]. Also, high glucose activates G protein, which stimulated cyclic

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adenosine 3, 5-monophosphate (cAMP) and then PKA activity [Park et al., 2004]. Previous studies reported the involvement of high glucose condition and AQPs. Antidiabetic drugs, troglitazone and tolbutamide, were observed to suppress AQP3 expression significantly [Asai et al., 2006]. Xenostoma is characterized by oral dryness in diabetic patients and it is because of an impairment of AQP5 translocation [Ishikawa et al., 2006]. AQP7 is expressed in capillary endothelia of adipose tissue (and cardiac and striated muscle) and is upregulated in white adipose tissue (WAT) as a response to streptozotocin-induced diabetes mellitus, which supports its role in glycerol metabolism [Skowronski et al., 2007]. In rats with streptozocin-induced diabetes, the level of AQP9 expression in the liver double that in normal rats, whereas the treatment of diabetic rats with long acting insulin restored the AQP9 level to the control levels [Carbrey et al., 2003; Hara-Chikuma and Verkman, 2006]. However, effect of high glucose on AQP8 (which is found mainly in hepatocytes) is unclear. Therefore, further study is needed to examine AQP8 has relation with high glucose in hepatocytes.

Dietary carbohydrates provide well over one-half of the energy needed for metabolic work, growth, and mechanical work in most warm-blooded animals. Chickens have higher metabolism rate, body temperature and digestion, absorption rate of carbohydrate and amino acid than mammals. And normally chickens have two times higher blood glucose level than mammals. Liver is a major site of lipogenesis in chickens but adipose tissue is a major site of lipogenesis in mammals [Pearce, 1977; Campbell et al., 1991]. A primary culture of hepatocytes has been used in many biophysiological studies on the liver function because it retains many of the liver-specific functions and responds to various hormones through the expression of the liver-specific functions [Lee et al., 2006a]. The primary chicken hepatocytes culture system used in this study also retains the in vitro differentiated phenotype typical of the liver, including albumin expression [Hou et al., 2001], P450 1A induction [Hou et al., 2001], tyrosine aminotransferase expression [Sasaki et al., 2000], and ascorbate recycling [Sasaki et al., 2001]. Therefore, this study examined the effect of high glucose levels on AQP8 and its related signaling pathways in primary cultured chicken hepatocytes.

MATERIALS AND METHODS

Materials

Two-week-old male White Leghorn chickens were obtained from the Dae Han Experimental Animal Co., Ltd. (Chungju, Korea). All the procedures for animal management were performed according to the standard operation protocols at Seoul National University. The appropriate management of the experimental samples and quality control of the laboratory facility and equipment were maintained. The Class IV collagenase and soybean trypsin inhibitor were purchased from Life Technologies (Grand Island, NY). The fetal bovine serum was purchased from Gibco (Rockville, MD). The SQ 22536, LY 294002, Akt inhibitor, PD 98059, SB 203580, SP 600125, staurosporine, 8 bromoadenosine 3′, 5′-monophosphate, and monoclonal anti-β-actin were obtained from Sigma Chemical Company (St. Louis, MO). The phospho-Akt(Thr^{308}, Ser^{473}), Akt, pan protein kinase C (PKC), PKCα, PKCδ, PKCζ, phospho-p44/42 MAPKs, p44/42 MAPKs, phospho-p38 MAPK, p38 MAPK, phospho-SAPK/c-jun NH_2-terminal kinase (JNK), SAPK/JNK, AQP8, dynein, and KLC1 (kinesin light chain) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The liquiscint was obtained from National Diagnostics (Parsippany, NY). The goat anti-rabbit IgG was purchased from Jackson Immunoresearch (West Grove, PA). All the other reagents were of the highest purity commercially available.

Primary Culture of Chicken Hepatocytes

The chicken liver cells were prepared and maintained as a monolayer culture, as described elsewhere [Hou et al., 2001]. Briefly, the chicken hepatocytes were isolated by perfusion with 0.05% collagenase from a liver that had been starved for 3 h. Hepatocytes with >90% viability, as verified by a trypan blue exclusion test, were used for subsequent plating. The hepatocytes were plated (5.0×10^5 cells/60-mm collagen-coated dish) with an incubation medium (Basal Medium Eagle supplemented with essential amino acids), containing 75 U/ml penicillin and 75 U/ml streptomycin, 1 μg/ml insulin, 10^{-12} M dexamethasone, 5 μg/ml transferrin, 10^{-8} M 3,5,3′-triiodi-L-thyronine (T3), and 5% calf serum, and incubated for 4 h at 37°C in 5% CO_2. In the

previous studies, primary cultured chicken hepatocytes [Sasaki et al., 2000; Hou et al., 2001; Lee et al., 2006a, 2006b], cultured cells from the ovarian surface epithelium (OSE) layer of the hen [Giles et al., 2006], and chicken renal proximal cells [Johnston and Laverty, 2007] were cultured at 37°C. In our preliminary studies to determine the temperature of culture since chickens have higher body temperature than mammals, we compared difference between 37 and 40°C. However, we did not find out any difference between 37 and 40°C (data not shown). Thus, we cultured primary chicken hepatocytes at 37°C in the present study. The medium was then replaced with a fresh incubation medium, and the hepatocytes were incubated for a further 20 h in order to achieve the monolayer culture. The experiments were then carried out.

Total Membrane Preparation for Western Blotting

The medium was removed and the cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation and resuspended in buffer A (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsufonyl fluoride (PMSF), 10 μg/ml leupeptin, pH 7.5). The resuspended cells were lysed mechanically on ice by trituration with a 21.1-gauge needle. The lysates were first centrifuged at 1,000*g* for 10 min at 4°C. The supernatants were then further centrifuged at 100,000*g* for 1 h at 4°C to prepare the cytosolic and total particulate fractions. The particulate fraction containing the membrane fraction was washed twice and resuspended in buffer A containing 1% Triton X-100. The protein level in each fraction was quantified using the Bradford procedure [Bradford, 1976].

Intracellular or Plasma Membrane Fractions Preparation

Membrane fractions enriched in plasma or intracellular microsomal membranes were prepared from hepatocytes by differential centrifugation as previously described method [Garcia et al., 2001]. Briefly, cells were washed and sonicated in 0.3 M sucrose containing 0.1 mM phenylmethanesulfonyl fluoride and 0.1 mM leupeptin. The plasma membrane fraction was obtained by centrifugation at 200,000*g* for 60 min on a discontinuous 1.3 M sucrose gradient. After removing the plasma membrane band, the sucrose gradient was sonicated, diluted to 0.3 M, and centrifuged at 17,000*g* for 30 min. The resulting supernatant was centrifuged at 200,000*g* for 60 min to yield the intracellular microsomal membrane fraction. The protein level in each fraction was quantified using the Bradford procedure [Bradford, 1976].

Immunofluorescence Confocal Microscopy

After a treatment with 30 mM glucose for 4 h, the hepatocytes were fixed with 3.5% (w/v) paraformaldehyde for 10 min at room temperature (25°C), permeabilized with 0.1% Triton X-100 for 5 min and incubated 1 h at room temperature (25°C) with the rabbit affinity-purified AQP8 antibody (10 μg/ml). After washing, the hepatocytes were incubated with FITC conjugated goat anti-rabbit secondary antibody (10 μg/ml) for 1 h. The fluorescence images were visualized by fluorescence microscopy (Flouview 300, Olympus, Japan).

cAMP Assay

The primary cultured chicken hepatocytes were incubated with 100 μM IBMX (3-isobutyl-1-methylxanthine) for 30 min at 37°C in order to inhibit cAMP degradation. The cells were then incubated with 30 mM glucose for 1 h at 37°C in a humidified 5% CO_2-95% air environment, and homogenized in DMEM containing 4 mM EDTA to inhibit cAMP phosphodiesterase activity using a Polytron PT 1200 (Brinkman Instrument, Westbery, NY). This was followed by 5 min incubation at 100°C. After centrifugation at 900*g* for 5 min, the supernatants were transferred into new tubes and stored at 4°C. The intracellular cAMP levels in the samples were examined using a [^{3}H]-cAMP assay system kit. The values are expressed as picomoles cAMP per milligram of protein.

PKC Assay

The hepatocytes grown in 60-mm plates were incubated with 30 mM glucose for 1 h and washed with ice-cold buffer [10 mM Tris HCl (pH 7.5), 0.25 M sucrose, 0.2 mM $CaCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, and 10 mM benzamidine]. The cells were then separated into the cytosolic and membrane fractions. Each fraction was then assayed for its PKC activity using a PKC enzyme system kit and the results are expressed as picomoles phosphate per milligram protein per minute.

Western Blot Analysis

The cell homogenates (40 μg of protein) were separated by 12 or 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The blots were then washed with tris-buffered saline Tween-20 (TBST) (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20). The membrane was blocked with 5% skimmed milk for 1 h and incubated with the proper primary antibody the dilutions recommended by the supplier. The membrane was then washed, and the primary antibodies were detected with goat anti-rabbit–IgG conjugated to horseradish peroxidase. Antibody incubations were performed at 4°C incubator. The bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, England).

LDH Release

The level of cell injury was assessed by measuring the level of LDH activity. The level of LDH activity in the medium was measured using a LDH assay kit (Iatron Lab, Tokyo, Japan). The amount of LDH released is expressed as a percentage of the control.

Statistical Analysis

The results are expressed as the mean ± the standard error (SE). All the experiments were analyzed by ANOVA. In some experiments, a comparison of the treatment means was made with the control using a Bonnferroni–Dunn test. A P-value < 0.05 was considered significant.

RESULTS

Effect of High Glucose on Translocation of AQP8

In order to examine the effect of high glucose on AQP8 translocation, the cells were incubated with 10–30 mM glucose for 4 h before harvesting for immunoblot analysis. As shown in Figure 1A, 30 mM glucose increased the translocation of AQP8 from the intracellular membrane to the plasma membrane compared with the control (10 mM). Moreover, 30 mM glucose-induced AQP8 translocation increased in a time (≥10 min) dependent manner (Fig. 1B). Using confocal microscopy analysis, it was also observed that 30 mM glucose translocated AQP8 (Fig. 1C). The amount of LDH released was not changed by 30 mM glucose treatment compared with the control (Fig. 1E). High glucose levels affect the osmolarity of medium. Thus, to determine whether the altered osmolarity may have had a further effect on AQP8 translocation, we exmamined the effect of 30 mM mannitol on AQP8 translocation. However, mannitol did not affect AQP8 translocation (Fig. 1D). In the present study, molarity of a basic culture medium was 296 mOsm. After treatment of glucose or mannitol into the 2 ml medium, final molarity of medium was 316 mOsm. In consistent with present observation, mannitol was usually used as osmotic control as shown in previous studies [Park et al., 2004; Yeshao et al., 2005; Duffy et al., 2006; Kim et al., 2006; Robert et al., 2006; Connell et al., 2007].

Involvement of cAMP, PI3 K/Akt, and PKC in High Glucose-Induced Translocation of AQP8

The effect of SQ 22536 (adenylate cyclase inhibitor, 10^{-6} M), LY 294002 (PI3 Kinase inhibitor, 10^{-6} M), and Akt inhibitor (10^{-5} M) were examined to determine the pathways involved in the high glucose-induced translocation of AQP8. All these inhibitors blocked the high glucose-induced translocation of AQP8 (Fig. 2A,C). Thirty millimolar glucose increased the cAMP level (Fig. 2B) as well as level of Akt phosphorylation (Thr 308) after the 1 h treatment, which was blocked by SQ 22536 (Fig. 3A). However, a pretreatment with the Akt inhibitor had no effect on the high glucose-induced increase in the cAMP level (Fig. 3B).

A pretreatment of staurosporine before the 30 mM glucose treatment for 4 h blocked the high glucose-induced translocation of AQP8 (Fig. 4A). Indeed, 30 mM glucose for 1 h increased the PKC activity (Fig. 4B) as well as the translocation of pan PKC and PKCα, δ, and ζ isotypes from the cytosol to the membrane compartment (Fig. 4C). On the other hand, a pretreatment with staurosporine prior to the 30 mM glucose treatment for 1 h did not block the high glucose-induced increase in the cAMP level (Fig. 4D) and Akt phosphorylation (Ser 473) (Fig. 4E).

Involvement of cAMP, PI3 K/Akt, and PKC in High Glucose-Induced Translocation of AQP8

The effects of MAPKs on high glucose-induced AQP8 translocation were examined by pretreating the cells with PD 98059 (MEK

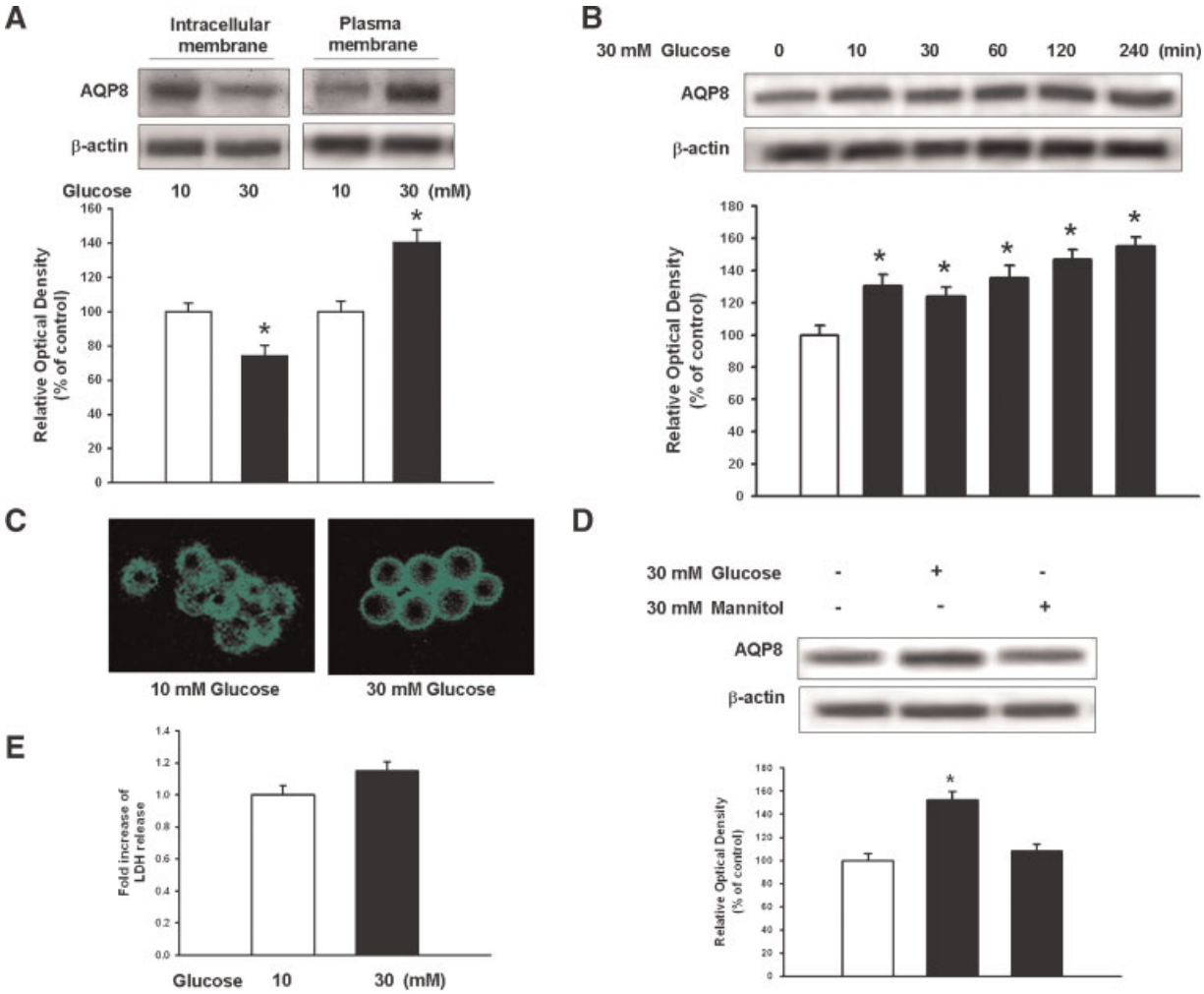

Fig. 1. Effect of high glucose levels on Aquaporin8 (AQP8) translocation. **A**: Chicken hepatocytes were incubated with 10–30 mM glucose for 4 h. The intracellular membrane and plasma membrane proteins were extracted and examined. Bands represent the 34 kDa of AQP8 and 41 kDa of β-actin. The example shown is a representative of four experiments. The **lower panels** denote the means ± SE of four experiments for each condition determined from densitometry relative to β-actin. $^*P < 0.05$ versus control. **B**: The cells were incubated with 30 mM glucose for various time points (0–240 min) and the level of AQP8 in the plasma membrane fraction was determined. The example shown is a representative of four experiments. The **lower panels** denote the means ± SE of four experiments for each condition determined from densitometry relative to β-actin. $^*P < 0.05$ versus control. **C**: The translocation of AQP8 by 30 mM glucose was observed under immunofluorescence confocal microscopy. The cells were incubated with 30 mM glucose for 4 h and then labeled with the anti-AQP8 antibody. **D**: The cells were treated with 30 mM glucose and 30 mM mannitol for 4 h and the level of AQP8 in the plasma membrane fraction was determined. The example shown is a representative of five experiments. The **lower panels** denote the means ± SE of five experiments for each condition determined from densitometry relative to β-actin. $^*P < 0.05$ versus control. **E**: The release of LDH was measured with 10–30 mM glucose, as described in Section ''Materials and Methods.'' The values are reported as a mean ± SE of four experiments with triplicate dishes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

blocker, 10^{-5} M), SB 203580 (p38 blocker, 10^{-6} M), and SP 600125 (JNK inhibitor, 10^{-6} M) for 30 min before the 30 mM glucose treatment for 4 h. As shown in Figure 5A, all these inhibitors blocked the high glucose-induced translocation of AQP8. Thirty millimolar glucose induced the phosphorylation of p44/42 MAPKs, p38 MAPKs, and JNK in a time-dependent manner (≥10 min) (Fig. 5B), which were blocked by the Akt inhibitor (Fig. 5C) and staurosporine (Fig. 5D). Each inhibitor itself did not affect AQP8 plasma membrane amount in heptocytes exposed to 10 mM glucose.

Involvement of Microtubule in High Glucose-Induced Translocation of AQP8

The role of microtubule in AQP8 translocation was examined by pretreating the cells with

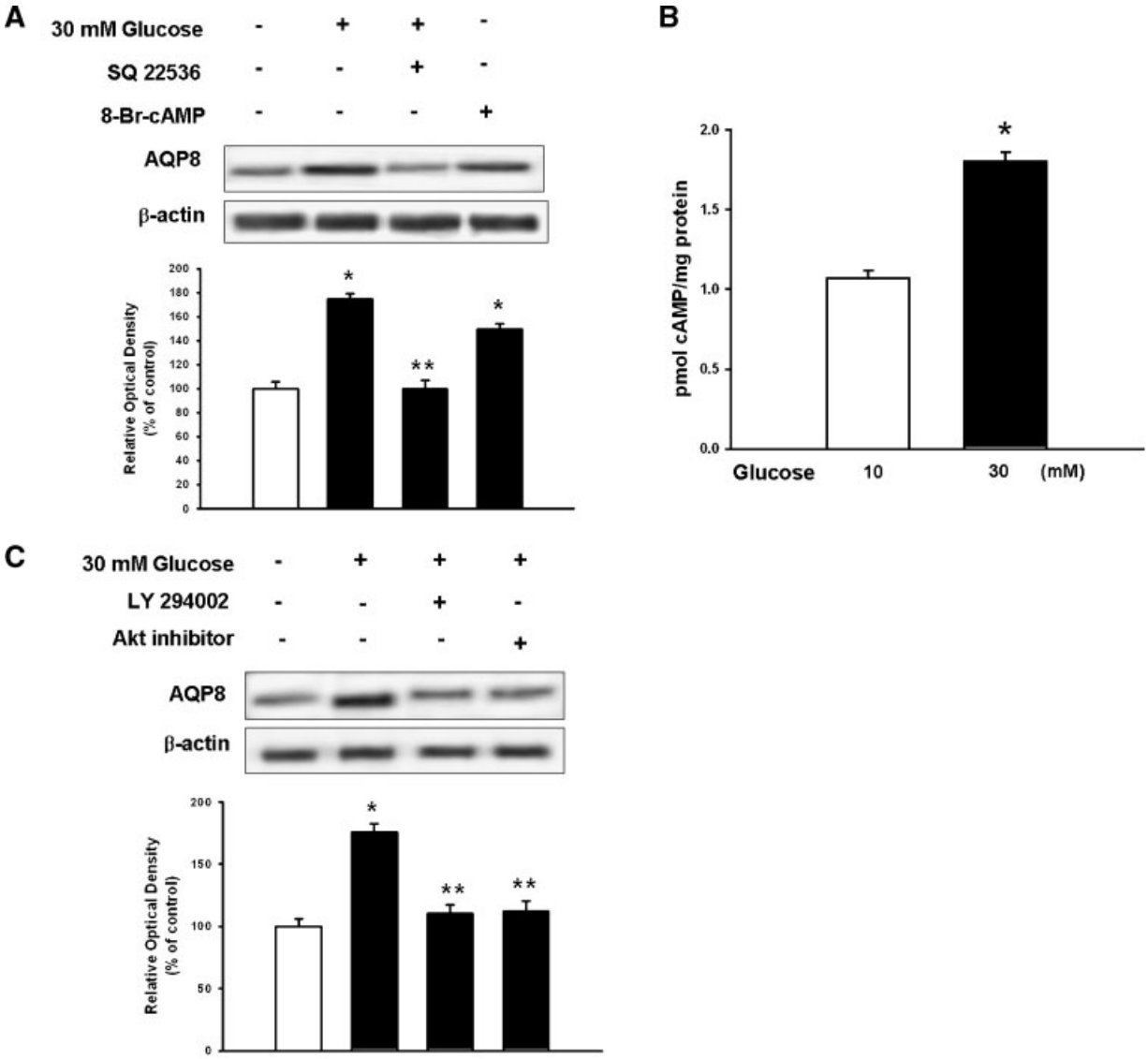

Fig. 2. Effect of cAMP and PI3K/Akt on the 30 mM glucose-induced AQP8 translocation. **A**: The cells were pretreated with SQ 22536 (10^{-6} M) for 30 min before treatment with 30 mM glucose for 4 h. The level of AQP8 in the plasma membrane fraction was determined. The example shown is a representative of four experiments. The **lower panels** denote the means ± SE of four experiments for each condition determined from densitometry relative to β-actin. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus 30 mM glucose alone. **B**: The cells were pretreated with IBMX (10^{-4} M) for 30 min, in order to prevent the degradation of cAMP into 5′-AMP, and treated with 30 mM glucose for 1 h. The cAMP assay was then carried out. The values are reported as a mean ± SE of four independent experiments with triplicate dishes. $^{*}P < 0.05$ versus control. **C**: The cells were pretreated with LY 294002 (10^{-6} M) and Akt inhibitor (10^{-5} M) for 30 min before treatment with 30 mM glucose for 4 h. The level of AQP8 in the plasma membrane fraction was determined. The example shown is a representative of four experiments. The **lower panels** denote the means ± SE of four experiments for each condition determined from densitometry relative to β-actin.$^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus 30 mM glucose alone.

nocodazole (microtubule blocker, 5×10^{-6} M). Nocodazole blocked the 30 mM glucose-induced AQP8 translocation (Fig. 6A). Protein level of kinesin and dynein (motor proteins involved microtubule movement) was increased by 30 mM glucose treatment (Fig. 6B).

DISCUSSION

The major findings reported in this paper are related to the regulatory mechanisms involved in hepatocytes AQP8. To our knowledge, this is the first study on the functional expression and

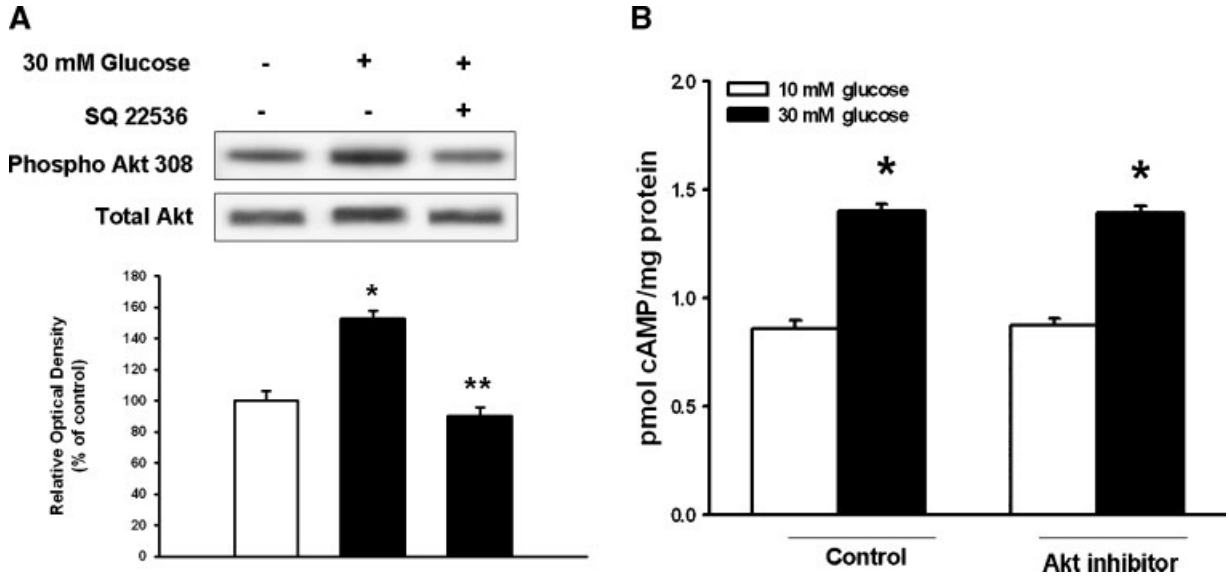

Fig. 3. Effect of cAMP on the Akt phosphorylation. **A**: The level of phosphorylation of Akt were determined after the chicken hepatocytes had been pretreated with SQ 22536 (10^{-6} M) for 30 min before treatment with 30 mM glucose for 1 h. The total protein was extracted and blotted. The example shown is a representative of four experiments. The **lower panels** denote the means ± SE of four experiments for each condition determined from densitometry relative to total Akt. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus 30 mM glucose alone. **B**: The cells were pretreated with IBMX (10^{-4} M) for 30 min in order to prevent the degradation of cAMP into 5′-AMP. They were then pretreated with an Akt inhibitor (10^{-5} M) for 30 min before treatment with 30 mM glucose for 1 h. A cAMP assay was then conducted. The values are reported as the mean ± SE of four independent experiments with triplicate dishes. $^{*}P < 0.05$ versus control.

regulation of the AQP8 protein in avian cells. This biochemical and immunofluorescence study indicates that hepatocyte AQP8 is primarily located within the cell, presumably in a vesicular compartment. Previous reports suggest that AQP8 is involved in water permeability across the canlicular plasma membrane, supporting its potential importance in canalicular bile formation [Carreras et al., 2003; Ferri et al., 2003; Marinelli et al., 2003; Tietz et al., 2005]. Also, AQP8-mediated water transports into and out of mitochondria may be particularly important for control of mitochondrial volume [Calamita et al., 2005]. In the basal state, water transport across the hepatocyte plasma membrane occurs mainly through a non-channel-mediated pathway because AQP8 is sequestered within an intracellular compartment [Gradilone et al., 2005b; Carreras et al., 2007]. In these experiments, 10 mM D-glucose was used as a control because this is equivalent to the serum glucose concentration in normal chickens (200–250 mg/dl) [Pearce, 1977]. Thirty millimolar D-glucose was used as the challenge. Upon exposure to high glucose levels, intracellular AQP8 was relocalized to the plasma membrane. However, increasing the mannitol concentration did not alter the translocation of AQP8 in primary cultured chicken hepatocytes. This suggests that high glucose concentrations do not act through an osmotic challenge but are more likely to act through altered cellular signaling.

In hepatocytes, high glucose levels increase the intracellular cAMP level, which in turn activates PKA. Dibutyryl cAMP (d-cAMP) can induce both AQP8, which is located primarily in the intracellular vesicles, and its relocalization to the plasma membrane [Gradilone et al., 2003]. Others suggested that AQP0 and AQP9 show no change after treatment with d-cAMP, whereas there is a fourfold increase in plasma membrane AQP8 [Huebert et al., 2002]. It was found that 30 mM glucose-induced AQP8 translocation occurs through the cAMP-dependent PKA signal transduction pathway. Moreover, cAMP has also been found to stimulate the vesicle trafficking to the hepatocyte plasma membrane of several other transporters [Garcia et al., 2001]. In addition, high glucose levels can activate hepatocyte PI3K, which mediates several signal transduction pathways in hepatocytes [Gradilone et al., 2005a], including some involved in the regulation of vesicle trafficking

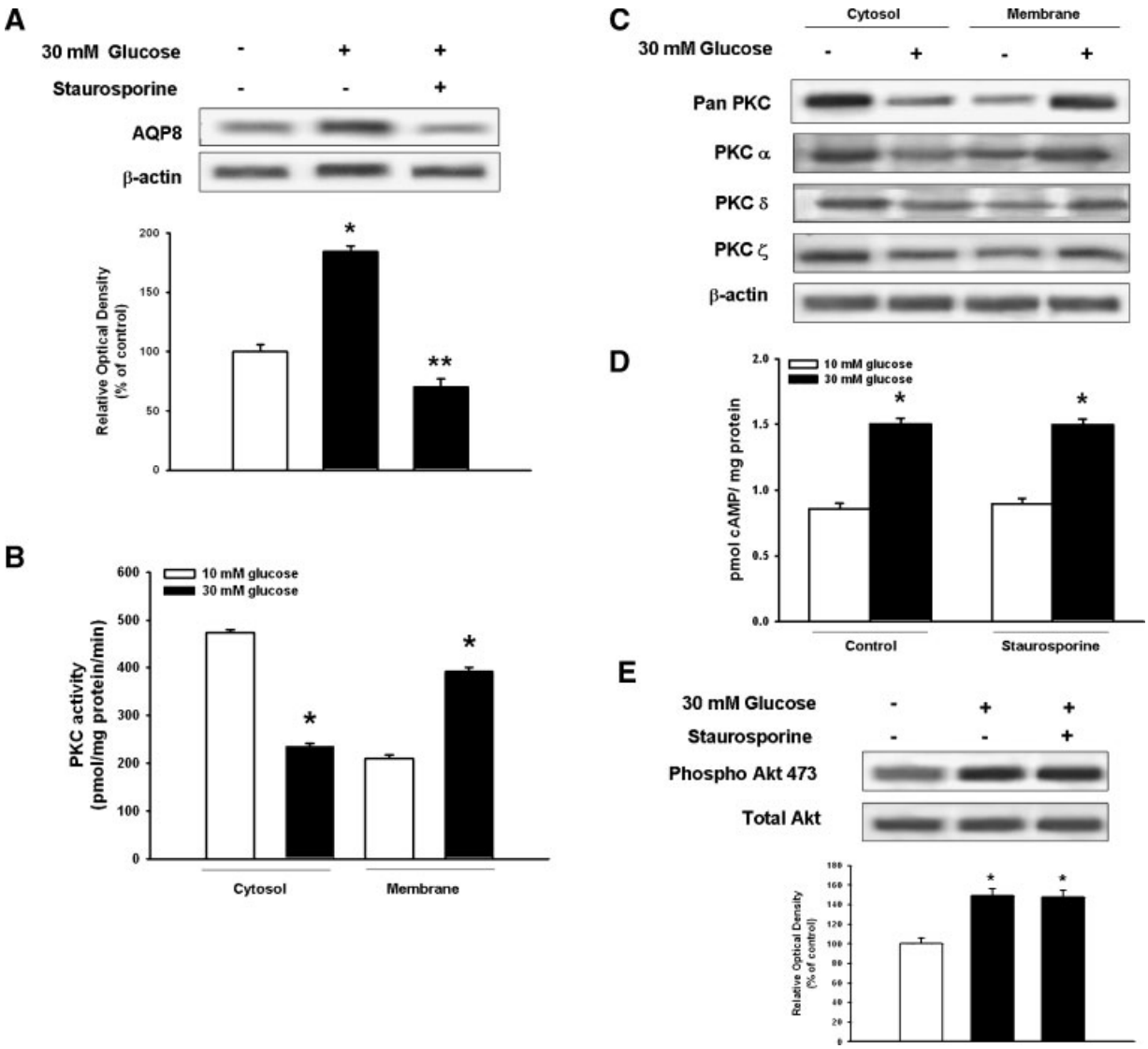

Fig. 4. Effect of PKC on 30 mM glucose-induced AQP8 translocation. **A**: The cells were pretreated with staurosporine (10^{-6} M) for 30 min before treatment with 30 mM glucose for 4 h and then harvested. The level of AQP8 in the plasma membrane fraction was determined. The example shown is a representative of four experiments. The **lower panels** denote the means ± SE of four experiments for each condition determined from densitometry relative to β-actin. $*P < 0.05$ versus control, $**P < 0.05$ versus 30 mM glucose alone. **B**: Effect of the 30 mM glucose on protein kinase C (PKC) activity was measured. The cells were pretreated with 30 mM glucose for 1 h and then scraped off into a tube after being washed with ice-cold PBS and homogenized. With these samples, the PKC activity was measured as described in Section ''Materials and Methods.'' The values are reported as the mean ± SE of four independent experiments with triplicate dishes. $*P < 0.05$ versus control. **C**: The cells were incubated with 30 mM glucose for 1 h and harvested. Pan-PKC, PKCα, -δ, and -ζ isoforms present in either the cytosolic and membrane compartment were detected by Western blotting. The bands represent 80–90 kDa for PKC pan, -α, -δ, and -ζ, and 41 kDa for β-actin. The example shown is a representative of five experiments. **D**: The cells were pretreated with staurosporine (10^{-6} M) for 30 min before treatment with 30 mM glucose for 1 h, a cAMP assay was then carried, and (**E**) the total protein was extracted and blotted with an antibody against Akt 473. The example shown is a representative of four experiments. The **lower panels** denote the means ± SE of four experiments for each condition determined from densitometry relative to total Akt. $*P < 0.05$ versus control. Each inhibitor itself did not affect AQP8 plasma membrane amount in heptocytes exposed to 10 mM glucose.

[Blommaart et al., 1997; Folli et al., 1997; Misra et al., 1999]. Interestingly, cross talk between cAMP and PI3K has been suggested, which is presumably mediated by cAMP activation of the downstream effectors of PI3K [Kagawa et al., 2002; Webster and Anwer, 1999]. In this study, AQP8 translocation was blocked by the cAMP inhibitor (SQ 22536), the PI3K inhibitor (LY

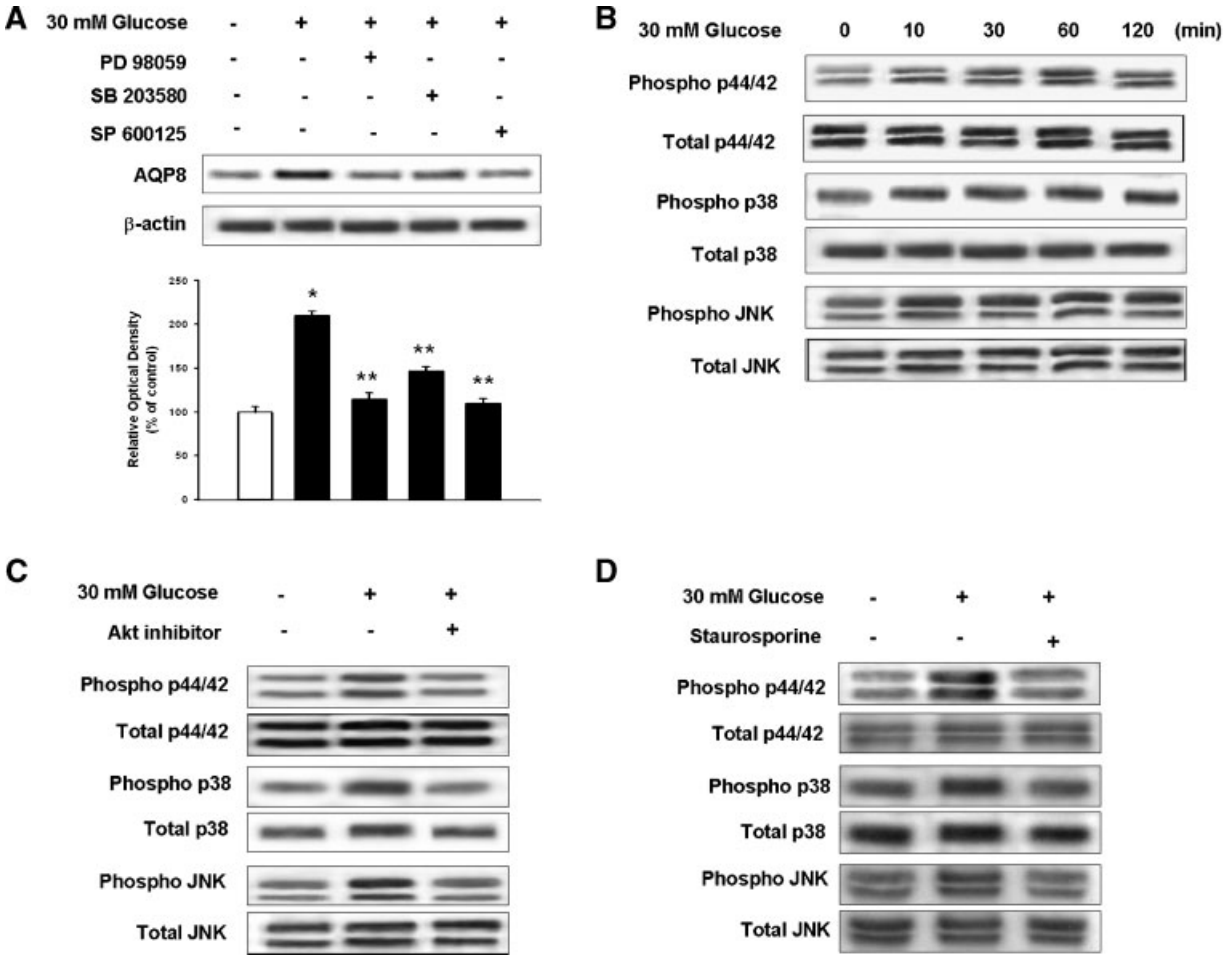

Fig. 5. Effect of the MAPKs on 30 mM glucose-induced AQP8 translocation. **A**: The cells were pretreated with PD 98059 (10^{-5} M), SB 203580 (10^{-6} M), and SP 600125 (10^{-6} M) for 30 min before treatment with 30 mM glucose for 4 h and were then harvested. The level of AQP8 in the plasma membrane fraction was determined. The example shown is a representative of four experiments. The **lower panels** denote the means ± SE of four experiments for each condition determined from densitometry relative to β-actin. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus 30 mM glucose alone. **B**: The time dependent effect of 30 mM glucose on the phosphorylation of p44/42 MAPKs, p38 MAPK, and JNK. The chicken hepatocytes were incubated with 30 mM glucose for 0–120 min and then harvested. The total protein was extracted and the level of p44/42 MAPKs, p38 MAPK, and JNK phosphorylation was determined. The cells were pretreated with the Akt inhibitor (10^{-5} M) (**C**) and staurosporine (10^{-6} M) (**D**) for 30 min before treatment with 30 mM glucose for 1 h. The level of p44/42 MAPKs, p38 MAPK, and JNK phosphorylation was determined. Each example shown is a representative of four experiments.

204002), and an Akt inhibitor. Overall, these results suggest the dual requirement of PKA and PI3K for the high glucose-induced trafficking of AQP8. These observations might reflect the need for co-operative action on a single downstream effector. Consistent with this, it has been reported that cAMP can activate PKB, a downstream PI3K effector, in primary cultured hepatocytes [Webster and Anwer, 1999]. On the other hand, PI3K might be essential for the translocation of some effector molecules to a subcellular localization where PKA exerts its action or is essential for inactivating an inhibitor of PKA activity. The interaction between PKA and PI3K in the regulated trafficking of the ATP-dependent bile acid transporter Bsep to the canalicular membrane domain of hepatocytes has already been suggested [Kagawa et al., 2002]. Therefore, it is possible that the high glucose-induced AQP8 translocation to the hepatocyte plasma membrane involves both the cAMP/PKA and PI3K signaling pathways in a co-operative manner. These results may also suggest that Ca^{2+}-dependent as well as Ca^{2+}-independent PKCs are involved in 30 mM glucose-induced

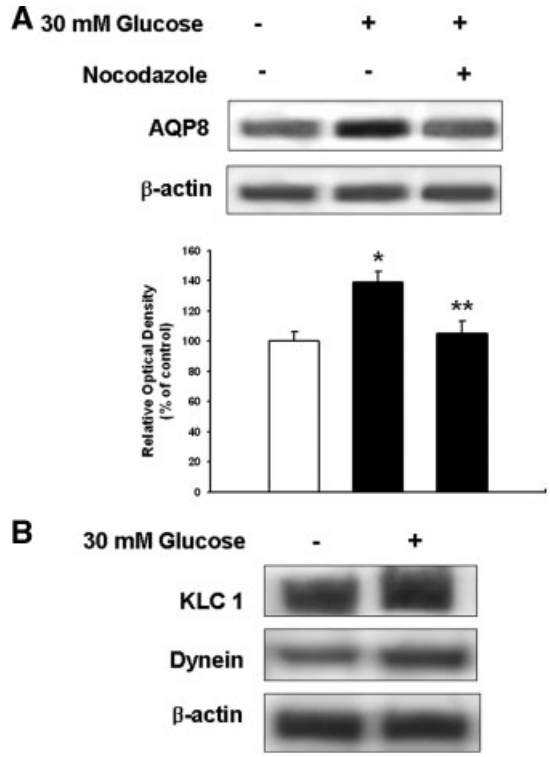

Fig. 6. Involvement of microtubule on AQP8 translocation. (**A**): Cell were pretreated with nocodazole (5×10^{-6} M) for 30 min before treatment with 30 mM glucose for 4 h and were then harvested. The level of AQP8 in the plasma membrane fraction was determined. The example shown is a representative of four experiments. The **lower panels** denote the means ± SE of four experiments for each condition determined from densitometry relative to β-actin. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus 30 mM glucose alone. **B**: The cells were treated with 30 mM glucose for 4 h. The cells were then harvested. The total protein was extracted and blotted with the antibodies against KLC 1, dynein, or β-actin. The bands represent 70 kDa for KLC1, 74 kDa for dynein, and 41 kDa for β-actin. The example shown is a representative of five experiments.

AQP8 translocation. The correlation between PKC and the cAMP/PKA signals was determined by examining the level of cAMP and Akt phosphorylation after a pretreatment with staurosporine. Staurosporine did not block the high glucose-induced increase in the cAMP level and Akt phosphorylation. These results suggest that AQP8 translocation increases through both PKC and cAMP/PKA signal molecules in response to 30 mM glucose, respectively.

In addition, these results provide the link between AQP8 expression and a MAPKs signaling cascade. MAPKs have been implicated in a wide range of cellular events, from growth factor-mediated proliferation to numerous stress responses [Nilssen et al., 2002]. In this study, the Akt inhibitor treatment prior to 30 mM glucose blocked the phosphorylation of p44/42 MAPKs, p38 MAPK, and JNK. This observation suggests significant cross talk between the PI3K and MAPKs pathway. Treatment with the PKC inhibitor before exposing the cells to 30 mM glucose also blocked MAPKs phosphorylation. This observation suggests PKC activated MAPKs phosphorylation like cAMP/PKA as the upstream molecule. The signaling events both upstream and downstream from the MAPKs in this model are currently under investigation. However, the AQP8 molecule lacks consensus protein kinase A phosphorylation sites [Ishibashi et al., 1997], which suggests that the effect of cAMP is not directed towards the AQP8 protein itself but rather to the protein mediators involved in vesicle trafficking. These experiments also show that the high glucose-induced increase in AQP8 translocation appears to be dependent on the integrity of the microtubules, as observed for some of the mentioned solute transporters. Other studies have revealed PI3K to be essential for the microtubule-based transcytosis of the bile acids and targeting of ATP-dependent transporters to the bile canalicular domain [Misra et al., 1998]. A previous study reported the presence of kinesin and dynein in AQP1-containing vesicles is supported by analogous data from cells of the rat renal collecting duct [Tietz et al., 2006]. The co-localization of intracellular vesicles containing AQP2 with the motor proteins dynein and dynactin, and the role they play in agonist-induced trafficking, has been well established [Marples et al., 1998]. Therefore, AQP8 translocation involves the microtubules. However, further study will be needed to provide more insight into the hepatocyte regulatory mechanisms of AQP8 as well as other hepatic AQPs under a high glucose level. In conclusion, this study showed that 30 mM glucose stimulated the AQP8 translocation to plasma membrane via cAMP, PI3K/Akt, PKC, MAPKs, and microtubule pathways in primary cultured chicken hepatocytes.

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