Induction of aquaporin 1 by dexamethasone in lipid rafts in immortalized brain microvascular endothelial cells

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Abstract

Water homeostasis in the brain is essential for brain function. We have studied how aquaporin (AQP) 1 expression in GP8 immortalized rat brain microvascular endothelial cells is regulated by glucocorticoid. AQP1 protein level was raised by dexamethasone treatment in a time- and concentration-dependent manner. The up-regulation of AQP1 protein by dexamethasone was associated with an increase of AQP1 mRNA level, with no change in the degradation rate of AQP1 mRNA. AQP1 was concentrated in detergent–insoluble fractions in the cells treated with or without dexamethasone, suggesting that function/trafficking of AQP1 may be regulated via the interaction with lipid rafts. Since glucocorticoid therapy has well known beneficial effects in the treatment of brain edema, the induction of AQP1 by dexamethasone raises a possibility that AQP1 plays a role in ameliorating brain edema.

Key words: Aquaporin 1, Brain microvessels, Lipid rafts, Caveolin, Glucocorticoid, Edema.

Running title: Glucocorticoid increases AQP1 in brain endothelia

Introduction

Regulation of water permeability across microvessels between blood and brain is essential for brain function, and its disruption by various brain disorders such as stroke, trauma, infection and metabolic disorders causes brain edema [13]. However, the molecular mechanisms of the regulation of water permeability are poorly understood.

Aquaporins (AQPs) are composed of a family of proteins identified as water channels [25]. At least 12 AQP subtypes have been identified in mammals: they share six transmembrane-spanning domains and Asn-Pro-Ala (NPA) sequences which form a water channel pore [1,2]. They have similar molecular weights approximately 30 kDa with amino acid similarity from 20% to 50%. The AQP family is subdivided according to characteristics: water selective channels (AQP1, 2, 4, 5 and 8) and channels transporting glycerol and other small solutes also called aquaglyceroporins (AQP3, 7, 9 and 10). AQP6 may transport chloride at low pH [8]. Each subtype has own cellular distribution and distinct regulatory mechanisms of their expression.

In the brain, AQP4 is mainly localized in astrocyte endfeet facing blood vessels and in ependymal cells, and is also expressed in microvascular endothelial cells at low level [4,31]. AQP1 is expressed in the choroid plexus that may play a role in cerebrospinal fluid formation [32]. The AQP1 expression in brain microvessels has not been detected in many species, whereas very low level of AQP was found in some, not all, samples of rat brain microvascular endothelial cells in primary culture and of the RBE4 rat brain microvessel endothelial cell line. [3,15,22,35]. In sections of normal human brain, low level of AQP1 was detected in the endothelium of a few (<33%) microvessels [20,26]. However, it is not known how its expression is regulated.

Lipid rafts are discrete membrane microdomains enriched in cholesterol and sphingolipids [5,28]. These microdomains provide platforms for signal transduction, and signaling molecules such as receptors, Src family tyrosine kinases, serine/threonine kinases, G proteins and actin binding proteins are recruited to these microdomains upon intra- and extra-cellular stimuli [29]. In addition, lipid microdomains have indispensable function in selection and trafficking of membrane proteins [10]. Caveolae are a subset of lipid raft microdomains characterized by flask-shaped invagination of 50-100 μm in diameter in the plasma membrane [30]. Caveolae are

formed from lipid rafts by polymerization of caveolins, hairpin-like palmitoylated integral membrane proteins that tightly bind cholesterol. Caveolae have a variety of function such as signal transduction, cholesterol transport and endocytosis that are distinct from those performed by the endocytosis pathway mediated by clathrin-coated vesicles. Some AQPs are known to interact with lipid rafts, and their trafficking is regulated by these domains. For example, AQP5 present in lipid rafts in rat parotid glands is translocated to the apical plasma membrane upon the stimulation of acetylcholine receptors of the glands [11]. In rat cardiac myocytes, AQP1 colocalizes with caveolin under isotonic condition [23]. Upon exposure of myocytes to hypertonic medium, AQP1 dissociated from caveolin and internalizes into intracellular compartments. Thus, elucidation of the AQP1 localization in lipid rafts may provide clues for understanding the intracellular trafficking of AQP1 and the regulatory mechanisms of its function.

From the clinical point of view, glucocorticoids are used for the treatment of brain edema especially caused by tumor, but its mechanism of action is not known [24]. Based on these observations, we have studied the expression of AQP1 and its regulatory mechanism by glucocorticoid in the brain microvessels. In addition, to confirm the possibility that the function and intracellular trafficking of AQP1 are regulated by lipid raft microdomains, we have studied whether AQP1 is localized in detergent-insoluble membrane fractions.

Materials and Methods

Materials

The affinity-purified immunoglobulin raised against a synthetic peptide of AQP1 was obtained from Alpha Diagnostic, San Antonio, USA. Anti-Na^+, K^+-ATPase α-subunit was from Upstate Biotechnology, Lake Placid, USA, anti-caveolin 1 was from Transduction Laboratories, Lexington, USA, anti-clathrin light chain was from Santa Crus Biotechnology, Santa Cruz, USA, anti-calnexin and anti-KDEL receptor were from Stressgen, Victoria, Canada, anti-von Willebrand factor was Dako, Glostrup, Denmark, anti-glyceraldehyde-3-phsphate dehydrogenase (GAPDH) was from Chemicon, Temecula, USA, Alexa488-labeled anti-rabbit IgG and Alexa 594-labeled anti-mouse IgG were from Molecular Probes, Eugene, USA. Horseradish peroxidase-labeled anti-rabbit Ig and anti-mouse Ig, PVDF membrane, nylon membrane, Rapid Hybri buffer and electronchemiluminescent reagent (ECL Plus) were from Amersham Pharmacia Biotech, Buckinghamshare, UK. Dexamethasone and Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F12) were from Sigma, St. Louis, USA. G418 was from Calbiochem, La Jolla, USA. Fetal calf serum was from Hyclone, Logan, USA. Trizol, DNase I, oligo$(dT)_{12\text{-}18}$ and reverse transcriptase (Superscript) were from Life Technologies, Tokyo, Japan. Taq polymerase (Ex Taq) was from Takara, Otsu, Japan, anti-Taq antibody from Toyobo, Osaka, Japan. GAPDH probe was from Clontech, Palo Alto, USA. Other chemicals were from Nacalai Tesque, Kyoto, Japan.

Preparation of brain microvessels

Cerebral microvessels were prepared from male Sprague Dawley rats using the albumin flotation and glass bead filtration techniques as described previously [14]. Briefly, the homogenate of cerebral cortices was passed through 700 μm, 200 μm and 100 μm nylon meshes, and centrifuged at 1,000 g for 10 min. The pellet was suspended in Ringer's solution containing 30% bovine serum albumin (BSA) and centrifuged at 1,000 g for 15 min. The resulting pellet was suspended in Ringer's solution containing 1% BSA and applied on a column of glass beads (0.2-0.5 mm in diameter). After washing the column with Ringer's solution, the microvessels attached to the glass beads were released by pipetting and collected by centrifugation.

Culture of GP8 cells

GP8 cells, immortalized rat brain microvascular endothelial cells made by introducing temperature-sensitive SV40 large T antigen [9], were cultured in DMEM/F12 containing 20% fetal calf serum, 2 mM glutamine, 1 ng/ml basic fibroblast growth factor and 200 μg/ml G418 on collagen coated dishes. The protein content was determined by the method of Lowry et al. [18].

RT-PCR and Northern blot

RNA of the cells was isolated by acid guanidine thiocyanate-phenol-chloroform method using Trizol. For reverse transcriptase-polymerase chain reaction (RT-PCR), total RNA (0.25 μg) was reverse transcribed and amplified with 1.25 units of Ex Taq pretreated anti-Taq antibody. The amplification reaction consisted of 22, 25 or 30 cycles of denaturation (94^{o}C, 30 sec), annealing (60^{o}C, 45 sec), and extension (72^{o}C, 60 sec). 5'- And 3'-primers were 89-108 and 432-412 of rat AQP1 (L07268), respectively. PCR products were separated by electrophoresis on 2% agarose gel containing ethidium bromide and photographed. The specificity of RT-PCR was confirmed by sequencing of the PCR product.

For northern blot analysis, 10 μg of total RNA was electrophoresed on 1% agarose gel was transferred to nylon membrane and hybridized with rat AQP1 probe (89-432 nt fragment of L07268) labeled with [α-^{32}P] in Rapid Hybri buffer, and subjected to autoradiography. After removing the probe, the membrane was rehybridized to GAPDH probe. The intensity of the band was estimated by Bio-Imaging analyzer (BAS 2000, Fuji Film, Tokyo, Japan).

Immunoblot

For immunoblot analysis, solubilized samples (20 μg protein) were separated by electrophoresis in 12% polyacrylamide gel containing SDS (SDS-PAGE), and transferred to a PVDF membrane. The membrane was sequentially incubated with anti-AQP1 antibody (2 μg/ml) and horseradish peroxidase-labeled anti-rabbit IgG, and detected by chemiluminescent reaction (ECL).

Immunohistochemical staining

For immunohistochemical staining, cultured cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with 10% goat serum. Samples were doubly stained with anti-AQP1 rabbit polyclonal antibody (2.5 μg/ml) and anti-KDEL receptor mouse monoclonal antibody (1/250) and with Alexa 488-labeled anti-rabbit IgG and Alexa 594-labeled anti-mouse IgG, and observed by fluorescence microscopy. Calnexin and von Willebrand factor were stained with respective rabbit polyclonal antibodies and Alexa 488-labeled anti-rabbit IgG.

Preparation of detergent insoluble fractions

Cultured GP8 cells were detached using PBS containing 0.5 mM EGTA, and collected by centrifugation at 500 g for 5 min. Cells were homogenized in TNE buffer (150 mM NaCl, 5 mM EDTA, 10 μg/ml leupeptin, 100 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 25 mM Tris, pH 7.5) using Dounce homogenizer. Homogenates were centrifuged at 500 g for 5 min. The supernatant was centrifuged at 100,000 g for 1 h and the resulting pellet was suspended in 500 μl TNE buffer, pH 11.0 containing 1% Triton X-100. After incubation on ice for 30 min, the suspension was mixed with the same volume of 80% sucrose, and was overlaid with 1.5 ml TNE buffer containing 36% sucrose and 2 ml 10% sucrose. After centrifugation at 100,000 g for 16 h at 4^{o}C, six fractions (750 μl) were collected from the top, diluted with 3 ml TNE buffer, pH 11.0, and centrifuged at 200,000 g for 30 min. Pellets were suspended in 50 μl of 2x sampling buffer, subjected to SDS-PAGE and detected by immunoblot with ECL detection.

Results

Presence of AQP1 in rat cerebral microvessels

We first examined mRNA and protein levels of AQP1 in the rat cerebral microvessels. PCR product of AQP1 was not detectable at 22 cycles as reported previously [15], whereas very low but significant level of PCR product was detected at 25 cycles in the brain microvessels (Fig. 1a, lane 5). The amount of the PCR product at 30 cycles (lane 6) of the microvessels was almost comparable with that of rat lung at 25 cycles (lane 8). Since PCR product was not detected when reverse transcription was omitted (lane 1-3), PCR products were amplified from cDNA reverse transcribed from mRNA but not from genomic DNA. AQP1 protein was also detected as a very faint band of 28 kDa with a smear band of ~42 kDa of glycosylated form by immunoblot, and its intensity of 25 μg protein was much less than that of 5 μg protein of rat lung (Fig. 1b). Thus, rat brain microvascular endothelial cells express AQP1 mRNA and protein but very low level.

Increase in AQP1 protein level in GP8 cells by glucocorticoid

Since the amount of the microvessels that can be prepared from rats is limited, the subsequent experiments were carried out using GP8, a cell line retaining the properties of the brain microvessels [9]. GP8 cells expressed glycosylated (~42 kDa) and unglycosylated (28 kDa) forms of AQP1, and both forms were increased qualitatively similar manners by dexamethasone. In contrast, protein level of a house keeping gene, GAPDH, was not increased by dexamethasone. Unglycosylated form AQP1 protein level was increased 5-10 fold after 24 hour by dexamethasone in time-dependent manner (Fig. 2a). AQP1 protein level was increased in a concentration-dependent manner by dexamethasone from 10^{-9} M reaching the maximum at 10^{-7} M (Fig. 2b).

Immunohistochemical study showed that weak but significant AQP1 staining was observed in the cell surface as well as in the perinuclear region of the control GP8 cells (Fig. 3). We have tried to identify the intracellular compartment of AQP1. Some parts of AQP1 staining overlapped with staining of KDEL receptor, a marker for Golgi apparatus (Fig. 3, a, b). The intensity of the AQP1 staining was different among cells suggesting that AQP1 expression in the brain endothelial cells may be dependent

on cell differentiation or stage of cell cycle. AQP1 staining in the cells surface and in the perinuclear region was increased by dexamethasone (10^{-6} M) treatment, confirming the increase in AQP1 expression by glucocorticoid observed in the biochemical study (Fig. 3). Calnexin, a maker for endoplasmic reticulum, was also present in the perinulear region, but its co-localization with AQP1 could not be determined because of lack of specific monoclonal antibody against calnexin or AQP1. GP8 cells were stained with von Willebrand factor, an endothelial maker, and its intensity tends to decrease in the presence of dexamethasone.

Induction of AQP1 mRNA in GP8 cells

Since AQP1 protein level increased in the presence of dexamethasone, we studied its mechanism. As shown in Fig. 4a, mRNA level in GP8 cells was increased from 1 hour after the addition of 1 μM dexamethasone reaching the maximum after 24 hour. Degradation rate of AQP1 mRNA was very slow and it was not changed by dexamethasone (Fig. 4b), indicating that glucocorticoid increased AQP1 expression by increasing the transcription of AQP1 gene but not by changing the stability of its transcript.

Localization of AQP1 in detergent insoluble fraction

Lipid raft microdomains including caveolae have unique property of detergent-insolubility and low density, and can be isolated by using density gradient centrifugation. To determine whether AQP1 is present in lipid microdomains, detergent-insoluble fractions were separated. Immunoblot analysis showed that both AQP1 and caveolin 1 distributed in light fractions 2 and 3. The AQP1 level was higher in fraction 2 than in fraction 3 whereas caveolin 1 level was higher in fraction 3 than in fraction 2, suggesting that a part of AQP1 may localize in lipid rafts other than caveolae. Caveolin 1 level was also increased by the treatment with dexamethasone. Na^+, K^+-ATPase and clathrin that localize in non-raft plasma membrane were mainly recovered in fraction 6 and 7, respectively (Fig. 5). Treatment of the cells with 1 μM dexamethasone for 24 hours increased AQP1 level but its distribution in detergent insoluble fraction was unchanged compared to control.

Discussion

In the present study, we found that AQP1 was expressed in isolated rat brain microvessels and in GP8 immortalized brain microvascular endothelial cells. AQP1 was predominantly localized in lipid rafts, and was increased by glucocorticoid. The increase in AQP1 expression was due to the increase in its gene transcription but not due to the increase in stability of the transcript. These results raise a possibility that AQP1 of brain microvessels may have a role in the regulation of water transport between blood and brain in physiological/pathological conditions, and that the beneficial effect of glucocorticoids in the treatment of brain edema may be correlated with the induction of AQP1 in brain microvessels. In addition, the localization of AQP1 in lipid rafts suggests that the function and intracellular trafficking of AQP1 are regulated by lipid microdomains in brain endothelial cells.

Low level of AQP1 in brain microvascular endothelial cells

In the brain, it has been considered that AQP1 is expressed only in choroid plexus but not in endothelial cells in normal human and animal brains. However, AQP1 was expressed in microvessel endothelia in human astrocytoma and metastatic carcinomas [26]. AQP1 was also detected in endothelial cells in glioblastoma transplanted into mouse brain [7]. In addition, AQP1 immunoreactivity was present in the endothelium of a few microvessels in the normal human brain [26], and tissue microarray study showed the presence of AQP1 in the microvessels in the human cerebrum and cerebellum [20]. Furthermore, AQP1 mRNA and protein are present at very low levels in rat brain microvessel endothelial cells, and are reduced by co-culture with rat astrocytes or by the addition of conditioned medium of astrocyte culture [6,16].

In the present study, we found low levels of AQP1 mRNA and protein by RT-PCR, immunoblot and immunohistochemistry (Fig. 1-3). The biochemical and histochemical identification of AQP1 in the present study indicates that the brain microvascular endothelial cells are capable to express AQP1. In addition, the low level of AQP1 expression in basal conditions in brain microvessels suggests that AQP1 is not important in water transport between blood and brain in physiological situations.

Regulatory mechanisms of AQP1 expression

AQP1 is expressed in various types of cell in many organs, and its expression changes in various physiological and pathological conditions. For example, AQP1 in the rat lung first appears late in gestation, increases prior to birth, and is sustained at high level in adult. However, its expression in the choroid plexus is at high level through out prenatal and postnatal life, while in periosteum, endocardium and coronal endothelium it increases transiently before birth and declines after birth [12]. On the other hand, AQP1 expression in the capillary endothelium of the lung increased in acute respiratory distress syndrome patients [17], whereas decreased following adenoviral infection in the mouse [34], which may be correlated with the dysfunction of water homeostasis occurred in these pathological states.

In the present study, expression of AQP1 was increased by dexamethasone by increasing transcription of its gene (Fig. 4). The promoter region of AQP1 gene contains glucocorticoid response elements, and the increased AQP1 expression by glucocorticoids was also observed in the fetal and adult rat lung, fetal kidney, peritoneum and erythroleukemia cells [21,33]. On the other hand, AQP1 expression was not changed by glucocorticoid in the adult kidney, forepaw of fetuses and red blood cells [12]. The increased expression of AQP1 by macrophage migration inhibitory factor (MIF) or tumor necrosis factor (TNF)-α was rather suppressed by glucocorticoids [17]. Thus, despite of the presence of glucocorticoid responsive element in AQP1 gene, AQP 1 expression is regulated in tissue- and cell-specific manners.

Localization of AQP1 in lipid rafts

We have shown AQP1 is present in detergent-insoluble lipid raft fractions. Although caveolin 1 was also recovered in the same fractions, the recovery of these proteins were different; AQP1 was recovered in lighter fractions than caveolin 1 suggesting that a part of AQP1 is localized in non-caveola raft microdomains.

In a biochemical study of the endothelial cells of the rat lung, 70% of AQP1 was found to be located in caveolae and the rest of 30% was in non-caveola plasma membrane [27]. Immunohistochemical study has shown that both of AQP1 and caveolin immunoreactivities are present on the cell surface as a fine punctate staining pattern that overlapped significantly [27]. In addition, AQP1 and caveolin are also present on perinuclear Golgi apparatus. In the present study, we found that AQP1 is located in lipid rafts in biochemical study, and is co-localized with KDEL receptor a

marker for Golgi apparatus, which may suggest that selection and intracellular trafficking of AQP1 is regulated by the interaction with lipid microdomains.

In the rat cardiac myocytes, AQP1 colocalizes with caveolin under isotonic condition [23]. Upon exposure of the myocytes to hypertonic medium, AQP1 was dissociated from caveolin and internalized into the intracellular compartments. These results suggest that the localization of AQP1 in the lipid microdomains is an important factor not only in its intracellular trafficking but also in its internalization.

Possible role of AQPs in the formation and therapy of brain edema

Accumulating evidence suggests that AQPs may play a role both in edema formation and amelioration. For example, in the AQP4 deleted mice, cerebral edema formation in response to water intoxication and ischemic stroke was reduced, and survival and neurological status were improved. On the contrary, in a freeze-injury model of vasogenic brain edema and in a brain tumor edema model, AQP4 deleted mice had worse clinical outcome [19].

The role of AQP1 in the brain microvascular endothelial cells is not clear at present. However, AQP1 is expressed in the endothelial cells in brain tumor [26], which may be involved in the edema formation but also may be involved in a compensatory mechanism to ameliorate edema. The expression of AQP1 was increased by dexamethasone in the present study. Since glucocorticoids are widely used to treat tumor edema, it would be an intriguing possibility that beneficial effects of glucocorticoids in brain edema might be related to the induction of AQP1 in the brain microvascular endothelial cells.

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Legend for figures

Fig. 1 Expression of AQP1 in rat brain microvessels.

a. AQP1 mRNA level in rat brain microvessels (1-6) and lung (7-9) was analyzed by RT-PCR. cDNA reverse transcribed from RNA (0.25 μg) (4-9) or RNA untreated with reverse transcriptase (1-3) was amplified by PCR 22 (1, 4, 7), 25 (2, 5, 8) or 30 (3, 6, 9) cycles. PCR product was separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. Data are from a representative of 3 experiments of different preparations of the tissues. b. AQP1 protein level was analyzed by immunoblot. Microvessels (25 μg of protein) (1) or lung (5 μg of protein) (2) were separated by 12% polyacrylamide gel electrophoresis, transferred to PVDF membrane, reacted with anti-AQP1 and horseradish peroxidase labeled anti-rabbit Ig, and immunoreactivity was detected by ECL. The positions of molecular weights of marker are indicated on kDa in the right.

Fig. 2. Increase in AQP1 protein level in GP8 cells by dexamethasone.

GP8 cells were cultured in the presence of 1 μM dexamethasone for various time (a), or in the presence of various concentrations of dexamethasone for 24 hours (b). Solubilized samples (20 μg protein) were separated by SDS-PAGE, transferred to PVDF membrane, and AQP1 protein levels were analyzed by immunoblot with ECL detection. GAPDH levels were also analyzed as a reference. The relative levels of unglycosylated form of AQP1 are shown in lower panels. Data are mean ± SEM (n=3) of a representative of three separate experiments with similar results.

Fig. 3. Immunohistochemical detection of the increase in AQP1 expression in GP8 cells by dexamethasone.

GP8 cells were cultured in the absence (a) or presence (b) of 1 μM dexamethasone for 1 day, and AQP1 and KDEL receptor (KDEL-R) were doubly stained as described in the text. Note that AQP1 staining was stronger in the cells cultured with dexamethasone than in control. Perinuclear region positive to AQP1 was also positive to KDEL-R, a marker for Golgi apparatus (arrows). Calnexin and von Willebrand factor (vWF) were also stained as markers for endoplasmic reticulum and for endothelial cell, respectively. Bar, 100 μm.

Fig. 4. Increase in AQP1 mRNA and its degradation rate in GP8 cells treated with dexamethasone.

GP8 cells were cultured in the presence of 1 μM dexamethasone and AQP1 and GAPDH mRNA levels were analyzed by northern blot (a). Degradation rate of AQP1 mRNA of the cells cultured in the presence (lower panel) or absence (upper panel) of dexamethasone (1 μM) for 3 hours was measured by northern blot of the cells incubated with actinomycin D (10 μg/ml) for periods indicated. The relative levels of AQP1 mRNA are shown in the bottom. ○, control; ●, dexamethasone. Data are from a representative of three separate experiments with similar results.

Fig. 5. Localization of AQP1 in lipid rafts.

Membrane fraction of GP8 cells cultured without (a) or with (b) dexamethasone (1 μM) was treated with 1% Triton X-100 on ice for 30 min, and separated by discontinuous sucrose density gradient centrifugation (10%, 36% and 40%) at 100,000 g for 16 h at 4^{o}C. Fractions were sequentially collected from the top (1) to the bottom (6), and the precipitate at the bottom was numbered 7. Each fraction was diluted with TNE (pH 11) and was precipitated by centrifugation. Samples were subjected to SDS-PAGE for immunoblot. Data are from a representative of three separate experiments with similar results.

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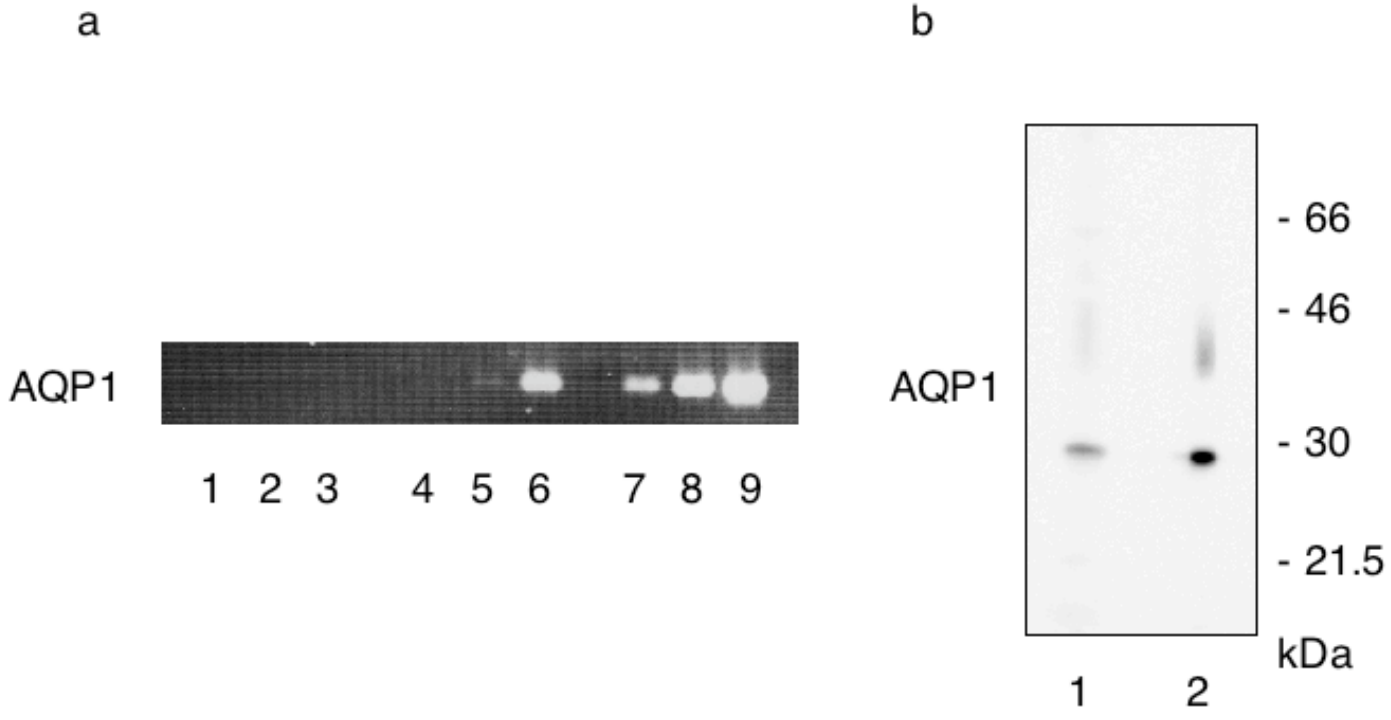

Fig. 1

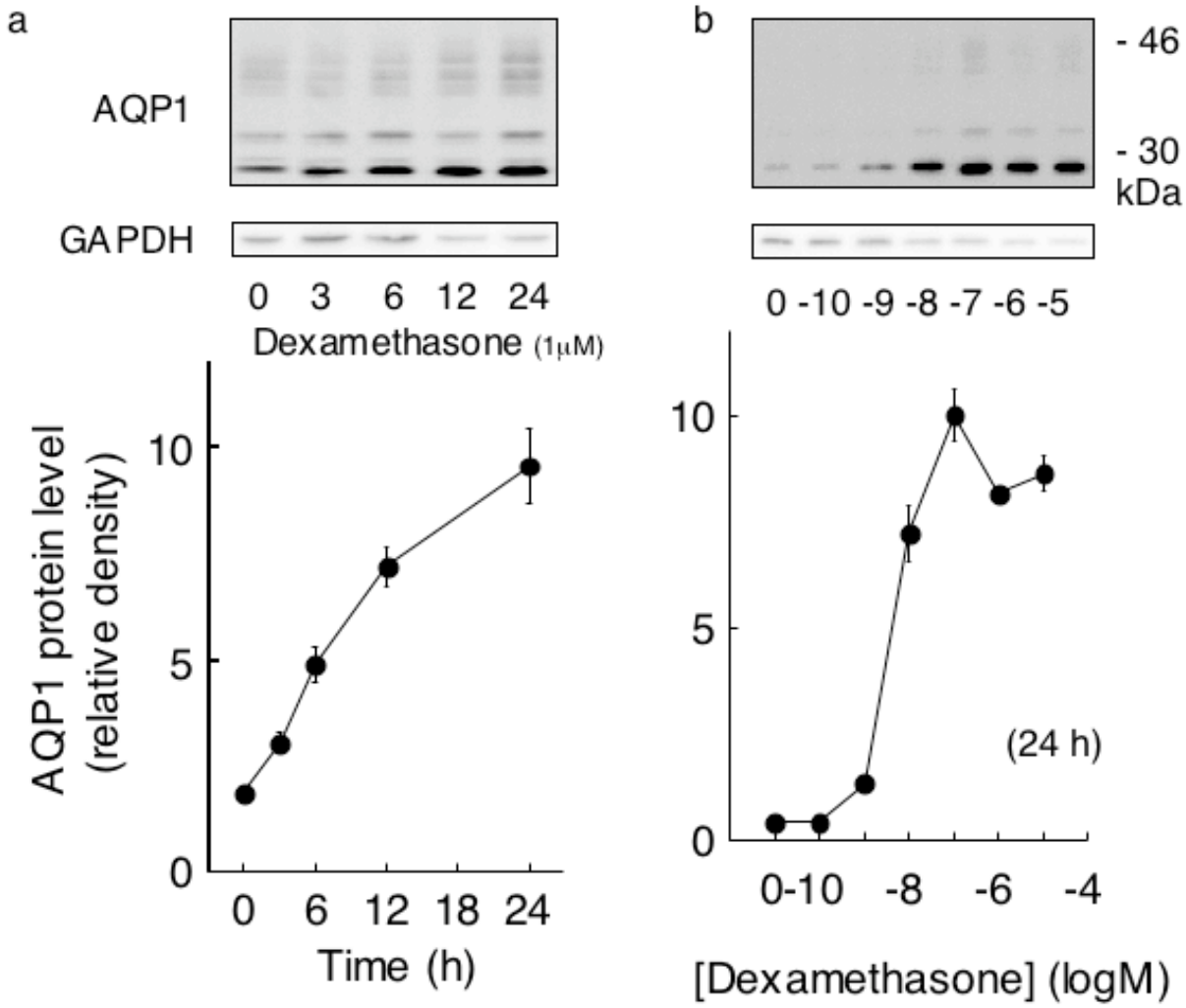
a
AQP1
GAPDH
0 3 6 12 24
Dexamethasone (1μM)
AQP1 protein level
(relative density)
10
5
0
0 6 12 18 24
Time (h)
b
- 46
- 30
kDa
0 -10 -9 -8 -7 -6 -5
10
5
0
(24 h)
0-10 -8 -6 -4
[Dexamethasone] (logM)

Fig. 2

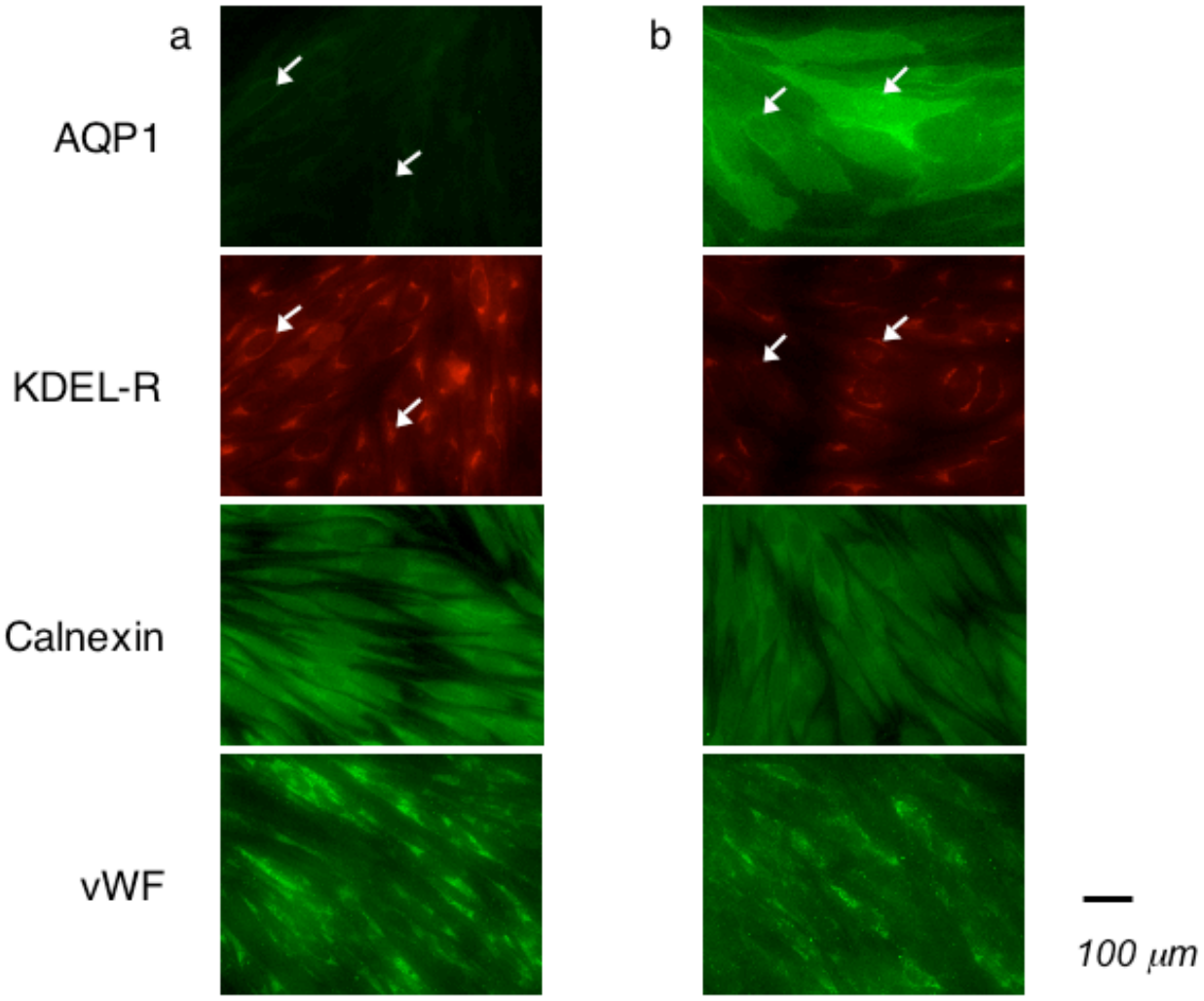

Fig. 3

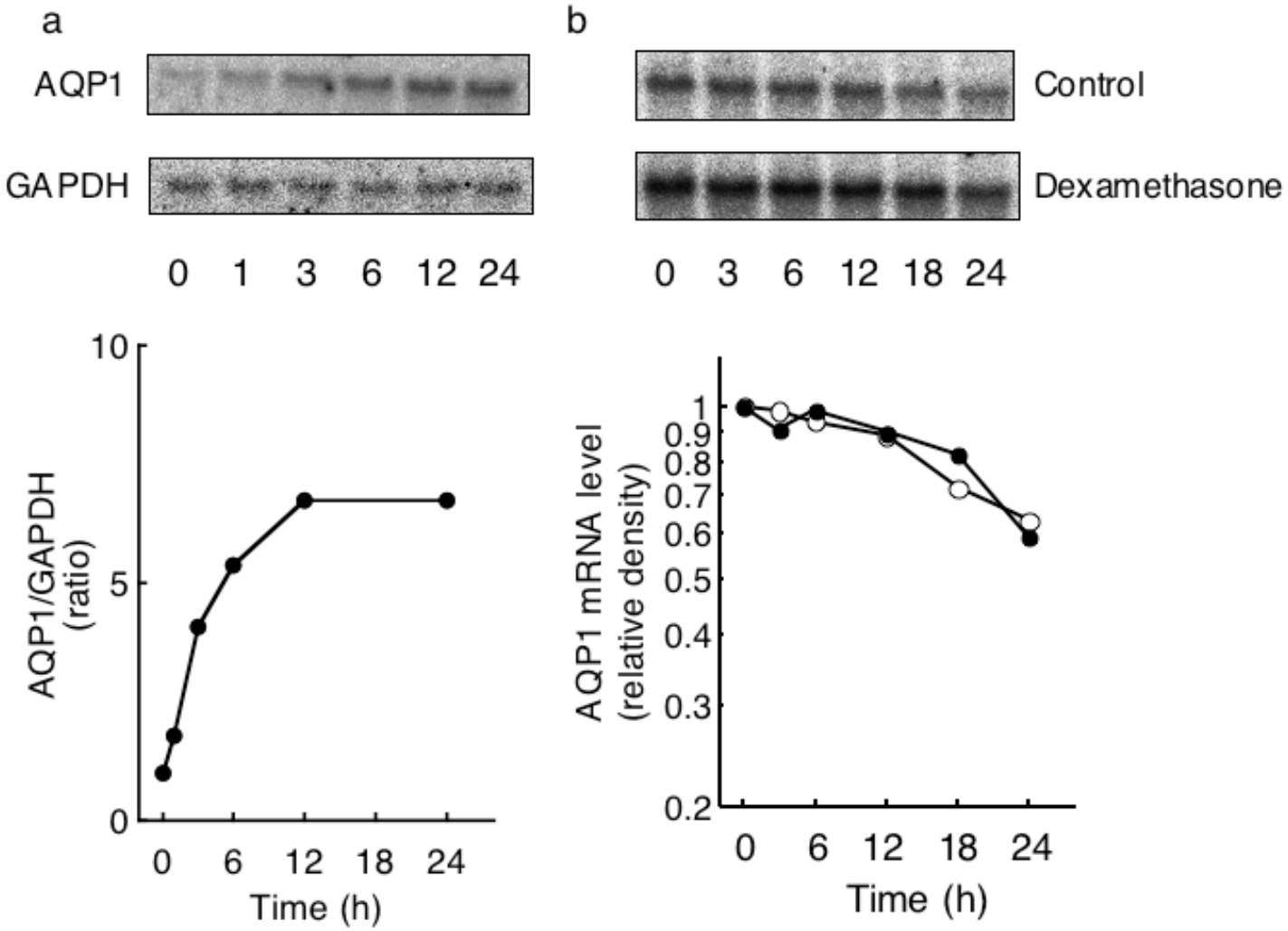
a
AQP1
GAPDH
0 1 3 6 12 24
b
Control
Dexamethasone
0 3 6 12 18 24
AQP1/GAPDH (ratio)
10
5
0
0 6 12 18 24
Time (h)
AQP1 mRNA level (relative density)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0 6 12 18 24
Time (h)

Fig. 4

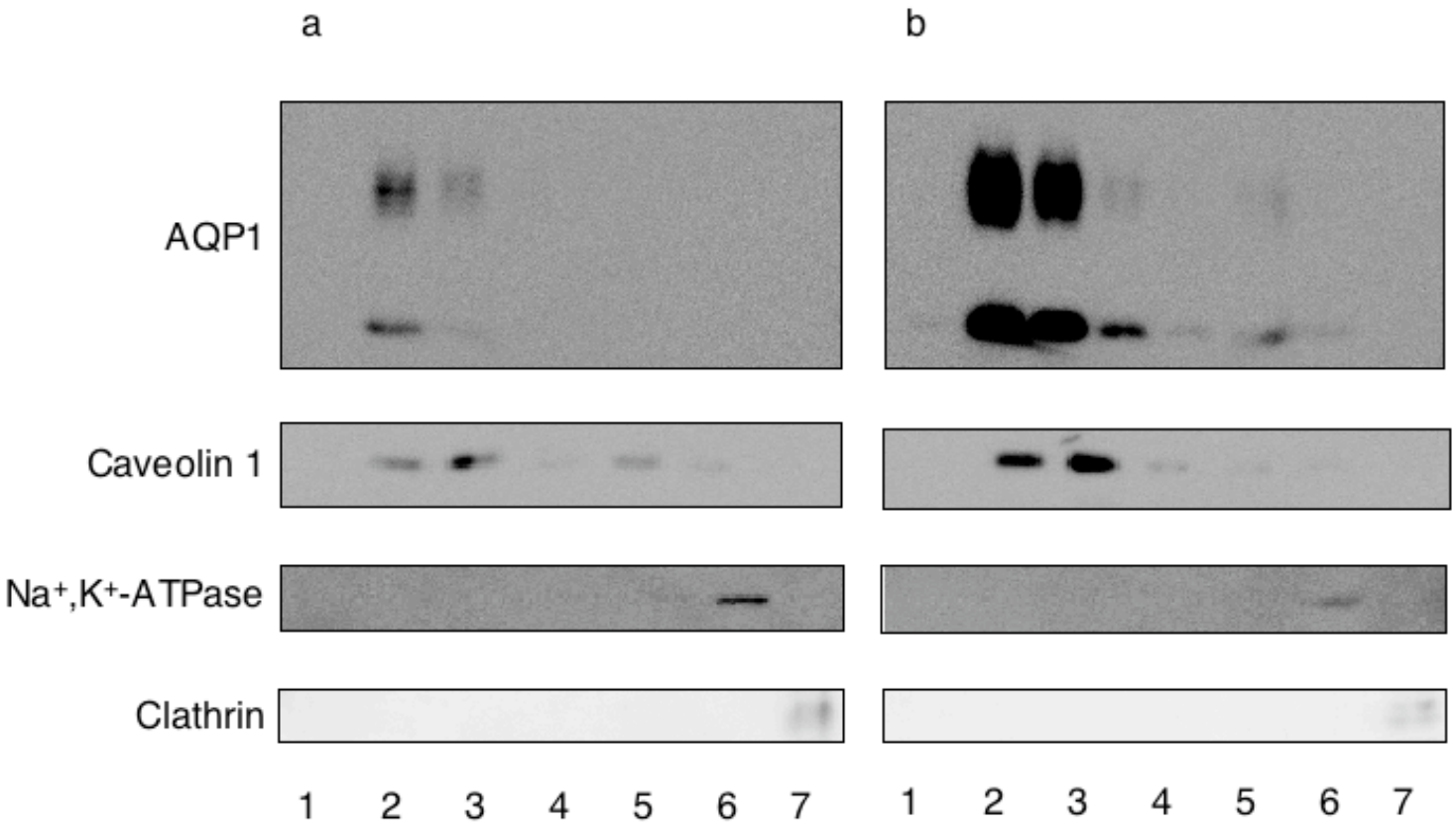

Fig. 5