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# Knockdown of aquaporin-8 induces mitochondrial dysfunction in 3T3-L1 cells

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ABSTRACT

*Background:* Aquaporin-8 (AQP8), a member of the aquaporin water channel family, is expressed in various tissue and cells, including liver, testis, and pancreas. AQP8 appears to have functions on the plasma membrane and/or on the mitochondrial inner membrane. Mitochondrial AQP8 with permeability for water, $H_2O_2$ and $NH_3$ has been expected to have important role in various cells, but its information is limited to a few tissues and cells including liver and kidney. In the present study, we found that AQP8 was expressed in the mitochondria in mouse adipose tissues and 3T3-L1 preadipocytes, and investigated its role by suppressing its gene expression.

*Methods:* AQP8-knocked down (shAQP8) cells were established using a vector expressing short hairpin RNA. Cellular localization of AQP8 was examined by western blotting and immunocytochemistry. Mitochondrial function was assessed by measuring mitochondrial membrane potential, oxygen consumption and ATP level measurements.

*Results:* In 3T3-L1 cells, AQP8 was expressed in the mitochondria. In shAQP8 cells, mRNA and protein levels of AQP8 were decreased by about 75%. The shAQP8 showed reduced activities of complex IV and ATP synthase; it is probable that the impaired mitochondrial water handling in shAQP8 caused suppression of the electron transport and ADP phosphorylation through inhibition of the two steps which yield water. The reduced activities of the last two steps of oxidative phosphorylation in shAQP8 cause low routine and maximum capacity of respiration and mitochondrial hyperpolarization.

*Conclusion:* Mitochondrial AQP8 contributes to mitochondrial respiratory function probably through maintenance of water homeostasis.

*General significance:* The AQP8-knocked down cells we established provides a model system for the studies on the relationships between water homeostasis and mitochondrial function.



## 1. Introduction

The major function of mitochondria is the production of ATP by oxidative phosphorylation. Through this process, a large amount of water is also formed. At the last step of the electron transport chain (ETC) mediated by cytochrome c oxidase (complex IV), electrons are finally passed to oxygen to yield water. In addition, ATP synthase-mediated production of ATP is coupled with equimolar formation of water. Thus, production of water as a by-product of ATP synthesis is inevitable and appropriate water handling is of crucial importance for the maintenance of mitochondrial integrity and function. Concerning the water transport across the mitochondrial membrane, channel mediated pathways such as aquaporins and the mitochondrial permeabilization pore have been postulated in addition to simple lipid bilayer diffusion. However, the details of such facilitated pathways remain largely unknown and more information has to be obtained.

Aquaporins (AQPs) are a family of membrane transport proteins which play a role in water transport across biological membranes. At least thirteen AQPs (AQP0～12) have been identified in mammals. Most of them are localized in the plasma membrane and are involved in water transport into and/or out of cells and specific

*Abbreviations:* TMRE, Tetramethylrhodamine Ethyl ester; FCCP, Carbonyl cyanide 4-(triflouromethoxy) phenylhydrazone

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functions of the cells; reabsorption of water in proximal tubular cells, water permeability of the blood-brain-barrier, and salivary secretion [1,2].

AQP8 was initially thought to be localized to the plasma membrane when it was cloned and identified in testis, liver, colon and heart [3–6]. But later it was found to be present on the inner mitochondrial membrane of liver, kidney and neural stem cells and was expected to play a role in unconditional transport of metabolic water produced in mitochondria [7]. AQP8 was recently reported to be involved in the mitochondrial transport of $H_2O_2$ in the hepatocytes [8] and $NH_3$ in the renal tubular cells [9] and hepatocytes [10]. However, the fact that AQP8 also shows high permeability to water comparable or superior to that of AQP1 [11] suggests its involvement in mitochondrial water flux. To achieve a comprehensive understanding of AQP8 function, accumulation of experimental evidence in various types of cells is needed.

The mitochondrial type AQP8 was distinct from the plasma membrane form in terms of molecular weight. In many cell types, AQP8 is simultaneously expressed in the plasma membrane and mitochondria. In the liver and testis, the two organs which show most active AQP8 synthesis, AQP8 was detected by western blot analyses as two bands; the band of about 28kDa was observed in the inner mitochondrial membrane-enriched fraction [12,13] and the other one of about 34kDa in the plasma membrane fractions [12,14–17]. The 34kDa band was converted to the 28kDa band by *N*-glycosidase treatment [16,17].

In this study, we found that, in mouse adipose tissues and 3T3-L1 preadipocytes, the mitochondrial type AQP8 is dominantly expressed. We therefore created an AQP8-knocked down cell line in which we have investigated the role of AQP8 on mitochondrial function, and showed that knockdown of AQP8 leads to significantly impaired mitochondrial function, suggesting a significant role for AQP8 for normal mitochondrial bioenergetic homeostasis.

## 2. Methods and materials

### 2.1. Cell and cell culture

Mouse 3T3-L1 preadipocytes obtained from Health Science Research Resources Bank (Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium high glucose (DMEM, GIBCO) supplemented with 100 Unit/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES and 10% calf serum at 37 °C under 5% $CO_2$ atmosphere.

### 2.2. Animals

Male BALB/cCrSlc and male C57BL/6J mouse at 8 weeks and 10 weeks of age were obtained from Charles River Laboratories International, Inc. and Sankyo Labo Service Corporation, Inc., respectively.

### 2.3. Preparation of mitochondria and mitoplast fractions

Cells washed with PBS were resuspended in mitochondrial isolation buffer (20 mM HEPES, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 1 mM PMSF, *p*H 7.6), incubated on ice for 20 min and homogenized using Biomasher (Nippi). Supernatant clarified at 800 g for 10 min was pelleted at 10,000 g for 20 min yielding a crude mitochondrial fraction. The pellet was resuspended in mitochondrial isolation buffer, clarified at 800 g for 20 min and pelleted at 10,000 g for 20 min resulting in an enriched mitochondrial fraction [18].

The preparation of mitoplast fraction was performed by using a detergent to remove the outer mitochondrial membrane. Digitonin was added to the mitochondrial fraction resuspended in mitochondrial isolation buffer to a final concentration of 0.6% (*w*/*v*). The suspension was incubated on ice for 15 min under gentle stirring. After dilution with 3 vol of isolation buffer, the suspension was centrifuged at 15,000 g for 10 min and the pellet was washed in mitochondrial isolation buffer resulting in an mitoplast fraction [8,9].

### 2.4. Western blotting

Sample preparation: Cells, mitochondria or mitoplasts were lysed in cold RIPA buffer consisting of 25 mM Tris–HCl, pH 7.4, 150 mM NaCl and 1% NP-40 (Thermo Scientific), supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Adipose tissues were homogenized in cold Lysis buffer (Cell Signaling), which consists of 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3Vo_4$ and 1 μg/ml leupeptin, supplemented with 1 mM PMSF. After centrifugation of the cell lysates or tissue homogenates at 14,000 g for 10 min at 4 °C, the protein concentration was measured using by DC™ Protein Assay Reagent (Bio-Rad).

Immunoblotting: Lysates of whole cells, mitochondria or mitoplasts (5 to 25 μg protein) or tissue homogenates (25 or 60 μg protein) were loaded on a 10-20% gradient or 12.5% SDS-Page gel (ATTO), separated and transferred onto a PVDF membrane (Hybond-ECL, GE Healthcare). The membrane was incubated with 2% ECL-Advance blocking agent (Amersham Scientifices) in TBS containing 0.1% tween 20 (TBS-T) for 1.5 to 2 hours at room temperature. After washing with TBS-T, the membrane was incubated with a goat polyclonal antibody specific for AQP8 (Santa Cruz Biotechnology) or Total OXPHOS Rodent WB Antibody Cocktail (Abcam) at 4 °C overnight. After washing with TBS-T, it was incubated with HRP-conjugated anti-goat IgG (Santa Cruz Biotechnology) or anti-mouse IgG (Cell Signaling). The blots were developed by a Pierce Western Blotting Substrate Plus (Thermo scientific). The luminescence-active bands were observed using a Lumi Cube plus (Liponics) and the band intensities were quantified using Image J. For re-probing with anti-beta-actin antibody (Santa Cruz Biotechnology), the antibodies on the membranes were stripped by incubating with 2% SDS/100 mM 2-mercaptoethanol in 62.5 mM Tris–HCl buffer (pH6.8) for 30 min at 55 °C.

Enzyme degradation: Cell lysates containing 150 μg protein were incubated with 1 U of *N*-glycosidase F (Roche) in 20 mM sodium phosphate buffer (*p*H 7.5) containing 10 mM EDTA, 0.1% SDS, 0.2% 2-mercaptethanol and 1.0% IGEPAL-630 at 37 °C for 24 to 72 h followed by immunoblotting as described above.

### 2.5. Immunofluorescence staining

The cells were rinsed with PBS and fixed in cold acetone for 10 min at 4 °C. Subsequently, the cells were washed in PBS and incubated with PBS containing 0.5% Triton X-100 for 4 min at room temperature. After washing with PBS, the cells were incubated with 10% fetal bovine serum (FBS) in PBS for 60 min. After removing the buffer, the cells were incubated for 60 min at room temperature with 0.2% FBS / 3% BSA in PBS containing goat polyclonal antibody specific for AQP8 (Santa Cruz Biotechnology) together with rabbit polyclonal antibody specific for VDAC1 (Abcam) or mouse monoclonal antibody specific for cytochrome c (Cell Signaling). After three times washing in PBS containing 0.1% Tween-20 (PBS-T), the cells were incubated for 60 min at room temperature with 0.2% FBS / 3% BSA in PBS containing Alexa Fluor 546-conjugated anti-goat antibody (Invitrongen) together with Alexa Fluor 488-conjugated anti-rabbit antibody (Invitrogen) for