Aquaporin 8 Regulates Mitochondrial Glycogen Phosphorylase Expression in C57BL6 Mice

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Abstract
Aquaporins (AQPs) are group of membranous water channel proteins that regulates water and small molecule movements through cell membrane. Among this group, aquaporin 8 which express in high abundance in colon, liver and kidneys. With the generation of aquaporin 8 knockout model, C57BL6 mice hepatic tissues were fractionated to purify mitochondrial fractions in both wild and AQP8 knockout model. Tandem mass spectrometric analysis revealed a significant downregulation of glycogen phosphorylase in AQP8 knockout mice. This finding was supported by western blotting. Immune-gold electron microscopy showed mitochondrial cisternae localization of glycogen phosphorylase in higher abundance than in knockout model. Present findings suggest a novel strong association between aquaporin 8 and mitochondrial glycogen phosphorylase which pinpoints indirect involvement of AQP8 in glycogen metabolism.

Introduction

Aquaporins (AQPs) are small integral proteins (monomer size 30 kDa) that have pivotal role as water channel and small molecule passage across the cells [1, 2]. This small protein molecule regulates water and small molecule influx and efflux through a well-known active transport machinery in addition to other steric factors and electrostatic molecular interactions [2]. Earlier experimental evidences demonstrated this pivotal role. For example, defective saliva secretion was reported in AQP 5 knockout mice[3]. Similarly, defective AQP1 knockout mice shows improper fluid absorption in the renal tubules[4].

All aquaporins are characterized by the presence of conserved structure of NPA (asparagine-proline-alanine) motif [5]. So far, 13 isoforms have been identified in mammals[2, 6, 7]. These aquaporins were showed to be widely distributed in several organs. For example, expression of AQP1, 2, and 8 in kidney's endothelial and

epithelial structure confirm their obvious role in fluid transport[8-10]. Water trafficking in the collecting duct is controlled by vasopressin-regulated AQP2 in addition to AQP3 and 4 as well [11-14]. Similarly, AQP0 was reported to be contributed in lacrimal secretions in the eye[15].

Among aquaporins, a subset group were reported to have a glycerol-transporting properties. This includes AQP 3, 7 and 9 [16]. While other studies documented the involvement of AQPs in transporting small molecules such as chloride or gases, including hydrogen peroxide, carbon dioxide, and ammonia [16-18].

Recently, there are enough evidences that AQPs also expressed significantly in cells that do not have a clear role in water transport such as skeletal muscle, leukocytes, neural cells, and adipose tissue. This observation triggered us to elucidate a novel role of AQP8 in alpha amylase regulation in colon in an earlier publication [19]. Interestingly, Calamita et al., reported the expression of AQP8 in the inner lining of the mitochondria with unclear function[20]. Taking in consideration that AQP8 is mainly integral protein, its expression in mitochondria suggests a functionality that needs to be investigated. The impetus of this work is to investigate the physiological function of AQP8 particularly in plasma membrane and mitochondria in C57BL6 mice.

Materials and methods

Animals

Wild and Aquaporin 8 (AQP8) C57BL6 knockout adult male mice (n=8/group) were used in the current study and bred at the animal care research center (Niigata University, Japan). Generation of AQP8 knockout mice model was validated and described in details elsewhere [21]. Mice were housed in individual cages in sterile environment with 12 hour light cycle and fed with a standard chow and filtered water *ad libitum*. Experimental animals were treated in accordance with ethics of animal center committee at school of medical and dental sciences, Niigata University.

Crude mitochondrial and plasma membrane- enriched fractionation

Mice were sacrificed by decapitation and 200 mg of liver were sliced into small pieces and rinsed in ice-cold PBS buffer. For the isolation of crude mitochondrial and plasma membrane- enriched fractions, liver samples were homogenized with a Potter-Elvehjem homogenizer (4-5 strokes in 1 min at 600 x g) in an isolation medium[20]. The isolation medium consists of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1mM EDTA and 5 mM EGTA pH 7.4 with protease inhibitors (1mM PMSF, 1ug/ul leupeptin, 1 ug/ul pepstatin A). The homogenate was centrifuged at 500 rpm for 10 min at

4˚C and the pellets representing cell debris (nuclei and unbroken cells) were discarded. Retrieved supernatant was further centrifuged at 1000 x g for 20 min at 4˚C and the related pellets representing crude mitochondrial fraction were washed twice at 1200 x g then pellets were resuspended in 500 ul isolation medium. Supernatant from previous step, containing plasma membrane- enriched fractions, were centrifuged at 17.000 x g for 30 min at 4˚C twice then resuspended again in 500 ul of isolation medium.

Protein quantification

The amount of total protein was quantified using modified Lowry's method [22]. In brief, 20 ul of 1% of sodium deoxycholate were added to same volume of diluted sample. Samples were vortexed and left in room temperature for 20 min. Next, 200 ul (36%) Trichloroacetic acid were added and mixture were vortexed and centrifuged at 7000 x g. supernatant were then aspirated using a vacuum needle and 100 ul of NaOH was added to the protein pellets followed by vortexing and incubation for 10 min at 37˚C. Suspended protein pellets were reacted with 1 ml alkaline mixture composed of 1% $CuCo_4$, 2% Na-K tartarate, 2.6% Sodium dodecyl sulfate, and 4% Na_2Co_3. Reaction mixture was kept in room temperature for 10 min followed by addition of 100 ul of 1 N phenol reagent. The mixture was incubated in room temperature for 45 min and then the absorbance was measured at 750 nm using Navaspec spectrophotometer (GE healthcare life science, Japan) in replicates [22].

Transmission electron microscopy

For transmission electron microscopy, both crude mitochondrial pellets and plasma membrane- enriched fractions were fixed in 2.5% glutaraldehyde in 0.1 mol/lM phosphate buffer (pH 7.2) for 3 hours, post fixed in 1% osmium tetroxide in 0.1 mol/lM phosphate buffer, washed twice in the same buffer. Samples were then dehydrated in ascending grades of ethyl alcohol and embedded in Oken EPOK812 resin (Oken Shoi Co., Ltd., Tokyo, Japan). Resin polymerization was accomplished in by incubation of the samples in oven at 60 °C, for 48 hours. Ultrathin sections (60 and 80 nm) were cut and transported to copper grids, stained with uranyl acetate and lead citrate. All samples were analyzed and photographically captured using Hitachi H-600A transmission electron microscope (Hitachi Co., Ltd., Tokyo, Japan)[23].

SDS-PAGE and gel slicing and endopeptidase digestion

Ten ug of crude mitochondrial pellets or plasma membrane-enriched fractions were run on 12.5% sodium dodecyl sulfate polyacrylamide gels .Gel was stained with Coomassie Brilliant Blue stain (CBB R-250, Wako, Japan) and silver staining to ensure

an accurate result. Designated area representing 2 cm and aligned around 110 KDa marker point was cut from the gel[24]. Gel slices were reduced with 10 mM dithiothreitol (DTT), alkylated with 55 mM iodoacetamide (IAA), and digested with 6 ng/ µl of trypsin overnight. Peptide was extracted with 0.3% formic acid and 5µl (0.25µg digested protein) from each sample was loaded onto nano-LC-ESI-IT-TOF-MS/MS (Hitachi NanoFrontier LD., Tokyo, Japan).

Western blot analysis

Twenty µg protein extract for glycogen phosphorylase and 10 µg for GAPDH diluted in 2x SDS sample buffer [0.125 M Tris-HCl (pH 6.8), 4 % SDS, 20% (w/v) glycerol, 0.01% bromophenol blue and 10% β mercabtoethanol] were electrophoresed on 12.5% SDS-polyacrylamide gel, and the bands were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA). PVDF membranes were then immersed in blocking buffer (10% nonfat milk, 0.05% Tween 20, 0.5% NaN3 in PBS) for 1 h. For primary antibody incubation, 10 µg/ml mouse polyclonal antibody to glycogen phosphorylase (pygl) (Abcam, Japan) or 1 µg/ml mouse monoclonal antibody to GAPDH (Ambion, Applied biosystem, Japan) was used. After several washing in 0.05% Tween 20 PBS, membranes were incubated for 1 h with goat anti-mouse immunoglobulins conjugated to peroidase-labeled dextran polymer for pygl and GAPDH, respectively (EnVision, DAKO Japan). Reaction was visualized by ECL chemiluminescence kit (Amersham Pharmacia Biotech, Tokyo)[19].

Immuno-electron (immune gold) microscopy

Mitochondrial and plasma membrane pellets were mixed with 3% formaldehyde, 0.1% glutaraldehyde, 10% saturated picric acid in 0.1M sodium cacodylate buffer, pH 7.4) for 5 hr at 48C. After successive 3 washes with 0.1 M sodium cacodylate with 270 mM sucrose for 60 minutes at 4 C. Pellets were then incubated with primary antibody (used in western blotting) diluted (1:15) with phosphate buffer saline (BPS). After 24 hrs incubation, samples were then rinsed with 0.1% tween 20 for 30 minutes and incubated with gold – conjugated secondary antibody (goat anti mouse 1:50) for 24 hrs. Following incubation, samples were rinsed 4 times with tween 20 and post fixed with 20% glutaraldehyde in BPS. After osmication in 1% osmium tetroxide for 1 hr, specimens were dehydrated with ethyl alcohol series from 50 to 100% and polymerized in UV light for 2 days. All samples were analyzed and photographically captured using Hitachi H-600A transmission electron microscope (Hitachi Co., Ltd., Tokyo, Japan)[25].

Protein identification by Ms/Ms ion search

Product ion data were searched against Uniprot *Mus Musculus* database (169347 entry) using a locally stored copy of the Mascot search engine (version 2.2, Matrix Science, London, UK)[19]. The following parameters were used for database search: precursor mass tolerance 0.3 Da, product ion mass tolerance 0.3 Da, 2 missed cleavages allowed, fully tryptic peptides only, fixed modification of Carbamoidomethyl (C), variable modifications of glutamine (Gln) to pyroglutamate (pyro-Glu) (N-term Q); glutamate (Glu) to pyroglutamate (pyro-Glu) (N-term E), Oxidation of histidine and tryptophan (HW); Oxidation of methionine (M), mass values of monoisotopic and peptide charge state of 2+ and 3+. Protein was accepted if at least 2 peptides pass identity and homology threshold of Mascot algorithm. The false discovery rate (FDR) against decoy database was below 5%.

Statistical analysis

All statistical analysis were done using R software version 3.4.3

Results

Purity of mitochondrial and plasma membrane enriched fraction

To investigate exact role of AQP8 on subcellular proteome expression mainly mitochondrial and other subcellular fractions, liver tissues were fractionated in sucrose buffer as reported earlier. The result showed a successful fractionation of both fractions with a purity of 89-92% for mitochondrial fraction. Purity of fraction were demonstrated by transmission electron microscopy as shown in figure 1. On the other hand, majority of remaining fraction was plasma membrane debri with other nuclear and cytosol compartments.

SDS-PAGE suggests differential expression of 110 KDa band

One dimensional SDS-PAGE allows simplification and better observation of protein pattern within extracted sample. In this experiment, a total protein amount of 10 ug was loaded in all fractions. Interestingly, the result showed a clear band close to molecular weight 110 KDa when staining with colloidal coomassie blue (Figure 2A). Taking in account that coomassie stain sensitivity is not high, SDS-PAGE was repeated with staining the gel using silver stain (Figure 2B). With the same pattern of the unknown band located at 110 KDa, the data showed that the initial observation was not biased by mis- quantification of protein or loading error, especially the fact that other band were with same staining intensities between fractions and replicates. In a next step, In-gel trypsin digestion followed by Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis was performed to identify the nature of this dysregulated protein.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

Data output results from each single analysis was combined for each group and refined based on a cutoff value (False discovery rate; FDR=0.05). Total number of protein and peptide identification from combined fractions is illustrated in figure (3A). Protein abundance of each protein was calculated based on the spectral count and further refined based on the cutoff scoring (spectral count more than 10^3). Interestingly, data showed a significant increase in glycogen phosphorylase abundance with 4.3 folds in the wild mitochondrial fraction compared to AQP8 knockout mitochondrial fraction. Peptides retrieved from mitochondrial fractions were 27 and 8 for the wild and AQP8 knock out model, respectively (figure 3B).

Immunological validation with western blotting

Targeting epitopes fragment (aa 797-847) representing the C-terminus of glycogen phosphorylase, an anti-PYGL antibody was used for western blotting. An appropriate amount of protein lysate (for either PYGL or GAPDH; see material and methods) were loaded on SDS-PAGE and transferred to PVDF membrane successfully (see figure 2C). After chemiluminescence activation, western blotting showed over 11 magnitude differences between PGYL expression in wild and knockout model (Figure 4).

Localization of glycogen phosphatase using Immuno-gold electron microscopy labelling

To confirm subcellular localization of PYGL expression, immune-gold labeling was performed. The binding of secondary antibodies to nano-gold particles allow subcellular localization of PYGL when transmission electron microscopy is used. This approach identified almost 2.3 increase in immuno-gold particles in wild mitochondrial cisternae compared to knockout model as shown in figure 5.

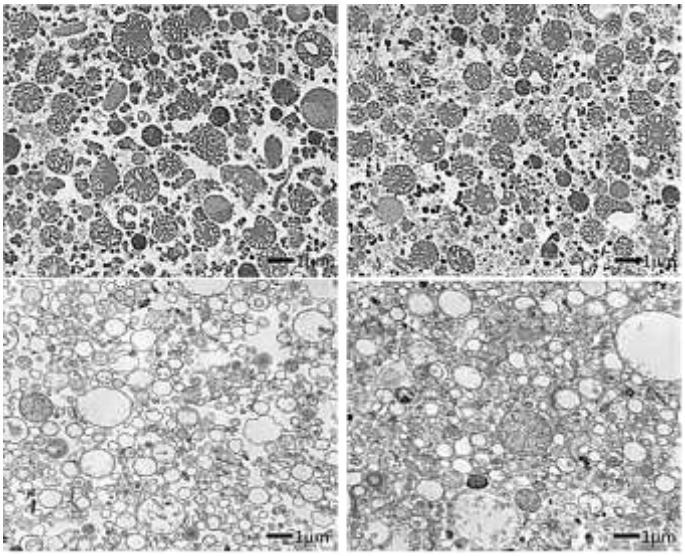

Figure 1: purity of mitochondrial and plasma membrane fractions in wild and AQP8 knockout mice by transmission electron microscopy. Clockwise from upper left; mitochondrial fraction in wild mice, AQP8 knockout mice, plasma membrane fraction in AQP8 knockout mice, and wild mice.

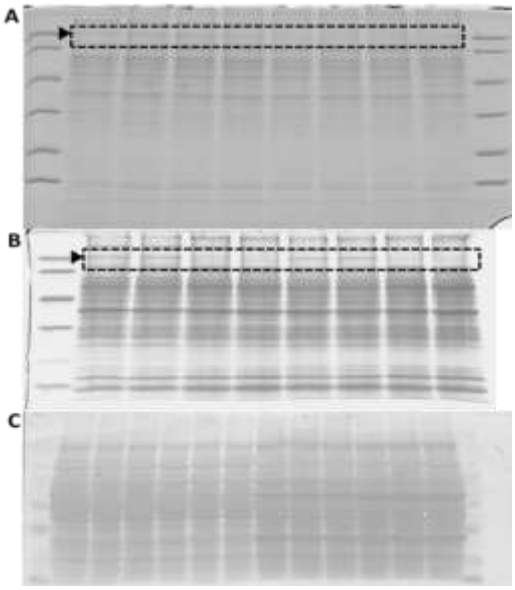

Figure 2: Fractionation of mitochondrial and plasma membrane fractions. A; SDS-PAGE stained with colloidal coomassie blue. Lane (1) high MW marker (250, 110, 50, 25, 10, and 5 KDa), lanes (2-5) wild mitochondrial fractions, lanes (6-9). B; same sample stained with silver staining. C; PVDF membrane electro-transfer prior to western blotting stained with colloidal coomassie blue.

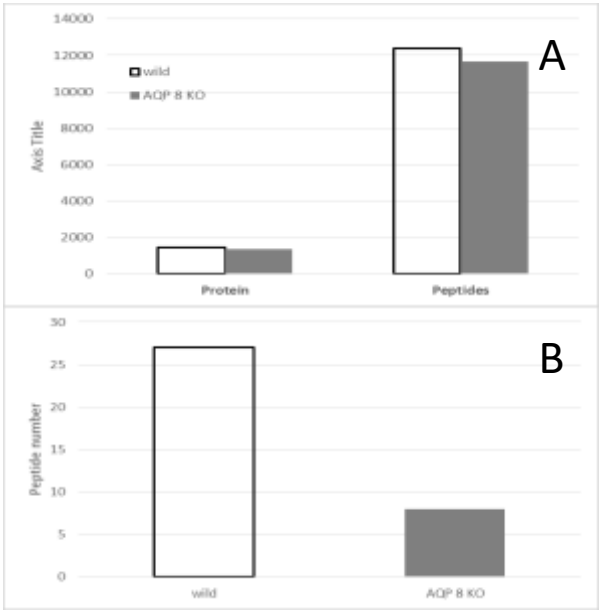

Figure 3: Protein and peptide identification of LC-MS/MS for wild and AQP8 Knockout mice. A represents confident protein and peptide identifications of all fractions (non- redundant). B represents attributed peptides of glycogen phosphorylase protein (PYGL) in both wild and AQP 8 knockout mice.

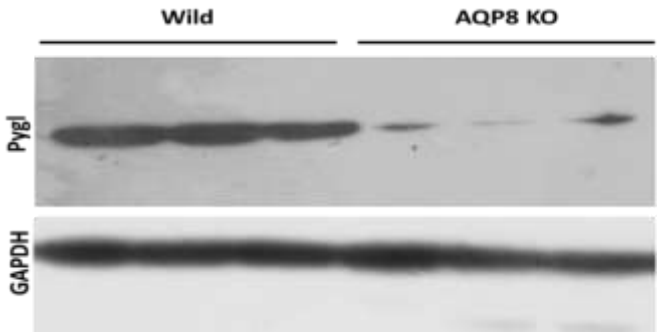

Figure 4: Western blotting of glycogen phosphorylase and GAPDH for both wild and aquaporin 8 knockout mice.

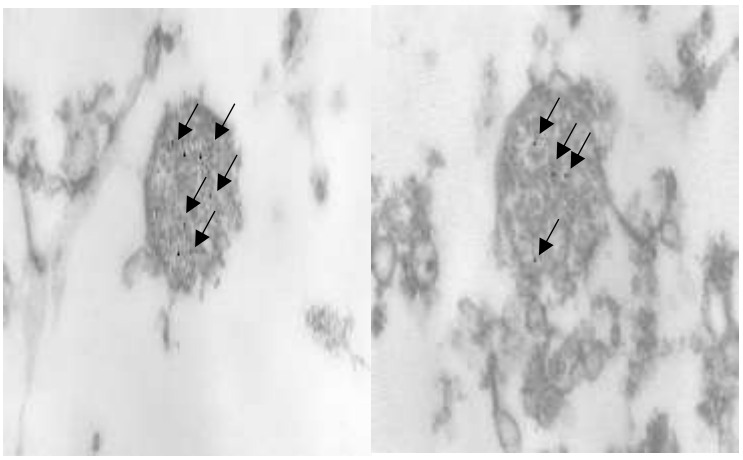

Figure 5: Immnuo-gold transmission electron microscopy against glycogen phophorylase showing immnuo- gold particle in mitochondrial cisternae of wild type (left) and AQP 8 knock out mice (right) with magnification power x 20000.

Discussion

While the anticipated mechanism of aquaporin in transepithelial fluid dynamics has been well documented earlier, several studies showed possible involvement of aquaporins in different physiological processes rather than water transport. A handful preliminary studies suggested possible implication of aquaporin in cancer progression[26], amylase metabolism[19], obesity[27], or wound healing[28]. This study investigated the possible physiological role of aquaporin 8 in mitochondria utilizing a high throughput liquid chromatography-mass spectrometric analysis (LC-MS/MS). The rough subcellular fractionation method segregates mitochondrial fraction apart from other subcellular fraction (plasma membrane debri, nuclear, ribosomes, and cytosol compartments) with an average purity of 90.5%. Silver stained SDS-PAGE of these fractions demonstrated a probable dysregulation in molecule(s) with molecular weight close to 110 KDa (reported later as exact molecular weight of glycogen phosphorylase). Interestingly, tandem mass spectrometry reveled 4.3 increase in glycogen phosphorylase abundance. Twenty seven high confidence peptides attributed to PYGL were detected in wild mice compared to 7 peptides in AQP8 knockout model. Taking in account that peptide abundance is directly correlated with protein abundance, the high sensitivity of the mass spectrometry depicts a possible role of AQP8 in glycogen phosphorylase. Glycogen phosphorylase (PYGL) is an

enzyme that hydrolyses glycogen into glycose[29]. This process facilitates exporting of glucose into other tissues[29]. It is important to note that regulation of glycogen metabolism here is controlled primarily by the level of free glucose level[30]. The association between glucose and water movement is demonstrated earlier as facilitated passive diffusion through (GLUT 1-4) or by active transport and has been linked earlier with aquaporin z [15]. One possible explanation is switching off the PYGL protein expression as a result of stagnant glucose movement resulted from AQP8 knockout or partial disruption of mitochondrial osmolality. To confirm this result, western blot analysis using a polyclonal antibody against PYGL was performed. The results showed a clear significant downregulation of PYGL in AQP8 knockout. Photometric measurement of band intensity revealed 11 fold differences between both experimental groups. The discrepancy between the mass spectrometric analysis and western blot analysis might be attributed to antibody reactivity or possible ion suppression for some PYGL peptides that have not been detected by LC-MS/MS. Aside from this discrepancy, a high relevance between aquaporin 8 and glycogen phosphorylase was confirmed immunologically. Immunogold electron microscopy, unambiguously, disclosed the subcellular localization off PYGL within mitochondrial cisternae. This finding was in line with our earlier results. Immunogold particles were 2.3 folds increased in wild group compared to knockout model (average count of 100 mitochondria/ individual). Although this results supports our earlier findings. It is thought that Immuno electron microscopy in this experiment was not fully optimized due to unavailability of PGYL antibody that suites this imaging method. This data suggests a provoked effect of AQP8 knockout on glycogen metabolism. To our knowledge, this finding has not been demonstrated earlier due to the paucity of research on aquaporin 8. A couple of earlier articles demonstrated the general link between aquaporin 2 and 4 and glycogen metabolism in prokaryotes and Caenorhabditis elegans [31]. A similar condition that might be explained by hypometabolic state of glycogen metabolism as a result of reduction of water permeability. This Compelling data warrant further investigation and augment the notion that aquaporin 8 is not only a water channel protein.

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الملخص العربي

الاكوابورين 8 ينظم تعبير الجلايكوجين فوسفوريليز في فئران C57BL6

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ألأكوابورينز هي مجموعة من البروتينات التي تشكل قناوات لعبور المياه عبرالأغشيه الخلويه حيث تنظم حركة المياه و الجزيئات الصغير. من بين هذه المجموعة، أكوابورين 8 التي يوجد بوفرة عالية في القولون والكبد والكلى. مع القدره علي انتاج جيل من فئران C57BL6 منزوعه لجين الأكوابورين 8. تم تجزئه الأنسجة الكبدية لهذه الفئران لتنقية الميتوكوندريا في كلا الفئران البريه و المنزوعه لجين AQP8 وبأستخدام كشف التحليل الطيفي الكتلي, أظهرت النتائج تعبير ضعيف لبروتين الجلايكوجين فوسفوريليز في الفئران منزوعة لجين AQP8. وقد أيدت هذه النتيجة بأستخدام تقنية الويسترن بلوت. كما أظهر المجهر الإلكتروني المناعي الدقيق وجود بروتين الجلايكوجين فوسفوريليز في سيستيرن الميتوكوندريا للفئران البريه بنسبه أعلي من مثيلاتها في مجموعة الفئران المنزوعه لجين AQP8.
تشير نتائجنا إلى وجود علاقة قوية جديدة بين أكوابورين 8 والميتوكوندريا الجليكوجين فسفوريلاز والتي تبرز مشاركة غير مباشرة للاكوابورين 8 في أيض الجليكوجين.