ATP6AP2/(Pro)renin Receptor Contributes to Glucose Metabolism via Stabilizing the Pyruvate Dehydrogenase E1 β Subunit*

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Background: The mechanism of efficient energy generation in the highly evolved mammalian retina remains incompletely understood. Here, we reveal an unsuspected role for (pro)renin receptor, also known as ATP6AP2, in energy metabolism. Immunoprecipitation and mass spectrometry analyses identified the pyruvate dehydrogenase (PDH) complex as Atp6ap2-interacting proteins in the mouse retina. Yeast two-hybrid assays demonstrated direct molecular binding between ATP6AP2 and the PDH E1 β subunit (PDHB). Pdhb immunoreactivity co-localized with Atp6ap2 in multiple retinal layers including the retinal pigment epithelium (RPE). ATP6AP2 knockdown in RPE cells reduced PDH activity, showing a predilection to anaerobic glycolysis. ATP6AP2 protected PDHB from phosphorylation, thus controlling its protein stability. Down-regulated PDH activity due to ATP6AP2 knockdown inhibited glucose-stimulated oxidative stress in RPE cells. Our present data unraveled the novel function of ATP6AP2/(P)RR as a PDHB stabilizer, contributing to aerobic glucose metabolism together with oxidative stress.

Significance: The present data provide a novel insight into the biological function of ATP6AP2 in the retina.

Aerobic glucose metabolism is indispensable for metabolically active cells; however, the regulatory mechanism of efficient energy generation in the highly evolved mammalian retina remains incompletely understood. Here, we reveal an unsuspected role for (pro)renin receptor, also known as ATP6AP2, in energy metabolism. Immunoprecipitation and mass spectrometry analyses identified the pyruvate dehydrogenase (PDH) complex as Atp6ap2-interacting proteins in the mouse retina. Yeast two-hybrid assays demonstrated direct molecular binding between ATP6AP2 and the PDH E1 β subunit (PDHB). Pdhb immunoreactivity co-localized with Atp6ap2 in multiple retinal layers including the retinal pigment epithelium (RPE). ATP6AP2 knockdown in RPE cells reduced PDH activity, showing a predilection to anaerobic glycolysis. ATP6AP2 protected PDHB from phosphorylation, thus controlling its protein stability. Down-regulated PDH activity due to ATP6AP2 knockdown inhibited glucose-stimulated oxidative stress in RPE cells. Our present data unraveled the novel function of ATP6AP2/(P)RR as a PDHB stabilizer, contributing to aerobic glucose metabolism together with oxidative stress.

Glucose is a major energy source for all mammalian cells in various organs. Glycolysis, linked to the tricarboxylic acid (TCA)2 cycle, breaks down glucose into the metabolites necessary for energy production through the mitochondrial respiratory (electron transport) chain via oxidative phosphorylation under the aerobic state. The retina is one of the most metabolically active tissues in the body, consuming more oxygen and glucose than even the brain, and also generates the glycolytic end product, lactate (1, 2). Phototransduction and visual cycle recovery processes, requiring a considerable amount of energy, take place in mitochondria-rich photoreceptor and retinal pigment epithelium (RPE). ATP6AP2 knockdown in RPE cells suggested a need for reducing the capacity linked to oxidative stress (7). (Pro)renin receptor (IPP)RR was originally found as an N-terminal truncated form of IPPRR, which is associated with vacuolar H+-ATPase (v-ATPase) and was termed ATP6 accessory protein 2 (ATP6AP2) (8, 9). Prorenin binding to its full-length receptor ATP6AP2/(P)RR simultaneously activates tissue renin-angiotensin system (RAS) and its own intracellular signal transduction. The dual activation of tissue RAS and RAS-independent signaling pathways, referred to as the receptor-associated prorenin system (RAPS), causes the molecular pathogenesis of end-organ damage, such as inflammation and angiogenesis, including diabetic retinopathy (10–13). On the other hand, v-ATPase is a multisubunit proton pump involved in diverse and fundamental cellular physiology, and v-ATPase-mediated acidification, requiring its accessory unit Atp6ap2, was shown to play a pivotal role in canonical and noncanonical Wnt signaling pathways (14–16). Furthermore, several studies on conditional knock-out mice specific for the heart and kidney cells revealed that Atp6ap2 is essential for v-ATPase-associated autophagy, leading to the maintenance of cell structure and survival (17–19). More recently, we have shown that Atp6ap2 interacts with partitioning defective 3 homolog, one of the™

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major cell polarity determinants, and contributes to laminar formation during retinal development in mice (20).

Atp6ap2 protein and mRNA were shown to be constitutively expressed in the mouse retina from developmental to mature stages (20). Given that ATP6AP2 is involved in multiple physiological cellular activities on top of the RAPS-related pathway, we hypothesized that ATP6AP2 plays some biological roles in the mature adult retina. In this study, we identified a novel ATP6AP2-binding protein in the retina, which is a subunit of the pyruvate dehydrogenase (PDH) complex, a key enzyme in energy metabolism linking glycolysis to the TCA cycle. We herein report the first evidence that shows the close association of ATP6AP2/(P)RR with the energy-generating pathway of glucose metabolism in the eye.

**EXPERIMENTAL PROCEDURES**

*Animals and Cell Lines—*C57BL/6j mice were obtained from CLEA Japan (Tokyo, Japan). Human embryonic kidney (HEK293T) and human RPE (hTERT-RPE1) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM and DMEM/F-12 (Wako Pure Chemical Industries, Osaka, Japan), respectively, supplemented with 10% fetal calf serum (Life Technologies) at 37 °C and 5% CO2.

*Reagents and Treatments—*Two siRNA oligos for suppressing the gene expression of ATP6AP2 (siRNA-1, HSC.RNAI.N005765.12.1; siRNA-2, HSC.RNAI.N005765.12.2) and a negative control siRNA oligo (DS NC1) were purchased from Integrated DNA Technologies (Coralville, IA) and used at 10 nM. Cells were transfected with siRNA using Lipofectamine RNAiMAX Reagent (Life Technologies), and plasmid DNA was transfected into HEK293T cells using HilyMax (Dojindo, Kumamoto, Japan) and into RPE cells using the Nucleofector 2b Device (Lonza, Basel, Switzerland) following the manufacturer’s protocols.

Cells were treated with 500 or 10 μM tyrosine kinase inhibitors AG18 or AG82 (Millipore, Billerica, MA) at 37 °C for 24 h. After treatment, cells were washed with medium and harvested for immunoblotting with anti-PDH antibody. To cover the handle region of the prorenin molecule, which is the binding site of (P)RR (11), decoy peptides NH2-RIFLKRMPSI-COOH as human PRRB were synthesized and purified using high pressure liquid chromatography on a C-18 reverse-phase column by GeneDesign (Osaka, Japan).

*Plasmid Construction and Mutagenesis—*Human ATP6AP2 cDNA (GenBank number NM_005765) was subcloned in the pCI vector with FLAG tag (Promega, Madison, WI). All deletion mutants from the ATP6AP2 expression construct were generated by PCR. Human PDHX, PDHA1, PDHB, DLD, and DLAT cDNAs (GenBank number NM_000284, NM_000925, NM_003477, NM_001931, and NM_000108) were obtained from the DNASU Plasmid Repository (Tempe, AZ) and subcloned into the pCMV tag 3B vector with Myc tag (Agilent Technologies, Santa Clara, CA). All constructs were sequence verified before use.

*Immunoprecipitation—*Mouse retinas were homogenized in TBS containing 1% Nonidet P-40 and protease inhibitors (Roche Applied Science). After preincubation of samples with Protein G beads (Life Technologies) for 1 h at 4 °C, antibodies were added and left overnight at 4 °C with gentle mixing. The beads were washed three times with the lysis buffer, and suspended in SDS sample buffer.

*Nanoscale Liquid Chromatography with Tandem Mass Spectrometry (NanoLC-MS/MS)—*Immunoprecipitated samples were resolved on a 4–15% Mini-PROTEAN TGX gels (Bio-Rad) and stained using a Mass silver stain kit (Wako Pure Chemical Industries). Gel slippage was reduced by 100 mM dithiothreitol and alkylated by 100 mM iodoacetamide. After washing, the gels were incubated with trypsin overnight at 30 °C. Recovered peptides were desalted by ZipTIP C18 (Millipore). Samples were analyzed by nanoLC-MS/MS systems (DiNa HPLC system, KYA Technologies, Tokyo, Japan; QSTAR XL, Life Technologies). Mass data acquisitions were piloted by Mascot software for matching proteins in the NCBI database.

Known-bait and Known-prey Yeast Two-hybrid Analyses—Known-bait and known-prey analyses were carried out using the Matchmaker GAL4 Two-hybrid System (Clontech, Mountain View, CA), according to the manufacturer’s instruction. ATP6AP2 (residues 16–275) and PDHB or other PDH complex genes were subcloned into either the bait vector pGBK7T or the prey vector pGADT7 (Clontech). Yeast AH109 cells were transformed sequentially with the bait and prey vectors. Transformants were selected for the bait (−tryptophan (TRP) selection) and prey (−leucine (LEU) selection) vectors. Yeast AH109 cells containing the bait and prey vectors grew in nutrition selection plates (− TRP, − LEU, − histidine (HIS), and − adenine (ADE) selection) with lacZ gene expression in blue by a filter lift assay for β-galactosidase activity.

*Immunoblot Analyses—*Cell and tissue extracts were lysed in SDS buffer and a protease inhibitor mixture (Roche Applied Science). Proteins were transferred to polyvinylidene fluoride membrane by electroblotting, and immunoblot analyses were performed as previously described (20). Membranes were incubated with the following primary antibodies: anti-ATP6AP2, anti-PDH (for human PDHB) antibodies (Sigma); anti-PDHA1, anti-PDHB (for mouse Pdhb), anti-DLAT, anti-HSP90 antibodies (Abcam, Cambridge, MA); anti-Na+/K+-ATPase α1 antibody (Millipore); anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology, Santa Cruz, CA); anti-β-actin antibody (Cell Signaling Technology, Danvers, MA); and anti-FLAG and anti-Myc antibodies (Wako Pure Chemical Industries). The signal was obtained by enhanced chemoluminescence (Western Lightning Ultra, PerkinElmer Life Sciences) and measured using a LAS-4000 mini-camera system (Fujifilm, Tokyo, Japan). The bands were analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

*Immunofluorescence Microscopy—*Mouse eyeballs were fixed in 4% paraformaldehyde for 30 min on ice, incubated in an increasing concentration of PBS/sucrose (10, 20, 30%), and embedded in Frozen Section Compound (Leica, Exton, PA) as previously described (20). Sections were probed with the following primary antibodies: rabbit anti-Atp6ap2 and mouse anti-Pdhb antibodies (Sigma). The secondary antibodies for fluorescent detection were Alexa Fluor 488 and 546 (Life Technologies). Nuclei were counterstained with DAPI (diamidino-
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Measurements of PDH Activity and Acetyl-CoA and Lactate Levels—After cells were transfected with siRNA or plasmid DNA for 48 h, the activity of PDH was measured by using a PDH Enzyme Activity Assay Kit (Millipore), and the intracellular levels of acetyl-CoA were detected by using an Acetyl-CoA Assay Kit (Biovision, Milpitas CA), following the manufacturer’s instructions. For PDH assays, purified mitochondria fractions from cultured cells were used as previously described (21). After 24 h of transfection, cells were replenished with fresh phenol red-free medium and incubated for 24 h, and the culture media were then collected for measurement of lactate levels. Lactate levels were determined by using a Lactate Assay Kit (Biovision), according to the manufacturer’s protocol. PDH activity and acetyl-CoA assays were normalized with protein concentrations, and lactate assay with the number of cells.

Intracellular Reactive Oxygen Species (ROS) Detection—ROS generation in RPE cells stimulated with or without a high concentration of glucose was measured using 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA, Life Technologies). After 4 days of culture in the medium containing 5.5 mM glucose, the RPE cells were maintained for 48 h at glucose concentrations of 5.5 or 30 mM in the presence of siRNA, 10 μM valsiartan (Sigma), or 1 μM (P)RR blocker. Cells of osmotic control group were exposed to 5.5 mM d-glucose plus 24.5 mM mannitol for 48 h. The cells were treated with 10 μM CM-H₂DCFDA and 1 μg/ml of Hoechst 33258 (Lonza) for 30 min at 37 °C. After the excess probe was washed out, fluorescence was quantified on a microplate reader (Infinite F200 PRO, Tecan, Männedorf, Switzerland) for CM-H₂DCFDA (λ_ex, 485 nm and λ_em, 535 nm) and DNA (λ_ex, 360 nm and λ_em, 465 nm). The results were expressed as a ratio relative to the cellular DNA contents.

Statistical Analyses—All results were expressed as mean ± S.E. Statistical analyses were performed using Student’s t test or analysis of variance followed by the Tukey-Kramer post hoc test. Differences were considered statistically significant when the p values were <0.05.

RESULTS

Identification of Atp6ap2-interacting Proteins in the Retina—To isolate Atp6ap2-interacting proteins from the mature adult retina, we first performed immunoprecipitation (IP) using anti-Atp6ap2 antibody and tissue extracts prepared from mouse retinas. The bound proteins were eluted, analyzed by electrophoresis, and detected by silver staining (Fig. 1A). Two significant protein bands corresponding to ~35 and 65 kDa were observed in the Atp6ap2 immunoprecipitates as compared with those with normal IgG (Fig. 1A), suggesting specific interactions between these proteins and Atp6ap2.

These two protein bands (Fig. 1A) were excised from the gel and trypsinized. The resulting peptides were analyzed by mass spectrometry (MS) and searched with the Mascot program. In total, nine proteins including Atp6ap2 (antigen) and one of the v-ATPase subunits were identified (Table 1). Interestingly, these putative interacting proteins included Dlat (dihydrolipoyl-S-acetyltransferase), Pdhb (PDH E1 β subunit), and Pdha1 (PDH E1α subunit), all of which compose the PDH complex that converts pyruvate into acetyl-CoA, linking glycolysis to the TCA cycle.

Direct Molecular Binding of ATP6AP2 with PDHβ—To validate the IP and MS results (Fig. 1A and Table 1), we carried out the following additional experiments. First, to investigate direct molecular interaction between ATP6AP2 and each component of the PDH complex including PDHX (PDH component X),
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ATP6AP2 grew well in nutrition selection plates with lacZ expression in blue, whereas those with empty bait or prey vectors and those with other cDNAs except for a positive control (p53 + T-antigen) showed no growth (Fig. 1B). These results indicated direct molecular binding between ATP6AP2 and PDHB.

Next, to confirm the protein interaction between the two molecules, we performed co-IP experiments using mouse retinal extracts as an input sample with anti-Atp6ap2 antibody and immunoblot analyses with anti-Pdhb antibody. In accordance with the yeast two-hybrid data (Fig. 1B), the anti-Atp6ap2 antibody demonstrated the precipitation of Pdhb protein from the retina, whereas the input retinal extracts as a positive control showed an immunoreactive band of the predicted size (Fig. 1C).

To further verify the molecular interaction between ATP6AP2 and PDHB, co-IP experiments followed by immunoblot analyses were performed with transfected HEK293T cells using antibodies against FLAG or Myc tag. We subcloned human ATP6AP2 and PDHB cDNAs into expression vectors containing FLAG and Myc, respectively, and co-transfected both of these constructs into HEK293T cells. IP with anti-Myc antibody from the co-transfected cell extracts demonstrated that PDHB could pull down ATP6AP2 (Fig. 1D). Additionally, we generated two deletion mutants of ATP6AP2 (Fig. 1D) to address a key domain to interact with the PDHB protein. Deletion of the N-terminal domain (ATP6AP2-ΔN) lost interaction with PDHB, whereas deletion of the C-terminal domain (ATP6AP2-ΔC) did not alter interaction with PDHB (Fig. 1D). These results suggested that the N-terminal domain in the ATP6AP2 protein is an essential region required for its direct binding with PDHB.

Co-localization of Atp6ap2 with Pdhb in the Retina—Previously, we reported that the Atp6ap2 protein is constitutively and ubiquitously expressed in the retina of adult mice (11, 20); however, no study on retinal localization of PDHB has ever been reported. To investigate the co-localization of Atp6ap2 with Pdhb, we performed double immunofluorescence in the mouse retina using the anti-Atp6ap2 antibody that recognizes the N-terminal domain, which is required for PDHB binding (Fig. 1D). Pdhb immunoreactivity was markedly detected in multiple cell types including RPE, photoreceptor (the inner segment), and ganglion cells, most of which demonstrated its co-localization with Atp6ap2 (Fig. 2, A–C).

When functioning as (P)RR to bind with prorenin, ATP6AP2 utilizes its N-terminal domain targeted to the extracellular space (22). To confirm intracellular localization of the N-terminal region of Atp6ap2, we carried out immunoelectron microscopy. Immunogold labeling revealed that the N-terminal domain of Atp6ap2 was present mainly in the cytosol of RPE (Fig. 2D) and other cells, in accordance with immunofluorescent co-localization results (Fig. 2, A–C). Subcellular protein fractionations of mouse retinal extracts followed by immunoblot analysis demonstrated that Atp6ap2 protein localized to both of membrane and cytosolic fractions (Fig. 2E).

Because RPE cells are one of the major cell types possessing a high oxidative capacity in the eye (5), we decided to use human RPE cell culture as a suitable tool for analyzing the potential role of ATP6AP2 in PDH-mediated aerobic metabolism, as shown...
in the following series of experiments (Figs. 3–5). Immunohistochemistry using human eye specimens confirmed the constitutive expression of ATP6AP2 protein in the RPE layer (Fig. 2, F and G).

Reduced PDH Activity and Acetyl-CoA Levels and Elevated Lactate Levels following ATP6AP2 Knockdown—To clarify the biochemical function of ATP6AP2 bound with PDHB, we carried out siRNA experiments to investigate whether ATP6AP2 knockdown affects PDH-related functions. We transfected either of two different sequences of siRNAs (siRNA-1 and -2) against ATP6AP2 into human RPE cells individually, and checked the knockdown efficiency. Both siRNAs significantly suppressed ATP6AP2 mRNA expression compared with control siRNA (Fig. 3A).

Using these siRNAs against ATP6AP2, we measured PDH enzymatic activity together with levels of acetyl-CoA and lactate, both of which are pyruvate metabolites, in siRNA-transfected RPE cells. Intriguingly, ATP6AP2 knockdown with siRNA-1 or -2 reduced PDH activity (Fig. 3A) and acetyl-CoA levels (Fig. 3D), but increased lactate levels (Fig. 3C) as compared with control siRNA. These results suggested that ATP6AP2 is involved in PDH activity that governs the conversion of pyruvate into aerobic (i.e. acetyl-CoA), but not anaerobic (i.e. lactate) metabolites.

Conversely, we next examined the effect of ATP6AP2 overexpression on the PDH-related parameters. ATP6AP2 plasmid DNA was successfully transfected into RPE cells (Fig. 3E); however, there were no significant differences between mock-transfected and ATP6AP2-overexpressed cells in either PDH activity or lactate levels (Fig. 3, F and G). These results suggested that endogenous ATP6AP2 levels are saturated or sufficient enough to control PDH activity.

Additionally, we performed siRNA experiments using HEK293T cells derived from the human embryonic kidney. ATP6AP2 mRNA expression was significantly suppressed by siRNAs compared with control (Fig. 3H). In contrast to human RPE cells, ATP6AP2 knockdown in human kidney cells did not show any significant changes in PDH activity or lactate levels (Fig. 3, I and J). These data suggested that the requirement of ATP6AP2 for PDH activity for aerobic metabolism is cell type-dependent.

Reduced PDHB Protein, but Not mRNA, Levels and Enhanced PDHB Phosphorylation following ATP6AP2 Knockdown—To elucidate the mechanism underlying reduced PDH activity due to ATP6AP2 knockdown, we checked protein levels of the PDH complex subunits including PDHA1, PDHB, and DLAT, all of which were detected by MS (Table 1). Knockdown of ATP6AP2, markedly impairing ATP6AP2 protein synthesis, did not alter PDHA1 or DLAT levels (Fig. 4A). By contrast, PDHB protein levels were substantially decreased by ATP6AP2 siRNAs compared with control siRNA (Fig. 4A), whereas mRNA expression levels were unchanged (Fig. 4B). The discrepancy between the protein and mRNA levels indicated that PDHB is regulated by ATP6AP2 at least in a post-transcriptional manner.

It was previously shown that a case of congenital PDH deficiency, associated with PDHB protein reduction despite no responsible gene mutations, exhibited a high basal level of tyrosine phosphorylation of PDHB protein leading to enhanced ubiquitination followed by proteasome-mediated degradation (23). Thus, we hypothesized that the post-transcriptional decrease in PDHB following ATP6AP2 knockdown (Fig. 4, A and B) is attributable to PDHB tyrosine phosphorylation, and examined the tyrosine-phosphorylated PDHB ratios via IP experiments. Cell lysates from ATP6AP2-knockdown RPE cells were immunoprecipitated with anti-PDHB antibody, and immunoblot analyses using anti-phosphotyrosine antibody were then carried out. ATP6AP2 knockdown reduced PDHB protein levels as compared with control; however, there were no apparent differences in levels of tyrosine phosphorylation, indicating that the PDHB phosphorylation was enhanced by ATP6AP2 knockdown (Fig. 4C).

To further determine whether PDHB tyrosine phosphorylation is required for the ATP6AP2 knockdown-mediated reduction of PDH protein levels (Fig. 4, A and C), we performed blocking experiments using the broad-spectrum tyrosine kinase inhibitor AG18. Treatment with AG18 almost completely recovered PDHB protein levels in RPE cells receiving ATP6AP2 knockdown (Fig. 4D). Treatment with another tyrosinase, AG82, also showed equivalent results with AG18 (data not shown). These data suggested that ATP6AP2 protects PDHB protein from a post-translational modification in which PDHB undergoes tyrosine phosphorylation and subsequent degradation in the absence of ATP6AP2, resulting in successful translocation of PDHB to the mitochondria, subsequent construction of the PDH complex and consequent preservation of its activity.

**TABLE 1**

| Gel band | Identified protein | Gene name | Molecular mass | No. of peptides | Accession number |
|----------|-------------------|-----------|----------------|-----------------|-----------------|
| A1       | Dihydrolipoyllysine residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial | Dlat | 67,942 | 26 | Q8BMF4 |
| A2       | Actin-binding LIM protein 2 | Ablim2 | 68,107 | 1 | Q8BL65 |
| B1       | Pyruvate dehydrogenase E1 component subunit β, mitochondrial | Pdhb | 38,937 | 14 | Q9DO51 |
| B2       | Pyruvate dehydrogenase E1 component subunit α, somatic form, mitochondrial | Pdh1a | 43,232 | 5 | P54586 |
| B3       | (Pro)renin receptor | Atp6ap2 | 39,092 | 10 | Q0CVN9 |
| B4       | 60 S ribosomal protein L6 | Rp6 | 33,510 | 1 | P47911 |
| B5       | 60 S acidic ribosomal protein P0 | Rppl0 | 34,216 | 1 | P14869 |
| B6       | F-actin-capping protein subunit α-2 | Capza2 | 32,967 | 2 | P47754 |
| B7       | V-type proton ATPase subunit d1 | Atp6v0d1 | 40,301 | 3 | P51863 |
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Suppression of Glucose-stimulated Oxidative Stress following ATP6AP2 Knockdown—Given that the PDH complex plays a pivotal role in linking glycolysis to mitochondrial aerobic metabolism and that excess glucose causes superoxide production through the respiratory chain, we hypothesized that ATP6AP2 contributes to glucose-stimulated oxidative stress via PDH activity. To examine whether ATP6AP2 knockdown suppresses the generation of ROS in RPE cells treated with high glucose, we measured ROS levels using the general oxidative stress indicator CM-H$_2$DCFDA. ROS generation in RPE cells, enhanced by high glucose application, was significantly suppressed by ATP6AP2 knockdown (Fig. 5A).

To rule out the possibility that the suppressive effect of ATP6AP2 knockdown on glucose-stimulated ROS generation (Fig. 5A) depended on its role as (P)RR in triggering the RAPS (i.e. tissue RAS activation together with RAS-independent signal transduction), we utilized the (P)RR blocker (11) and the angiotensin II type 1 receptor blocker valsartan, both of which can eliminate ROS generation in the downstream of RAPS and RAS, respectively. Data obtained using either of the two blockers (Fig. 5B) were equivalent to the original data (Fig. 5A). These results indicated that glucose-induced oxidative stress was suppressed by ATP6AP2 knockdown independently of the RAPS- and RAS-related pathways.

DISCUSSION

Post-translational modifications such as phosphorylation, glycosylation, and ubiquitination can modify protein functions and dynamically coordinate their signaling networks. Rapid and reversible modulation of molecular activities by post-translational modifications is necessary for adaptation to changing cellular environments and is accomplished by modifying protein stability, subcellular localization, and protein-protein interaction (24–27). In the present study, ATP6AP2 was shown to interact directly and co-localize in the retina with the PDHB subunit of the PDH complex (Fig. 1 and 2), a key enzyme in energy metabolism linking glycolysis to the aerobic energy-generating pathway. Importantly, ATP6AP2 regulated the tyrosine phosphorylation of PDHB in an inhibitory manner, contributing to its protein stability (Fig. 4). Accordingly, knockdown of ATP6AP2 in RPE cells decreased PDH enzymatic activity together with acetyl-CoA levels while increasing lactate levels, showing a predilection to seemingly anaerobic glycolysis (Fig. 3). Moreover, down-regulated PDH activity due to ATP6AP2 knockdown suppressed glucose-induced ROS generation in RPE cells (Fig. 5). However, the changes in PDH activity (Fig. 3B) and associated reactions (Figs. 3, C and D, 5A) were relatively small compared with the ATP6AP2 knockdown efficiency (Fig. 3A). This mode of regulation is consistent with previous findings that little or no expression of PDHB protein still maintained PDH activity almost by half (23), suggesting that ATP6AP2 plays a role as a modulator in the energy-generating pathway. In addition to the RAPS-related pathological roles in retinal inflammation and angiogenesis (10–13) as well as the physiological function as a cell polarity determinant during retinal development (20), our current study provided a novel insight into the biological function of ATP6AP2 in the retina. ATP6AP2 was newly characterized as a PDHB protein stabilizer associated with PDH enzymatic activity, thus contributing to aerobic glucose metabolism together with oxidative stress (Fig. 6).

Aerobic metabolism can efficiently create a large amount of ATP via oxidative phosphorylation in mitochondria following the combustion of carbohydrates in the presence of oxygen. The PDH complex, a multienzyme complex that catalyzes the

FIGURE 2. Co-localization of Atp6ap2 with Pdhb in the retina. A–C, double labeling of Pdhb (green) and Atp6ap2 (red) together with DAPI (blue). A’–C’, images show higher magnification. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar = 30 μm. D and D’, immunogold labeling for Atp6ap2 in the cytosol of RPE cells. Scale bar = 1 μm. E, mouse retinal homogenate was fractionated to the cytosol and membrane followed by SDS-PAGE and immunoblot analyses using anti-Atp6ap2, anti-Hsp90 (cytosolic marker), and anti-Na$^+/K^+$-ATPase α1 (plasma membrane marker) antibodies. F, Atp6ap2 immunostaining (purple) in human RPE. G, no staining with normal IgG. Scale bar = 15 μm.

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oxidative decarboxylation of pyruvate to yield acetyl-CoA, is essential for regulating cellular fuel metabolism and acid-base equilibrium. The complex comprises three catalytic components including PDH (E1), DLAT (E2), and DLD (E3) combined with the E3-binding protein PDHX. The E1 enzyme is a heterotetramer of two E1 α subunits (PDHA1) and two E1 β subunits (PDHB) with thiamine pyrophosphate as a cofactor for pyruvate decarboxylation. Mutations in this complex are the major cause of primary lactic acidosis, which is complicated by neurological presentations due to degenerative changes in the central nervous system where high energy from the aerobic oxidation of glucose is indispensable (28, 29).

Interestingly, even though PDHB is expressed ubiquitously, the Pdhb mutant zebrafish demonstrated the retina-specific
mitochondrial diseases that the eye is regarded as the initial or most prominently affected organ (39). Inconsistency with the retina-specific phenotype of Pdhb mutant vertebrates (30), the ATP6AP2 knockdown-mediated reduction of PDH activity was shown to be cell type-dependent (Fig. 3), suggesting that the newly characterized function of ATP6AP2 as a PDH stabilizer may possibly be confined exclusively to metabolically active cells such as in the retina.

Acute and chronic excess glucose leads to devastating changes in energy metabolism. Under the aerobic state, a rapid and sustained rise in pyruvate as a product of cytosolic glycolysis accelerates the PDH-mediated conversion of pyruvate to acetyl-CoA followed by mitochondrial NADH-dependent ATP synthesis together with surplus superoxide leakage, leading to oxidative stress that plays a central role in the pathogenesis of diabetes (40, 41). Reasonably, the antioxidative effect of ATP6AP2 may be of particular relevance in the retina.

FIGURE 3. Reduced PDH activity and acetyl-CoA levels and elevated lactate levels following ATP6AP2 knockdown in RPE cells. A, gene expression levels of ATP6AP2 in ATP6AP2-knockdown RPE cells. Real-time PCR amplification of ATP6AP2 after transfection with ATP6AP2 or negative control siRNA (fold-change = 0.12 for siRNA-1 and 0.16 for siRNA-2). B, PDH enzymatic activity in ATP6AP2-knockdown RPE cells (control = 1.32 ΔA450/mg of protein/min, siRNA-1 = 1.16 ΔA450/mg of protein/min, siRNA-2 = 1.17 ΔA450/mg of protein/min) (n = 8). C, lactate levels in ATP6AP2-knockdown RPE cell culture medium (control = 2.51 μmol/10^6 cells, siRNA-1 = 3.49 μmol/10^6 cells, siRNA-2 = 3.48 μmol/10^6 cells) (n = 14). D, acetyl-CoA levels in ATP6AP2-knockdown RPE cells (control = 11.77 pmol/mg of protein, siRNA-1 = 7.15 pmol/mg of protein, siRNA-2 = 7.96 pmol/mg of protein) (n = 10). E, gene expression levels of ATP6AP2 in ATP6AP2-overexpressed RPE cells. Real-time PCR amplification of ATP6AP2 after transfection with ATP6AP2 or mock plasmid DNA (ATP6AP2, fold-change = 3.6) (n = 5). F and G, PDH enzymatic activity (mock = 1.23 ΔA450/mg of protein/min, ATP6AP2 = 1.18 ΔA450/mg of protein/min) and lactate levels (mock = 2.52 μmol/10^6 cells, ATP6AP2 = 2.36 μmol/10^6 cells) in ATP6AP2-overexpressed RPE cells (n = 8–12). H, gene expression levels of ATP6AP2 in ATP6AP2-knockdown HEK293T cells. Real-time PCR amplification of ATP6AP2 after transfection with ATP6AP2 or control siRNA (fold-change = 0.14 for siRNA-1 and 0.10 for siRNA-2) (n = 4). I and J, PDH enzymatic activity (control = 0.46 ΔA450/mg of protein/min, siRNA-1 = 0.47 ΔA450/mg of protein/min, siRNA-2 = 0.45 ΔA450/mg of protein/min) and lactate levels (control = 1.87 μmol/10^6 cells, siRNA-1 = 1.95 μmol/10^6 cells, siRNA-2 = 1.97 μmol/10^6 cells) in ATP6AP2-knockdown HEK293T cells (n = 8–12); *, p < 0.05; **, p < 0.01.

FIGURE 4. Reduced PDH protein, but not mRNA, levels and enhanced PDH phosphorylation following ATP6AP2 knockdown. A, immunoblot (IB) analyses for ATP6AP2, PDHA1, DLAT, PDHB, and β-actin proteins in ATP6AP2-knockdown RPE cells. Relative intensity = 0.49 ± 0.12 for siRNA-1, and 0.60 ± 0.18 for siRNA-2, compared with control (n = 3). B, gene expression levels of PDHB in ATP6AP2-knockdown RPE cells. Real-time PCR amplification of PDHB after transfection with ATP6AP2 or negative control siRNA (fold-change = 0.97 for siRNA-1 and 1.05 for siRNA-2). C, co-IP of ATP6AP2-knockdown RPE cell extracts using anti-PDH antibody followed by SDS-PAGE and IB analyses using anti-PDH and anti-phosphotyrosine (p-tyrosine) antibodies. Relative intensity = 1.24 ± 0.10 for siRNA-1, and 1.25 ± 0.21 for siRNA-2, compared with control (*, p < 0.05, n = 4). D, IB analyses using anti-PDH antibody following treatment with or without the tyrosine kinase inhibitor AG18 for ATP6AP2-knockdown RPE cells. Relative intensity = 0.55 ± 0.12* for siRNA-1, 0.65 ± 0.08* for siRNA-2, 0.98 ± 0.14 for control + AG18, 0.94 ± 0.09 for siRNA-1 + AG18, and 0.99 ± 0.05 for siRNA-2 + AG18, compared with control (*, p < 0.05, n = 4). Densitometry values of PDHB and tyrosine-phosphorylated PDHB (p-PDH) normalized to β-actin and total PDHB (t-PDH), respectively, are shown in parentheses (A, C, and D).

phenotype characterized by a decrease in motion vision followed by loss of light perception, resulting from retinal degeneration due to energy insufficiency (30). In contrast, vertebrates deficient in Pdha1, Dld (mouse), and Dlat (zebrafish) exhibited prenatal to premature death, suggesting the systemic requirement of the mitochondrial oxidative pathway is dependent on these subunits of the Pdh complex (31–33). The Dlat mutant animals, although lethal, also showed retinal impairment to blindness during lifetime (33). Thus, the Pdhb mutant model serves as a fascinating example of the dysfunction of a ubiquitous protein leading to a tissue-specific abnormality. The retina consumes an exceptionally high amount of energy in the processes of phototransduction and visual cycle recovery (3, 4, 34–37). Indeed, mitochondria-rich photoreceptors are among the most metabolically active cells in the body (34, 38, 39). Furthermore, ophthalmologic manifestations such as optic neuropathy and retinitis pigmentosa are so common to several
ity (Fig. 3B), causing a metabolic shift from aerobic cellular respiration to anaerobic glycolysis (Fig. 6). This mechanism of action is compatible with and explained by several previous data showing that PDH was responsible for mitochondrial ROS generation and that inhibition of PDH activity in turn mitigated oxidative stress (42–44). The blocking experiments to rule out the involvement of the RAPS-related pathways (Fig. 5B) further confirmed the regulatory role of ATP6AP2 in PDH-mediated aerobic metabolism and ROS generation (Fig. 6).

During evolution, the respiratory and circulatory systems combined with efficient energy generation have been acquired and modified to adapt properly to various environmental changes related to oxygen concentration. Energy metabolism is particularly active in the vertebrate retina, especially RPE and photoreceptor cells receiving ample oxygen supply from the well-developed vasculature of the choroid, which has never been provided for the invertebrate retina with photoreceptors arrayed in front (i.e. back to front). Interestingly, the N-terminal amino acid sequence of ATP6AP2, which interacts with PDHB (Fig. 1D), is highly conserved in vertebrates, but not in invertebrates (45), suggesting that the vertebrate (or at least the mammalian) N-terminal sequence of Atp6ap2 allows high oxygen consumption in the retina by ensuring Pdh enzymatic activity against Pdhb protein degradation. Ironically, as the price for the ATP6AP2-dependent efficient energy generation acquired in human eyes, oxidative stress has emerged as the underlying pathogenesis of diabetic retinopathy in the current era of excessive eating.

When functioning as (P)RR to bind with its ligand prorenin, ATP6AP2 is known to have its N-terminal domain targeted to the extracellular space (i.e. to the ER lumen during synthesis) or released in a C-terminal truncated form as soluble (P)RR (11, 13, 22). In contrast, our ultrastructural immunohistochemistry demonstrated that the N-terminal region of Atp6ap2 was present mainly in the cytosol of RPE cells (Fig. 2D). Single-pass molecules with their C-terminal domains targeted to the ER lumen are topologically classified into type II transmembrane proteins. Indeed, as an accessory subunit for v-ATPase for urinary acidification, Atp6ap2 (C-terminal region) was shown to be detectable within the Golgi apparatus and at the luminal surface of intercalated cells in the kidney collecting duct (46). Our previous data on Atp6ap2 interaction with the partitioning defective 3 homolog (20) suggested that Atp6ap2 had its N-terminal domain targeted to the cytosol harboring partitioning defective 3 homolog. Therefore, it is reasonable to speculate that the direction of Atp6ap2 to penetrate the lipid membrane may differ depending on its multiple functions.

ATP6AP2 plays a pivotal role in the activation of RAPS, thus contributing to the molecular pathogenesis of end-organ damage such as inflammation and angiogenesis including diabetic retinopathy (10–13). In concert with the present findings on its association with oxidative stress (Fig. 5), ATP6AP2 is considered to be pathogenic with its N-terminal domain bound to prorenin and PDHB alike, triggering the distinctly different pathways related to the pathogenesis (i.e. retinal RAPS activation and mitochondrial ROS generation, respectively). Given that the N-terminal truncated form of ATP6AP2 is required for the v-ATPase-mediated physiological functions (17–19), the
current data provided a significant biological rationale for specifically targeting the N-terminal amino acid sequence of ATP6AP2 for the treatment of diabetic retinopathy. In conclusion, our present findings revealed the brand-new function of ATP6AP2/(P)RR associated with PDH enzymatic activity as a protein stabilizer for PDHB, thus regulating the energy-generating pathway of glucose metabolism as well as oxidative stress in the eye.

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