Brief Ultrarapid Communication

The (Pro)renin Receptor/ATP6AP2 is Essential for Vacuolar H\(^+\)-ATPase Assembly in Murine Cardiomyocytes

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Rationale: The (pro)renin receptor [(P)RR], encoded in ATP6AP2, plays a key role in the activation of local renin-angiotensin system (RAS). A truncated form of (P)RR, termed M8.9, was also found to be associated with the vacuolar H\(^+\)-ATPase (V-ATPase), implicating a non–RAS-related function of ATP6AP2.

Objective: We investigated the role of (P)RR/ATP6AP2 in murine cardiomyocytes.

Methods and Results: Cardiomyocyte-specific ablation of Atp6ap2 resulted in lethal heart failure; the cardiomyocytes contained Rab7- and lysosomal-associated membrane protein 2 (LAMP2)-positive multivesicular vacuoles, especially in the perinuclear regions. The myofibrils and mitochondria remained at the cell periphery. Cardiomyocyte death was accompanied by numerous autophagic vacuoles that contained undigestible cellular constituents, as a result of impaired autophagic degradation. Notably, ablation of Atp6ap2 selectively suppressed expression of the V\(\text{O}_2\) subunits of V-ATPase, resulting in deacidification of the intracellular vesicles. Furthermore, the inhibition of intracellular acidification by treatment with bafilomycin A1 or chloroquine reproduced the phenotype observed for the (P)RR/ATP6AP2-deficient cardiomyocytes.

Conclusions: Genetic ablation of Atp6ap2 created a loss-of-function model for V-ATPase. The gene product of ATP6AP2 is essential for cell survival. (Circ Res. 2010;107:30-34.)

Key Words: V-ATPase • autophagy • heart failure • bafilomycin • renin-angiotensin system

Activation of the (pro)renin receptor [(P)RR], the gene product of ATP6AP2, plays a key role in the local renin-angiotensin system (RAS). We have shown that (P)RR activation is involved in the development of cardiac fibrosis and proteinuria in hypertension and diabetes. Interestingly, a truncated form of (P)RR, termed M8.9, was also found to be associated with the vacuolar H\(^+\)-ATPase (V-ATPase), implicating a non–RAS-related function of the gene products of ATP6AP2. In the present study, we show that gene products of ATP6AP2 are essential for cardiomyocyte survival via regulating V-ATPase function.

Methods

We generated conditional knockout (CKO) mice in which exon 2 of the Atp6ap2 gene was flanked by loxP sites (Online Figure I). Atp6ap2-floxed mice were bred with mice that expressed the Cre recombinase under the control of the cardiomyocyte-specific α-myosin heavy chain (α-MHC) promoter (Online Figure II). The resulting Atp6ap2\(^{lox/Y}\); αMHC-Cre\(^{+}\)mice represent cardiac-specific Atp6ap2 CKO mice. The control male mice were littermates that were heterozygous for α-MHC-Cre (αMHC-Cre \(^{+}\); Atp6ap2\(^{+}\)), thereby excluding Cre-mediated toxicity as the basis for phenotypic disparity. An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results and Discussion

The CKO mice were born at the expected mendelian frequency, without any gross cardiac anomalies being noted in the newborn mice, although cardiomyocyte-specific ablation of Atp6ap2 inevitably resulted in heart failure and the mice died within 3 weeks of birth (Figure 1a through 1c). Ventric-
ular functions were severely impaired on postnatal day (PD)18 (fractional shortening, 12.87 ± 4.73% versus 27.69 ± 5.8% for CKO versus control; n = 3; *P < 0.05) (Figure 1d, Online Video I). The CKO mice showed significantly increased ratios of heart weight–to–body weight beginning on PD14 (Figure 1e). The levels of cardiac stress markers, including atrial natriuretic peptide, brain natriuretic peptide, α-skeletal actin, connexin, connective tissue growth factor, and monocye chemoattractant protein-1, were increased as early as PD10 (Figure 1f). Histological examination of the CKO mice on PD18 revealed that clusters of degenerating cardiomyocytes with extensive vacuolation, especially in the perinuclear region, were embedded in areas of replacement fibrosis (Figure 2a and 2b). Electron microscopic examination of the CKO cardiomyocytes revealed perinuclear accumulations of numerous multivesicular vacuoles (Figure 2c and 2d). The myofibrils and mitochondria were located exclusively at the cell periphery. In addition, we observed large, electron-dense autophagic vacuoles that contained partially digested or undigested cellular constituents, such as mitochondria and aberrant vacuoles, scattered in the field of cell debris around the perinuclear region (Online Figure III). The accumulation of microtubule-associated protein 1 light chain 3 (LC3)-II (a phosphatidylethanolamine conjugate) and p62/SQSTM1, as well as the induction of genes in response to amino acid starvation (eg, the genes for asparagine synthetase, activating transcription factor 4, and C/EBP homologous protein) reflected defective autophagic protein degradation in the CKO mice (Figure 2e and 2f).

To examine the underlying cellular mechanism responsible for cardiac death, we examined the role of the ATP6AP2 protein in the function of V-ATPase, which maintains a
luminal acidic environment in the intracellular vesicular compartments (Figure 3a). Mouse embryonic fibroblasts (MEFs) were obtained from male mice that were hemizygous for the floxed Atp6ap2 allele. The Atp6ap2 gene was ablated by infecting the MEFs with the Cre adenovirus (Ad-Cre) (Online Figure IVa). Quantitative PCR and Western blot analyses showed that ~90% of the ATP6AP2 protein was missing in the floxed MEFs after Ad-Cre treatment, as compared with the wild-type (WT) MEFs (Online Figure IVb and Figure 3b). V-ATPase is a large multisubunit complex that is organized into the V1 and VO sectors. In mammals, the V1 sector is composed of 8 different subunits (A through H), whereas the VO sector contains 6 different subunits (a, c, c', d, e, and the accessory subunit Ac456) (Figure 3a). Western blot and immunohistochemical analyses revealed that the levels of subunits a1, a2, and c were significantly decreased in the floxed MEFs after Ad-Cre infection, as compared with the WT MEFs (Figure 3b and 3c). In contrast, the level of V1 subunit E2 was unaffected. Consistent with these findings, LysoTracker staining revealed that the loss of ATP6AP2 was accompanied by impaired vesicular acidification (Figure 3c). Taken together, these findings suggest that genetic ablation of ATP6AP2 selectively affects the stability and assembly of the VO subunits, thereby compromising vesicular acidification.

Consistent with the findings observed for cultured cells, ATP6AP2-depleted hearts revealed that the characteristic perinuclear vacuoles in the cardiomyocytes were positive for late endosomal/lysosomal markers RAB7 and/or LAMP2 (Figure 3d). The levels of the c-subunit of the VO sector but not the E2 subunit of the V1 sector markedly reduced in the CKO cardiomyocytes.

To investigate whether disruption of intracellular acidification accounts for the phenotype of the ATP6AP2-deficient cardiomyocytes, we treated cultured cardiomyocytes with bafilomycin A1 or chloroquine. Sequential time-lapse microscopic analysis revealed that intracellular vacuoles accumulated over time (Figure 4; Online Video II). These vacuoles were positive for RAB7 (Figure 4). Interestingly, Atp6ap2 mRNA expression in the cultured cardiomyocytes was strikingly upregulated after treatment with either bafilomycin A1 or chloroquine (Online Figure V).

The biogenesis of the multisubunit complex of V-ATPase requires the coordinated association of V1 subunits, which are synthesized in the cytosol, with VO subunits, which are targeted to the vacuolar membrane. Studies in yeast cells have shown that the loss of a V1 subunit has little effect on the stability of the remaining V1 subunit, whereas the loss of any single VO subunit affects the stability and assembly of the remaining VO subunits. In yeast, several additional genes (Vma12p, Vma21p, and Vma22p) that are required for V-ATPase assembly have been identified. The VO subunits were detected at greatly reduced levels in the mutant cells that lacked these assembly factors, an effect that is similar to that observed after the loss of a VO subunit.

Interestingly, there is no known yeast homolog of the mammalian ATP6AP2. It is possible that ATP6AP2 is an assembly chaperone of V-ATPase, representing a function that is unique to mammals. An alternative scenario is that ATP6AP2 is a component of the VO sector itself rather than an assembling factor. Atp6ap2 mRNA expression was upregulated in cells that were treated with bafilomycin A1 or chloroquine, which suggests that ATP6AP2 senses the acidity levels of the intracellular compartments and accordingly regulates V-ATPase activity.
In conclusion, the gene product of \textit{Atp6ap2} is considered to act in 2 ways: (1) as (P)RR, exerting an RAS-related function; and (2) as the V-ATPase–associated protein, exerting a non–RAS-related function that is essential for cell survival. The phenotypes observed after genetic ablation of \textit{Atp6ap2} are ascribed to V-ATPase loss of function. Further characterizing the function of ATP6AP2 as an assembly chaperone of V-ATPase and the pathological function of ATP6AP2 is indispensable for the assembly of V-ATPase. a, Structure of the V-ATPase and putative colocalization of ATP6AP2 and the V-ATPase. TM & CD indicates transmembrane and cytosolic domain; and ECD, extracellular domain. b and c, The levels of \(V_{a}\) subunits are decreased significantly in the floxed MEFs after deletion of the \textit{Atp6ap2} gene. The \(V_{i}\) subunit \(E2\) appears to be unaffected. c, Defective acidification in the floxed MEFs after deletion of the \textit{Atp6ap2} gene. Scale bars: 50 \(\mu\)m. d, Immunofluorescence staining of left ventricular tissues from WT and CKO mice on PD18. The vacuoles in the actinin-positive cardiomyocytes are RAB7-positive and LAMP2-positive. The levels of the c-subunit of the \(V_{o}\) sector of the V-ATPase are markedly reduced in the CKO cardiomyocytes, whereas the depletion of ATP6AP2 had no effect on the expression of the subunit \(E2\) in cardiomyocytes. Nuclei were counterstained with DAPI (blue). Scale bar: 10 \(\mu\)m.

**Figure 3.** ATP6AP2 is indispensable for the assembly of V-ATPase. a, Structure of the V-ATPase and putative colocalization of ATP6AP2 and the V-ATPase. TM & CD indicates transmembrane and cytosolic domain; and ECD, extracellular domain. b and c, The levels of \(V_{a}\) subunits are decreased significantly in the floxed MEFs after deletion of the \textit{Atp6ap2} gene. The \(V_{i}\) subunit \(E2\) appears to be unaffected. c, Defective acidification in the floxed MEFs after deletion of the \textit{Atp6ap2} gene. Scale bars: 50 \(\mu\)m. d, Immunofluorescence staining of left ventricular tissues from WT and CKO mice on PD18. The vacuoles in the actinin-positive cardiomyocytes are RAB7-positive and LAMP2-positive. The levels of the c-subunit of the \(V_{o}\) sector of the V-ATPase are markedly reduced in the CKO cardiomyocytes, whereas the depletion of ATP6AP2 had no effect on the expression of the subunit \(E2\) in cardiomyocytes. Nuclei were counterstained with DAPI (blue). Scale bar: 10 \(\mu\)m.

**Figure 4.** Inhibition of vesicular acidification by bafilomycin A1 or chloroquine mimics the in vivo cardiac phenotype that results from ablation of \textit{Atp6ap2}. The rat cardiomyocytes were treated with bafilomycin A1 (100 nmol/L) or chloroquine (10 \(\mu\)mol/L) for 24 hours and then stained either with RAB7 plus DAPI or LysoTracker Red. Note that treatment with bafilomycin A1 or chloroquine reproduces the RAB7-positive intracellular vesicles by inhibiting vesicular acidification. Scale bars: 100 \(\mu\)m (left panels) and 20 \(\mu\)m (center and right panels).
(P)RR would require rescue experiments with the WT protein and with mutant proteins that lack the domain responsible for binding renin and prorenin.

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Disclosures
None.

References

Novelty and Significance

What is Known?
- The renin-angiotensin system, is encoded by the Atp6ap2 gene.
- The gene products of Atp6ap2 are associated with V-ATPase, which maintains an acidic environment in the lumen of intracellular vesicular compartments.

What New Information Does This Article Contribute?
- Cardiomyocyte-specific ablation of Atp6ap2 caused fulminant heart failure.
- Ablation of Atp6ap2 selectively suppressed protein expression of the V0 subunits of V-ATPase, resulting in deacidification of intracellular vesicles.

- The phenotypes observed after genetic ablation of Atp6ap2 are ascribed to V-ATPase loss of function.

The gene products of Atp6ap2 have 2 distinct functions. Their first role in regulating the renin-angiotensin system pathway is well established, but a secondary role in regulating V-ATPase (and other cellular roles) has not been proposed or investigated in vivo. In the present study, we generated a mouse with a cardiac-specific deficiency in Atp6ap2. The Atp6ap2-disrupted cardiomyocytes showed extensive vacuolation, a phenotype that could be reproduced by pharmacologically inhibiting intracellular acidification. We demonstrated for the first time that Atp6ap2 might be an essential assembly chaperone of mammalian V-ATPase and that genetic ablation of Atp6ap2 created a loss-of-function model for V-ATPase.
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Full Methods

*Animals.* All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the Keio University School of Medicine.

*Generation of Atp6ap2 conditional knockout mice.* The 13.3-kb genomic fragment that contains exons 2 and 3 of the *Atp6ap2* gene was obtained from a mouse BAC clone (RP23-339K2). A targeting vector was designed to insert a *loxP* and *frt*-flanked PGK-*neo* cassette upstream of exon 2, and a third *loxP* site downstream of exon 2 of the targeted gene. The targeting vector was electroporated into C57/BL6 (B6) mouse-derived embryonic stem (ES) cells. Correctly targeted ES cells were injected into recipient blastocysts, and chimeric mice were bred with C57BL/6 mice, to establish a colony. The mutant mice were then bred with mice that expressed the Flp recombinase, to remove the *frt*-flanked *neo* cassettes. The resulting male *Atp6ap2*flx/Y mice (with *loxP* sites and a single *Frt* site remaining upstream of exon 2, and a second *loxP* site located downstream of exon 2) were bred with female mice that expressed cell-type-specific Cre recombinase.

*Cardiomyocyte culture.* Neonatal ventricular myocytes from 1- to 2-day-old Sprague-Dawley rats were subjected to Percoll gradient centrifugation and differential plating in order to enrich for cardiac myocytes and deplete nonmyocyte populations.1

*Histology.* Hearts were fixed overnight in 10% formalin at 4°C, dehydrated with 70% ethanol, mounted in paraffin, and sectioned (5-µm thickness). Heart sections were stained with hematoxylin and eosin (HE) or with Masson trichrome for fibrosis.2 Low and high-magnification fields of short-axis views were analyzed using a BIOREVO (BZ-9000; Keyence, Japan).

For immunostaining, hearts were fixed in 4% paraformaldehyde, then successively infiltrated with 30% sucrose in PBS, embedded in OCT compound (Miles), and stored frozen. Sections of 6 micrometer thickness were mounted on gelatin-coated slides, and then stained with hematoxylin. Sections were stained immunochemically as described previously.3 The immunofluorescence of cultured cells were performed as previously described.4 For the labeling of acidic organelles, cells were incubated with LysoTracker (Molecular Probes) for 30 minutes, and then fixed with 4% paraformaldehyde in PBS (pH 7.4). Fluorescence images were acquired with a confocal microscope, LSM 510 (Carl Zeiss).

For transmission electron microscopy, tissue was minced and fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M cacodylate buffer, prepared according to standard protocol; and
electron microscopy was performed using the RMC MT6000 ultramicrotome, and visualized using the Hitachi H7500 electron microscope and the 2K × 2K Gatan CCD camera.

For sequential time-lapse microscopic analysis, bafilomycin A1-, chloroquine-, or vehicle-treated cardiomyocytes were observed every 20-30 minutes over 48-72 hours using Leica FW4000.

**Antibodies.** Antibodies against V-ATPase subunits and RAB7 were described previously. Other primary antibodies used were monoclonal antibodies for LAMP2 and actin (DSHB and Abcam, respectively), anti-tubulin and GAPDH antibody (Cell Signaling Technology), anti-ATP6AP2 antibody (R&D Systems), anti-p62 C terminus polyclonal antibody (Progen), anti-LC3 (kind gift from Dr. Komatsu, the Tokyo Metropolitan Institute of Medical Science). Fluorescent dye or enzyme-linked secondary antibodies were obtained from Jackson ImmunoResearch.

**Gene expression.** Total RNA was purified using TRIzol reagent according to manufacturer’s instructions. For RT-PCR, total RNA was used for reverse transcriptase using random hexamer primers. Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). Predesigned gene-specific primer and probe sets (TaqMan Gene Expression Assays) were used. The 18s ribosomal RNA was amplified as an internal control.

**Western blotting.** Protein lysate preparation and immunoblotting procedures were performed as previously described. Total lysates were applied at 10-20 μg/lane on 5-20% gradient polyacrylamide gel (Daiich Pure Chemicals) and transferred to PVDF membrane with 0.2 μm pore size. The immunoblots were performed with aforementioned antibodies. Dulbecco’s modified Eagle medium (DMEM), minimal essential medium (MEM) and fetal bovine serum (FBS) were from Invitrogen. The protein blot was developed with an ECL detection kit (GE Healthcare) or chemiluminescence (Amersham Biosciences), and images were obtained using an image capture system (model LAS1000 and LAS3000 luminomager; Fujifilm). The intensities of bands were measured and analyzed using Image Gauge Software (Fujifilm).

**Echocardiography.** Mice were anesthetized by inhalation of 1.5% isoflurane, and then immobilized on a positionable platform in the supine position. Short-axis echocardiography and Doppler echocardiographic measurements were made using the Vevo 660 system (VisualSonics) with a 600 series real-time microvisualization scanhead probe. The left ventricular (LV) internal end-systolic diameter and LV end-diastolic diameter (LVESD and LVEDD, respectively) were measured using the leading-edge convention adopted by the American Society of Echocardiography. LV fractional shortening (FS) was calculated according to the formula: FS (%) = [LVEDD –
LVESD/LVEDD] × 100.

Statistics. Values are presented as mean ± SEM. Statistical significance was evaluated using 2-tailed, unpaired Student’s t tests for comparisons of 2 mean values. Multiple comparisons involving more than 3 groups were performed using ANOVA. A P value less than 0.05 was considered statistically significant.
Online Figure Legends

Online Figure I

Strategy used to generate a conditional \textit{Atp6ap2} allele. The genomic structure, targeting vector, and targeted allele are shown. CDS, coding sequence; UTR, untranslated region; FRT, flipase recognition target.

Online Figure II

Female \textit{Atp6ap2}-floxed mice were bred with male mice that expressed the Cre recombinase under the control of the cardiomyocyte-specific $\alpha$-myosin heavy chain (\(\alpha\)MHC) promoter.

Online Figure III

Ultrastructure of ATP6AP2-depleted cardiomyocytes. a, A giant autophagolysosome with undigested cytosolic constituents. b, A cytosolic inclusion composed of concentric lamellae body which is often found in lysosomal disease and chloroquine induced cardiomyopathy (arrow) c, Mitophagy (arrowhead). Scale bars: 1 \(\mu\)m.

Online Figure IV

\(a\), PCR genotyping of MEF DNA reveals a deletion of floxed \textit{Atp6ap2} contingent on coinheritance of Cre recombinase. \(b\), Quantitative PCR analysis reveals \(>90\%\) elimination of \textit{Atp6ap2} from floxed MEFs after Ad-Cre treatment.

Online Figure V

Pharmacologic inhibition of intracellular acidification significantly up-regulates \textit{Atp6ap2} mRNA expression in cultured cardiomyocytes. \(*P < 0.05\) vs. WT control (unpaired Student’s \(t\)-test).
Online Video Legends

Online Video I

Echocardiographic data in WT (a) and cardiomyocyte-specific Atp6ap2 CKO (b) mice on PD18. Echocardiography measurements using left ventricular trace on B-mode images depicts severely impaired ventricular function.

Online Video II

Sequential time-lapse observation of the cultured cardiomyocytes. a, Cardiomyocytes treated with vehicle. b, Cardiomyocytes treated with bafilomycin A1. c, Cardiomyocytes treated with chloroquine. Time scale: 6 hours per second.
Supplementary References


Online Figure I

**Atp6ap2 allele**

- Targeting vector: PGKDTpA
- Neo
- Targeted allele
- Targeted allele (neo out)
- Deleted allele

**Markers and Distances:**
- ATG
- BglII
- loxP
- FRT
- UTR
- CDS
- 394 bp
- 3.0 kb (short arm)
- 1.0 kb (deletion)
- 9.3 kb (long arm)
- 381 bp
- 591 bp
- 2866 bp

Markers and distances are indicated with arrows and distances in base pairs.
Online Figure II

\[ Atp6ap2^{lox\ neo/\ 0} \]

\[ X^{\ lox\ neo\ Y} \]

\text{neo cassette deleter}

\[ XX \]

\[ Atp6ap2^{lox/\ Y} \]

\[ XX^{\ lox} \]

\[ \alpha-\text{MHC-Cre}^{+/0} \]

\[ Atp6ap2^{lox/\ Y} \]

\[ X^{\ lox\ Y} \]
Online Figure III
Online Figure IV

(a) DNA fragments from F2/R3 and F2/R11 genotypes with Ade-Cre and Flox alleles. The band at 500 bp is indicated.

(b) Quantitative expression of Atp6ap2 mRNA in WT and Flox genotypes with Ade-Cre treatment. The fold change is indicated, with a * symbol for statistical significance.
Online Figure V

**Atp6ap2 mRNA**

<table>
<thead>
<tr>
<th>Control</th>
<th>Bafilomycin</th>
<th>Chloroquine</th>
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<tr>
<td>0.8</td>
<td>3.2</td>
<td>1.8</td>
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(fold)

Arbitrary unit

* p < 0.05