A Common Genetic Variant in the 3′-UTR of Vacuolar H+-ATPase ATP6V0A1 Creates a Micro-RNA Motif to Alter Chromogranin A Processing and Hypertension Risk

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Background—The catecholamine release-inhibitor catestatin and its precursor chromogranin A (CHGA) may constitute “intermediate phenotypes” in the analysis of genetic risk for cardiovascular disease such as hypertension. Previously, the vacuolar H+-ATPase subunit gene ATP6V0A1 was found within the confidence interval for linkage with catestatin secretion in a genome-wide study, and its 3′-UTR polymorphism T+3246C (rs938671) was associated with both catestatin processing from CHGA and population blood pressure. We explored the molecular mechanism of this effect by experiments with transfected chimeric photoproteins in chromaffin cells.

Methods and Results—Placing the ATP6V0A1 3′-UTR downstream of a luciferase reporter, we found that the C (variant) allele decreased overall gene expression. The 3′-UTR effect was verified by coupled in vitro transcription/translation of the entire/intact human ATP6V0A1 mRNA. Chromaffin granule pH, monitored by fluorescence of CHGA/EGFP chimera during vesicular H+-ATPase inhibition by bafilomycin A1, was more easily perturbed during coexpression of the ATP6V0A1 3′-UTR C-allele than the T-allele. After bafilomycin A1 treatment, the ratio of CHGA precursor to its catestatin fragments in PC12 cells was substantially diminished, though the qualitative composition of such fragments was not affected (on immunoblot or matrix-assisted laser desorption ionization (MALDI) mass spectrometry). Bafilomycin A1 treatment also decreased exocytotic secretion from the regulated pathway, monitored by a CHGA chimera tagged with embryonic alkaline phosphatase. 3′-UTR T+3246C created a binding motif for micro-RNA hsa-miR-637; cotransfection of hsa-miR-637 precursor or antagonist/inhibitor oligonucleotides yielded the predicted changes in expression of luciferase reporter/ATP6V0A1/3′-UTR plasmids varying at T+3246C.

Conclusions—The results suggest a series of events whereby ATP6V0A1 3′-UTR variant T+3246C functioned: ATP6V0A1 expression probably was affected through differential micro-RNA effects, altering vacuolar pH and consequently CHGA processing and exocytotic secretion. (Circ Cardiovasc Genet. 2011;4:381-389.)

Key Words: chromaffin ■ catecholamine ■ adrenal ■ hypertension ■ vacuolar pH

Chromogranin A (CHGA), a member of the chromogranin/secretogranin family of neuroendocrine secretory proteins, is the precursor to several bioactive peptides including the catecholamine release-inhibitory catestatin (human CHGA352–372).1,2 Catestatin secretion may be an “intermediate phenotype” in the analysis of genetic risk for cardiovascular disease such as hypertension. Previously, the vacuolar H+-ATPase subunit gene ATP6V0A1 was found within the confidence interval for linkage with catestatin secretion in a genome-wide study, and its 3′-UTR polymorphism T+3246C (rs938671) was associated with both catestatin processing from CHGA and population blood pressure. We explored the molecular mechanism of this effect by experiments with transfected chimeric photoproteins in chromaffin cells.

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ATP6V0A1 (NC_000017), initially isolated in 1995, encodes the α1 subunit of the vacuolar (V) H+-translocating ATPase heteromultimeric complex, which mediates acidification of eukaryotic intracellular organelles; the α1 subunit is a 116-kDa integral membrane protein that participates directly in H⁺ translocation. The pH of organelles along the secretory pathway decreases progressively from the endoplasmic reticulum to the secretory granule, and chemical (bafilomycin A1) inhibition of the vacuolar H⁺-ATPase impairs chromaffin granule formation and catecholamine storage and secretory protein trafficking into the regulated pathway. In the present study, we explore the molecular mechanism whereby ATP6V0A1 influences CHGA and catestatin concentrations and their ratios. It was plausible to hypothesize that changes in control of vacuolar pH would influence such traits through the effect of the secretory pathway pH to modulate either precursor proteolytic processing or exocytotic secretion. Through the use of transfected chimeric photoprotein reporters in chromaffin cells, our results reveal that ATP6V0A1 3′-UTR variant T+3246C alters gene expression through differential binding to a particular micro-RNA, thereby altering vacuolar pH and consequently the processing of CHGA to catestatin.

**Methods**

See the online-only Data Supplement Methods section for construction of human expression plasmids and cell culture and transfection.

**Luciferase Reporter Activity Assay**

After transfection and cell growth over an 8- to 24-hour time course, cells were lysed with passive lysis buffer (Promega) for sequential measurement of luciferase enzymatic activity and total protein concentration. Luciferase enzymatic activity was measured using the Luciferase Assay System (Promega) on a Luminometer Autolumat 953 (EG&G Berthold). EAP activities are measured in the culture supernatant and the cell lysate. Exocytotic secretion of the transfected/expressed EAP chimera was provoked by the potent regulated pathway stimulus Ba²⁺ (2 mM/mL), which acts by blocking influx of cellular K⁺ and hence cell membrane repolarization. A detailed protocol was described previously. The secretion rate is calculated as supernatant EAP activity as a percentage of total enzymatic activity (cell plus supernatant). The “sorting index” is calculated as a function of increase in secretion rate after stimulation of the regulated exocytotic pathway by Ba²⁺: (stimulated minus basal)/basal.

**Immunoblot Analysis**

Proteins from PC12 cell lysates were separated in a 10% SDS-PAGE (Novex precast gel; Invitrogen) gel and electrohoresis transferred to nitrocellulose membranes (Protran, BA85; Whatman Inc, Florham Park, NJ). The membrane was blocked with 5% (wt/vol) powdered/dry milk in Tris-buffered saline with 0.1% Tween-20. After incubation with primary antibody (rabbit anti-rat catestatin), the membrane was washed and incubated with secondary antibody (horseradish peroxidase–conjugated donkey anti-rabbit). The membrane was then developed by the Supersignal West picochemiluminescent substrate (Pierce, Rockford, IL). Anticatin was used as an internal control for immunoreactive band and quantification was done on the software Quantity One (Bio-Rad).

**Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry**

PC12 cells were treated with vehicle (DMSO) or bafilomycin A1 (100 nM/mL; from Streptomyces griseus; Sigma B1793), which is a specific inhibitor of the vacuolar-type ATPase (V-ATPase) and was shown to inhibit vacuolar acidification as well as dense core granule formation and secretory protein trafficking effectively. After exposure for 22 hours, cells were lysed, precleared with normal rabbit serum, and then subjected to immunoprecipitation with anti-rat catastatin antibody. Antigen-antibody complexes were then isolated using protein G plus/protein A agarose; the complexes were washed several times, and the bound peptides were eluted with acetonitrile/water/trifluoroacetic acid. Eluted peptides were concentrated by lyophilization and subjected to matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analysis: reflectron mode to scan the 1000 to 2100 Da range and linear mode to scan the 2000 to 25 000 Da range, on a Voyager De STR MALDI-TOF mass spectrometer (Applied Biosystems) at a proteomics core facility (http://massspec.ucsd.edu/bioms/). Resulting peptide masses were analyzed in the program Protein Prospector (http://prospector.ucsf.edu) to identify the fragments of rat Chga.
The structure of the reporter plasmid is given in online-only Data Supplement Figure I. A 3′-UTR variant transfection into PC12 cells (n = 4 per group at each time point). Transcription is driven by the SV40 early promoter. The structure of the reporter plasmid is given in online-only Data Supplement Figure I.

Figure 1. ATP6V0A1 3′-UTR variant T+3246C (rs938671) influences gene expression. In each case, results are plotted as mean±SEM. A. Luciferase reporter/3′-UTR variant transfection into PC12 cells (n = 4 per group at each time point). Transcription is driven by the SV40 early promoter. The structure of the reporter plasmid is given in online-only Data Supplement Figure I. After transfection into PC12 cells, cellular luciferase activity was measured. The two 3′-UTR allelic plasmids gave rise to significantly different reporter activities, with wild-type 3′-UTR allelic plasmids giving rise to significantly higher luciferase activity than the variant (C-allele) 3′-UTR.

Results

ATP6V0A1 3′-UTR Variant T+3246C Influences Gene Expression

Evidence From Isolated 3′-UTR Segments on Transfected 3′-UTR/Luciferase Reporter Plasmids

Wild-type (T+3246) versus variant (+3246C) 1458 bp ATP6V0A1 3′-UTRs were ligated into the reporter plasmid pGL3-Promoter, downstream of the luciferase reporter gene (online-only Data Supplement Figure I). After transfection into PC12 cells, cellular luciferase activity was measured. The two 3′-UTR allelic plasmids gave rise to significantly different reporter activities, with wild-type > variant (ie, T>C) and progressively greater differences in expression as a function of increasing time after transfection (P<0.003) (Figure 1A). The T>C pattern of expression was not dependent on the exact promoter driving transcription because T>C differential expression remained when the promoter was changed from SV40 to CMV (new normalized luciferase activity, 6.52±0.25E−6 for T/wild-type versus 5.01±0.19E−6 for C/variant; P=8.64E−4 between wild-type/T and variant/C alleles).

Evidence From Coupled In Vitro Transcription/Translation of the Full-Length ATP6V0A1 cDNA

Wild-type (T +3246) or variant (+3246C) ATP6V0A1 cDNAs driven by an SP6 promoter were expressed in rabbit reticulocytes in vitro. The wild-type (T-allele) cDNA exhibited greater expression than the variant (C-allele) when visualized by either the avidin/biotin method or specific anti-ATP6V0A1 immunoblotting (Figure 1B).

ATP6V0A1 T+3246C and Chromaffin Granule pH

First, we used the fluorescence of transfected/expressed human CHGTA/EGFP to calibrate pH within PC12 chromaffin granules, in which we confirmed its subcellular localization (in a punctate subplasmalemmal distribution characteristic of chromaffin granules) and estimated basal/resting granular pH.
at 5.8±0.3 (Figure 2A). We then cotransfected/expressed full-length human ATP6V0A1 cDNA in two 3'-UTR allelic versions (T3246C). When granular pH was perturbed (alkalized) by Bafilomycin A1 (100 nmol/L), the acute rate of rise in pH was greater (P<0.0001) for the C-allele (4.62±0.10 %/sec) than the T-allele (3.34±0.01 %/sec).

Disruption of Secretory Granule Core Acidification Alters Trafficking of CHGA Into the Regulated Secretory Pathway as Well as CHGA Proteolytic Processing

Proteolytic Processing: Bafilomycin A1 Alters CHGA Processing in Chromaffin Cells
To determine whether vacuolar pH influences CHGA processing, we used immunoblots and MALDI-TOF mass spectrometry to probe the effects of V-ATPase inhibition with 22 hours of exposure to bafilomycin A1 (100 nmol/L). Anticathestatin immunoblots on PC12 lysates visualized fragments from 20 to 75 kDa (Figure 3A), whereas MALDI-TOF probed derivatives from 1 to 25 kDa (Figure 3B). No qualitative fragment changes (creation or abolition) were found between bafilomycin A1 and vehicle. However, quantification of immunoblots suggested substantially reduced CHGA (∼75 kDa) coupled with similar or increased amounts of immunoreactive fragments after exposure to bafilomycin A1. Thus, the ratio of CHGA/fragments was significantly (P=7.2E-05) decreased.

V-ATPase with bafilomycin A1 (100 nmol/L). After 18 hours of preexposure of CHGA-EAP-expressing PC12 cells to bafilomycin A1, Ba2+ induced CHGA-EAP secretion was inhibited by approximately one-half (Figure 4). In contrast, unstimulated (basal) release of the fusion protein was unchanged during bafilomycin A1. Interaction of secretory stimulus and pH change (P=9.12E-08), coupled with the decline in sorting index, indicates that inhibition of V-ATPase disrupts the progress of CHGA-EAP into the regulated secretary pathway, probably diverting the chimera into the constitutive (unregulated) pathway of secretion or even out of the secretory pathway altogether.

Thus, loss of the vacuolar pH gradient appears to enhance CHGA processing while impairing its regulated secretion.

ATP6V0A1 3'-UTR Variant T+3246C Functions Through Micro-RNA Hsa-miR-637

Computation
The local region of the 3'-UTR surrounding T+3246C is highly conserved (indeed, invariant) in other sequenced primate species, except for either T or C in the SNP position (online-only Data Supplement Figure II). miRNA motif predictions suggested that T+3246C is located in a region complementary to hsa-miR-637, with a superior match for the C (variant) allele (Figure 5A), raising the possibility of diminished mRNA translation18 for transcripts bearing the C allele. The predicted minimum folding energy of hsa-miR-637 differed between T (ie, in the mRNA) and C: −24.8 versus −27.3 kcal/mol. The lower minimum folding energy of the C allele would predict better binding of hsa-miR-637 to

Figure 2. Chromaffin granule pH (pHves) in PC12 cells: Estimation by fluorescence intensity of a transfected/expressed human CHGA/EGFP chimera and effects of ATP6V0A1. A, CHGA/EGFP fluorescence as a function of pH. PC12 cells expressing CHGA/EGFP were subjected to pH calibration as described in the Methods section. Cells display punctate fluorescence in a subplasmalemmal distribution typical of chromaffin granules as well as a proportional log/linear relationship between fluorescence intensity and pH over the range pH=5.0 to 8.0 (n=10 determinations at each calibration pH). The resting/basal intragranular pH (pHves) was estimated by interpolation at pH=5.8±0.3. B, ATP6V0A1 and chromaffin granule pH. CHGA/EGFP was cotransfected/coexpressed with the full-length ATP6V0A1 cDNA (3'-UTR T+3246C alleles) to monitor pHves using fluorescence intensity, as described in the Methods section and in A. Fluorescence was monitored every 30 seconds over a 25-minute period after exposure to bafilomycin (100 nmol/L), with n=6 replicates per allele group.
the +3246C mRNA and hence more efficient translational repression (or perhaps even degradation) of the variant/C mRNA, a finding consistent with decreased reporter expression by the variant/C luciferase/3'-UTR plasmid (Figure 1).

Hsa-miR-637 Mimicry

To validate a differential hsa-miR-637 effect on T/H110013246C, a specific 24-mer miRNA precursor was cotransfected with the luciferase/3'-UTR plasmid into PC12 cells. Exogenous/cotransfected hsa-miR-637 precursor decreased reporter expression (Figure 5B). The expression difference between T and C was maintained after additional/exogenous hsa-miR-637, and the % difference between 2 genotypes (T/C) was amplified appreciably (32.8% in the negative controls versus 41.6% during hsa-miR-637 treatment). By 2-way ANOVA, \( P = 6.67 \times 10^{-4} \) for variant, \( P = 3.07 \times 10^{-13} \) for inhibitor, and \( P = 4.25 \times 10^{-6} \) for interaction.

Hsa-miR-637 Antagonism

Inhibition of hsa-miR-637 by a specific “antagomir” increased reporter expression (Figure 5C). In the baseline state (negative control group), the expression pattern was C<T, consistent with the initial transfection experiments (Figure 1). After knockdown of hsa-miR-637, however, this difference was reversed to C>T.

Both enhancement and inhibition results for hsa-miR-637 were consistent with the hypothesis that the C<T expression pattern occurs because of higher affinity of the C allele for hsa-miR-637, with consequent translational repression. By 2-way ANOVA, \( P = 6.67 \times 10^{-4} \) for variant, \( P = 3.07 \times 10^{-13} \) for inhibitor, and \( P = 4.25 \times 10^{-6} \) for interaction.

Discussion

Overview

In the present study, we aimed to clarify the mechanism whereby 3'-UTR (T+3246C; rs938671) genetic variation at the vacuolar H^+-ATPase subunit ATP6V0A1 influences CHGA/catestatin secretion and consequently BP in the population. We began by isolating the ATP6V0A1 3'-UTR onto a luciferase reporter gene, revealing that +3246C decreased reporter expression (Figure 1A); the effects on gene expression were documented by coupled in vitro transcription/
Inhibiting the vacuolar H\(^+\)-ATPase alters trafficking of the CHGA-EAP chimera into the regulated secretory pathway

![Graph showing the effect of bafilomycin A1 on CHGA-EAP trafficking](image)

**Figure 4.** V-ATPase inhibition by bafilomycin A1 alters trafficking of CHGA to the regulated secretory pathway in chromaffin cells. Secretion of EAP enzymatic activity of chimeric CHGA-EAP photoproteins exposed for 18 hours to bafilomycin A1 (10 nmol/L) or vehicle (DMSO) is shown. Expression of each chimera is driven by the strong pCMV promoter. Units for basal secretion and stimulated secretion are percentages (of cell total stores). Sorting index is a dimensionless ratio. Values represent mean±SEM for n=3 replicates per condition.

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Translation (Figure 1B), and we observed directionally coordinate effects on luciferase reporter activity in vitro and catastatin in vivo (online-only Data Supplement Figure III). The +3246C allele also impaired control of vacuolar pH during disruption of the H\(^+\) gradient (Figure 2). When V-ATPase was inhibited by bafilomycin A1, proteolytic cleavage of CHGA appeared to be enhanced in chromaffin cells (Figure 3). Bafilomycin A1 also impaired CHGA-EAP chimera sorting into chromaffin granules for regulated secretion (Figure 4). Computation suggested that T+3246C disrupted a micro-RNA recognition motif for hsa-miR-637 (Figure 5); data from overexpression or knockdown of hsa-miR-637 were consistent with an enhanced effect of the micro-RNA on the C allele, to account for its diminution in *ATP6V0A1* expression. Taken together, the results suggest that a 3'-UTR variant influences *ATP6V0A1* expression through altered microRNA recognition, thereby changing vesicular pH with consequences for CHGA processing and regulated secretion, ultimately influencing BP in the population.

**Secretory Protein Processing and/or Trafficking: Actions of the Vacuolar H\(^+\)-ATPase**

Previous research revealed a statistical association of *ATP6V0A1* 3'-UTR variant T+3246C with altered plasma concentration of CHGA and its fragment catastatin. Because *ATP6V0A1* is an essential component of the vacuolar H\(^+\)-ATPase, a change in CHGA/catastatin secretion could be envisioned to result from 2 kinds of alterations: proteolytic cleavage of CHGA to its fragments and/or regulated secretory pathway trafficking of the granin.

The ratio of CHGA/fragment concentrations in chromaffin cells was substantially reduced after exposure to bafilomycin A1, as shown in the quantified immunoblot (Figure 3A); this decreased precursor/product ratio is consistent with enhanced CHGA processing to catastatin. However, bafilomycin A1 has a variety of reported effects on secretory protein processing.\(^{19,20}\) Such variable results may be dependent on a variety of factors, such that secretory protein substrates are cleaved by various sets of proteases at different pH optima. In the case of CHGA, particular involved proteases may favor pH optima higher than that within secretory granules (in situ pH \(\approx 5.5\)), such as prohormone convertase PC1 (optimum pH=6.0),\(^{21,22}\) thereby predicting better efficiency when pH is elevated by bafilomycin A1. After bafilomycin treatment, why do fragments increase only moderately whereas CHGA decreases more substantially? CHGA is proteolytically cleaved to other fragments that cannot be captured by anticatestatin because CHGA is the precursor of several other peptide fragments (eg, pancreastatin, vasostatin).

To understand exocytosis from chromaffin granules, we followed CHGA secretion with a chemiluminescent EAP reporter fused in-frame to its carboxy-terminus. The EAP tag has multiple advantages, including high sensitivity and low background.\(^{13}\) Bafilomycin A1 impaired secretion, and in the regulatory pathway (Figure 4). This result may explain why concentrations of CHGA and catastatin are lower in +3246C population. Other protein/protein interactions influence secretory pathway acidification; for example, disrupted granule acidification caused by furin ablation reduced secretion of insulin in mice;\(^{23}\) and V-ATPase activity regulation by HRG-1 influences trafficking of a membrane protein (transferrin receptor).\(^{24}\)

**3'-UTR Variant T+3246C as a Micro-RNA Target Site Polymorphism**

Micro-RNAs are a class of noncoding, small RNAs that regulate gene expression by complementary base pairing to target motifs, typically in 3'-UTR regions of mRNAs, with consequent transcript cleavage (in the case of a perfect match) or translational repression (in the case of a partial match). SNPs that reside in miRNA target motifs\(^{25}\) may either abolish existing binding sites or create novel binding sites. Such variants are potentially implicated in a broad range of human traits\(^{26}\) despite stronger negative selection in miRNA sites than in other motifs of the 3'-UTR.\(^{27}\) In our case, BP-associated variant +3246C (rs938671) in the *ATP6V0A1* 3'-UTR created a binding site for hsa-miR-637. We confirmed predictions for such a site by both overexpression and knockdown of hsa-miR-637: Differential expression between reporters with wide-type and variant 3'-UTRs was magnified after overexpressing hsa-miR-637 and was rescued or even reversed after knockdown hsa-miR-637 (Figure 5). These results suggest that *ATP6V0A1* T+3246C does affect hsa-miR-637 binding to modulate gene expression. We also detected partial homology of the T+3246C region to another micro-RNA motif (hsa-miR-331), but the selective antagonist for that miRNA did not influence expression of the transfected luciferase/3'-UTR
reporter plasmids (data not shown). Inspection of NCBI GEO data sets reveals abundant endogenous ATP6V0A1 expression in the adrenal gland (GDS1464) as well as clonal chromaffin cells (GDS2555).  

Vacuolar H⁺-ATPase Functions

The vacuolar H⁺-ATPase (V-ATPase) is a multisubunit complex that has important roles in the acidification of a variety of several intracellular compartments as well as extracellular milieu. The ATP6V0A1 that we investigate in this work encodes the α₁ subunit that is involved in the V0 translocation, integral membrane) domain. There are 4 known isoforms of the “α” subunit (α₁ through α₄) that have differential cellular localizations. Even the α₁ subunit itself may have several alternative splice forms that are targeted to different membrane compartments of the cell. Such diversification may lend far-ranging functions to the H⁺-ATPase.

In Drosophila, a functional role for the V-ATPase V0 domain α₁ subunit occurs in late stages of synaptic vesicle exocytosis; this mutation in subunit α₁ does not affect neurotransmitter content but impairs evoked synaptic transmission. In mice, the α3 isoform may have a regulatory function in exocytotic secretion of insulin but not insulin processing. In addition to secretion, the V-ATPase participates in protein degradation and membrane fusion. Thus the vacuolar H⁺-ATPase is involved in a variety of physiological and anatomic systems, including the heart, kidney, and skeletal muscle.

Advantages and Limitations of The Study

We undertook functional studies at a positional candidate genetic locus on chromosome 17q, ATP6V0A1 to document biological mechanisms underlying the statistical genetic association of marker and trait. Thus, ATP6V0A1 appears to be a trans-QTL (quantitative trait locus) for CHGA/catestatin secretion and BP, whereas T+3246C (rs938671) may represent a trans-QTN (Quantitative Trait Nucleotide) at that locus. The use of luciferase and EAP as reporter genes allowed us to quantify expression and secretion and so document effects of the variant as well as the transacting factor hsa-miR-637, thereby permitting quantitative verification to augment the plausibility of our conclusions.

Although T+3246C/rs938671 is a human variant, the functional studies conducted were performed in rodent (rat PC12 cell) pheochromocytoma cells because the PC12 line is well established for studies of secretory protein traffic.
Human MIR637, the gene encoding hsa-miR-637, is located on human chromosome 19p in the fifth intron of DAPK3. Although miR-637 is not yet well characterized in the rat, the homologous intronic region of rat Dapk3 displays 82% sequence identity with human miR637. Intriguingly, rat Dapk3 is also within a QTL region for BP.40

Catecholamine storage depends on the vacuolar H\(^+\)-ATPase,\(^{12}\) but our transient transfection studies typically target only a minor percentage of plated cells and thus did not permit a more global analysis of catecholamine metabolism. Finally, we did not examine the effect of other genetic variants at the positional candidate locus, \(ATP6V0A1\), although T\(^{+}\)-3246C (rs938671) was selected for study because it is the only common (minor allele frequency >5%) variant observed thus far in the transcript region (exons) of Caucasians (CEU), the source population for the original linkage and association result.\(^6\) Finally, future studies with appropriate animal models may assist in confirming the H\(^+\)-ATPase mechanism in vivo.

**Conclusions and Perspectives**

The \(ATP6V0A1\) 3′-UTR common polymorphism T\(^{+}\)-3246C (rs938671) is associated with CHGA/catestatin secretion and systemic BP in the population. We explored precisely how such genetic variation affected sympathochromaffin exocytosis. As shown in online-only Data Supplement Figure IV, our results support the viewpoint that T\(^{+}\)-3246C is located in a binding motif for micro-RNA hsa-miR-637, at which the C allele may impair translation of the \(ATP6V0A1\) mRNA. Because \(ATP6V0A1\) is a necessary subunit of the vacuolar H\(^+\)-ATPase, its reduced expression may thus impair the physiological acidification of intracellular organelles such as chromaffin granules. Consequently, the processing of CHGA may be enhanced and the secretion of proteins/peptides such as CHGA/catestatin may be impaired. The results shed new light on the role of chromaffin granule acidification in processing and trafficking of secretory peptides/proteins, pointing to new molecular strategies for probing autonomic control of the circulation and ultimately the susceptibility to and pathogenesis of cardiovascular disease states such as hypertension.

We explored molecular mechanisms of how a polymorphism (T\(^{+}\)-3246C) in the 3′-UTR of the proton translocating ATPase (V-ATPase) subunit \(ATP6V0A1\) influences human cardiovascular traits, including chromogranin A (CHGA) and BP. First, with the use of a luciferase reporter plasmid, we found that the C (variant) allele of T\(^{+}\)-3246C (rs938671) decreased overall gene expression. The 3′-UTR effect was verified by coupled in vitro transcription/translation of the entire/intact human \(ATP6V0A1\) mRNA. Second, by overexpression and knockdown of a specific miRNA, we found that T\(^{+}\)-3246C disrupted a miRNA recognition motif for hsa-miR-637. Third, since \(ATP6V0A1\) is an important component of the V-ATPase, chromatin granule pH was monitored by fluorescence of a CHGA/EGFP chimera: The +3246C allele impaired control of vacuolar pH. Increased (caused by the C allele) was then mimicked by the V-ATPase inhibitor bafilomycin A1. After bafilomycin A1, the ratio of CHGA precursor to its catestatin fragment was diminished. Bafilomycin A1 also decreased exocytotic secretion from the regulated pathway. The results point to new molecular and mRNA translational strategies for probing autonomic control of the circulation and ultimately the susceptibility to and pathogenesis of cardiovascular disease states such as hypertension.

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**Disclosures**

None.

**References**

Here we explored molecular mechanisms of how a polymorphism (T+3246C) in the 3′-UTR of the proton translocating ATPase (V-ATPase) subunit ATP6V0A1 influences human cardiovascular traits, including chromogranin A (CHA) and BP. First, using a luciferase reporter plasmid, we found that the C (variant) allele of T+3246C (rs938671) decreased overall gene expression. The 3′-UTR effect was verified by coupled in vitro transcription/translation of the entire/intact human ATP6V0A1 mRNA. Second, by over-expression and knockdown of a specific microRNA, we found that T+3246C disrupted a microRNA recognition motif for hsa-miR-637. Third, since ATP6V0A1 is an important component of the V-ATPase, chromatin granule pH was monitored by fluorescence of a CHGA/EGFP chimera: the +3246C allele impaired control of vacuolar pH. Fourth, increased pH (caused by the C allele) was then mimicked by the V-ATPase inhibitor bafilomycin A1. After bafilomycin A1, the ratio of CHGA precursor to its catestatin fragment was diminished. Bafilomycin A1 also decreased exocytotic secretion from the regulated pathway. The results point to new molecular and mRNA translational strategies for probing autonomic control of the circulation, and ultimately the susceptibility to and pathogenesis of cardiovascular disease states such as hypertension.
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SUPPLEMENTAL MATERIAL

Construction of human expression plasmids.

ATP6V0A1 3’-UTR/luciferase expression reporter. The 1458 bp 3’-UTR of human ATP6V0A1 was amplified from human genomic DNA with polymerase chain reaction primers incorporating XbaI restriction sites, facilitating ligation into the unique XbaI site in the luciferase reporter plasmid pGL3-Promoter (Promega, Madison, Wisconsin) where the XbaI site is just downstream (3’) of the firefly luciferase reporter open reading frame (ORF) and upstream of the polyadenylation signal (pA from SV40). The eukaryotic expression plasmid is depicted in supplemental Figure 1. Transcription is driven by the SV40 early promoter, just upstream (5’) of the luciferase cassette. Correct orientation (5’ to 3’) of the insert was verified by sequencing, and creation of the variant +3246C polymorphism was achieved by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, California), followed by sequence verification. In some experiments, the SV40 promoter was replaced by the strong eukaryotic CMV promoter (from pcDNA3.1/Invitrogen).

ATP6V0A1 eukaryotic expression. Full-length human ATP6V0A1 cDNA (accession number BC032398) was obtained from Open Biosystems (Huntsville, Alabama), in eukaryotic expression plasmid pCMV-SPORT6, thus under the control of the strong eukaryotic pCMV promoter. Site-directed mutagenesis was used to create 3’-UTR variant +3246C.

CHGA/EAP secretion reporter. A truncated form of embryonic alkaline phosphatase (EAP) was fused in-frame to the carboxyl terminus of full-length human CHGA (NM_001275), as described previously 1. In these plasmids, expression was driven by the strong pCMV promoter (in a pcDNA vector).
**Cell culture and transfection.** Plasmids for transfection were grown in the recombination-deficient *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, California), and supercoiled plasmid DNA was purified on columns (Qiagen, Valencia, California). PC12 rat pheochromocytoma cells were cultured as described previously. One day before transfection, PC12 cells were split onto poly-L-lysine (Sigma, St. Louis, Missouri)-coated 12- or 24-well Costar polystyrene plates (Corning, Patchogue, New York). Cells were transfected with 1.0 μg (12-well plate) or 0.5 μg (24-well plate) of supercoiled plasmid DNA per well, using the TransFectin Lipid Reagent (Bio-Rad, Hercules, California).

**References**


**ATP6V0A1 3’-UTR variant T+3246C (rs938671):**
Effect on transfected luciferase reporter expression in chromaffin cells

![Diagram](attachment:image.png)

**ATP6V0A1 3’-UTR 1458 bp insert:**
Wild-type versus variant

*Supplemental Figure 1*
**ATP6N1** 3’-UTR variant T3246C (rs938671): Conserved local sequence region across primate species

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human wild-type (T)</td>
<td>GAGTGGCTTCTCCCTGTCATCCCCAGGGGTCATAGGATA</td>
</tr>
<tr>
<td>Human variant (C)</td>
<td>GAGTGGCTTCTCCCTGTCACCCCCAGGGGTCATAGGATA</td>
</tr>
<tr>
<td>Chimp</td>
<td>GAGTGGCTTCTCCCTGTCATCCCCAGGGGTCATAGGATA</td>
</tr>
<tr>
<td>Orangutan</td>
<td>GAGTGGCTTCTCCCTGTCATCCCCAGGGGTCATAGGATA</td>
</tr>
<tr>
<td>Rhesus</td>
<td>GAGTGGCTTCTCCCTGTCACCCCCAGGGGTCATAGGATA</td>
</tr>
<tr>
<td>Conserved (*)</td>
<td>****************************************</td>
</tr>
</tbody>
</table>

**Bold (↑):** T+3246C
**ATP6V0A1 3’-UTR variant T3246C:**
Coordinate effects on expression of transfected luciferase reporter in chromaffin cells and plasma catestatin in vivo

**Human plasma catestatin, nM**
(epitope: CHG A361-372)

**ATP6V0A1 3’-UTR/luciferase reporter activity**
(normalized to beta-galactosidase; 24 hours)

Luciferase $p<0.001$
Catestatin $p=0.020$

Suppl. Figure 3
**ATP6V0A1:**
Post-transcriptional, cellular, physiological and disease consequences of common genetic variant T+3246C in the 3’-UTR

**Concept:**
- Gene
  - ↓ mRNA
  - ↓ Protein
  - ↓ Cellular function
  - ↓ Precursor traits
  - ↓ Disease trait

**Application to ATP6V0A1:**
- ATP6V0A1 3’-UTR +3246C allele
  - ↓ hsa-miR-637
  - ↓ ATP6V0A1 translation
  - ↓ V-ATPase activity
  - ↑ Vacuolar pH
    - ↑ CHGA processing
    - ↓ Granin secretion
    - ↓ CHGA/catestatin ratio in plasma
    - ↓ CHGA & catestatin concentrations
    - ↓ Blood pressure
    - ↓ Hypertension

**Time**

**Mechanism**

Supplemental Figure 4