Original Article

Mice overexpressing chromogranin A display hypergranulogenic adrenal glands with attenuated ATP levels contributing to the hypertensive phenotype

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Objective: Elevated circulating chromogranin A (CHGA) is observed in human hypertension. CHGA is critical for granulogenesis and exocytosis of catecholamine stores from secretory large dense core vesicles (LDCV). This study aims to understand the morphological, molecular and phenotypic changes because of excess CHGA and the mechanistic link eventuating in hyper-adrenergic hypertension.

Methods: Blood pressure and heart rate was monitored in mouse models expressing normal and elevated level of CHGA by telemetry. Catecholamine and oxidative stress radicals were measured. Adrenal ultrastructure, LDCV content and mitochondrial abundance were compared and respiration analyzed by Seahorse assay. Effect of CHGA dosage on adrenal ATP content, electron transport chain components and uncoupling protein 2 (UCP-2) were compared *in vivo* and *in vitro*.

Results: Mice with excess-CHGA displayed hypertensive phenotype, higher heart rate and increased sympathetic tone. They had elevated plasma catecholamine and adrenal ROS levels. Excess-CHGA caused an increase in size and abundance of LDCV and adrenal mitochondria. Nonetheless, they had attenuated levels of ATP. Isolated adrenal mitochondria from mice with elevated CHGA showed higher maximal respiration rates in the presence of protonophore, which uncouples oxidative phosphorylation. Elevated CHGA resulted in overexpression of UCP2 and diminished ATP. *In vitro* in chromaffin cells overexpressing CHGA, concomitant increase in UCP2 protein and decreased ATP was detected.

Conclusion: Elevated CHGA expression resulted in underlying bioenergetic dysfunction in ATP production despite higher mitochondrial mass. The outcome was unregulated negative feedback of LDCV exocytosis and secretion, resulting in elevated levels of circulating catecholamine and consequently the hypertensive phenotype.

Keywords: ATP, chromaffin granules, chromogranin A dosage, hypertension, uncoupling protein 2

Abbreviations: BAC, bacterial artificial chromosome; CHGA, chromogranin A; CHGB, chromogranin B; Dbh, dopamine beta-hydroxylase; LDCV, large dense core vesicles; NPY, neuropeptide Y; OCR, oxygen consumption rate; Scg2, secretogranin 2; Th, tyrosine hydroxylase; UCP2, uncoupling protein 2; Vmat, vesicular monoamine transporter 1

INTRODUCTION

Chromogranin A (CHGA) is an index member of the granin family ubiquitously expressed in cells of the neuroendocrine system and is involved in granulogenesis of the large dense core vesicles (LDCV) [1,2]. In specialized secretory cells including neuroendocrine and nonneuroendocrine cells, LDCV are key organelles for storage and secretion of hormones and neuropeptides. Chromaffin granules are LDCV of the adrenal chromaffin cells and their biogenesis involves the granin proteins: CHGA, chromogranin B (CHGB) and VGF- nerve growth factor (VGF-nonacronymic) [3–5]. The granins help concentrate catecholamine and other solutes within the LDCV by reducing osmotic pressure and prevent the vesicles from bursting [6,7]. This role of CHGA is suggested by the phenotypic features of the *Chga* −/−(KO) mice as these mice have attenuated size and number of chromaffin granules, augmented circulating catecholamine and hypertension [3].

CHGA is co-stored and co-released into circulation with catecholamine and other neurotransmitters from sympathoadrenal cells [8]. Studies over the past few decades have established the role of CHGA and its peptides (derived by proteolysis) in various pathophysiological conditions. Although levels of plasma chromogranin A are largely heritable and elevated in established essential hypertension, the elevation is not seen in early association with genetic risk of

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essential hypertension. Plasma and vesicular stores of CHGA is increased in patients with established essential hypertension [9]. The elevation in CHGA is not simply a response to hypertension as CHGA is not elevated in patients with secondary hypertension, nor is its level altered by antihypertensive treatment. Also, in the hypertensive SHR rodents, increased medullary stores of CHGA is observed [10]. Studies in established hypertension patients provoked for an adrenal medullary response, sympathetic axonal stimulation and sympathetic neuronal suppression results in increased vesicular stores of CHGA and an adrenergic origin of augmented CHGA release. Unlike patients with established hypertension, still normotensive subjects with a familial history of hypertension (FH^+) do not have augmented adrenomedullary stores of CHGA. The full-length CHGA molecule, initiates biogenesis of dense-core secretory vesicles in the adrenal medulla and postganglionic sympathetic axons and therein storage of catecholamine. Marked exocytotic sympathoadrenal stimulation results in co-secretion from granule stores of catecholamine with CHGA along with its processed catestatin peptide [11,12]. The secreted catecholamine activates cardiovascular target cells and augments blood pressure. Therefore, an increase in blood pressure caused by the action of catecholamine is tightly coupled to the biogenesis of dense-core granules, which is regulated by CHGA. CHGA is cleaved by proteases in the granule to generate catestatin, a peptide that works as an antagonist at nicotinic cholinergic receptors, thus relieving high blood pressure.

In individuals with normal blood pressure with (FH^+), catestatin levels are lower compared with those of normotensive controls without family history of hypertension (FH^-) [13]. In normotensive (FH^+) individuals with attenuated catestatin levels, both plasma CHGA and catecholamine levels are increased [11]. This could be because of processing defect of CHGA or alternatively, these individuals may have a pathological upregulation of cholinergic input, which could lead to enhanced exocytosis of immature chromaffin granules resulting in secretion of unprocessed CHGA and thus, diminished catestatin [14]. Uninhibited stimulation of chromaffin granule exocytosis with lowered catestatin levels accounts for the higher sympathoadrenal activity that leads to increased levels of circulating catecholamine and hypertension in these patients. Essential hypertension displays late penetrance and develops in the fourth, fifth and sixth decade of life. However, diminished catestatin peptide is an intermediate phenotype that predicts the future development of hypertension and is seen in yet normotensive FH^+ individuals very early on in life [13]. CHGA correlates with diastolic pressure in grouped normal and essential hypertensive patients suggesting that an excess of exocytotic sympathoadrenal tone may be involved in the initiation or maintenance of essential hypertension. Renal failure results in increased circulating CHGA levels [15] and several polymorphisms in the *CHGA* gene are associated with hypertensive renal disease [16,17]. In mice and humans, the expression of plasma catecholamine is U-shaped as a function of *CHGA* gene dose, with a reciprocal pattern of adrenal epinephrine stores (in an inverted U-shape) suggesting dysregulated storage and release of catecholamine as the underpinning of the blood pressure (BP) trait pattern [18]. CHGA in excess accompanies increased releasable catecholamine stores, which in turn elevates BP [19].

The packaging and release of CHGA from the chromaffin granules into circulation is a tightly regulated process, which involves calcium (Ca^{2+}) and ATP [20,21]. Chromaffin granules store high concentration of ATP, catecholamine and other neurotransmitters; an interaction between ATP and catecholamine also helps maintain vesicular osmolarity [22]. Therefore, understanding the influence, excess CHGA may have on vesicular contents such as catecholamine, other granins, neuropeptides, Ca^{2+} and ATP can shed light on pathophysiological mechanisms in hypertension and hypertensive-kidney disease.

ATP is synthesized by the ATP synthase, an enzyme localized in the inner mitochondrial membrane. As electrons are transferred along the electron transport chain (ETC) and the protons pumped from the matrix into the intermembrane space, a proton gradient is generated across the inner mitochondrial membrane-activating ATP synthase. The flow of protons back into the mitochondrial matrix via ATP synthase provides energy to form ATP. However, the proton backflow to the matrix is not always coupled with ATP generation and sometimes results in a loss of free energy as heat. This is referred to as uncoupling of the cellular oxidative phosphorylation process and the mitochondrial uncoupling proteins (UCPs) are responsible for this uncoupling and proton backflow bypassing ATP synthesis [23]. This study showed that CHGA overexpression in mouse adrenal gland was associated with an increase in expression of the mitochondrial UCP2 protein and decreased ATP levels. This possibly caused deregulation of negative feedback of exocytosis and therefore, excessive catecholamine release into circulation resulting in the observed hypertensive and hyperadrenergic phenotype of mice overexpressing CHGA.

METHODS

Animal studies

All the animal studies were performed following protocols approved by the Institutional Animal Care and Ethics Committee at the University of California at San Diego. Mice were housed in pathogen-free environment and fed with murine chow diet and water ad libitum. Special care was taken during experimental procedures to minimize stress and discomfort to the animals. All the mice had mixed background with 50% C57BL6 and 50% 129SvJ, which was routinely confirmed by genome scan analysis at Jackson Laboratories (Bar Harbor, Maine, USA). Adult mice, 12–16 weeks of age weighing 25–30 g were used for experiments.

Generation of two-copy and four-copy humanized mice

'Humanized' *CHGA* mice were generated by BAC transgenesis [3]. 2-Copy mice had only two copies of the human *CHGA* transgene and lacked mouse *Chga* alleles. 4-Copy mice were generated by breeding wild-type mice (with two copies of mouse *Chga* gene) with two-copy humanized *CHGA* mice, following the protocol detailed previously [18]. Both the human and mouse *chromogranin A* genes were

expressed in all neuroendocrine tissues of the four-copy strain. Mice were regularly genotyped for presence of human and mouse chromogranin A alleles by PCR amplification with human and mouse *chromogranin A* gene-specific primers [24]. Expression of the *CHGA* transgenes and the *Chga* alleles in the four-copy and in humanized mice has the expected pattern of fidelity of expression specific to all neuroendocrine tissues and lack of expression in tissues such as spleen and liver like in wild-type mice [24]. The expression of CHGA and blood pressure is comparable in two-copy humanized and two-copy wild-type mice and detailed in our previous studies [18,24].

Telemetric blood pressure measurement

Continuous monitoring of BP and heart rate in conscious experimental mice was carried out with an invasive intra-arterial telemetric BP measurement method using Physio-Tel telemetry system from Data Sciences International (DSI, St. Paul, Minnesota, USA). A TA11PA-C20 transmitter with a catheter was surgically inserted into the left carotid artery of the mice. Cages were kept on an antenna that captured and relayed signals to a processor attached with a computer for data viewing and analysis (Ponemah 5.2; DSI). Mice were rested and recovered for 10–12 days' postsurgery before recording. BP and heart rate were continuously recorded for a period of 24 h at a frequency of 2000 Hz/s. The BP and heart rate for every time point were calculated from 10 s of continuous recording every 5 min and averaging every hour data [25].

Transmission electron microscopy of mice adrenal glands

The mice were euthanized with isoflurane and perfused through the heart with modified Karnovsky's fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 mol/l sodium cacodylate buffer, pH 7.4) and then the adrenal glands were dissected. Glands were kept in 2% OsO_4 in 0.15 mol/l sodium cacodylate buffer followed by 2% uranyl acetate and subsequently subjected to dehydration with graded series of alcohol and infiltration with Durcupan epoxy resin. Ultrathin sections were cut at 60 nm and mounted on 300 mesh copper grids. Sections were stained with 2% uranyl acetate and Sato's lead stain for better contrast [26]. Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope equipped with an Eagle 4k HS digital camera (FEI, Hilsboro, Oregon, USA) for imaging. At least four animals were used for each cohort and 20 micrographs from each mouse were examined for the ultrastructure of adrenal medulla. DCV were analyzed for their abundance, granule size and granule percentage area.

Catecholamine, reactive oxygen species, creatinine, calcium and ATP measurements

Mice were euthanized after deep anesthesia with isoflurane (Baxter) followed by cervical dislocation. Blood was drawn by cardiac puncture using a heparinized 22-gauge needle (heparin sodium 1000USP U/ml) and a 1 ml syringe and collected into a 4 ml blood collection tubes (BD vacutainer K2 EDTA 7.2 mg plus). The tube was centrifuged at 3000 rpm for 5 min and the plasma supernatant collected. After the blood draw, mice were dissected to harvest adrenal glands. Both plasma and excised adrenal tissue were frozen in liquid nitrogen and stored at −80 °C until further experiment. Catecholamine was measured in plasma and adrenal extracts by high performance liquid chromatography coupled to an electrochemical detector as previously described [24]. The reactive oxygen species (ROS)/reactive nitrogen species (RNS)-free radical activity was measured in adrenal gland extracts using the OxiSelect In Vitro ROS/RNS assay kit from MyBiosource (San Diego, California, USA; Cat# MBS168257) according to manufacturer's instructions. To detect ROS in live PC12 cells, a fluorescent cellular ROS detection assay kit (ABCAM; Cat# ab113851) was used. Plasma creatinine was measured from 10 μl plasma sample volumes using electrospray mass-spectrometry LC-MS at the University of Alabama Birmingham -O'Brien core. Total tissue Ca^{2+} level in adrenal gland extracts was measured following manufacturer's protocol (Cayman Chemicals, Ann Arbor, Michigan, USA; Cat# 701220) and values are expressed as nmol/l/μg protein. The ATP content in freshly prepared adrenal extracts and in cultured cell extracts was measured by a fluorometric assay using ATP detection kit from Biovision Inc. (Milpitas, California, USA; Cat# K354) and expressed as nmol/μg protein.

Real time-PCR and western blotting analysis

The relative abundance of mRNAs in tissues was measured by SYBR Green qRT-PCR and normalized by housekeeping RNAs (18SrRNA). Total RNA was extracted from adrenal glands using RNeasy tissue kit (Qiagen). Threshold cycle (Ct) was determined for both the specific target RNA as well as the housekeeping RNA, and the difference in Ct (target RNA versus housekeeper RNA) is normalized to the average for that state by the comparative Ct (i.e. $2^{-\Delta\Delta Ct}$) method [27]. Expression of chromogranin B (*Chgb*), secretogranin 2 (*Scg2*), vesicular monoamine transporter 1 (*Vmat1*), neuropeptide Y (*Npy*), tyrosine hydroxylase (*Th)* and dopamine beta- hydroxylase (*Dbh*) were examined and primer sequences are provided as a supplemental Table 1, http://links.lww.com/HJH/A896.

Adrenal glands from two-copy and four-copy mice were homogenized in RIPA buffer (150 mmol/l NaCl, 20 mmol/l Tris, 10 mmol/l EDTA) and total protein estimated by the Bradford method. Total protein (5–10 μg) was separated on SDS-PAGE and immunoblotted. The membranes were probed with primary antibodies against chromogranin A [24], Ox-Phos complex [28] (cat# 45-8199 Invitrogen), CHGB, SCG2, UCP2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotech, Dallas, Texas, USA; Cat# sc18235; sc53441, sc6525, sc32233) [29–32] and visualized by chemiluminescent detection method using horseradish peroxidase (HRP)-conjugated secondary antibodies specific for primary antibodies. Densitometric analysis of the band intensity was performed using NIH ImageJ software.

Measurement of mitochondrial DNA copy

Total DNA was extracted from two-copy and four-copy mouse adrenal glands using DNeasy tissue kit following manufacturer's instructions (Qiagen, Waltham, Massachusetts, USA). qPCR analysis was done using 2.5 ng of total DNA as template and primers specific for mitochondrial

(*MtND1*) and nuclear (*Gapdh*) genes. Relative amount of mitochondrial DNA to that of nuclear DNA was calculated by $2^{-\Delta\Delta Ct}$ method and changes were expressed in arbitrary units (AU).

Isolation of mitochondria from mouse adrenal glands

All instruments, glassware and reagents were prechilled to 4 °C. Mice were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine via intraperitoneal injection followed by cervical dislocation. Adrenal glands were immediately removed and placed in cold (0 °C) isolation buffer containing 210 mmol/l Mannitol, 70 mmol/l Sucrose, 5 mmol/l HEPES, 1 mmol/l EGTA, and 0.5% BSA. The pH was adjusted to 7.4. Samples were then homogenized in 500 μl of isolation buffer for 5–15 s while chilled. The homogenate was centrifuged at 600*g* for 10 min at 4 °C. Supernatant was transferred into a 1.5 ml tube and centrifuged at 11 000*g* for 10 min at 4 °C. The resulting pellet was re-suspended in 1 ml of cold isolation buffer; then spin down at 11000*g* for 10 min at 4 °C. The pellet was re-suspended in minimal amount of cold isolation buffer and dispersed with 200 μl pipette. Mitochondria concentration was determined by DC protein assay (Bio-Rad, Hercules, California, USA).

Mitochondrial metabolic analysis

Mitochondria respiration was measured using Seahorse XFe 96 Extracellular Flux Analyzer. 2 × mitochondria assay solution (MAS) containing 70 mmol/l sucrose, 220 mmol/l mannitol, 10 mmol/l KH_2PO_4, 5 mmol/l $MgCl_2$, 2 mmol/l HEPES, 1 mmol/l EGTA, and 0.2% (w/v) fatty acid-free BSA was made in distilled-deionized H_2O. The pH was adjusted to 7.2 with 0.1 mol/l KOH at 37 °C and filter-sterilize the solution. Mitochondria isolated from adrenal glands were diluted to a needed concentration (0.16 μg/μl) in cold 1x MAS with substrate (succinate 10 mmol/l) and initial condition (rotenone 2 μmol/l). Diluted mitochondria (25 μl) were then seeded to each well of the XF^e 96-well cell culture microplate (Seahorse Bioscience, Santa Clara, CA, USA), whereas the plate was on ice. After spinning down at 2000 *g* for 20 min, 155 μl of prewarmed (37 °C) 1× MAS-containing substrate succinate (10 mmol/l) and complex I inhibitor rotenone (2 μmol/l) was added to each well. The plate was then transferred to the Seahorse XFe/XF96 Analyzer. Mitochondrial oxygen consumption rates (OCRs) were measured at the basal state and states initiated by sequentially adding ADP (2 mmol/l), oligomycin (2 μmol/l), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 4 μmol/l), and antimycin A (4 μmol/l) to each well, as per previously published protocols [33].

PC12 cell culture and chromogranin A overexpression

Rat chromaffin cells of adrenal pheochromocytoma origin (PC12 cells) were grown and maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% bovine serum and 10% horse serum and pen-strep on poly- L-lysine coated culture plates. To overexpress CHGA, PC12 cells were transfected with *CHGA*-WT-pCMV6XL5 construct using lipofectamine reagent (BioRad, Hercules, California, USA). Control cells were transfected with empty vector pcDNA3.1(−). Cells were harvested for protein 48 h posttransfection. Cultured PC12 cells were also treated with UCP2 inhibitor genipin (50 μmol/l) 6 h after transfection with vectors and cultured for another 24 h [34–36]. Cells were harvested and analyzed to check CHGA, ATP and ROS levels. Control cells were treated with an equal volume of culture media as used to dilute genipin.

Statistical analysis

Data were analyzed by Student's *t*-test and ANOVA and expressed as mean ± SEM wherever applicable using Kaleidagraph (Synergy Software, Reading, Pennsylvania, USA) and/or SPSS-23 (Chicago, Illinois, USA) software. Continuous recordings data of BP and heart rate was analyzed by repeated measures ANOVA using linear mixed model. Results from at least three independent experiments were considered for analysis of in-vitro data. Data with a '*P*' value <0.05 were considered significant.

RESULTS

Phenotypic changes because of overexpression of chromogranin A *in vivo*

Expression of CHGA was compared in mice expressing two copies of the human *CHGA* gene and mice with four copies of *chromogranin A* gene (two copies each of mouse *Chga* and human *CHGA*). In the adrenal glands of four-copy mice, CHGA expression was significantly increased (Fig. 1a) [18]. Densitometric analysis showed an almost two-fold increase in CHGA protein in adrenal extracts of four-copy mice (2.06 ± 0.08) as compared with two-copy mice (1.12 ± 0.06, $P < 0.001$, Fig. 1b). Continuous telemetric recording of BP and heart rate revealed that four-copy mice had significantly elevated SBP, DBP and heart rate compared with mice expressing basal levels of CHGA. The average SBP/DBP and heart rate in four-copy mice were 138.24 ± 7.84/103.28 ± 5.62 mmHg, 581.05 ± 39.89 bpm compared with 119.05 ± 7.78/86.603 ± 6.29 mmHg, 492.087 ± 39.5323 in two-copy (Fig. 1c–e). Telemetric analysis showed attenuated variation in diurnal SBP in mice with excess CHGA (Fig. 1f). The four-copy mice also showed loss of diurnal DBP variation compared with the 7 mmHg dip seen in two-copy mice (Fig. 1g).

Compared with two-copy mice, the four-copy mice were hyperadrenergic with elevated plasma catecholamine (Fig. 2a) and reciprocal diminished catecholamine stores in adrenal glands (Fig. 2b). The ROS levels were elevated 1.3-fold in adrenal glands of four-copy mice (Fig. 2c) indicative of increased oxidative stress. The kidney function was estimated by measuring renal clearance of creatinine by a method that is not confounded by circulating noncreatinine Jaffe chromogens in rodents and the estimated glomerular filtration rate (eGFR) was unchanged in 12–16-week-old mice because of overexpression of CHGA (Fig. 2d).

Morphometric analysis of adrenal chromaffin cells

Comparison of ultrastructure morphology of adrenal glands of two-copy and four-copy mice showed significant differences in overall morphology of LDCVs in the chromaffin cells (Fig. 3a). Excess CHGA (four-copy mice) increased

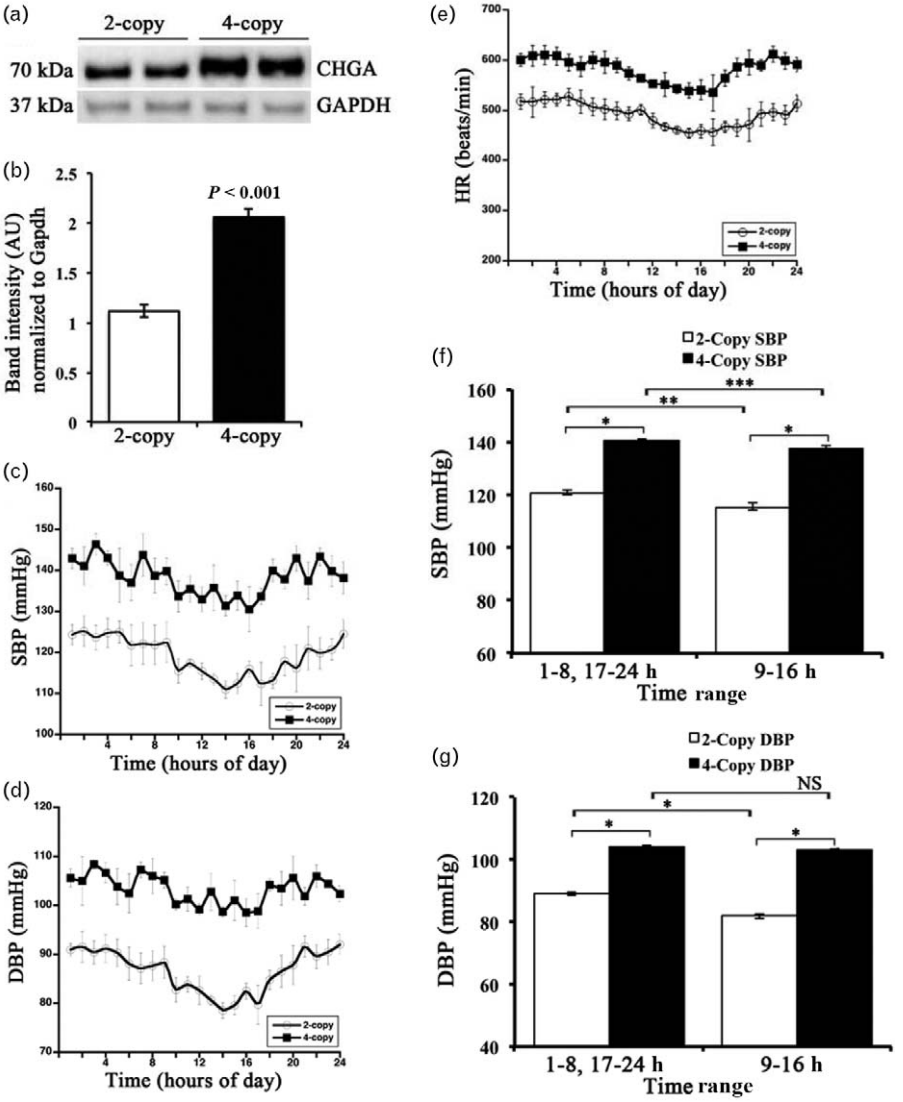

FIGURE 1 Overexpression of chromogranin A is associated with attenuated nondipping hypertensive phenotype. The four-copy mice that over-express CHGA carry two copies each of the mouse *Chga* and the human *CHGA* genes. The 'humanized' two-copy mice carry and express only two copies of the human *CHGA* gene in lieu of mouse *Chga* alleles. (a) Tissue extracts were prepared from adrenal glands of two-copy and four-copy mice. Total protein (5 μg) was electrophoresed and probed for CHGA expression using anti-CHGA antibody. In four-copy mice, CHGA expression was nearly doubled compared with expression in the two-copy mice. Housekeeping protein GAPDH was used as loading control. (b) Graph showing densitometric quantitative measurement of CHGA expression normalized to GAPDH. Continuous telemetric recordings of BP and heart rate for 24 h in both stains of mice (two-copy and four-copy; each $n = 4$) were performed. Average data for each hour is plotted and the error bars show ±SEM. The data analyzed by repeated measure ANOVA using linear mixed model, showed significant differences in SBP, DBP and heart rate between the two mouse strains. (c) Average SBP was elevated by 19.19 mmHg in four-copy (138.24 ± 1.98 mmHg) compared with that of two-copy mice (119.05 ± 1.98 mmHg); (time-F = 3.22, $P < 0.001$; strain-F = 46.77, $P < 0.001$). (d) DBP was also significantly higher by 16.68 mmHg in four-copy mice (103.28 ± 5.6 mmHg) compared with two-copy (86.6 ± 6.3 mmHg); (Time- F = 4.45, $P < 0.001$; Strain- F = 54.42, $P < 0.001$). (e) The heart rate of two-copy mice (492 ± 11.49 bpm) was significantly lower by ~89 bpm compared with mice over-expressing CHGA (581 ± 16.25 bpm; time-F = 5.28, $P < 0.001$; strain-F = 29.98, $P = 0.002$). The 24-h period of telemetric BP recording was stratified into three blocks of time intervals (1–8, 9–16 and 17–24 h) and the SBP/DBP plotted. (f) Both strains of mice display diurnal SBP dipping (during hours 9–16), however, this dipping in SBP is attenuated in four-copy mice (***$P = 0.05$) compared with two-copy mice (**$P = 0.004$). The difference between the dipping (hours 10–17) and nondipping period (1–8 and 17–24) is 5.4 mmHg for two-copy versus 2.9 mmHg in case of four-copy mice. The SBP is significantly elevated for four-copy compared with two-copy mice (*$P < 0.0001$). (g) The hypertensive four-copy mice showed loss of diurnal DBP variation ($P = 0.3$). The difference in DBP for four-copy versus two-copy mice is significant (*$P < 0.0001$). The two-copy mice display a 7 mmHg dip in DBP. CHGA, chromogranin A.

granule number and size. Total number of LDCV per μm^2 area was higher in four-copy mice (26.630 ± 1.17) compared with two-copy mice (15.58 ± 1.24, $P < 0.001$, Fig. 3b). Also, average chromaffin granule size increased considerably in four-copy mice ($11698 \pm 670.69\,nm^2$) compared with that of two-copy mice ($7799.8 \pm 705.04\,nm^2$, $P < 0.0005$, Fig. 3c). The total granule area/percentage cytosolic area was significantly higher in four-copy versus two-copy mice (36.640 ± 1.18 versus 22.749 ± 1.96 $P < 0.001$, Fig. 3d).

Expression of other dense core vesicle proteins is changed because of excess chromogranin A

Granin family members-CHGB and SCG2 are also constituents of LDCV. Therefore, their expression in both two-copy and four-copy mice was measured at both RNA and

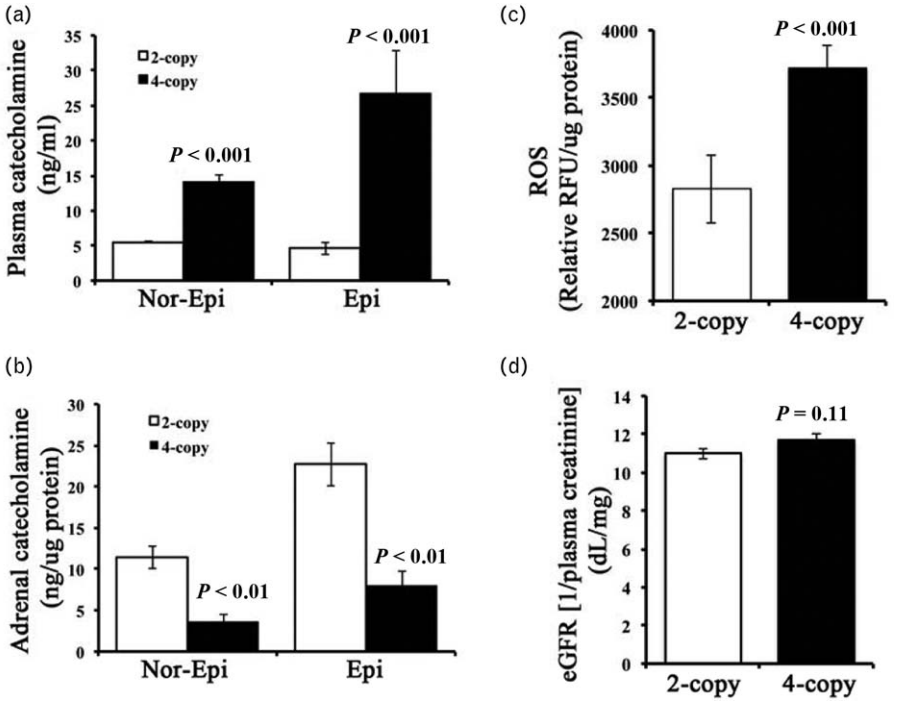

FIGURE 2 Adrenal chromogranin A overexpression elevated sympathetic activity and oxidative stress while kidney function was unaffected. (a) Plasma from both two-copy and four-copy strains of mice were measured for catecholamine levels. Circulating levels are elevated because of CHGA over-expression suggesting augmented release of catecholamine, $N=8$. (b) Adrenal gland extracts of four-copy mice have attenuated levels of epinephrine and norepinephrine compared with two-copy mice, indicative of dysregulated storage and release of catecholamine from LDCV, $N=8$. (c) Elevated CHGA in adrenal glands resulted in accumulation of ROS, which is 1.3-fold higher in four-copy mice versus two-copy mice. Data is one representative of three independent experiments, $N=7$. (d) Renal function (estimated GFR) measured as a function of renal clearance of creatinine remains unchanged in 12–16-week-old mice because of overexpression of CHGA, $N=8$. CHGA, chromogranin A; GFR, glomerular filtration rate; LDCV, large dense core vesicles; ROS, reactive oxygen species.

protein level. qPCR analysis of the transcripts showed no significant difference in expression (Fig. 4a), however, protein analysis showed considerable decrease in CHGB and SCG2 protein levels because of CHGA overexpression (Fig. 4b), confirmed by densitometric analysis (Fig. 4c). Real-time qPCR expression analysis of neuropeptide Y (*Npy*) another prominent protein of DCV and catecholamine biosynthetic pathway genes tyrosine hydroxylase (*Th*) and dopamine beta-hydroxylase (*Dbh*) did not vary significantly between the two groups of mice (Fig. 5). However, expression of the vesicular monoamine transporter (*Vmat1*) required to transport catecholamine into vesicles was significantly augmented in the adrenal glands of four-copy mice (Fig. 5).

Level of calcium and ATP in adrenal glands

Calcium and ATP are important components of the neurosecretory chromaffin cells involved in the process of granule formation and exocytosis. ATP is a key regulator of the CHGA packaging into DCVs, whereas, Ca^{2+} is involved in docking of the vesicles on cell membrane and the final exocytosis process. Colorimetric assay for Ca^{2+} showed no significant difference in total Ca^{2+} level because of excess CHGA (1.7706 ± 0.34 in two-copy versus 2.0845 ± 0.33 mmol/l/μg protein in four-copy mice, $P=0.52$, Fig. 6a). There was, however, a significant decline in total ATP concentration in adrenal glands of four-copy mice (0.0196 ± 0.0009 nmol/μg protein) compared with the two-copy mice (0.0392 ± 0.0049 nmol/μg protein, $P<0.009$, Fig. 6b).

ATP diminution and mitochondrial respiration

Diminution in ATP levels in the adrenal glands of four-copy mice directed us to examine mitochondrial abundance, which was significantly augmented in the adrenal glands of four-copy compared with two-copy mice ($P<0.001$; Fig. 7a) as revealed by qPCR analysis of mitochondrial DNA (mtDNA). Expression of oxidative phosphorylation (Ox-Phos) complex enzymes (complex II–V) responsible for electron transfer across inner mitochondrial membrane and final ATP synthesis was considerably increased in four-copy mice (Fig. 7b and c). Thus, despite increased expression of Ox-Phos enzymes and mitochondrial abundance, there was a considerable decline in ATP content in the adrenal gland because of CHGA overexpression.

Next, mitochondrial function was evaluated by analyzing the key parameters of mitochondria respiration, which were determined directly by measuring oxygen consumption in fresh mitochondria isolated from adrenal glands using Seahorse XFe 96 Extracellular Flux Analyzer. We sequentially measured basal OCR in a coupled state with substrate (succinate), followed by ADP-initiated phosphorylating respiration, oligomycin (ATP synthase inhibitor)-induced State 4o, and FCCP-stimulated maximal uncoupled respiration. OCR in the state of basal respiration with ADP was modestly higher, but not statistically significant in the

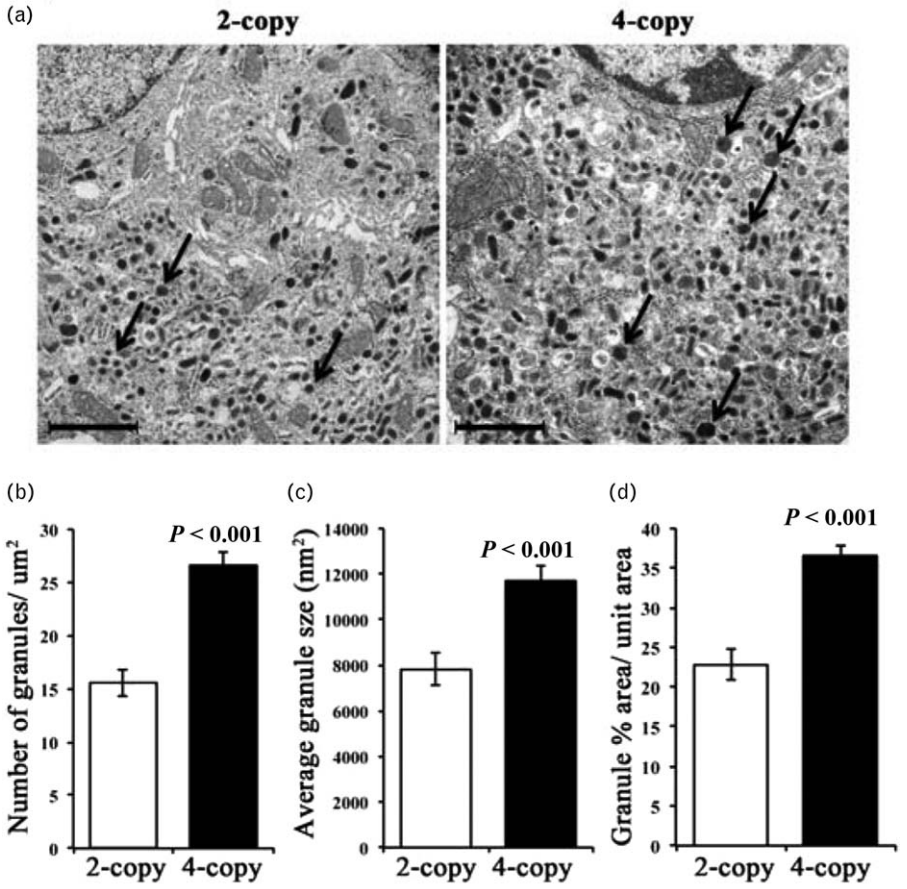

FIGURE 3 Augmented chromogranin A expression leads to increase in number and size of chromaffin granules. (a) Adrenal glands from both two-copy and four-copy strains of mice were prepared for transmission electron microscopy and morphometric analysis. LDCV in the chromaffin cells (indicated by arrows) located in the medullary region were counted for their abundance and size. Data was collected from ~20 micrographs for each type of mouse (bar scale = 1 μm). (b) Granule abundance per unit area (μm^2) in the cytoplasm of adrenal medullary cells of four-copy mice was significantly higher compared with two-copy mice. (c) Average size of the chromaffin granules (nm^2) in the adrenal medulla of four-copy mice significantly increased compared with that of two-copy mice. (d) Area covered by LDCV (% area) per unit area was significantly higher in four-copy mice compared with two-copy. CHGA, chromogranin A; LDCV, large dense core vesicles.

adrenal mitochondria from mice overexpressing CHGA than those expressing normal level of CHGA. Treatment with oligomycin abolished the increase in oxygen consumption confirming that the increase was specific to the mitochondria. To determine the maximal OCR that the cells can sustain, the proton ionophore (uncoupler) FCCP was injected resulting in stimulation of OCR, which occurs as the mitochondrial inner membrane becomes permeable to protons and electron transfer is no longer constrained by the proton gradient across the mitochondrial inner membrane. This maximal respiration rate uncoupled with ATP synthesis-induced by FCCP was significantly higher ($P = 0.037$) in the four-copy group than the two-copy group, (Fig. 7d). Finally, respiration was returned to baseline after injecting antimycin A (inhibitor of complex III of the ETC) treatment, confirming that the increase in OCR mediated by FCCP exposure, was because of increased oxygen consumption by the mitochondria [37].

High maximal respiration rates suggest increased spare capacity in the mitochondria to respond to increase demand for ATP. Given that ATP levels were decreased in four-copy group despite higher maximal respiratory rates, we examined whether uncoupling in the mitochondrial ETC might be responsible for the decrease in ATP. Therefore, we examined the expression of mitochondrial uncoupling protein 2 (UCP2), which is the most widely expressed uncoupling protein in various organ systems in mammals. Expression of both the monomeric and dimeric forms of UCP2 in adrenal extracts was significantly elevated whenever CHGA was overexpressed (Fig. 7e and f; for UCP2 monomer and dimer forms).

Recapitulation *in vitro* in PC12 cells overexpressing chromogranin A of the in-vivo uncoupling protein 2 upregulation

Observation of concurrent UCP2 and CHGA expression upregulation from the animal studies was confirmed in cell culture. CHGA was overexpressed in PC12 cells by transfection with a construct constitutively overexpressing human *CHGA* under the influence of a CMV promoter. Elevated CHGA expression was observed in transiently transfected cells corroborating in-vivo findings, a two-fold increase in UCP2 protein expression was also observed (Fig. 8a and b). CHGA overexpression also brought down the ATP level significantly in PC12 cells ($P < 0.026$) and treatment with UCP2 pharmacological inhibitor genipin

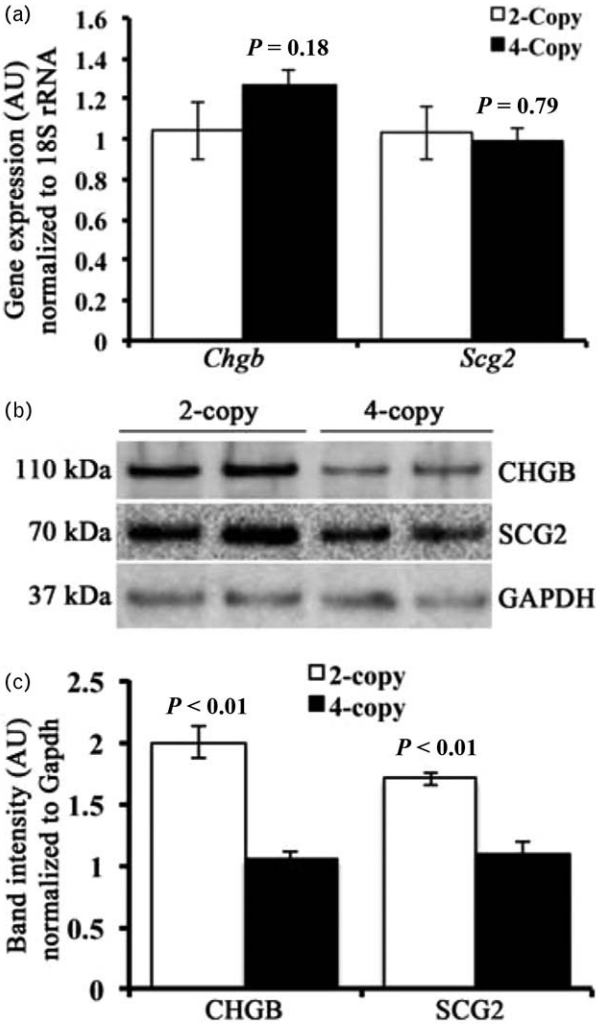

FIGURE 4 Homeostasis of granin expression in the large dense core vesicles of adrenal glands. (a) qPCR analysis of other granin family members – chromogranin B (*Chgb*) and secretogranin 2 (*Scg2*) mRNA levels showed no difference in expression between the two-copy and four-copy mice adrenal glands. Data were normalized against 18S ribosomal RNA. (b) Total adrenal extracts were also analyzed by western blots for expression of CHGB and SCG2 proteins. Both CHGB and SCG2 protein expression was significantly reduced in four-copy mice that express excess CHGA indicating, translational regulation of total granin expression. Even loading was confirmed by probing blots with housekeeping protein GAPDH. (c) Bar graph showing quantitative difference in protein expression of CHGB and SCG2. CHGA, chromogranin A; CHGB, chromogranin B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

resulted in restoration of ATP levels ($P < 0.002$; Fig. 8c). In PC12 cells, we could not recapitulate the in-vivo finding of elevated ROS, as a result of CHGA over expression (Fig. 8d).

DISCUSSION

CHGA, the most abundant granin family member situated in chromaffin granules, the organelles for catecholamine storage and exocytotic release is implicated in various pathophysiological conditions. Studies in human and animal models have established a role for *CHGA* and its proteolytically derived peptide catestatin in hypertension, heart failure, diabetes, brain disease and end-stage renal disease (ESRD) [11,16,17,38]. In mouse models with varied copy number of this gene and thus varied CHGA expression, it is

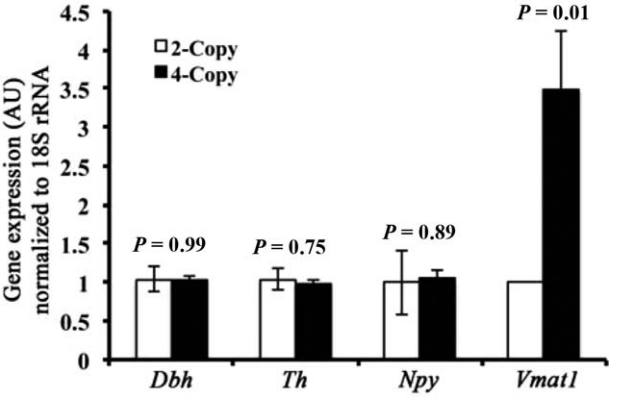

FIGURE 5 Influence of chromogranin A overexpression on large dense core vesicles constituents. qPCR analysis normalized to 18S rRNA did not show any significant change in expression of *Npy* and catecholamine biosynthetic pathway genes – *Th* and *Dbh,* because of overexpression of CHGA. Significant increase in vesicular amine transporter *Vmat1* was observed in the adrenal glands of four-copy compared with that of two-copy mice. CHGA, chromogranin A.

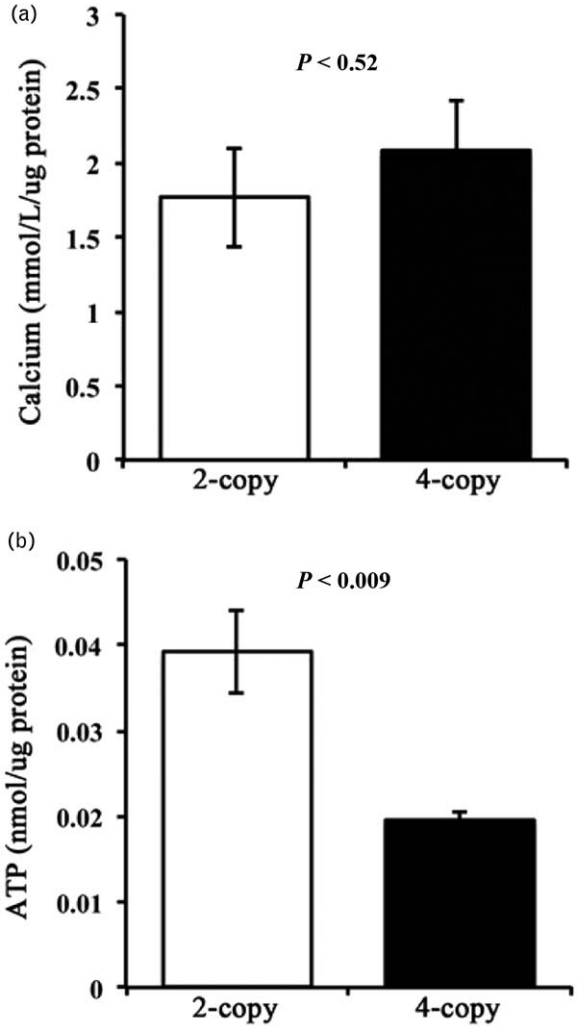

FIGURE 6 Chromogranin A overexpression does not affect calcium levels but diminished ATP in mouse adrenal gland. Adrenal extracts were assayed for calcium and ATP levels. (a) No significant change in total calcium was observed. (b) A significant attenuation in ATP level was observed in the adrenal extracts of four-copy (n = 13) compared with two-copy (n = 15) mice.

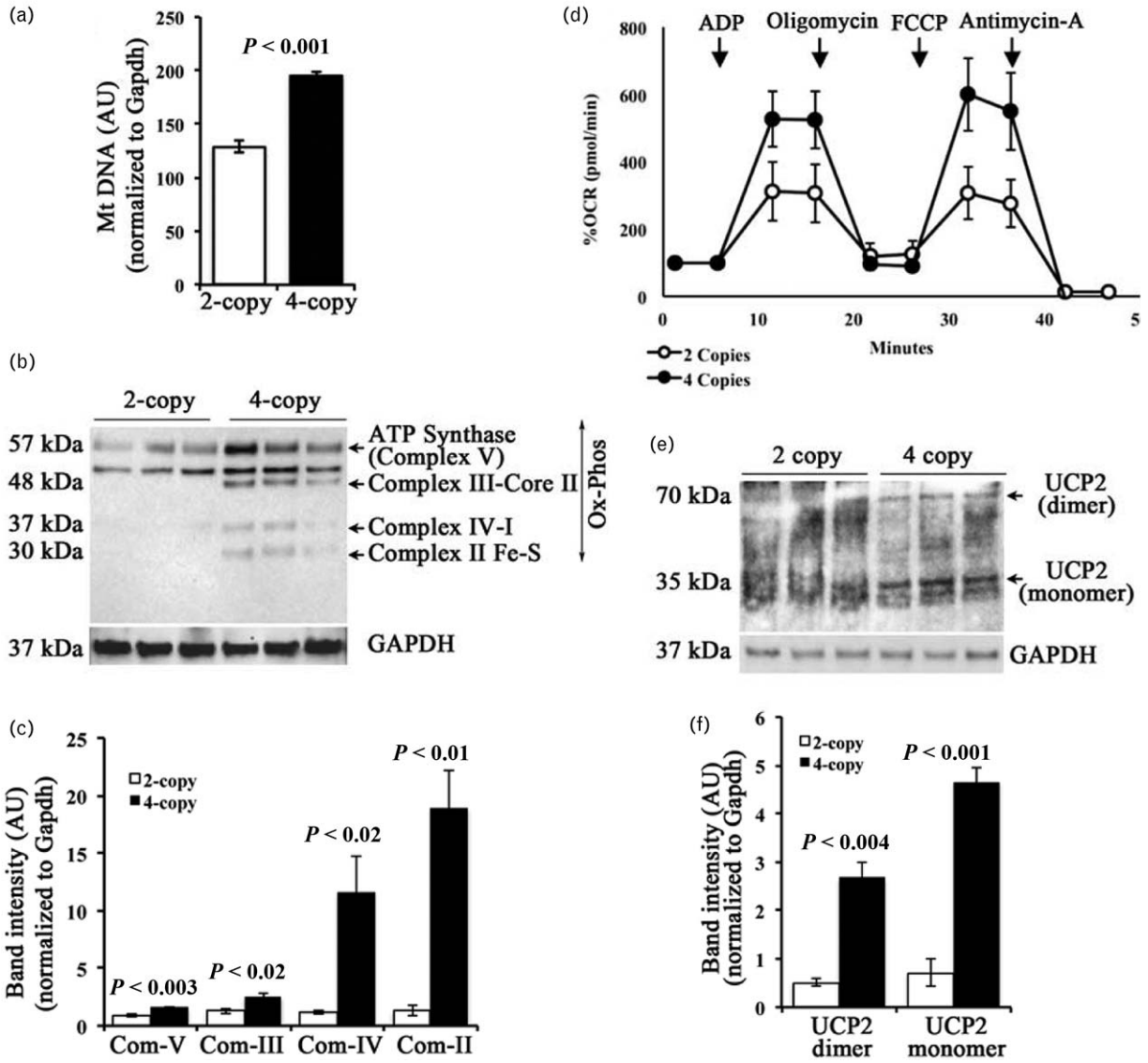

FIGURE 7 Mitochondria and Ox-Phos complex proteins increased because of elevated chromogranin A, concomitant with uncoupling protein 2 overexpression and dysregulated mitochondrial bioenergetics. Mitochondrial respiration response to an uncoupler of mitochondrial oxidative phosphorylation was elevated by CHGA overexpression. (a) Total DNA was extracted from adrenal glands of two-copy and four-copy mice and qPCR analysis was done using mitochondrial gene-specific primers (MtND1). Mitochondrial DNA content was significantly higher in four-copy mice compared with two-copy mice. *Gapdh*-specific primers were used as control for nuclear gene expression. (b) Expression of the mitochondrial oxidative phosphorylation (Ox-Phos) complexes was analyzed by western blot. The protein levels of complex II–V were significantly increased in mice overexpressing CHGA compared with two-copy mice. Even loading was confirmed by normalizing to GAPDH protein. (c) Densitometric analysis confirmed significant elevation in the Ox-Phos complex enzymes in adrenal extracts of four-copy mice. (d) Basal and stimulated OCR was measured in isolated mitochondrial preparations from adrenal glands of four-copy (solid circles) and two-copy (open circles) *CHGA* mice using Seahorse XFe 96 Extracellular Flux Analyzer. Data analyzed from 48 wells for each group are summarized from six (two-copy) and seven (four-copy) independent experiments and presented as percentage OCR normalized to non-ADP-treated levels. Data was analyzed by independent samples *t*-test. Under basal conditions, the energetic demand of the isolated mitochondria was comparable in mitochondria of both strains of mice ($P = 0.279$). To enhance substrate utilization by the mitochondria, along with substrate succinate, ADP or FCCP was injected. Both ADP and FCCP stimulated OCR compared with basal ($P < .001$). Statistical analysis showed that whenever normalized to basal level, the FCCP-induced increase of OCR was higher for the four-copy group ($P = 0.037$). (e) In the adrenal extracts of four-copy mice overexpressing CHGA a significant elevation in UCP2 protein expression was observed compared with two-copy mice. Arrows indicate dimer (~70 kDa) as well as monomer (~35 kDa) bands of the UCP2 protein. Probing blot with GAPDH shows equal loading. (f) Densitometric analysis of the band intensities observed in the western blots confirm significant increase in both the monomer and dimer forms of UCP2 in adrenals of four-copy mice. CHGA, chromogranin A; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UCP2, uncoupling protein 2.

observed that CHGA expression has a biphasic correlation with the BP phenotype. Both high-circulating levels as well as absence of CHGA is associated with elevated BP suggesting that an optimum level of CHGA is a determinant of the eventual BP phenotype. Also, in a human cohort stratified based on levels of circulating CHGA, in individuals with both low and elevated levels of CHGA augmented BP response to cold-pressor environmental stress test is observed [18]. Therefore, we evaluated in a mouse model, overexpressing CHGA the consequence to chromaffin granule morphology and phenotype. The comparator strain with normal CHGA expression used for this study was the humanized *CHGA* mouse model. The humanized *CHGA* model allows us to address the in-vivo role of the human *CHGA* gene. The mouse and human *chromogranin A* genes share only ~46% homology. The human BAC construct

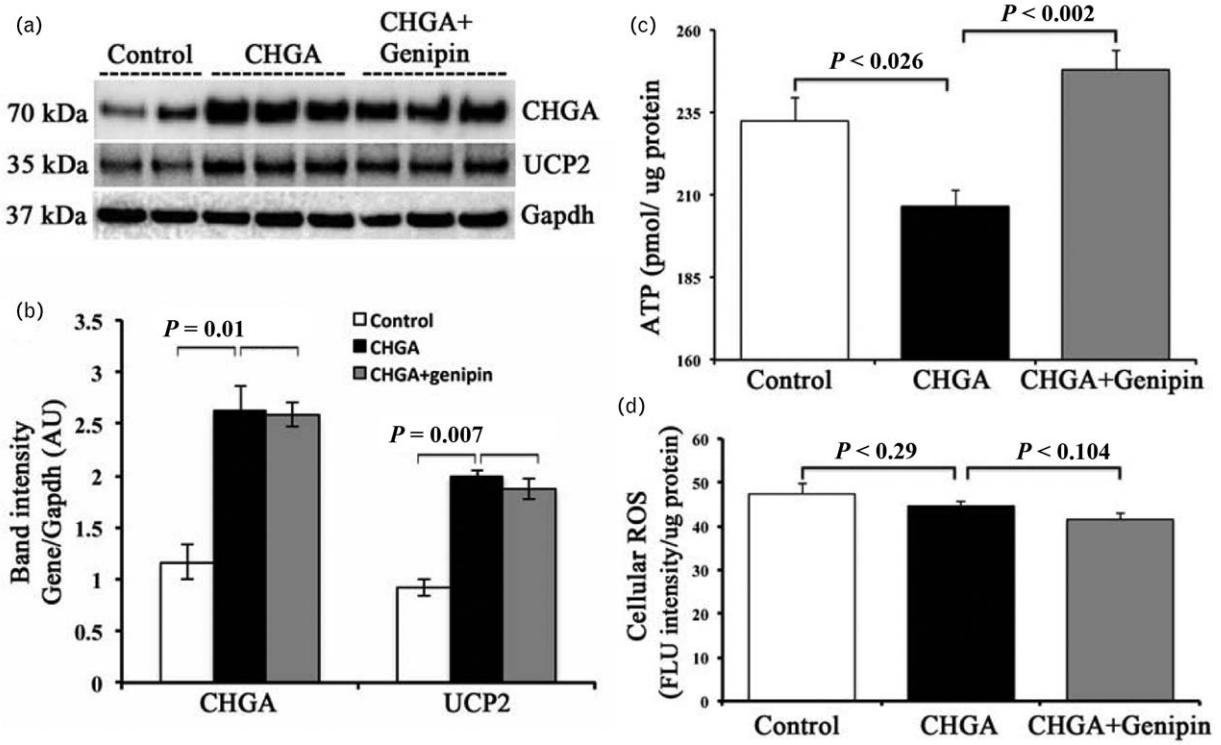

FIGURE 8 Cultured rat chromaffin cells overexpressing *CHGA* have elevated uncoupling protein 2 and diminished ATP level. (a) PC12 cells were cultured and transfected with either empty vector (pcDNA3.1) or a construct expressing *CHGA* driven by CMV promoter (*CHGA*-WT-pCMV6XL5) with or without genipin treatment (50 μmol/l). Western blot analysis of transfected cell extracts showed ~2-fold increase in CHGA expression in PC12 cells transfected with *CHGA* construct compared with control cell extracts. With CHGA overexpression concomitant increase in UCP2 protein expression is observed. GAPDH was used as a loading control. (b) Densitometric analysis of western blot results showing significant increase in CHGA with or without genipin treatment. UCP2 expression was increased significantly because of CHGA overexpression. (c) CHGA overexpression in PC12 cells caused a significant decline in ATP level. Pharmacological inhibition of UCP2 with genipin (50 μmol/l), restored the ATP levels. (d) No significant change in cellular ROS level was observed because of either CHGA overexpression or genipin treatment. CHGA, chromogranin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species; UCP2, uncoupling protein 2.

carrying the *CHGA* gene complements loss of mouse *Chga* and permits more specific studies of the function of the human *CHGA* gene *in vivo*, under circumstances where studies in humans are not permissible or possible. This humanized *CHGA* mouse model, therefore, serves to differentiate between regulation of human and mouse gene. We have earlier detailed the phenotype of the humanized *CHGA* model and compared it to the wild-type mice, both strains are normotensive with similar SBP, DBP and carry two copies of the human or mouse chromogranin A alleles, respectively [24].

Previously, we have monitored BP of four-copy mice with CHGA overexpression, by tail-cuff measurements and reported that they are hypertensive [18]. The gold-standard method of monitoring BP and heart rate by continuous telemetric recording was employed in this study and confirmed elevated SBP, DBP as well as increased heart rate in mice expressing elevated levels of CHGA. Telemetric recording showed that in four-copy mice, daytime dipping in DBP was absent, matching the nocturnal nondipping phenotype of hypertensive patients [39]. Various studies show the nondipping BP pattern to be associated with increased target-organ damage and cardiovascular morbidity. Nondipper hypertension is frequently accompanied by endothelial dysfunction and activation [40,41]. Type 1 diabetic patients who did not display dipping showed a two-fold increase in risk of developing hypertension [42]. Among patients with postural tachycardia syndrome, a nondipping BP profile is associated with a reduced orthostatic sympathetic reactivity not accounted for by autonomic neuropathy. In this cohort, dippers differed significantly from nondippers in that they had significantly greater orthostatic heart rate increment and orthostatic plasma norepinephrine increase [43].

As previously reported, elevated circulating and diminished adrenal catecholamine levels is observed possibly because of dysregulated storage and release of LDCV contents in four-copy mice resulting in hypertensive and hyperadrenergic phenotype [18]. Elevated CHGA levels also resulted in oxidative stress. The renin–angiotensin–aldosterone system plays a pivotal role in hypertension [44]. Angiotensin II is a major regulator of aldosterone synthesis and its secretion, is also known to facilitate ROS generation in adrenal cortical cells. Aldosterone, a mineralocorticoid produced and secreted by the adrenal cortex, plays important roles in the control of BP via regulation of sodium and water homeostasis. Excessive production and secretion of aldosterone, results in sodium retention and systemic arterial hypertension and increase. Intermittent hypoxia elevates BP and increases plasma catecholamine in rats via ROS-dependent enhanced synthesis and secretion of neuropeptide Y (NPY) from the adrenal medulla. The vasoconstrictor peptide NPY is a sympathetic neurotransmitter that colocalizes with catecholamine in the adrenal medulla,

has been implicated in BP regulation during persistent stress. It was possible to block hypoxia-induced increase in BP by treatment with antioxidants. The antioxidants reversed hypoxia-induced increases in ROS, which in turn blocked NPY production in the adrenal medulla and thus, prevented BP elevation [45].

Although elevated CHGA may augment adrenal chromaffin granule catecholamine storage in humans, we have previously reported a reciprocal defect in catestatin formation [18]. Diminished catestatin results in loss of inhibition of epinephrine secretion, a prediction consistent with elevation of plasma epinephrine in humans with higher CHGA levels. Patient adrenal biopsies have not yet been studied for effect of excess CHGA on chromaffin granule morphology and the only in-vitro study on the effect of excess CHGA on DCV packaging of catecholamine and exocytosis utilized CHGA–EGFP fusion protein [46]. It is known that CHGA inside the DCVs of neurosecretory cells help in the packaging of catecholamine, ATP, other secretory proteins and biogenesis of the granules itself [47]. As in *Chga-KO* and in transgenic mice with decreased CHGA expression, there is a significant decline in granule content [3,48] we hypothesized that an increase in CHGA expression would most likely increase the number of granules. We investigated as to what mechanism might lend excess CHGA (that has low affinity and high capacity to bind to catecholamine in the DCV) to the hypertensive hyper-adrenergic phenotype? This study reports on the in-vivo effect of excess CHGA on chromaffin granule morphology and composition. In the adrenal medulla of four-copy (excess-CHGA) mice there was a significant increase in number and size of DCVs. In the knockout mice lacking CHGA, expression of other granin family proteins –CHGB and SCG2 levels displayed a compensatory increase [3]. Mice with elevated CHGA had diminished adrenal CHGB and SCG2 protein levels reflecting homeostatic regulation of total granin content in adrenomedullary cells by translational regulation as the transcript levels did not differ but protein levels did between the mouse strains. *Vmat1* the amine transporter situated on the vesicular membrane was markedly elevated in four-copy mice, which fits with the role of Vmat1 receptor protein in facilitating transport of excess catecholamine into the vesicle for packaging [49]. Within the DCVs, high concentration of ATP together with catecholamine, other hormones, amines and enzymes maintain structural integrity of the granules and osmotic balance [22]. A high concentration of Ca^{2+} is responsible for the movement, exocytosis and release of catecholamine from the vesicle into circulation [50]. Although the Ca^{2+} levels remained unchanged in adrenal glands of mice over-expressing CHGA, a decline in ATP content was observed. A recent study proposed that the association of ATP with catecholamine reduces their osmotic forces, permitting the extraordinary accumulation of amines within chromaffin granules [51]. Therefore, diminished ATP could contribute to increased release of catecholamine stores and thus, lend itself to the hyperadrenergic phenotype of the four-copy mice [18]. The increased exocytotic release of catecholamine in four-copy mice also expends ATP and this could result in the observed decreased levels of ATP [52]. ATP is the first component released from the DCVs and then is followed by release of all other vesicular constituents during exocytosis [53]. ATP, after its release into the extracellular environment, is degraded by ectonucleotidases into adenine and phosphate. These in turn act as signaling molecules in an autocrine or paracrine fashion through the purinogenic G-protein-coupled P2Y receptors regulating granule exocytosis by a negative feedback mechanism [54,55]. Diminished ATP in four-copy mice could also be because of modulation of V-ATPase activity that is critical to maintain vesicular pH and thus, exocytosis of catecholamine [56]. This decline in ATP as seen in excess-CHGA mice could thus, compromise the negative feedback mechanism regulating release of catecholamine again resulting is an increase in catecholamine release and the observed hypertensive phenotype in excess-CHGA mice [18].

Although in mice over-expressing CHGA, adrenal ATP content was decreased, the Ox-Phos enzymes involved in the cellular ATP synthesis, were unexpectedly increased. This is accompanied by an increase in the number of mitochondria organelles in the four-copy mice, possibly because of higher synthetic activity demands of the chromaffin cells. Usually, during the transfer of electrons along the ETC, a proton gradient is generated between the inner mitochondrial membrane space and the matrix. This proton gradient is neutralized by FoF1-ATP synthase, transporting protons into the matrix and thus, generates ATP. We investigated whether the dysregulation of ATP synthesis because of CHGA over-expression despite mitochondrial copy number increase could be attributed to uncoupling of proton backflow. Proton backflow is at times uncoupled with ATP generation by the mitochondrial uncoupling proteins (UCPs) resulting in a loss of free energy as heat. Mitochondrial UCPs belong to a large family of transmembrane carrier proteins involved in proton conductance, thermogenesis as well as anion transport across the inner mitochondrial membrane [23,57]. In mammals, UCP2 is the most ubiquitously expressed member of this family, involved in proton backflow and thereby reducing excess proton motive force in the mitochondrial inner membrane space [58]. It also can sense mitochondrial oxidative stress and controls mitochondrial ROS production [58]. As ATP was diminished in adrenal glands of mice overexpressing CHGA, we hypothesized that UCP2 might be playing a role in this process. UCP2 and its functions have been extensively studied in relation to obesity and obesity-induced diabetes, immunity, cardiovascular and many other diseases. However, the involvement of this uncoupling protein in the adrenal gland bioenergetics remains unexplored. UCP2 expression was markedly increased in adrenal glands of four-copy mice as well as in PC-12 cells overexpressing CHGA. This phenomenon has also been reported in another important secretory pathway involved in insulin secretion. In-vitro culture of the β cells show an increase in the UCP2 expression caused by a decline in ATP levels [59]. Also in the UCP2-deficient Langerhans cells isolated from *UCP2-KO* mice, ATP level is increased [60]. Our functional mitochondrial data also revealed higher FCCP-stimulated uncoupled respiration reflecting higher maximal respiratory rates in the adrenal mitochondria from mice expressing excess-CHGA compared with those expressing normal levels. This is consistent with the results indicating higher

mitochondrial mass in the four-copy group. Moreover, the relative increase in FCCP-stimulated uncoupled rates versus the basal ADP rates in the four-copy group is suggestive of not only increased reserve capacity but also reflects underlying bioenergetic dysfunction in ATP production despite higher mitochondrial mass, which can be explained by the higher UCP2 expression observed in the four-copy group [37]. It is possible that pancreastatin, a diabetogenic peptide, derived from full-length CHGA is playing a role in elevating UCP-2 expression as is in the case of rat adipocytes [61].

Although, UCP2 dysregulation has been implicated in several physiological and pathological studies, few reports have contradicted the role of UCP2 in mitochondrial uncoupling and ATP synthesis. In the *UCP2-KO* mice, no difference is observed in the ATP/ADP ratio and proton leak compared with the wild type mice [62]. In the rat, skeletal muscle elevated level of UCP2 or UCP3, did not change the mitochondrial proton conductance [63]. Pecqueur *et al.* [64] showed that UCP2 promotes mitochondrial fatty-acid oxidation by limiting pyruvate catabolism. Several others also dispute a role for UCP2 in the mitochondrial uncoupling process [65–67]. These inherent differences in observations are because the studies were done with living cells and isolated mitochondria resulting in unclear evidence for the uncoupling role of UCP2, further extensive studies are required to establish role of UCP2 [68]. Though there exists a debate about the role of UCPs in uncoupling process, researchers have established the role for these proteins in modulating mitochondrial ROS generation. UCPs, mainly UCP2 and UCP3 are responsible for mitochondrial proton leak and thus, reduce the proton motive force and prevent excess ROS generation by the ETC [58]. UCP2 in fact, senses the level of mitochondrial oxidative stress and controls ROS generation by a local feedback mechanism and protects the cells from oxidative damage [68–70]. At low levels of ATP, mitochondrial membrane potential is likely to be increased resulting in an elevation in the mitochondrial ROS generation [70]. In *Chga-KO* mice, an elevation in ROS levels and decline in ATP levels is observed [71]. Significant increase in ROS levels is observed in excess-CHGA (four-copy) mice suggesting that oxidative stress lends itself to the pathogenic phenotype, we could not, however, recapitulate this in our in-vitro experiment, possibly because of absence of regulatory factors present *in vivo*. In our study, decline in ATP level in four-copy mice, could possibly be attributed to increased expression of UCP2. Cell culture studies also showed concomitant up-regulation of CHGA and UCP2 contributing to diminished ATP. Pharmacological inhibition of UCP2 with genipin restored ATP levels.

The limitation of this study is the use of whole versus the medullary adrenal extracts, as separation of the cortex and medulla of a mouse adrenal gland is technically challenging. It cannot be concluded that increased number of mitochondria because of overexpression of CHGA is due to increased biogenesis or decreased turnover (via autophagy). UCP2 is shown to be involved in the mitochondrial autophagy regulation [64]. Future work exploring how mitogenesis and autophagy pathways may be regulated by CHGA would be of interest. The molecular mechanism responsible for CHGA-induced upregulation of UCP2 also holds relevance.

Thus adrenal glands of mice with excess-CHGA are hypergranulogenic with attenuated ATP stores suggesting dysregulated and uncoupled oxidative phosphorylation because of upregulation of UCP2 expression. The reduced ATP in turn contributes to decreased negative feedback of secretory vesicle exocytosis and deregulated catecholamine release, resulting in the hyperadrenergic and hypertensive phenotype.

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Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Referee 1

The authors examined the role of chromogranin A (CHGA) in blood pressure and heart rate control in mice expressing elevated level of CHGA. They suggest bioenergetics dysfunction in ATP production related to elevated CHGA leading to increased circulating catecholamine and hypertensive phenotype. It would be important to confirm whether elevated CHGA hypertensive patients could have a similar mechanistic dysfunction and which treatment could be effective.

Referee 2

The authors compare mouse models expressing two copies and four copies of chromogranin A (CHGA) allowing elevated levels of CHGA to be compared to lower levels. The authors explore the functional consequences of increased CHGA levels and implicate over expression of uncoupling protein 2 with a reduction in ATP.