Original Article

Chromogranin A pathway: from pathogenic molecule to renal disease

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Background: Chromogranin A (CHGA) is an index granin protein critical for biogenesis and exocytotic release of catecholamine storage granules. It is elevated in plasma of patients with sympathetic over-activity and kidney dysfunction. Several *CHGA* polymorphisms are associated with hypertensive kidney disease. Previously, we unraveled the molecular mechanism by which CHGA expression is regulated in African Americans carrying a genetic variation associated with hypertensive chronic kidney disease (CKD).

Method: Experimental CKD mouse model were created by 5/6th nephrectomy (Npx) using wild-type and *Chga−/−* knockout mouse strains to delineate the role of CHGA in CKD.

Result: Wild-type-Npx mice expressing *Chga* developed exacerbated azotemia and fibrosis as compared with their knockout-Npx counterparts. Gene expression profiling revealed downregulation of mitochondrial respiratory complexes genes consistent with maladaptive mitochondria in wild-type-Npx mice, contrasted to knockout-Npx. In healthy individuals, an inverse relationship between circulating CHGA levels and glomerular function was observed. *In vitro,* mesangial cells treated with CHGA-triggered nitric oxide release by a signaling mechanism involving scavenger receptor SR-A. The CHGA-treated and untreated mesangial cells displayed differential expression of cytokine, chemokine, complement, acute phase inflammatory and apoptotic pathway genes. Thus, build-up of plasma CHGA because of kidney injury served as an insult to the mesangial cells resulting in expression of genes promoting inflammation, fibrosis, and progression of CKD.

Conclusion: These findings improve understanding of the role of elevated CHGA in the progression of CKD and reveal novel pathways that could be exploited for therapeutic strategies in hypertensive kidney disease.

Keywords: chromogranin A, chronic kidney disease, hypertensive kidney disease, mesangial cell, nephrectomy

Abbreviations: AASK, African American Study of Kidney Disease and Hypertension; BP, blood pressure; CHGA, chromogranin A; CKD, hypertensive chronic kidney disease; CRL-1927, mouse mesangial cells; ESRD, end-stage renal disease; GFR, glomerular filtration rate; HR, heart rate; iNOS, nitric oxide synthase; KO, knockout (*Chga−/−*); LPS, lipopolysaccharide; NO, nitric oxide; Npx, 5/6th nephrectomy; ROS, reactive oxygen species; SR-A, scavenger receptor; WT, wild-type (*Chga+/+*)

INTRODUCTION

The growing prevalence of CKD is a major public health challenge, associated with high morbidity and is at risk of cardiovascular disease [1]. Catecholamine a measure of the sympathetic nerve over-activity, is associated with mortality and cardiovascular outcomes in ESRD [2]. Chromogranin A (CHGA) is an index member of the granin family co-stored and released into circulation with catecholamine from secretory granules of chromaffin cells and postganglionic sympathetic neurons [3]. A relationship has been established between CHGA, hypertension and kidney disease. Kidney dysfunction contributes to increasing levels of plasma CHGA [4,5] and several *CHGA* gene polymorphisms contributing to traits of autonomic blood pressure control, hypertension, and hypertensive kidney disease have been identified [6–10]. In the African American Study of Kidney Disease and Hypertension (AASK) cohort, glomerular filtration rate (GFR) decline in CKD was associated with a polygenic trait with contributions from several genes involved in catecholamine biosynthesis, storage and catabolism including *CHGA* [8]. We previously unraveled the molecular mechanism by which CHGA expression is regulated in the African American carriers of a genetic variation in the *CHGA* 3′-UTR region that is associated with hypertensive nephropathy [11]. In this study, we seek to understand the role of CHGA in CKD progression.

The nephrectomy (Npx) mouse model, which represents progressive kidney dysfunction because of reduced renal parenchyma was used to investigate the influence of CHGA on kidney function [12]. In this study, it was observed that mice expressing *Chga* that underwent Npx surgery

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developed more severe azotemia and fibrosis compared with their *Chga*−/− (knockout) counterparts. We profiled by microarray differential gene expression in both strains of mice with or without surgery and observed downregulation of mitochondrial respiratory complexes genes. In healthy individuals, a negative inverse correlation between circulating CHGA levels and glomerular filtration was observed. Loss of kidney function led to a significant elevation of plasma CHGA and to understand its consequence, mesangial cells in culture were treated *in vitro* with CHGA protein. This triggered NO release and activated genes involved in its biosynthesis. The signal transduction mechanism leading to NO production involved activation of scavenger receptor and expression of genes leading to kidney disease progression.

METHODS

Animal husbandry, nephrectomy, and estimated glomerular filtration rate measurements

Mice were housed in a 12-h light (0600–1800 h) and 12-h dark cycle under pathogen-free conditions, maintained on a normal chow-diet and allowed to have water ad libitum. Experiments were carried out in accordance with the IACUC at UCSD.

Progressive hypertensive-kidney disease model, mimicked features of human CKD and was created sequentially: briefly, the upper and lower poles of the left kidney (two-thirds) were resected. After 1 week, the remaining right kidney was removed through a right paramedian incision after ligation of the right renal artery, vein, and ureter [13]. Sham surgery was performed by manipulation of the renal pedicle in age and sex-matched mice and used as controls. The mice were anesthetized with ketamine/xylazine (100 and 10 mg/kg, respectively) and via precision vaporizer isoflurane (1.25–1.5% in 100% oxygen). Buprenorphine (0.1 mg/kg) was given two to three times per day, for 3 days, to mice during the recovery. To avoid overdosing of ketamine/xylazine, the strategy of half dosage of standard ketamine/xylazine (50 and 5 mg/kg, respectively) and isoflurane (1.25–1.5% in 100% oxygen) was administered prior to operation. Standard processes for preoperational and postoperational animal care was applied. The surgery when done in two stages improves the survival chances of the mouse. This is in accordance with published literature.

The animals are then followed sequentially for 10 weeks, with weekly assessments of blood pressure and hazard ratio using tail-cuff apparatus, as described previously [14]. Weekly blood sampling of operated mice was done by tail vein incision to obtain plasma for creatinine measurements [15]. Measurements of eGFR were estimated by renal clearance of creatinine by a method not confounded by circulating noncreatinine Jaffe chromogens in rodents. Plasma creatinine was measured using electrospray mass-spectrometry LC-MS and 10 μl sample volumes, at the UAB-O'Brien core.

At the terminal stage of the experiment, mice were euthanized by deep anesthesia in isoflurane and blood was collected by a transthoracic ventricular puncture in EDTA-tubes. The remnant kidney tissue samples were dissected and suspended in RNA later (Life Technologies, California, USA) for subsequent RNA extraction or in formaldehyde for histological analysis. The kidneys were fixed in 10% formalin and embedded in paraffin wax. Kidney sections on slides were stained with three color Masson's Trichrome stain. To quantify fibrosis, digitized images were analyzed using ImageJ software (NIH) to calculate collagen volume fraction as percentage area of collagen divided by total section area. At least 15 images from each group were analyzed. Plasma CHGA was measured using a mouse ELISA kit kindly provided by Corti [16].

Microarray analysis

Kidney tissue 10 weeks post-Npx/sham surgery in four cohorts: *Chga* +/+ sham/Npx; *Chga* −/− sham/Npx were harvested and their transcriptome analyzed in quadruplicates. Total RNA was isolated using a RNeasy Kit (QIAGEN, Germantown, Maryland, USA). In a parallel experiment, RNA from CRL-1927 cells (four replicates, with or without CHGA treatment *in vitro*) was isolated. The RNA quality was assessed by using an Agilent Model 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) and labeled and hybridized to a mouse 12-plex array (100718_MM9_EXP_HX12, Nimblegene/Roche, Madison, Wisconsin, USA). The array was scanned on a GenePix 4000B scanner (Molecular Devices, Sunnyvale, California, USA) and data extracted using Arraystar 4 (DNAStar Inc., Madison, Wisconsin, USA). Raw intensity data were analyzed using a Bayesian variance modeling approach in VAMPIRE using median normalization and an FDR of 0.05. Principal component analysis and unsupervised hierarchical clustering using Euclidean distance were performed, and violin plots were generated using GeneSpring 14.5 (Agilent Technologies). The list of genes with altered expression between the wild-type-Npx and knockout-Npx, or with CHGA treatment *in vitro*, was subjected to gene ontology and pathway mapping using GeneGo's Metacore software (St. Joseph, Mississippi, USA). Microarray data are available at GEO Accession Number (GSE106300).

Cell culture and nitric oxide assay

Mouse kidney glomerular mesangial cells (ATCC CRL-1927) were grown in 3 : 1 mixture of DMEM high glucose and Ham's F12 (Corning Cellgro) with 14 mmol/l Hepes, 5% fetal bovine serum and penicillin/streptomycin/glutamine at 37 °C in 5% CO_2. Cells were treated with various stimuli in fresh culture media and incubated for up to 24 h. Tissue culture supernatant was collected, diluted 1 : 1 with reaction diluent, filtered through Amicon Ultra-0.5 ml centrifugal filters (Ultracel, 10K, Millipore, Burlington, Massachusetts, USA) and the flow through media used to determine total NO according to the manufacturer's instruction (KGE001, R&D Systems, Inc., Minneapolis, Minnesota, USA). Experiments were performed on at least two unique cell preparations. Data are representative of a typical experiment and are given as a mean of triplicates ± SEM.

Chromogranin A and estimated glomerular filtration rate measurements in individuals

Individuals were volunteers from Southern California and gave informed written consent; the protocol was approved by the UCSD Human Research Protection Program. CHGA and estimated GFR were measured in this large cohort of European ancestry as previously described [17]. None of these healthy individuals had a history of kidney dysfunction, GFR was estimated from plasma creatinine, age, sex and body size, using the simplified NIDDK Modification of Diet in Renal Disease algorithm: GFR (ml/min) = (186) × (pCr1.154) × (Age0.203) × (0.742 [if female]) × (1.21 [if black]).

Statistical methods

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. Results from at least three independent experiments were considered for analysis of in-vitro data. Data with a '*P*' value less than 0.05 were considered significant.

RESULTS

Chga+/+-Npx model mimics clinical observation of inverse correlation between circulating chromogranin A levels and glomerular filtration rate

To evaluate the effect of circulating CHGA on glomerular function, both the wild-type and knock out strains of mice were subjected to either Npx or sham surgery and assessed weekly for their plasma creatinine and BP levels for up to week 10 postsurgery. Both strains of mice with Npx displayed decreased renal function with the progression of time compared with mice that underwent sham surgery. Both strains did not display a significant difference in the build-up of plasma creatinine at 4 weeks post-Npx. After 4 weeks, the creatinine levels in the wild-type-Npx continued to increase whereas the knockout-Npx did not display further deterioration of kidney function, potentially indicating a detrimental effect of CHGA in the progression of CKD (Fig. 1a). Wild-type-Npx mice displayed elevated plasma levels of the CHGA, contributed in part because of increased sympathetic activity and also in part failure of kidneys to filter CHGA (Fig. 1b). High BP is a hemodynamic characteristic of CKD and accelerates the progression of kidney dysfunction by worsening glomerular injury and proteinuria. The phenotype of knockout mice is hypertensive prior to surgery [18]. Kidney dysfunction because of Npx elevated BP in both strains of Npx mice and week 5 post-Npx, no significant difference in BP between strains was observed (Fig. 1c), like the knockout-Npx mice, the wild-type-Npx were hypertensive (BP ~150 mmHg). To investigate whether CHGA levels are predictive of kidney disease progression in humans, we measured plasma CHGA in individuals with known GFR (Fig. 1d) and a significant negative correlation was observed. Therefore, in both mice and humans, an inverse correlation suggested a decrease in GFR with increasing plasma CHGA concentration.

Renal fibrosis augmented by chromogranin A

Renal fibrosis causing stiffness and loss in structural integrity of the kidney greatly affects survival outcomes [19]. To assess fibrosis, sections of kidneys from wild-type and knockout mice 10 weeks' post-sham/Npx operation were stained with Masson's trichrome. Both knockout and wild-type Npx showed excess deposition of extracellular collagenous tissue, whereas staining was not present in kidneys from sham-operated mice. Strikingly, matrix deposition in the wild-type kidney was much more prominent with almost no empty extracellular space in kidney parenchyma as compared with the knockout-Npx (Fig. 2) consistent with worsened kidney function associated with wild-type-Npx mice. Collagen volume fraction (CVF) as calculated from Masson Trichrome Stained kidney tissue sections (Fig. 2) was significantly increased in wild-type-Npx group compared with WT-sham (Supplementary Data 3, http://links.lww.com/HJH/B168).

Chromogranin A imparts differential gene expression in kidneys subjected to injury

Gene expression profiling by microarray was performed on kidney tissue at week 10 to assess the effects of strain and operation. Each group (*Chga*+/+sham, *Chga*+/+Npx, *Chga*−/− sham, and *Chga*−/− Npx) constituted of four animals. The analysis focused on genes differentially expressed between the wild-type-Npx and knockout-Npx as we found little difference between wild-type and knockout mice in the sham state. Figure 3a is the Principal Component Analysis plot of the samples; a Venn diagram shows the number of genes altered by Npx in wild-type or knockout mice (Fig. 3b), as well as the genes altered by genotype in sham or Npx mice (Supplementary Data 1, http://links.lww.com/HJH/B166). The heat map provides pictograph of all the altered genes (Fig. 3c). The GO enrichment reports for the 612 genes altered in wild-type-Npx vs. knockout-Npx indicate that genes involved in extracellular matrix remodeling are significantly enriched (Supplementary Data 2, http://links.lww.com/HJH/B167). The data also suggested that genes with oxidoreductase activity or associated with the mitochondrion are also enriched. These families of genes were inspected individually, and those representing ECM, transport, and mitochondrial genes are shown as violin plots (Fig. 4a). Globally, genes in the extracellular matrix, transporters and mitochondrial families are elevated by Npx in both wild-type and knockout mice. These findings are in agreement with the histological analysis (Fig. 2) showing fibrosis because of Npx. Several mitochondrial protein mRNAs (*Lyrm1, Hsd3b1, Mrps33, Gm6181, Gm11810, Hsd3b5, Amacr, Lypla1, Ndufaf1, Auh, Immp2l, Gm4952, Agxt2l1, Nme1, Mpv17l, 8430408g22rik, Cox6a2, Acot13, Gm6462, Gm7180, Mrpl33, Gm2382)* are overexpressed in wild-type-Npx; however, the plot depicting expression of the electron transport chain and ATP synthase genes, showed decreased expression selectively in wild-type-Npx but not knockout-Npx. This might suggest that mitochondrial

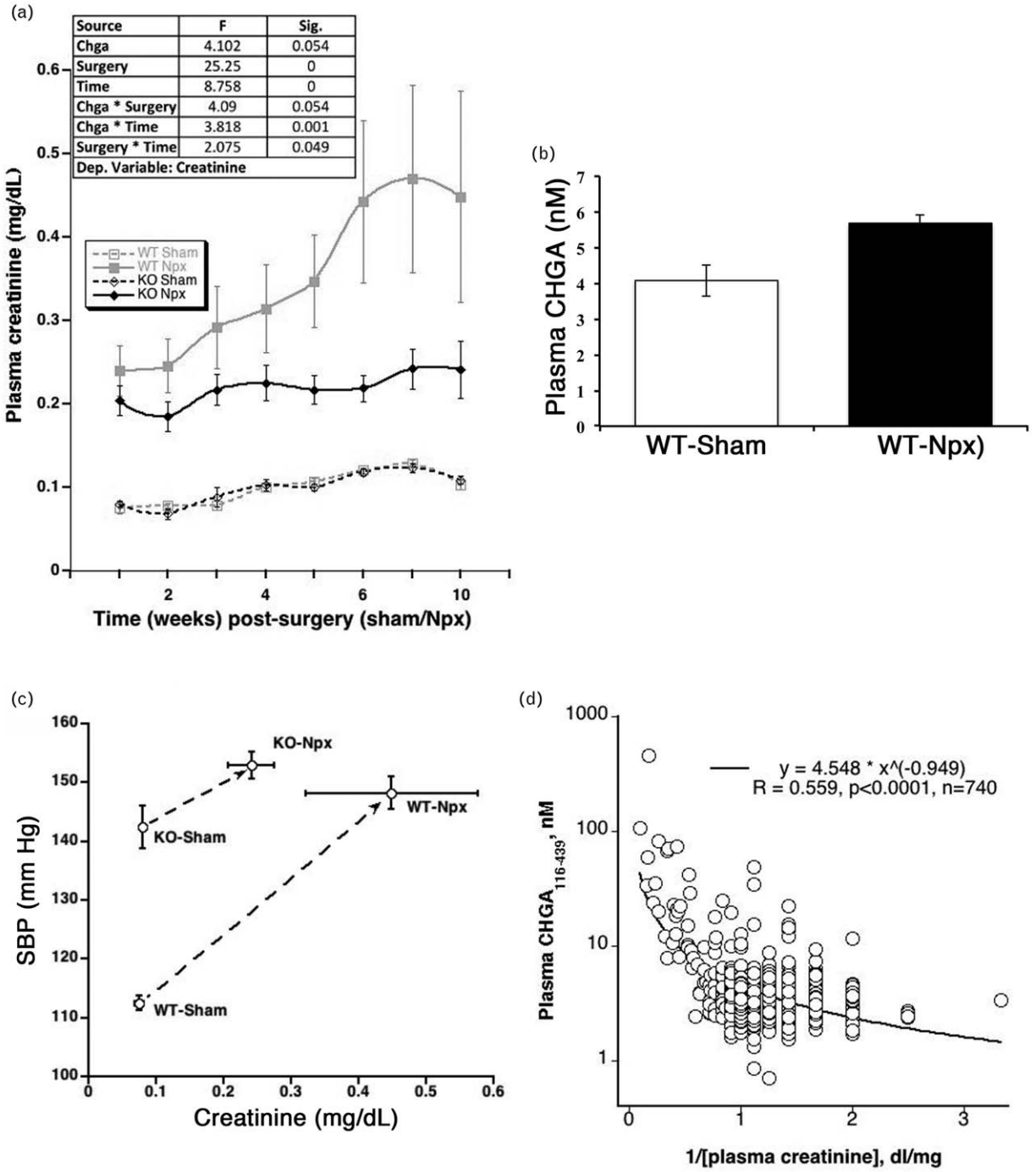

FIGURE 1 Impact of chromogranin A on glomerular function. (a) Time course on the effect of CHGA accumulation on eGFR. wild-type and knockout mice ($N = 12$ each of the four groups), 8 weeks of age, were subjected to sham or Npx surgery and their weekly eGFR was estimated by measuring the plasma creatinine levels. The data was analyzed by repeated measure ANOVA using linear mixed model and showed significant differences in creatinine measurements between the two mouse strains subjected to Npx. (b) Elevated CHGA in the CKD mouse model. The wild-type (WT)-Npx mice showed 1.4-fold elevated plasma CHGA level as compared with sham-operated mice. (c) Ten weeks post-Npx, the BP did not differ significantly between Npx strains; however, WT-Npx have significantly augmented azotemia compared with knockout (KO)-Npx suggesting BP-independent mechanism of CHGA pathogenesis. (d) Circulating CHGA concentration and human eGFR. A large cohort of healthy individuals of European ancestry were measured for plasma CHGA and eGFR. A significant negative correlation was observed suggesting decrease in glomerular function with increasing plasma CHGA concentration. ANOVA, analysis of variance; BP, blood pressure; CHGA, chromogranin A; eGFR, estimated glomerular filtration date; Npx, nephrectomy.

efficiency is reduced in wild-type-Npx but somehow protected in knockout-Npx. The expression data for the gene family members that were significantly altered in the Npx samples are shown separately (Fig. 4b), highlighting the different response of the wild-type and knockout mice to kidney injury. *ECM* genes are more highly expressed in wild-type-Npx than knockout-Npx consistent with the histology. Transporter and mitochondrial genes show greater variance in the expression for the wild-type-Npx than the knockout. However respiratory complex genes are significantly downregulated in wild-type-Npx vs. knockout-Npx consistent with the entire gene family data (Fig. 4a)

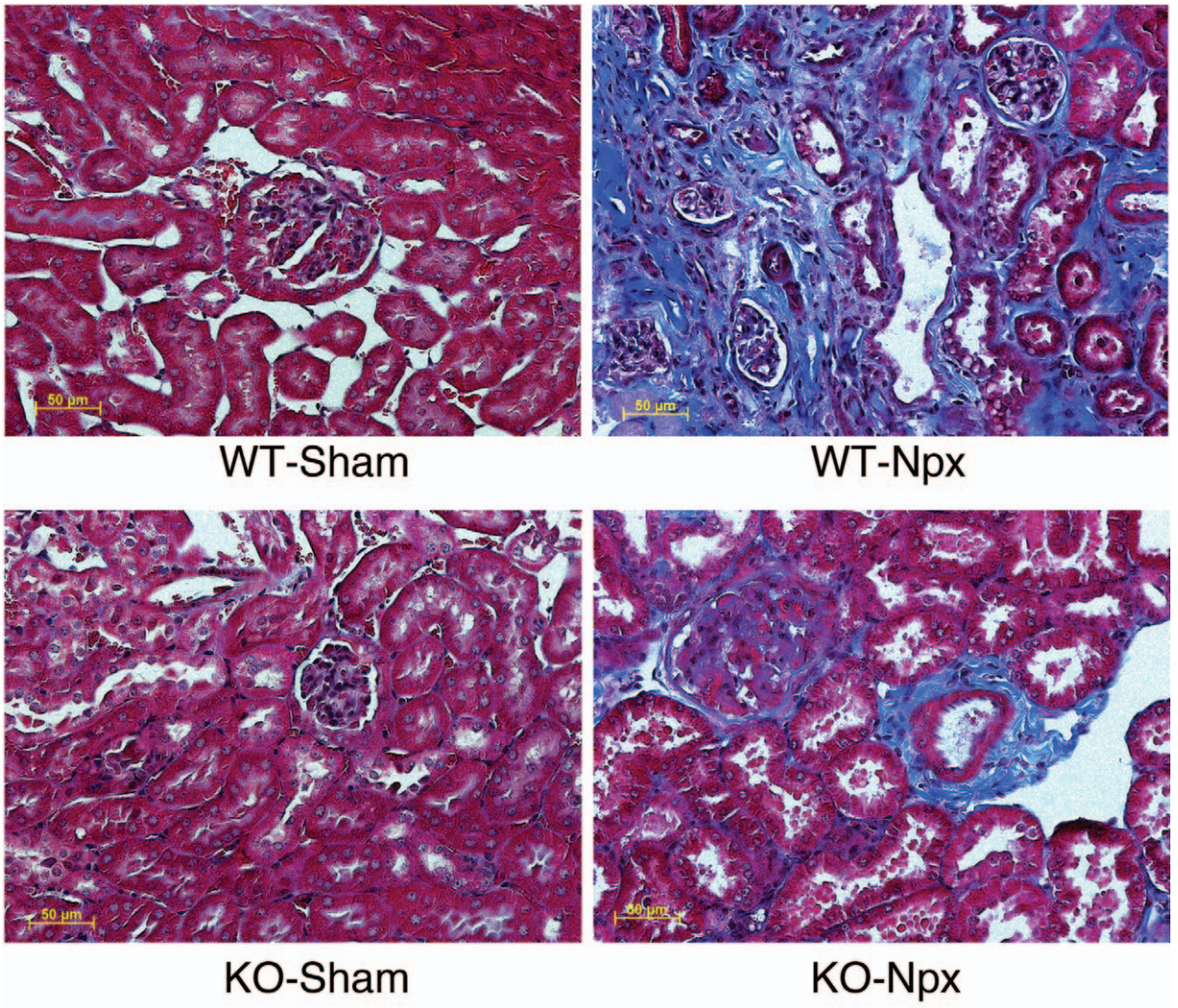

FIGURE 2 Chromogranin A accumulation because of injury augments renal fibrosis. The kidney tissue was harvested 10-weeks postsurgery (sham/Npx) and histology sections were stained by Masson's trichome. All pictographs have the same magnification. KO, knockout; Npx, nephrectomy; WT, wild-type.

suggesting impaired mitochondria in WT- Npx mice but protection in the knockout.

Chromogranin A treatment of mesangial cells triggers nitric oxide synthesis

As CHGA builds up in the plasma because of loss of kidney function (Fig. 1b and d), we investigated the response of kidney cells to elevated CHGA. CRL-1927 mouse mesangial kidney cells were incubated with recombinant CHGA protein and the release of NO measured over time (Fig. 5a). In the presence of CHGA, the NO secretion was 2.9-fold and 2.1-fold higher than untreated cells at 8 and 24 h respectively. LPS, a potent activator of the immune system resulted in 5.9 and 3.8-fold stimulation over untreated cells at the same time points. The stimulation of NO release by mesangial cells treated with CHGA was by upregulation of nitric oxide synthase (iNOS) transcription (Fig. 5b) and was abolished by a specific iNOS pharmacological inhibitor L-NIL. The signal transduction mechanism leading to NO release involved protein tyrosine kinase and de novo protein synthesis, as it was blocked by genistein and cycloheximide (Fig. 5c). The CHGA-induced NO release was completely blocked by preincubating cells in the presence of the scavenger receptor (SR-A) ligand fucoidan. Poly-inosinic acid the ligand for both toll-like receptor 3 ligand (TLR3), and SR-A also blocked NO production to some extent (Fig. 5d). The activation of iNOS expression was specific to full-length CHGA protein; CHGA's peptide derivatives – catestatin, vasostatin hydrophobic loop, and WE-14 failed to stimulate NO release (data not shown). Tropomyosin, a protein that has a coiled-coil structure like full-length CHGA, also stimulated NO release whereas poly (Glu)n peptide with a random coil form was ineffective (Fig. 5e).

Concerted transcription of genes involved in nitric oxide production and pro-inflammatory markers in mesangial cells treated with chromogranin A

Following CHGA or mock treatment of CRL-1927 cells, gene expression was analyzed by microarray. A two-stage

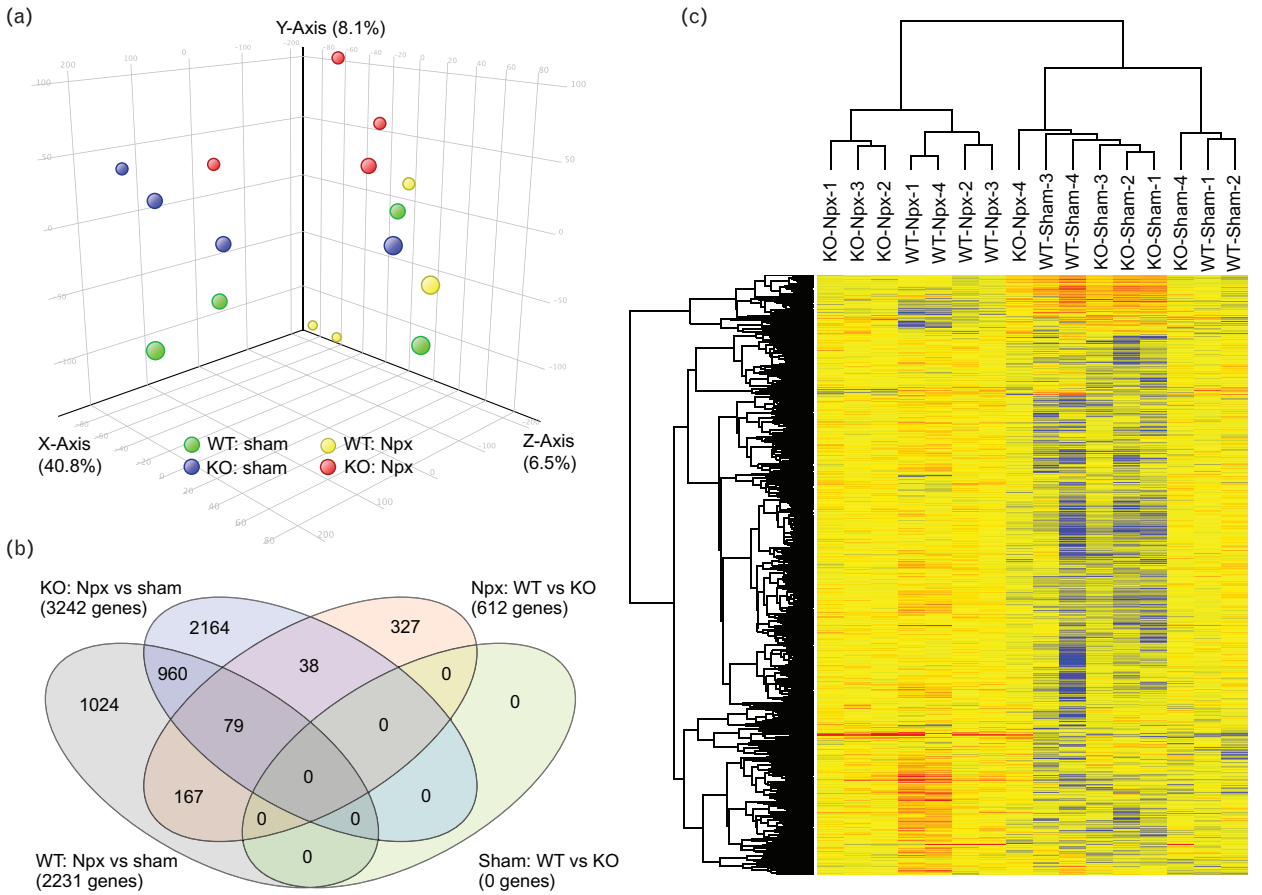

FIGURE 3 Transcriptome analysis of the remnant kidney. (a) PCA plot of the kidney tissue of mice subjected to sham and Npx surgery. (b) Venn diagram showing the number of genes altered by Npx in wild-type (WT) or knockout (KO) mice, as well as the genes altered by genotype in sham or Npx mice. (c) The heatmap depicts all the altered genes. All the sham samples cluster together, as do most Npx samples. Npx, nephrectomy.

amplification of NO synthesis was brought about by an increase in transcription of both the arginine transporter *Slc7a7* and *iNOS* (Fig. 6a). L-Arginine transported by Slc7a7 serves as a substrate for iNOS to catalyze the production of NO. Expression of several solute carrier family genes was also upregulated in CHGA-treated cells (Fig. 6b). Differential expression of pro-inflammatory genes was also observed in mesangial cells including the upregulation of

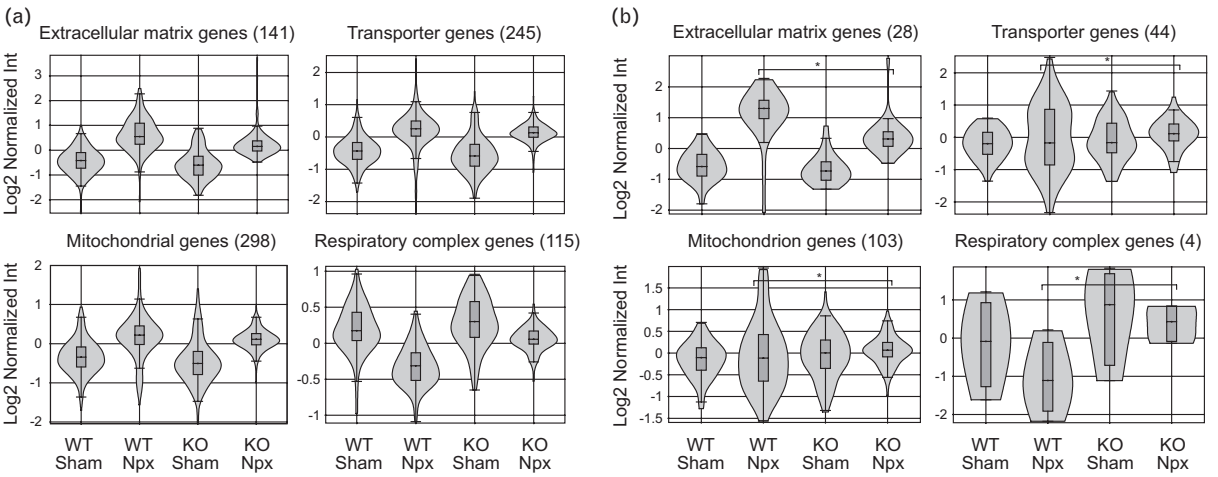

FIGURE 4 Differential expression of genes by strains in response to nephrectomy. (a) Transcriptome analysis of ECM, transport, and mitochondrial gene families significantly altered in the complete dataset shown as violin plots. Most were up because of Npx in both wild-type (WT) and knockout (KO) strains. The plot showing electron transport chain and ATP synthase genes were down in WT-Npx but not KO-Npx suggesting reduced mitochondrial efficiency in WT-Npx but somehow protected mitochondrial function in KO-Npx. (b) Gene families differentially expressed between WT-Npx and KO-Npx. There was no difference between wild-type and knockout mice in the sham state. Expression of ECM genes were higher in wild-type than knockout. Expression of transporter and mitochondrial genes showed greater variance in WT-Npx. Respiratory complex genes were downregulated in WT-Npx vs. KO-Npx similar to (a), again suggesting impaired mitochondria in WT-Npx. CHGA, chromogranin A; Npx, nephrectomy.

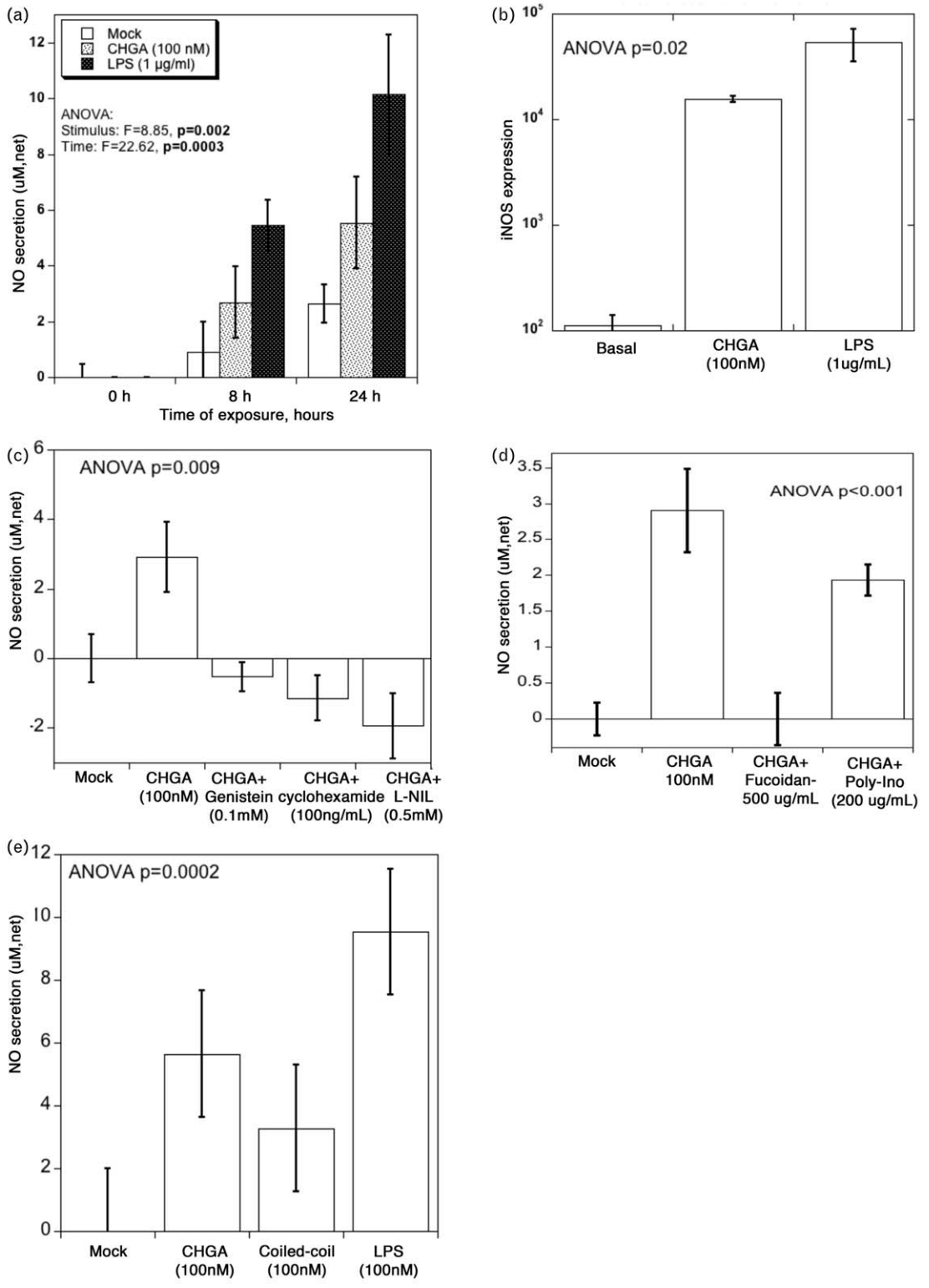

FIGURE 5 Chromogranin A triggers nitric oxide release from mouse glomerular mesangial cells. (a) CRL-1927 cells were treated with CHGA (100 nmol/l) or LPS (1 µg/ml) for 8 and 24 h, and the tissue culture media collected to measure total NO. The assay determined NO concentration based on the enzymatic conversion of nitrate to nitrite by nitrate reductase, followed by a colorimetric detection by Griess reagent. Control media was from cells left untreated. Net secretion was calculated by subtracting zero-hour basal secretion from all other time points. (b) Cells were treated with CHGA or LPS for 24 h, and the total RNA extracted and processed for real-time PCR. Relative amount of the iNOS mRNA was quantified as iNOS/beta actin ratio. (c) Specificity of CHGA induced NO secretion. Cells were treated with CHGA in presence and absence of L-NIL, cycloheximide and genistein for 24 h, and net NO secretion was measured from culture media. (d) Cells were pretreated with SR-A blocker fucoidan or poly-ionosine for 1 h prior to the addition of CHGA. After 24 h tissue culture supernatant was harvested and the released NO measured. (e) Cells were treated with full length CHGA, a coiled-coil protein tropomyosin, poly- L-glutamic acid or LPS for 24 h and the tissue culture supernatant collected to measure total NO. CHGA, chromogranin A; LPS, lipopolysaccharide; NO, nitric oxide; Npx, nephrectomy; WT, wild-type.

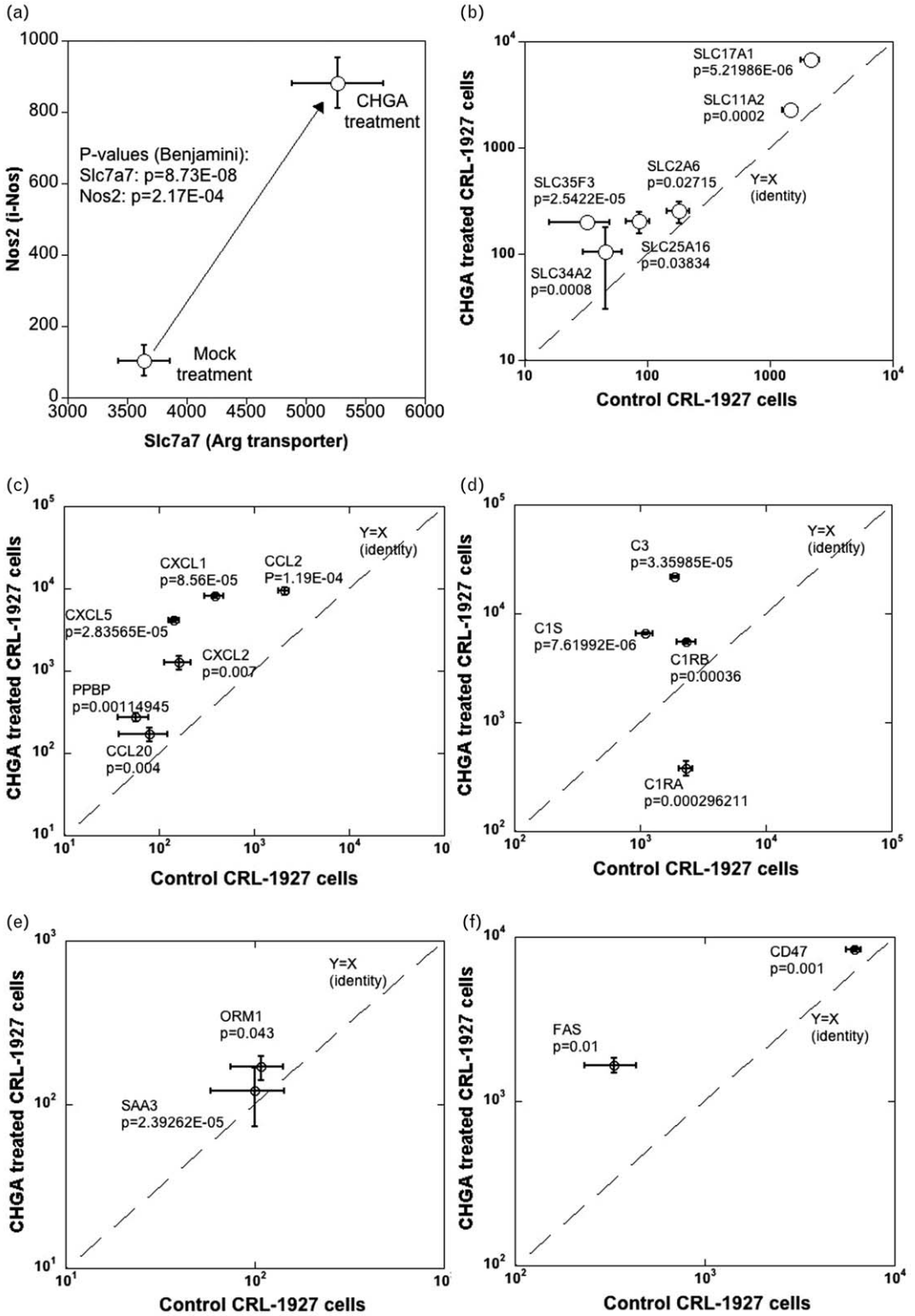

FIGURE 6 Chromogranin A impacts transcription of genes involved in NO synthesis and proinflammatory markers. The mesangial cells were treated with CHGA (100 nmol/l) or untreated for 24 h, harvested and transcriptomes compared. (a) A two-stage amplification of transcripts involved in NO synthesis. Both the precursor Arg transporter and NO biosynthesis enzyme iNOS showed concomitant over-expression. (b) Several solute carrier transport family genes are over-expressed. Similarly, CHGA-treated cells overexpress: (c) cytokine and chemokines family transcripts, (d) complement family transcripts, (e) acute phase inflammatory markers, (f) transcripts related to cellular apoptosis. CHGA, chromogranin A; NO, nitric oxide.

several chemokine and cytokine genes (Fig. 6c). Three of the four complement family members were over-expressed in CHGA-treated cells, and the fourth was under-expressed (Fig. 6d). Acute phase response genes (*Saa3* and *Orm1*) are upregulated in response to CHGA treatment as in case of serious injury and inflammation (Fig. 6e). Oxidative stress is a major cause of damage associated with elevated NO that leads to apoptosis and *Fas* expression plays a central role in this process. *Fas* expression was upregulated significantly because of CHGA treatment (Fig. 6f).

Thus, this study describes the association between hypertensive CKD and CHGA, a gene known to play critical roles within the sympathoadrenal response.

DISCUSSION

Epidemiological studies have demonstrated an excessive risk of CKD progression to ESRD among African Americans, even after controlling for confounding factors [20,21]. In this population, several potential disease-susceptibility loci and gene polymorphisms for hypertensive-CKD leading to ESRD have been identified including *CHGA*, the adrenergic catecholamine storage protein [8–10,22–25]. This study supports observations in humans that loss of kidney function leads to build-up of plasma CHGA and triggers mesangial cell activation via scavenger receptor, mounting an oxidative stress response potentiating inflammation and fibrosis ensuing in the disease phenotype.

Sympathetic activation because of kidney failure results in hypertension and cardiovascular complications in many of these patients [26,27]. CHGA and its hypotensive peptide derivative catestatin have been implicated in both the heritability [28] and pathogenesis of essential hypertension [29–31]. CHGA catalyzes the biogenesis and regulated exocytosis of catechaolamine storage granules in neuroendocrine cells, and thus contributes to blood pressure homeostasis [32,33]. Acetylcholine stimulation results in the simultaneous release of catecholamine and CHGA and the secreted catecholamine goes on to trigger cardiovascular target cells to increase blood pressure [3]. In this study, we explored the role of the *CHGA* in kidney disease. Kidneys have an essential role in clearing proteins including CHGA from blood; however, because of loss of kidney function, there is build-up of CHGA in plasma of patients [34] and in WT-Npx mice (Fig. 1b). Our data indicate that postkidney injury, elevated levels of CHGA in plasma is detrimental to recovery of kidney function (Fig. 1). Also stress induced by nephrectomy augmented sympathetic response contributing to CHGA release from adrenergic stores, which along with loss of GFR elevated circulating CHGA in mice.

Studies in other rodent models have also shown association between sympathetic nervous system activity and rate of progression of kidney disease. In obese spontaneously hypertensive rats, progressive increase in blood pressure, cardiac remodeling and renal injury were mediated by renal sympathetic activation as they were attenuated by renal sympathetic denervation [35]. In 5/6th nephrectomized rats sympathectomy attenuated hypertension; however, the level of proteinuria in these rats was reduced to a greater extent than what would be expected solely on the basis of lower blood pressure confirming involvement of sympathetic nervous system [36]. Campese's study [37] indicates that increased sympathetic nervous system activity contributes to the pathogenesis of hypertension and progressive glomerulosclerosis in rats with the renal ablation model of chronic renal failure. In the mouse AKI model, elevated neurohormonal signaling of the sympathetic nervous system and the endothelin system play a driving force in kidney and heart damage involving GPCR-G$\beta\gamma$ signaling [38].

Our findings suggest the probable chain of events involves increased sympathetic response, diminished kidney filtration leading to accumulation of CHGA in plasma, triggering augmented expression of pro-fibrotic and inflammation genes resulting in progression of CKD. These findings are in line with the patient cohort confirming the inverse correlation between GFR and plasma CHGA levels (Figs. 1 and 4).

Previous studies have reported that plasma CHGA is elevated in CKD patients [4] and several *CHGA* gene, promoter and 3′UTR polymorphisms have been associated with hypertensive kidney disease [8,9]. CHGA acts via glomerular endothelium to regulate kidney function, and plasma CHGA correlates positively with endothelin-1 and negatively with GFR [17]. In African Americans with hypertensive-ESRD, *CHGA* has been identified as a disease-susceptiblity locus, and these patients also show an 'intermediate' biological phenotype of diminished circulating levels of the catestatin (the hypotensive peptide derived from CHGA that serves as a nicotinic cholinergic receptor antagonist) [9]. Our study suggests that although injured kidneys contribute to sympathetic activation and elevated BP, the disease outcome is not entirely a result of sympathetic activation. In both strains of Npx mice, the sympathetic response is elevated; however, loss of kidney function is dramatically accelerated in mice expressing *Chga*. Similar findings have been observed previously in 5/6th nephrectomized rats whose sympathetic overactivity in early phase after nephrectomy is closely related to progression of CKD; however, is independent of changes in blood pressure [39]. In the hypertensive and hyperadrenergic *Chga*−/− mice, glomerular hyperfiltration is observed [11].

The differential response by both strains to surgery is parsed by mitochondrial dysfunction in mice expressing *Chga*, and as a result expression of the electron transport chain and ATP synthase genes are down in wild-type-Npx but not knockout-Npx (Fig. 4). Overall, in wild-type-Npx as compared with knockout-Npx, mitochondrial genes are overexpressed, and this generates superoxide radicals leading to oxidative stress and inflammation. These CHGA-induced mitochondrial genes' transcriptional response was not observed *in vitro* potentially because of the absence of regulatory factors present *in vivo*. The kidney receives 20% of cardiac output and consumes 10% of the body's oxygen to perform its functions, and mitochondrial dysfunction is recognized as a leading factor to many chronic and acute renal diseases [40]. CHGA triggers oxidative stress injury and inflammation leading to energy shortage from either impaired mitochondrial biogenesis or ATP energetics. This could result in the recruitment of

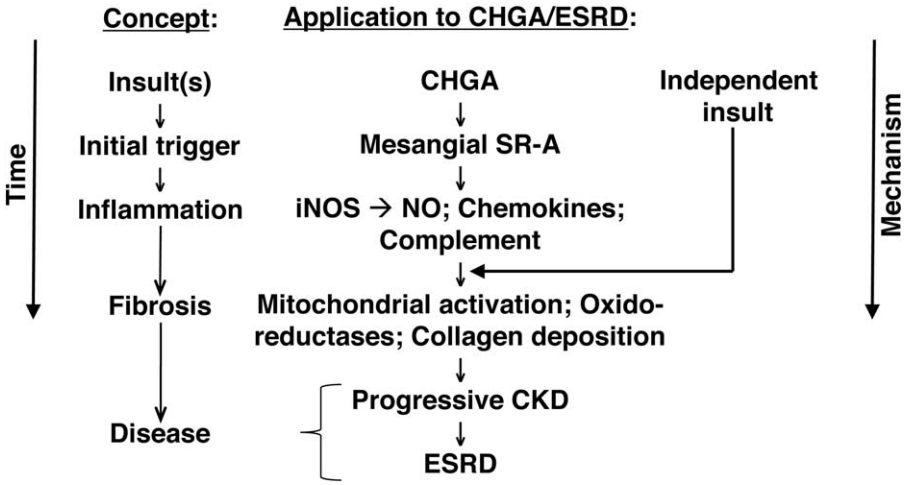

FIGURE 7 CKD/ESRD and the CHGA pathway: from pathogenic molecule to disease. CHGA, chromogranin A; CKD, hypertensive chronic kidney disease; ESRD, end-stage renal disease.

immune cells, inflammatory cytokines accumulation, apoptosis, and tissue injury. CHGA triggers mesangial cells to release NO by activating *iNOS* (*Nos2*) expression (Fig. 5). Under normal physiological condition, the kidneys generate reactive oxygen species (ROS), such as NO in amounts that are beneficial to maintain electrolyte homeostasis and extracellular fluid volume [41]. However, inflammatory conditions promote the induction of *iNOS* expression and the generation of much larger amounts of NO, which participates in modulating aspects of inflammatory processes. NO now binds to ferrous heme sites and reacts with biological molecules to disrupt iron–sulfur centers and reacts with catecholamine and generates other reactive oxygen and nitrogen molecules resulting in pathophysiological processes [40].

The activation of Toll-like receptor 4 (TLR4 or human ortholog SR-A) plays a fundamental role in pathogen recognition and activation of innate immunity [42,43]. Anders proposed that some inflammation also accompanies nonpathogen injury to the kidney because damaged renal tissues activate innate immunity by releasing immune-stimulatory 'danger signaling' molecules. The resulting nonpathogen signaling turns maladaptive causing renal tissue scarring. This happens because intrarenal inflammation is unable to attenuate the metabolic, hemodynamic, toxic, or autoimmune drivers of kidney injury [44]. Our findings (Fig. 5) suggest CHGA might be such a signaling molecule activating TLR 4/SR-A, which is known to bind to endotoxin LPS. LPS similarly via TLR triggers an inflammatory response of chemokines and cytokines [45]. TLRs on or in kidney cells transduce the recognition of such danger signals into the secretion of cytokines, chemokines, acute phase inflammatory makers and apoptosis genes as is observed in CRL-1927 cells treated with CHGA. TLR4 has an important role in renal interstitial fibrosis and CKD progression [46].

In conclusion, our results are consistent with the model (Fig. 7) depicting the chain of events from kidney injury to the development of CKD. Data from mice support observations in humans that after loss of kidney function, build-up of plasma CHGA activates via SR-A mesangial cells, mounting an oxidative stress response potentiating inflammation and fibrosis eventuating in CKD phenotype. These findings should provide new inroads into therapeutic approaches for a currently intractable health disparity.

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Conflicts of interest

There are no conflict of interest.

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