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RESEARCH PAPER

Inactivation of Cys^{674} in SERCA2 increases BP by inducing endoplasmic reticulum stress and soluble epoxide hydrolase

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Background and Purpose: The kidney is essential in regulating sodium homeostasis and BP. The irreversible oxidation of Cys^{674} (C674) in the sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) is increased in the renal cortex of hypertensive mice. Whether inactivation of C674 promotes hypertension is unclear. Here we have investigated the effects on BP of the inactivation of C674, and its role in the kidney.

Experimental Approach: We used heterozygous SERCA2 C674S knock-in (SKI) mice, where half of C674 was substituted by serine, to represent partial irreversible oxidation of C674. The BP, urine volume, and urine composition of SKI mice and their littermate wild-type (WT) mice were measured. The kidneys were collected for cell culture, Na^+/K^+-ATPase activity, protein expression, and immunohistological analysis.

Key Results: Compared with WT mice, SKI mice had higher BP, lower urine volume and sodium excretion, up-regulated endoplasmic reticulum (ER) stress markers and soluble epoxide hydrolase (sEH), and down-regulated dopamine D_1 receptors in renal cortex and cells from renal proximal tubule. ER stress and sEH were mutually regulated, and both upstream of D_1 receptors. Inhibition of ER stress or sEH up-regulated expression of D_1 receptors, decreased the activity of Na^+/K^+-ATPase, increased sodium excretion, and lowered BP in SKI mice.

Conclusions and Implications: The inactivation of SERCA2 C674 promotes the development of hypertension by inducing ER stress and sEH. Our study highlights the importance of C674 redox status in BP control and the contribution of SERCA2 to sodium homeostasis and BP in the kidney.

Abbreviations: 4-PBA, 4-phenylbutyric acid; DHE, dihydroethidium; ER, endoplasmic reticulum; RPT, renal proximal tubule; sEH, soluble epoxide hydrolase; SERCA2, sarcoplasmic/endoplasmic reticulum calcium ATPase 2; SKI, SERCA2 C674S knock-in; TPPU, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea; WT, wild-type.

1 | INTRODUCTION

Hypertension affects about 1 billion adults worldwide and is an important risk factor for coronary heart disease, stroke, and chronic kidney disease (Arnett & Claas, 2018; Ezzati & Riboli, 2012). The increasing incidence of hypertension requires the development of more effective strategies to prevent and treat hypertension. Despite extensive research in this field, the detailed mechanisms involved have not been fully understood. The nervous system, the vascular system and the kidney are all involved in long-term regulation of BP, in which the kidney plays a key role by regulating sodium excretion. Renal sodium retention expands extracellular fluid volume and increases BP, which in return is offset by pressure-induced natriuresis under physiological conditions. If the natriuretic response to the elevated BP is insufficient to lower BP to normal, hypertension occurs.

Altered calcium handling occurs in essential hypertension and calcium channel blockers are one of the first-line treatment options for hypertension (Maranta, Spoladore, & Fragasso, 2017). The **sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA)**, encoded by ATP2A1–3 genes in a species- and tissue-specific manner, is a key enzyme to maintain calcium homeostasis by taking up calcium from intracellular space to sarcoplasmic reticulum and endoplasmic reticulum (ER). The use of the SERCA inhibitor, **thapsigargin**, confirms the participation of SERCA in BP control by inducing vasodilation and neurohumoral activation (Schneider et al., 2015; Zhang & Stern, 2017). However, the contribution of renal SERCA to the control of BP has not been assessed.

The *S*-glutathiolation of the amino acid residue Cys^{674} (C674) in SERCA is key to increase the activity of SERCA2 under physiological conditions, but this post-translational protein modification is prevented by the irreversible oxidation of C674 thiol in pathological situations characterised by high levels of ROS, such as aging, diabetes mellitus and atherosclerosis (Qin et al., 2013; Tang et al., 2010; Tong, Hou, Jourd'heuil, Weisbrod, & Cohen, 2010; Ying et al., 2008), which are all risk factors for hypertension. Similar to other risk factors of hypertension, the irreversible oxidation of C674 in SERCA2 that causes its inactivation of *S*-glutathiolation was increased in hypertensive mice induced by a high fat, high sucrose diet (Qin et al., 2014; Weisbrod et al., 2013). We hypothesize that the chronic inactivation of SERCA2 C674 by its irreversible oxidation, contributes to the later development of hypertension. This study was designed to elucidate whether inactivation of the redox site C674 of SERCA2 in pathological situations affects BP and whether the SERCA2 in the kidney is involved.

What is already known

- The effects of inhibiting SERCA demonstrate that SERCA controls BP via vasodilation and neurohumoral activation.

What this study adds

- The redox state of Cys^{674} in SERCA2 is essential for BP control.
- Renal SERCA2 participates in BP control by regulating sodium homeostasis.

What is the clinical significance

- Increased levels of ROS irreversibly oxidize Cys^{674} in SERCA2 and induce the development of hypertension.
- Inhibiting soluble epoxide hydrolase or ER stress contributes to controlling hypertension by regulating sodium homeostasis.

2 | METHODS

2.1 | Animals

All animal care and study protocols complied with the guidelines of the ethical use of animals and were approved by the Animal Care and Use Committee (Third Military Medical University, Chongqing, China). All efforts were made to minimize animal suffering and reduce the number of animals used. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath & Lilley, 2015) and with the recommendations made by the *British Journal of Pharmacology*. Experimental protocols and design adheres to *BJP* guidelines (Curtis et al., 2015).

All mice used in this study were of C57BL/6J background (RRID: IMSR_JAX:000664, https://www.jax.org/strain/000664) and were originally obtained from The Jackson Laboratory (Bar Harbor, USA). Mice were bred and housed in the laboratory animal room at Chongqing University under specific pathogen-free conditions. Mice were kept in open polypropylene cages with clean chip bedding. The animal room was maintained at a controlled temperature (22 ± 3°C) and a 12-hr cycle of light and dark. Five mice in each cage were free to drink water and were fed on a regular diet (0.3–0.8% sodium, 1.0–1.8% calcium, 0.6–1.2% phosphorus, Beijing Keao Xieli Feed Corporation, China) unless otherwise indicated. To obtain tissues, all mice were killed by i.p. injection with 2,2,2-tribromoethanol (250 $mg \cdot kg^{-1}$, Sigma-Aldrich, Cat# T48402).

2.2 | Angiotensin II-induced hypertension

Angiotensin II was used to induce hypertension and oxidative stress in LDL receptor-deficient mice (The Jackson Laboratory, RRID: IMSR_JAX:002207, https://www.jax.org/strain/002207) originally designed for modelling aortic aneurysms. Male mice at 8 weeks old (18–22 g) were fed on a Western diet (21% w/w milk fat, 0.15% w/w cholesterol, Beijing Keao Xieli Feed Corporation) for 1 week and then were randomized to receive subcutaneous implantation of ALZET

osmotic pumps (Model 2004, DURECT Corporation, USA), delivering angiotensin II (1.44 $mg \cdot kg^{-1} \cdot day^{-1}$, dissolved in PBS) or PBS for 4 weeks (n = 5 per group). Before implantation, mice were given buprenorphine (0.08 $mg \cdot kg^{-1}$, subcutaneous) as analgesic, and during pump implantation, they were anaesthetized with isoflurane (1.5%) inhalation. The mouse back was depilated with depilatory ointment. After disinfection with iodophor, the pump was implanted subcutaneously into the back of the mouse. The incision was sealed with sterile sutures and disinfected with iodophor. Buprenorphine (0.08 $mg \cdot kg^{-1}$, s.c., every 8 hr) was given to the mouse the first day after surgery. The mice were monitored every day in the first week after operation. Mice were killed by i.p. injection with 2,2,2-tribromoethanol (250 $mg \cdot kg^{-1}$) and perfused with ice-cold PBS, and then the kidneys were embedded in optimum cutting temperature formulation for detecting ROS and SERCA2 C674 irreversible oxidation.

2.3 | BP measured by tail-cuff plethysmography

BP was measured in conscious mice using tail-cuff plethysmography (Softron Biotechnology, China) between 9 a.m. and 11 a.m. Each mouse was warmed at 37°C for 10 min to detect tail artery pulsations and to acquire pulse levels and then was put in a plastic restrainer with its tail wrapped in a cuff containing a pneumatic pulse sensor. To minimize stress-induced fluctuations on BP, all mice were acclimated to the procedures for 1 week, before actual data collection. The arterial BP was measured at least five times, and systolic BP, diastolic BP, and mean BP were recorded.

2.4 | Measurement of ROS generation

Dihydroethidium (DHE, APEXBIO, Cat# C3807) was used to detect intracellular oxidants, mainly superoxide. In brief, fresh frozen sections of renal cortex from angiotensin II or PBS-infused mice were incubated with 15-μM DHE at 37°C for 40 min and then washed with PBS to remove free DHE. Fluorescence at λ605 nm was recorded by fluorescence microscopy (Leica DM6, Germany), and the average DHE fluorescence was calculated. To avoid different fluorescence background, the average value of the PBS group was used to normalize the data in each measurement.

2.5 | Histology and immunohistochemistry

Kidneys were dissected and fixed in 10% formalin for 2 days, followed by 30% sucrose solution for 1 day before embedding in optimum cutting temperature formulation to prepare serial frozen sections. Masson's trichrome staining was used for morphological analysis. The antibody-based procedures used in this study comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). For immunohistochemistry, sections were heated for antigen retrieval in 10-mM citrate acid buffer before being blocked in goat serum. Primary antibodies against SERCA2 C674-SO_3H (Ying et al., 2008; C674-SO_3H antibody was a custom polyclonal antibody from Bethyl Laboratories, Inc.), **soluble epoxide hydrolase** (**sEH**; Santa Cruz, Cat# sc-87099, RRID:AB_2293440), or **dopamine D_1 receptors** (Abcam, Cat# ab81296, RRID:AB_2814742) were incubated overnight at 4°C according to the staining procedure of HRP-DAB kit (CWBIO, Cat# CW0125M). IgG acted as a negative control. The positive areas (brown) of cortex tubules were scored by four individual observers blinded to the experimental groups using a scale of 0 to 4, where 0 equals *negative intensity*, 1 *weak intensity*, 2–3 *medium intensity*, and 4 *strong intensity*.

2.6 | SERCA2 C674S knock-in mouse construct

The construct of the SERCA2 C674S knock-in (SKI) mouse (RRID: MGI:5780876) was generated as previously described (Thompson et al., 2014). Briefly, the genetic variation is TGT at C674 to TCC (Ser^{674} [S674]) in exon 14 of SERCA2. The presence of the C674S mutation was verified by sequencing of both genomic DNA and cDNA from heart. Homozygous SKI mice died before birth, so only heterozygous SKI mice that express 50% of C674 and 50% of S674 were used in this study, and their littermate wild-type (WT) mice without S674 were used as controls.

2.7 | Mice treated with inhibitors for sEH or ER stress

Both male WT and SKI mice at 12–16 weeks old (25–30 g) were randomly divided into PEG400 solvent control group or treatment group (n = 5 per group). In treatment group, mice were given either a sEH inhibitor, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU, 0.3 $mg \cdot kg^{-1} \cdot day^{-1}$; Zhou et al., 2017), or ER stress inhibitor, 4-phenylbutyrate (4-PBA, 320 $mg \cdot kg^{-1} \cdot day^{-1}$) individually for 1 week via oral gavage. Both TPPU and 4-PBA were dissolved in PEG400. The BP in conscious WT and SKI mice was measured by tail-cuff plethysmography. Urine was collected in a metabolic cage, and 24-hr urinary sodium excretion and other urinary compounds were measured by the AU5800 clinical chemistry analyser (Beckman Coulter, USA).

2.8 | Primary renal proximal tubule cell culture

Primary renal proximal tubule (RPT) cells from 4- to 8-week-old male WT or SKI mice were isolated with a modified method (Terryn et al., 2007). Briefly, the sliced renal cortex was digested in collagenase II (Worthington Biochemical Corporation, Cat# LS004176) solution at 37°C for 30 min before being ground on the first nylon sieve (100 mesh) and the second nylon sieve (200 mesh). The solution collected by flushing the second sieve in the reverse direction was centrifuged for 10 min at 1,000 x g, and the pellet was suspended in

DMEM/F12 medium (Hyclone, Cat# SH30023.01) and cultured at 37°C in a humidified atmosphere containing 5% CO_2, supplemented with 10% FBS (ExCell Bio, Cat# FSP500), 10 μg·L^{-1} **EGF** (Novoprotein, Cat# CH28), 5 mg·L^{-1} **insulin** (Solarbio, Cat# I8830), 5 mg·L^{-1} transferrin (Solarbio, Cat# T8010), 4 mg·L^{-1} **dexamethasone** (Solarbio, Cat# D8040), and 1% penicillin–streptomycin. The medium was replaced every 2 days, and confluent monolayer was formed 7 days later. Cells from passages 0 to 1 were used.

2.9 | Na^+/K^+-ATPase activity assay

Na^+/K^+-ATPase activity of primary RPT cell membranes was assessed by using Na^+/K^+-ATPase analysis kit (Nanjing Jiancheng Bioengineering Institute, Cat# A070-2) with a modified method (Chen et al., 2016) using malachite green dye to measure inorganic phosphate (Pi) released from ATP. Na^+/K^+-ATPase activity was corrected for protein concentration and expressed as μmol phosphate released per mg protein per min.

2.10 | Real-time quantitative PCR

Total RNA was isolated from the renal cortex using TRIzol reagent (Invitrogen, Cat# 15596026) and retro-transcribed to cDNA using cDNA PCR kit (Takara Bio Inc., Cat# RR037Q). Real-time quantitative PCR was performed using SYBR-Green-based detection (Takara Bio Inc., Cat# RR420L) with primers commercially synthesized (Sangon Biotech, China) and listed in Table S1. The following cycling conditions were used: denaturation, annealing, and extension at 95°C, 57°C, and 72°C for 10, 30, and 10 s, respectively, for 40 cycles. β-actin was used as internal control. Relative expression of mRNA was analysed using the comparative Ct method ($2^{-\Delta\Delta Ct}$), and the relative copy number of SERCA was analysed using a $2^{-\Delta Ct}$ method.

2.11 | Western blot

The renal cortex or RPT cells cultured in 0.2% FBS DMEM/F12 overnight were lysed in RIPA buffer (Enogene, Cat# E1WP106). Experimental details of Western blots are in accordance with *BJP* guidelines (Alexander et al., 2018). Proteins were separated by SDS-PAGE electrophoresis using standard methods, transferred to PVDF membrane, and immunoblotted with specific antibodies overnight at 4°C against SERCA2 (C498 antibody was a custom polyclonal antibody from Bethyl Laboratories, Inc.), p-PERK (Thr981, Santa Cruz, Cat# sc-32577, RRID:AB_2293243), BIP (EnoGene, Cat# E90019, RRID:AB_2814761), ATF6 (EnoGene, Cat# E90009A, RRID:AB_2814762), CHOP (EnoGene, Cat# E90221, RRID:AB_2814763), sEH (Abcam, Cat# ab155280, RRID:AB_2814743), D1 receptors (Abcam, Cat# ab81296, RRID:AB_2814742), β-actin (EnoGene, Cat# E12-051-3, RRID:AB_2814764), GAPDH (EnoGene, Cat# E1C604-1, RRID:AB_2814765), followed by incubation with HRP-conjugated goat-anti-rabbit secondary antibody (Sino Biological Inc., Cat# SSA003, RRID:AB_2814815) 1 hr at room temperature. Proteins were visualized with an X-ray film system (Fujifilm, Japan) or ChemiDoc™ Touch System (Bio-Rad, USA). Band density was quantified by NIH ImageJ software (RRID:SCR_003070, https://imagej.net/) and normalized to GAPDH or β-actin.

2.12 | Intracellular calcium measurements

Primary RPT cells were subcultured onto cover slides overnight in a 24-well plate. According to the manufacturer's procedure, the cells were loaded with 4-μM Fluo-4 AM (Solarbio, Cat# F8500) at 37°C for 20 min, then were retained for 40 min after adding fivefold volume of HBSS containing 1% FBS, and were washed three times with HEPES buffer (10-mM HEPES, 1-mM Na_2HPO_4, 137-mM NaCl, 5-mM KCl, 1-mM $CaCl_2$, 0.5-mM $MgCl_2$, 5-mM glucose, 0.1% BSA; pH 7.4). The fluorescence signals with excitation λ494 nm and emission λ516 nm were recorded by fluorescence microscopy (Leica DM6), and the average fluorescence signals were analysed by Las X software (Leica, Germany).

2.13 | Immunofluorescence staining

Primary RPT cells were incubated with specific antibodies for sEH, D_1 receptors, or cytokeratin 18 (Bioss Antibodies, Cat# BSM-33103M, RRID:AB_2814819) for 12 hr, followed by Alexa Fluor 488 goat anti-rabbit IgG (H + L; Jackson ImmunoResearch, Cat# 111-545-144, RRID:AB_2338052) or Cy3-conjugated AffiniPure goat anti-mouse IgG (H + L; Proteintech, Cat# SA00009-1, RRID:AB_2814746) for 2 hr. Nuclei were stained with DAPI (Solarbio, Cat# C0065) for 10 min. The fluorescence signals were monitored by microscopy (Leica DM6) and analysed by Las X software (Leica).

2.14 | Primary RPT cells treated with TPPU or 4-PBA

Primary RPT cells isolated from 4- to 8-week-old male WT or SKI mice were cultured in DMEM/F12 medium and treated with 1-μM TPPU or 0.5-M 4-PBA for 48 hr, before being collected for measurement of Na^+/K^+-ATPase activity and protein expression. PEG400 served as a solvent control.

2.15 | Adenoviral infection in rat RPT cells

The rat RPT cells were infected with adenovirus sEH or empty adenovirus at 50 MOI per cell in DMEM/F12 respectively without serum and antibiotics for 3 hr before adding DMEM/F12 containing 2% FBS for 2 days.

2.16 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). All studies were designed to generate groups of equal size, using randomisation and blinded analysis. Group size is the number of independent values, and statistical analysis was carried out using these independent values. Sample sizes in each groups subjected to statistical analysis were determined based on our previous studies, preliminary results, and power analysis (Curtis et al., 2015). Statistical analysis was undertaken only using these independent values with $n \geq 5$. Fold change over control was used in Western blot and fluorescent image analysis to avoid the larger variation among different experiments. The mean values of the control group were normalized to 1. In the figures, the *Y* axis shows the ratio of the experimental group to that of the corresponding matched control values and is labelled as "fold matched control values." All results were expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 7.00 (RRID:SCR_002798, http://www.graphpad.com/). Unpaired Student's *t* test was used to analyse data from two groups. When *F* achieved statistical significance and there was no significant variance heterogeneity, two-way ANOVA with Bonferroni post hoc analysis was used to analyse the differences among multiple groups. For determining whether groups differ, the level of probability was set at $P < .05$ to constitute the threshold for statistical significance. No data were excluded from any study.

2.17 | Materials

The following compounds were supplied by Sigma-Aldrich - 4-PBA, Cat# SML0309; angiotensin II, Cat# A9525; buprenorphine, Cat#BP1062. The sHE inhibitor TPPU was provided by Dr. Lee and Dr. Hammock (University of California-Davis, CA)

2.18 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (RRID:SCR_013077) (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos, et al., 2019; Alexander, Fabbro, et al., 2019).

3 | RESULTS

3.1 | Inactivation of SERCA2 C674 increases BP and decreases urine output and sodium excretion

The irreversible oxidation of C674 in SERCA2 was increased in the aorta of hypertensive mice induced by high fat, high sucrose diet (Qin et al., 2014; Weisbrod et al., 2013). As shown in Figure S1A,B, in the renal cortex of angiotensin II-induced hypertensive mice (SBP in mmHg, PBS 99.6 ± 2.8 vs. angiotensin II 133.6 ± 3.3, $n = 5$, $P < .05$), we observed increased superoxide production and increased irreversible oxidation of C674, as detected by the specific antibody targeting SERCA2 C674-SO_3H that was previously fully validated (Qin et al., 2013; Tang et al., 2010; Tong et al., 2010; Ying et al., 2008). To address if the C674 of SERCA2 is key to control BP and whether its irreversible oxidation under hypertension-prone conditions drives the

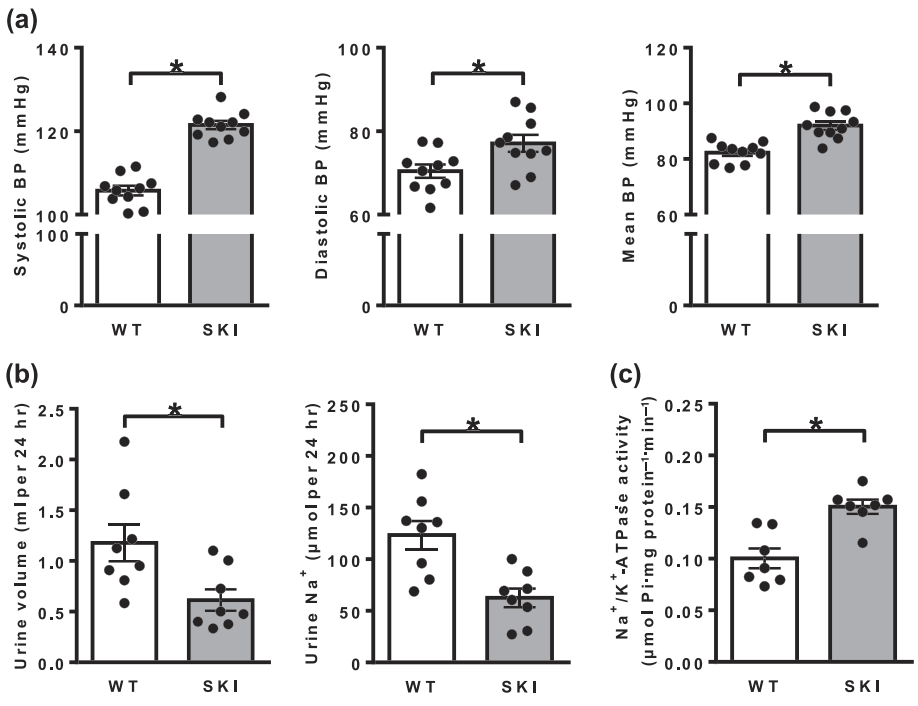

FIGURE 1 Inactivation of SERCA2 C674 increases BP and decreases urine output and sodium excretion. (a) BP. $^*P < .05$, significantly different as indicated, $n = 10$. (b) 24-hr urine volume and urine sodium excretion, $^*P < .05$, significantly different as indicated, $n = 8$. (c) Na^+/K^+-ATPase activity in primary RPT cells. $^*P < .05$, significantly different as indicated, $n = 7$. All data are presented as means ± SEM, unpaired Student's *t* test

direction of hypertension, we used heterozygous SKI mice, where half of the C674 was substituted by S674 to represent the partial irreversible oxidation of C674. In the heterozygous SKI mice, body weight was not different from that in the WT mice (WT 27.2 ± 0.67 g vs. SKI 27.5 ± 0.53 g, n = 20), but BP was increased, as shown in Figure 1a. SKI mice also had decreased daily urine volume and daily urine sodium excretion, compared with WT mice (Figure 1b). The concentrations of urinary sodium and other components were not different between WT mice and SKI mice (Table S2), indicating that the decreased urinary sodium excretion was due to the decrease of urine volume. Overall, there was no difference in renal histological structure between WT mice and SKI mice (Figure S2). The renal proximal tubule in the renal cortex accounts for 60–80% reabsorption of filtered sodium (Burnier, Bochud, & Maillard, 2006), in which the Na^+/K^+-ATPase is the most important pump to transport sodium from proximal tubule to extracellular space to reduce urinary sodium excretion (Harris & Zhang, 2012) and is responsible for sodium homeostasis. Activation of Na^+/K^+-ATPase in the proximal tubule induces the retention of water and sodium (Te Riet, van Esch, Roks, van den Meiracker, & Danser, 2015), so we measured the activity of Na^+/K^+-ATPase in the cultures of primary RPT cells. As shown in Figure 1c, the activity of Na^+/K^+-ATPase was higher in SKI RPT cells than in WT RPT cells. These results suggest that an enhancement of water and sodium retention may account for the elevated BP in SKI mice.

3.2 | Inactivation of SERCA2 C674 increases intracellular calcium level in RPT cells

C674 inactivation could occur in both SERCA2a and SERCA2b, two major isoforms of SERCA2. There was no difference in the mRNA abundance of both SERCA2a and SERCA2b in renal cortex between WT mice and SKI mice, although the relative copy number of SERCA2b was about 100 times higher than that of SERCA2a (Figure 2a), consistent with previous report that SERCA2b as a housekeeping gene is the predominant one among all SERCA isoforms in the kidney, while other isoforms are negligible (Guo et al., 2016). The substitution of S674 for C674 did not affect the protein expression of SERCA2 in the renal cortex and primary RPT cells (Figure 2b), suggesting that the elevation of BP in SKI mice was not due to the different expression levels of SERCA2 in the renal cortex. The major function of SERCA2 is to take up intracellular calcium into sarcoplasmic reticulum and ER to maintain calcium homeostasis. Next, we used Fluo-4 to detect intracellular calcium. As shown in Figure 2c, the intracellular calcium level of SKI RPT cells was higher than that of WT RPT cells, indicating that inactivation of C674 inhibited the normal function of SERCA2 in RPT cells without affecting the expression of SERCA2.

3.3 | The sustained induction of ER stress by inactivation of SERCA2 C674 accounts for the elevated BP

We further explored the causes of water and sodium retention and elevated BP in SKI mice. SERCA, including SERCA2, is the only enzyme in the ER to take up intracellular calcium into ER to maintain ER calcium stores, which is essential for ER normal function, and sustained ER calcium depletion causes ER stress (Mei, Thompson, Cohen, & Tong, 2013). ER stress is an important contributor to hypertension (Mohammed-Ali et al., 2017; Wang et al., 2017; Yum et al., 2017). We previously reported that endothelial cells from SKI mice had depleted ER calcium stores (Thompson et al., 2014), implying that inactivation of C674 could induce ER stress. In the renal cortex (Figure 3a) and primary RPT cells (Figure 3b), compared with the samples from WT mice, SKI mice had higher protein expression levels of

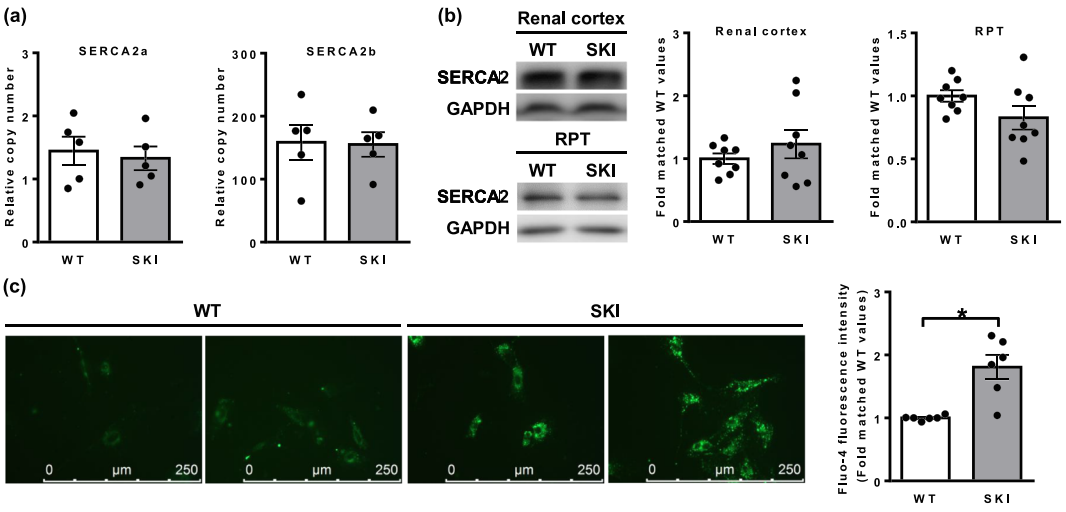

FIGURE 2 Inactivation of SERCA2 C674 increases intracellular Ca^{2+} levels in RPT cells. (a) The mRNA levels of both SERCA2a and SERCA2b in the renal cortex. n = 5. (b) SERCA2 protein levels in the renal cortex and primary RPT cells. n = 8. (c) Intracellular Ca^{2+} detected by Fluo-4 in primary RPT cells. $^*P < .05$, significantly different as indicated, n = 6. All data are presented as mean ± SEM, unpaired Student's t test

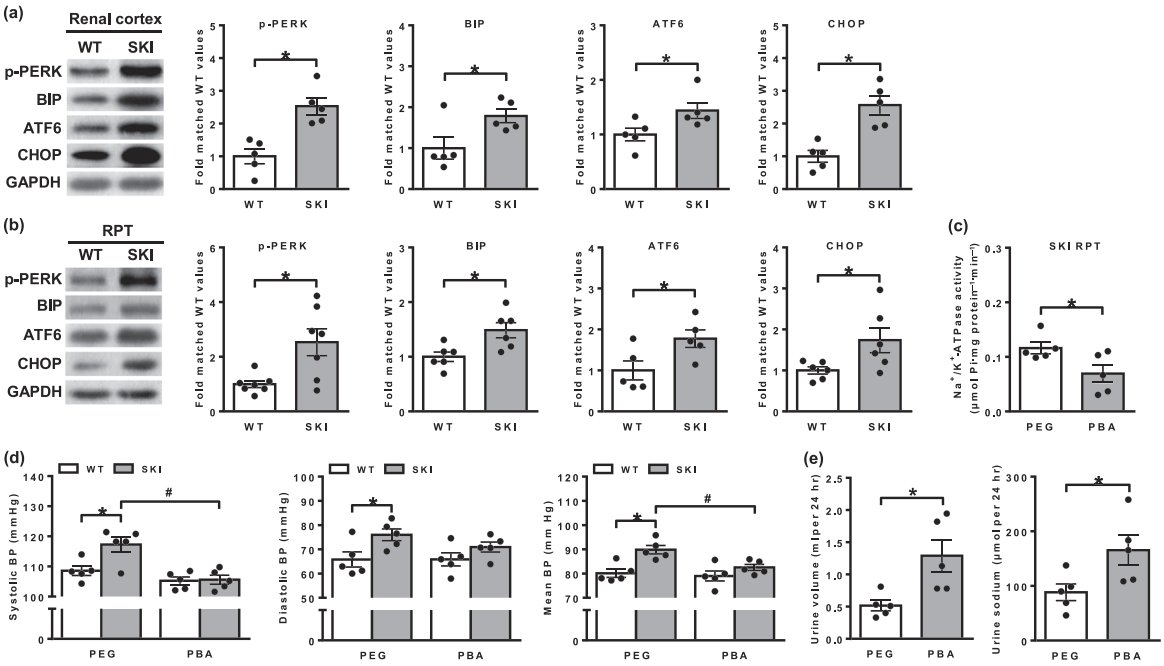

FIGURE 3 The sustained induction of ER stress by inactivation of SERCA2 C674 accounts for the elevated BP. (a) Representative Western blots of ER stress markers from the renal cortex and quantification of band intensities in graph. Mean ± SEM, unpaired Student's *t* test, $^{*}P < .05$, significantly different as indicated, $n = 5$. (b) Representative Western blots of ER stress markers from primary RPT cells and quantification of band intensities in graph. Mean ± SEM, unpaired Student's *t* test, $^{*}P < .05$, significantly different as indicated, p-PERK, $n = 7$; BIP, $n = 6$; ATF6, $n = 5$; CHOP, $n = 6$. (c) Na^+/K^+-ATPase activity in primary SKI RPT cells treated with 4-PBA. Mean ± SEM, unpaired Student's *t* test, $^{*}P < .05$, significantly different as indicated, $n = 5$. (d) The effects of 4-PBA on BP. Mean ± SEM, two-way ANOVA, $^{*}P < .05$, significantly different as indicated,; $^{\#}P < .05$, significantly different as indicated, $n = 5$. (e) The effects of 4-PBA on SKI urine volume and urine sodium secretion. Mean ± SEM, unpaired Student's *t* test, $^{*}P < .05$, significantly different as indicated, $n = 5$

ER stress markers, p-PERK, BIP, ATF6, and CHOP. To determine if the sustained induction of ER stress in SKI mice accounts for the elevated BP, we applied the ER stress inhibitor, 4-PBA. The increased activity of Na^+/K^+-ATPase in primary SKI RPT cells was reversed by 4-PBA (Figure 3c). Furthermore, treatment in vivo with 4-PBA for 1 week reversed the elevated BP in SKI mice to levels similar to those in WT mice treated with solvent control, but it did not affect BP in WT mice (Figure 3d). 4-PBA therapy also reversed the decreased daily urine volume and urinary sodium excretion in SKI mice (Figure 3e). These results indicate that the elevation of BP in SKI mice is caused by persistent ER stress, which enhances Na^+/K^+-ATPase activity in primary RPT cells to cause the retention of water and sodium.

3.4 | Inactivation of SERCA2 C674 up-regulates the expression of sEH and down-regulates the expression of D_1 receptors

In the renal cortex, a number of physiological systems control sodium excretion and BP, including the renin-angiotensin system, intrarenal dopaminergic neurons, a range of renal sodium transporters (Wang, Luo, et al., 2010; Harris & Zhang, 2012; te Riet et al., 2015), and some other important factors, such as sEH, endothelin-1, and caveolin-1. We screened genes to determine which factors contribute to the reduction of water and sodium excretion and elevated BP in SKI mice and found that both sEH and D_1 receptors were affected. Compared with WT mice, the sEH of SKI mice increased and D_1 receptors decreased at the level of mRNA (Figure S3), which were further supported at the level of protein in both renal cortex and primary RPT cells (Figure 4). sEH was mainly located in the RPT of kidney (Figure S4; Liu, 2018) and this enzyme metabolizes epoxyeicosatrienoic acids to less active dihydroxyeicosatrienoic acids (He, Wang, Zhu, & Ai, 2016), and thus inhibits sodium excretion by enhancing the activity of Na^+/K^+-ATPase in RPT (Dos Santos, Dahly-Vernon, Hoagland, & Roman, 2004; Imig, 2000; Imig, 2004). On the contrary, activation of D_1 receptors promotes sodium excretion by inhibiting Na^+/K^+-ATPase in RPT (Zeng & Jose, 2011; Harris & Zhang, 2012). Therefore, the increase of sEH and the decrease of D_1 receptors in SKI RPT cells could reduce sodium excretion by enhancing the activity of Na^+/K^+-ATPase, thus causing the retention of water and sodium, and ultimately increasing BP.

3.5 | Inhibition of ER stress reverses the expression of sEH and D_1 receptors in SKI RPT cells

Next, we examined whether persistent induction of ER stress explained the increase in sEH expression and the decrease in D_1

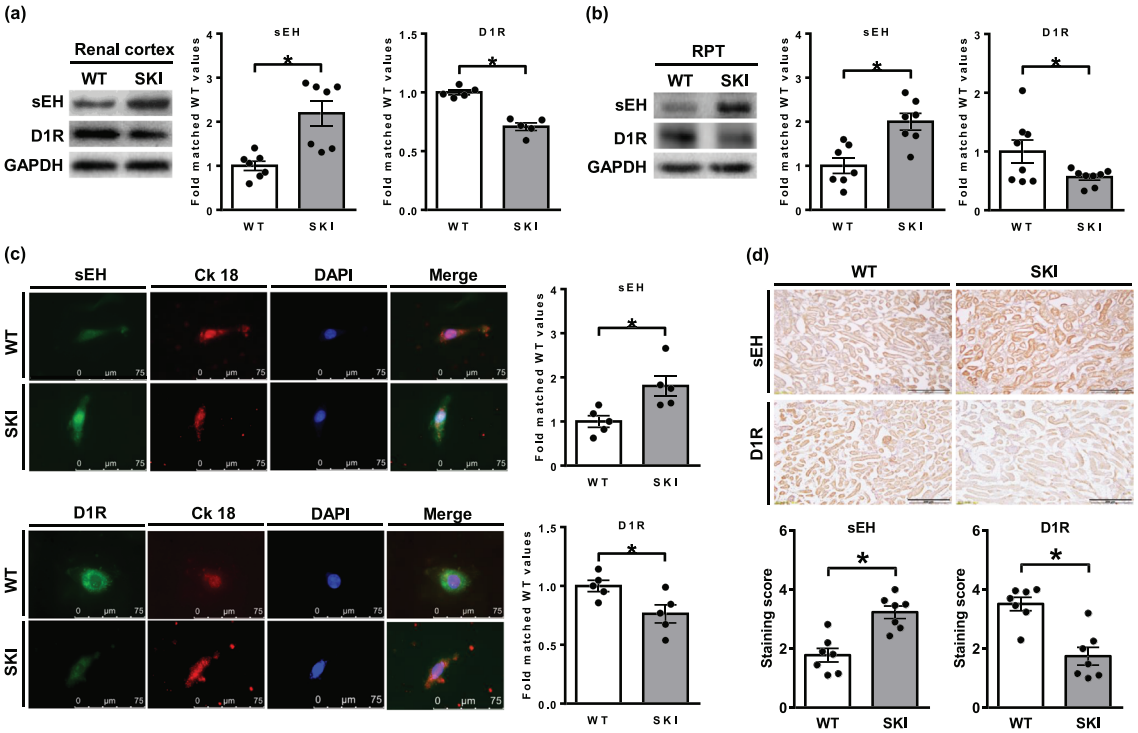

FIGURE 4 Inactivation of SERCA2 C674 up-regulates sEH expression and down-regulates expression of D_1 receptors. (a) Representative Western blots of sEH and D_1 receptors from the renal cortex and quantification of band intensities in graph. $^*P < .05$, significantly different as indicated, sEH, $n = 7$; D_1 receptors, $n = 5$. (b) Representative Western blots of sEH and D_1 receptors from primary RPT cells and quantification of band intensities in graph. $^*P < .05$, significantly different as indicated, sEH, $n = 7$; D_1 receptors, $n = 8$. (c) Representative picture of immunofluorescence of sEH and D_1 receptors in primary RPT cells. Quantification in graph, $^*P < .05$, significantly different as indicated, $n = 5$. Scale bars = 75 μm. (d) Representative picture of immunohistochemical staining of sEH and D_1 receptors in frozen sections of kidney. Quantification in graph, $^*P < .05$, significantly different as indicated, $n = 7$. Scale bars = 200 μm. All data are presented as mean ± SEM, unpaired Student's *t* test

receptor expression in SKI RPT cells. In WT RPT cells, both p-PERK and BIP were down-regulated by 4-PBA, and sEH showed a trend towards decrease, while ATF6, CHOP, and D1R were not affected by 4-PBA (Figure S5). However, 4-PBA reversed the elevation of BP in SKI mice, down-regulated ER stress markers and sEH expression, and up-regulated the reduced D_1 receptor expression in SKI RPT cells (Figure 5). These results suggest that the persistent ER stress in the renal cortex of SKI mice controls sodium secretion and BP by regulating sEH and D_1 receptors.

3.6 | sEH inhibitor suppresses ER stress and reverses the elevated BP in SKI mice

Several studies indicate that sEH could regulate ER stress (Bettaieb et al., 2017; Harris et al., 2015; Wagner, McReynolds, Schmidt, & Hammock, 2017), so we applied the sEH inhibitor TPPU to RPT cells from both WT mice and SKI mice. In WT RPT cells, TPPU itself did not affect the expression of sEH compared with solvent control, nor did it affect the expression of ER stress markers and D_1 receptors

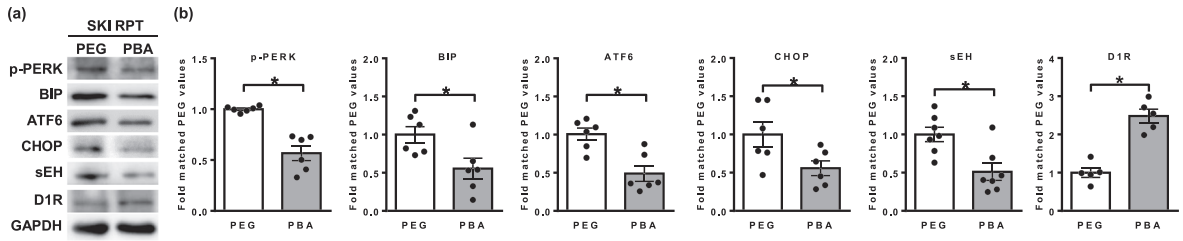

FIGURE 5 Inhibition of ER stress reverses the expression of sEH and D_1 receptors in primary SKI RPT cells. Representative Western blots from primary RPT cells treated with 4-PBA (a) and quantification of band intensities in graph (b). $^*P < .05$, significantly different as indicated, p-PERK, $n = 6$; BIP, $n = 6$; ATF6, $n = 6$; CHOP, $n = 6$; sEH, $n = 7$; D_1 receptors, $n = 5$. All data are presented as mean ± SEM, unpaired Student's *t* test

(Figure S6). Although TPPU did not affect sEH expression, it did reverse the increased ER stress markers and decreased D_1 receptor expression in SKI RPT cells (Figure 6a,b). The increased Na^+/K^+-ATPase activity in SKI RPT cells was also reversed by TPPU (Figure 6c). As observed with 4-PBA, treatment in vivo with TPPU for 1 week reversed the elevated BP and the decreased daily urine volume and urinary sodium excretion in SKI mice (Figure 6d,e). These results suggest that up-regulation of sEH is a major cause of elevated BP in SKI mice.

3.7 | sEH up-regulates ER stress markers and down-regulates expression of D_1 receptors

To investigate the roles of the up-regulated sEH in SKI mice, we overexpressed sEH in rat RPT cells. As shown in Figure 7, compared with empty adenovirus control, overexpression of sEH increased the expression of ER stress markers, while decreasing the expression of D_1 receptors. This indicates that while ER stress and sEH might regulate each other, but both are upstream of D_1 receptors.

4 | DISCUSSION

The role of SERCA and its incorrect regulation in the disease environment have been well described in a recent review (Chemaly, Troncone, & Lebeche, 2018). The participation of SERCA in BP control has been confirmed by using thapsigargin, a non-specific inhibitor of SERCA isoforms (Schneider et al., 2015; Zhang & Stern, 2017). The role of different SERCA isoforms in BP control is far from clear. SERCA2 encoded by the ATP2A2 gene is predicted to link to hypertension (Ohno et al., 1996). A reduced expression of SERCA2 is observed in hypertensive rodents and an A724A polymorphism in SERCA2 exon 15 links to BP level in hypertensive patients (Dupont et al., 2012; Huisamen et al., 2012; Kiec-Wilk et al., 2007), implying a potential contribution of SERCA2 to BP control. C674 in the SERCA2 is evolutionarily conserved from invertebrates to mammals, locates at the cytoplasmic side, surrounded by positively charged arginine, which makes its sulfhydryl group exist in an ionic state and is easily attacked by high levels of ROS which causes its irreversible oxidation (Adachi et al., 2004). The redox status of C674 is closely related to cardiovascular health and disease (Lancel et al., 2009; Tong et al., 2010), but its roles in the kidney are not clear. We have found significant irreversible oxidation of C674 in the renal cortex of spontaneous hypertensive rats (data not shown). We have presented here evidence that, in the renal cortex of angiotensin II-induced hypertensive mice, there was irreversible oxidation of C674, suggesting its involvement in hypertension. Using heterozygous SKI mice to represent the partial irreversible oxidation of C674 under hypertension-prone conditions, we found that inactivation of C674 did increase BP, which could be reversed by a classical ER stress inhibitor or sEH inhibitor. We would therefore suggest that, in hypertensive conditions with increased ROS, the chronic inactivation of SERCA2 C674 induces ER stress and elevates sEH activity. These changes inhibit expression of D_1 receptors and enhance the activity of Na^+/K^+-ATPase in the renal cortex, thereby reducing sodium excretion and leading to the retention of water and sodium, ultimately increasing BP.

The link between SERCA2 and ER stress has been reported earlier. The decreased expression of SERCA2 in the renal cortex is associated with induction of ER stress in diabetic mice (Guo et al., 2017). Our data show that, without affecting renal cortical SERCA2 expression, the inactivation of C674 in the SERCA2 can also lead to persistent ER stress, which is the cause of elevated BP in SKI mice. This is consistent with the report that ER stress is an important factor leading to hypertension (Young, 2017). Inhibition of ER stress decreases BP in hypertensive rodents (Mohammed-Ali et al., 2017; Wang et al., 2017;

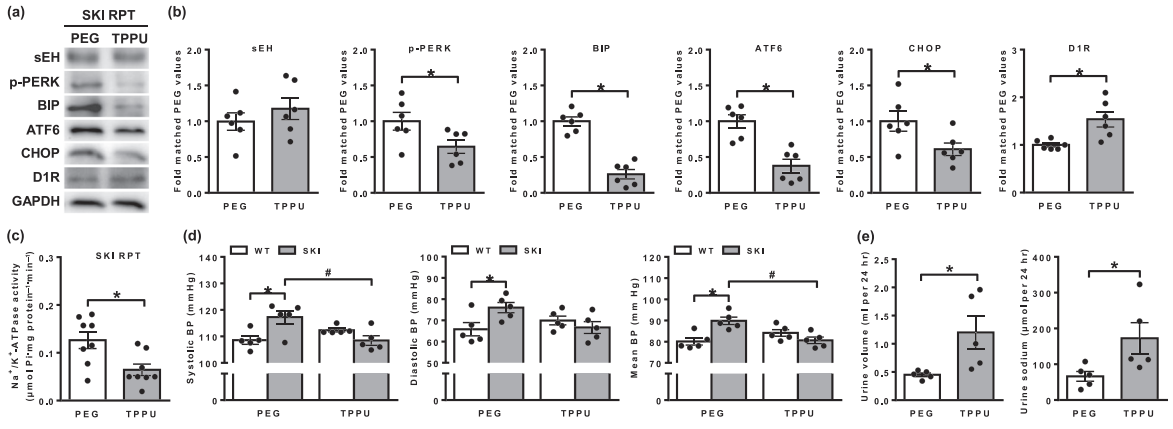

FIGURE 6 The sEH inhibitor TPPU suppresses ER stress and reverses the elevated BP in SKI mice. Representative Western blots from primary RPT cells treated with TPPU (a) and quantification of band intensities in graph (b). Mean ± SEM, unpaired Student's *t* test, $^*P < .05$, significantly different as indicated, $n = 6$. (c) Na^+/K^+-ATPase activity in primary SKI RPT cells treated with TPPU. Mean ± SEM, unpaired Student's *t* test, $^*P < .05$, significantly different as indicated, $n = 8$. (d) The effects of TPPU on BP. Mean ± SEM, two-way ANOVA, $^*P < .05$, significantly different as indicated; $^\#P < .05$, significantly different as indicated, $n = 5$. (e) The effects of TPPU on SKI urine volume and urine sodium secretion. Mean ± SEM, unpaired Student's *t* test, $^*P < .05$, significantly different as indicated, $n = 5$

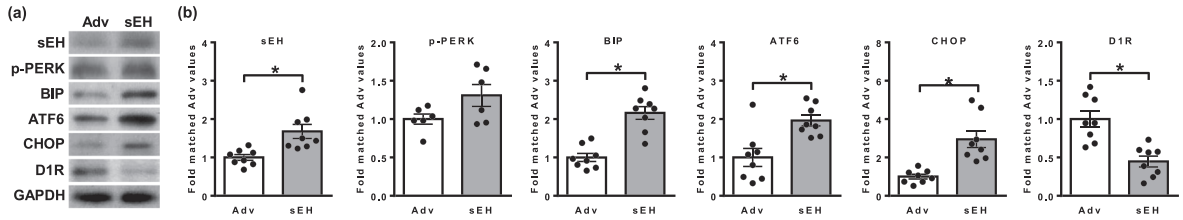

FIGURE 7 sEH up-regulates ER stress markers and down-regulates D_1 receptor expression. (a) Representative Western blots from cultured RPT cells with overexpression of sEH. (b) Quantification of band intensities in graph. $^*P < .05$, significantly different as indicated, sEH, $n = 8$; p-PERK, $n = 6$; BIP, $n = 8$; ATF6, $n = 8$; CHOP, $n = 8$; D_1 receptors, $n = 8$. All data are presented as mean ± SEM, unpaired Student's t test

Yum et al., 2017), but the underlying mechanisms are not clear. Our data provide a possible mechanism whereby persistent ER stress in the renal cortex interacts with sEH to inhibit the expression of D_1 receptors and to increase the activity of Na^+/K^+-ATPase and leads to retention of water and sodium, thereby increasing BP.

Renal sEH is up-regulated in several hypertensive models, and inhibition of sEH lowers their BP (Liu, 2018), similar to our results in SKI mice. In this present report we have not investigated how SERCA2 may regulate sEH. The ER stress marker ATF6 is a nuclear transcription factor, whose activation increases sEH expression through a putative ATF6 binding motif on the sEH promoter (Zhang et al., 2012). In addition to ATF6, sEH can be regulated by c-Jun binding to the AP-1 site of sEH promoter (Ai et al., 2007; Zhang, Ai, Tanaka, Hammock, & Zhu, 2010) or by a SP-1-dependent DNA methylation of the sEH promoter (Ai et al., 2007; Zhang et al., 2010). We show here that SERCA2 in the renal cortex could regulate sEH by controlling ER status. Persistent ER stress induced by C674 inactivation can lead to up-regulation of sEH in the renal cortex, which in return can also cause ER stress. The potential regulatory effect of renal sEH on ER stress has not been reported. TPPU alleviates ER stress of liver and hepatic fibrosis in CCl_4-treated mice (Harris et al., 2015). Podocyte-specific sEH deficiency reduces ER stress and renal fibrosis under hyperglycaemia (Bettaieb et al., 2017). Inhibition of sEH stabilizes epoxy fatty acids to reverse ER stress (Wagner et al., 2017). Similar to these findings, we confirm that sEH can positively regulate ER stress in the renal cortex. Although sEH and ER stress can regulate each other, we infer that increased ER stress precedes the up-regulation of sEH in SKI mice, based on the basic role of SERCA2 in maintaining ER function.

We find that ER stress and sEH are both upstream of effects on D_1 receptors and Na^+/K^+-ATPase. There are many studies on the regulation of Na^+/K^+-ATPase in RPT by sEH and D_1 receptors (Imig, 2000; Dos Santos et al., 2004; Imig, 2004; Zeng & Jose, 2011; Harris & Zhang, 2012), so we have not further explored their regulatory mechanisms. In the kidneys of hypertensive models, the expression and function of D_1 receptors are impaired (Lokhandwala & Hussain, 2000; Wang, Li, Jose, & Ecelbarger, 2010). The D_1 receptors include D_{1A} and D_{1B} receptors and the D_{1A} receptor gene is expressed more than that for the D_{1B} receptor in the renal proximal tubule. Both heterozygous mice and homozygous mice with D_{1A} receptor deletion exhibit decreased renal sodium transport and hypertension (Albrecht et al., 1996). However, the mechanisms by which ER stress and sEH regulate D_1 receptors are not known. In hypertensive rodents, increased ROS in RPT suppresses D_1 receptor expression, and the antioxidant Tempol restores the expression of D_1 receptors and normalizes BP (Banday, Lau, & Lokhandwala, 2008). ROS suppresses D_1 receptor expression in RPT through transcription factors, such as AP1 (Banday & Lokhandwala, 2015) and NF-κB (Banday, Fazili, & Lokhandwala, 2007). In return, D_1 receptors can suppress ROS production in RPT (Yang et al., 2015). We found that ROS levels were increased in SKI RPT cells (data not shown) and we are exploring whether ROS or other factors mediate the regulation of D_1 receptors by ER stress and sEH.

We have found significant irreversible oxidation of SERCA2 C674 in various tissues from pathological models with increased ROS (Qin et al., 2013; Tang et al., 2010; Tong et al., 2010; Ying et al., 2008), implying that C674 was inactivated extensively throughout the body, which is why we constructed whole-body SKI mice. Single replacement of C674 with S674 is lethal, emphasizing the importance of C674. We cannot rule out the contribution of other systems to the elevated BP in SKI mice, such as the nervous system and vascular system, which are both likely to be involved in the process of BP elevation in SKI mice. Based on our previous research on the vascular system (Qin et al., 2013; Tang et al., 2010; Tong et al., 2010; Ying et al., 2008), we are particularly exploring the mechanism of its involvement in BP control in SKI mice.

Inactivation of SERCA2 C674 in SKI mice induced higher BP, lower urine volume and sodium excretion, and up-regulated ER stress markers and sEH. We confirmed that ER stress and sEH were mutually regulated, and pharmacologically targeting either of them could decrease BP in SKI mice through maintaining sodium homeostasis, which suggests inhibitors of ER stress or sEH as potential diuretics in hypertension. Activation of SERCA2, especially SERCA2b, might also have beneficial effects to maintain sodium homeostasis in hypertension. CDN1163 is a non-specific SERCA activator (Cornea et al., 2013),Its effect on hypertension has not been reported. Currently, we are testing whether CDN1163 could reverse the increased BP in SKI mice. However, as the chemical structure of CDN1163 contains potentially cytotoxic groups, there is still a need to develop other chemicals to stimulate SERCA2.

In summary, our data provide direct evidence of SERCA2 in maintaining BP and highlight the importance of the redox status of C674 in SERCA2 for BP control. We have shown here that the irreversible oxidation of SERCA2 C674 not only indicates increased levels

of ROS but also directly promotes the development of hypertension by inducing ER stress and sEH. One possible mechanism is to inhibit the expression of D_1 receptors in the renal cortex and to enhance the activity of Na^+/K^+-ATPase, which leads to water and sodium retention and elevates BP. Pharmacological inhibition of sEH activity or ER stress, or activation of SERCA2, may exert therapeutic benefits in hypertension under these conditions.

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AUTHOR CONTRIBUTIONS

G.L., P.H, J.Y., C.Z., and X.T. participated in the research design, performed the data analysis and interpretation, and wrote the manuscript; G.L., F.W., X.J., Y.Q., and Z.Q. conducted the experiments; K.S.S.L. and B.H. prepared TPPU and commented on the manuscript. All authors reviewed and revised the final version of this manuscript and approved its submission.

CONFLICT OF INTEREST

The University of California holds patents on sEH inhibitor (TPPU) and cardiovascular health.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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