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Original Article

Tempol treatment normalizes membrane expression of epithelial transport proteins in the kidney of salt-loaded hypertensive diabetic db/db mice

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Abstract: Objective: Hypertension exacerbates the progression and severity of diabetic kidney disease. In this study, we addressed the hypothesis that tempol acts at multiple segments of the nephron to normalize the abundance of sodium coupled epithelial transport proteins in the luminal plasma membrane to mitigate high blood pressure in salt-loaded hypertensive diabetic db/db mice. Methods: Soluble and membrane fractions from freshly homogenized kidney cortex tissue samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and probed for specific proteins by Western blotting. Western blotting for specific urinary extracellular vesicle (uEV) markers and nanoparticle tracking analysis was performed to characterize each uEV preparation from each group. A one-way ANOVA was performed to determine statistical significance between three groups (hypertensive diabetic db/db mice treated with vehicle, hypertensive diabetic db/db mice treated with tempol, and wild-type mice). Results: Tempol treatment reduced systolic blood pressure in hypertensive diabetic db/db mice compared to db/db mice that received vehicle. We observed attenuated membrane protein expression of the sodium hydrogen exchanger 3 (NHE3), sodium potassium chloride co-transporter (NKCC2), sodium chloride cotransporter (NCC), and epithelial sodium channel (ENaC) in the kidney of salt-loaded hypertensive diabetic db/db mice infused with tempol by osmotic minipump for 5 days compared to hypertensive diabetic db/db mice infused with vehicle. Also, the infusion of tempol in hypertensive diabetic db/db mice reduced the augmented protein expression of protein kinase c (PKC) epsilon observed in the vehicle treated hypertensive diabetic db/db kidney when compared to the healthy wild-type kidney. The amount of uEV and their size profiles were comparable between the three groups. Conclusions: This study demonstrates that tempol down-regulates epithelial transport mechanisms in each segment of the nephron and normalizes salt-induced high blood pressure in diabetic animals presumably in a PKC dependent manner.

Keywords: Tempol, diabetes, hypertension, epithelial transport mechanism, sodium

Introduction

Several studies have shown that tempol has beneficial effects to mitigate outcomes associated with kidney injury. Tempol administration was demonstrated to have a protective effect against ischemic injury in an animal model of renal ischemia/reperfusion (I/R) injury [1]. In that study tempol administration was shown to reduce tubule dilation, swelling and necrosis after renal I/R injury trough a phosphatidylinositol 3-kinase/protein kinase B (PKB)/nuclear factor erythroid 2-related factor 2 (PI3K/Akt/Nrf2) and caspase-3 dependent manner. In a follow-up study, Zhang et al. showed that tempol protects against acute kidney injury (AKI) by reversing the reduction of p-Akt, p-mTOR, and phosphorylated glycogen synthase kinase 3 beta (p-GSK3β) expression and reversing the reduction of afferent arteriolar contraction seen with renal I/R injury [2].

Oxidative stress is known to play an important role in the pathophysiology of diabetic nephropathy (DN), the leading cause of end-stage kidney disease. Multiple studies have investigated the beneficial effects of tempol in mitigating the progression of DN. Ranjbar et al. showed that tempol ameliorates diabetic kidney disease in adult male rats injected with streptozotocin [3].

In another study by Peixoto et al., tempol was shown to reduce albuminuria associated with the reduction of podocyte apoptosis and decrease oxidative stress in a poly (ADP-ribose) polymerase 1 (PARP1) dependent manner [4]. A study by De Blasio et al. showed that tempol attenuates diabetes induced upregulation of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase isoforms, reduces diabetes-induced glomerular injury, and blunts tubulo-interstitial fibrosis and pro-inflammatory cytokine expression in male Sprague-Dawley rats injected with streptozotocin [5]. Wu et al. showed that tempol reduces high glucose induced lipid accumulation in cultured mouse podocytes [6]. A study by Luan et al. suggested that tempol protects kidneys by reducing the expression of transforming growth factor β1, and type IV collagen [7]. Rodriguez et al. showed that chronic tempol treatment reduces renal hemodynamic effects induced by a heme oxygenase inhibitor [8].

Multiple studies suggested that tempol is effective in mitigating high blood pressure. Tempol was previously shown to attenuate high blood pressure and reduce protein expression of the renal myristoylated alanine-rich C-kinase substrate (MARCKS) and epithelial sodium channel (ENaC) alpha subunit which were augmented after salt-loading. Data from that study suggested that the mechanism may involve changes in the profiles of lipids enriched in extracellular vesicles (EVs) released from renal epithelial cells into the urine of salt-loaded hypertensive 129SV mice [9]. In a different study, intrarenal medullary interstitial infusion of tempol was shown to prevent an angiotensin II-dependent increase in blood pressure and normalize cardiac hypertrophy [10].

In this study we hypothesized that tempol mitigates diabetes induced augmentation of epithelial transport proteins throughout the nephron and overall high blood pressure induced by salt-loading in a PKC dependent manner. The results of this study will help elucidate the mechanism by which tempol can mitigate high blood pressure secondary to diabetes.

Materials and methods

Animals

Seven-week old male and female diabetic db/db mice (BKS.Cg-Dock7m +/+ Leprdb 116/J; Stock No: 000642) were purchased from Jackson Laboratories (Bar Harbor, ME).

Ethical approval

All animal studies were performed according to a protocol approved by the University of Florida, Gainesville, FL Institutional Animal Care and Use Committee (IACUC) (Protocol Number 202011157).

Animal diet and metabolic cage study

Adult diabetic db/db mice and wild-type mice were switched from a normal salt diet (0.4% NaCl) and maintained for 10 days on a high salt gel-based diet (4% NaCl) prepared using agar, AIN-93G Diet (TD.94045.PWD) (Envigo; Madison, WI, USA), and an appropriate amount of NaCl in di$H_2$0. During this time the mice were individually housed in metabolic cages (Ancare; Bellmore, NY, USA) and maintained on a 12 hr light/12 hr dark cycle. Water was given ad libitum and urine was collected daily. Urine collections were performed between 10-11 am to reflect urine produced mostly during the active cycle of the mice.

Tempol and vehicle administration

Four adult salt-loaded hypertensive diabetic db/db mice were given tempol through osmotic mini pumps (Alzet model 2002, ALZET Osmotic 103 Pumps, Cupertina, CA, USA) according to the manufacturer's instructions. Tempol was administered at a rate of 0.5 μL/h for 5 days. The same number of salt-loaded hypertensive diabetic db/db mice were given sterile saline (Vehicle) through osmotic mini pump at the same rate and duration. Subcutaneous implantation of osmotic minipumps occurred between 2:30 and 4:30 pm under isoflurane. At the end of the infusion period, all mice were euthanized by cervical dislocation between 4:30 and 7:30 pm.

Systolic blood pressure measurements

Systolic blood pressure in male and female db/db mice and wild-type mice was measured using a mouse tail cuff IITC MRBP System (Life Science Inc., Woodland Hills, CA, USA) after salt-loading. Blood pressure measurements were taken during the active cycle of the mice between 6-8 pm.

Table 1. Sources of antibodies used in this study

Antibody	Manufacture	Catalogue Number/Reference
ENaC alpha	StressMarq	SMC-242D
NHE3	Novus Biologicals	NB110-81529
NKCC2	StressMarq	SPC-401
PKG	Cell Signaling Technologies	3248
HSP70	Cell signaling Technologies	4872
Annexin A2	Cell Signaling Technologies	8235
MARCKS	abcam	Ab72459
Furin	Cell Signaling Technologies	64709
PKC alpha	Cell Signaling Technologies	2056
PKC delta	Cell Signaling Technologies	9616
PKC epsilon	Cell Signaling Technologies	2683

PKG, protein kinase G; NHE3, sodium hydrogen exchanger 3; NKCC2, sodium potassium chloride co-transporter; NCC, sodium chloride cotransporter; ENaC, epithelial sodium channel; MARCKS, myristoylated alanine-rich C-kinase substrate; PKC, protein kinase c.

Urinary sodium measurements

Urinary sodium levels were measured using a SmartLyte Electrolyte Analyzer (Diamond Diagnostics, Holliston, MA, USA). Samples were centrifuged at 13,000 rpm for 10 min before being diluted in urine diluent (Diamond Diagnostics), and then measured.

Isolation of uEVs

A total of 13 mL of urine from each group of mice was subject to centrifugation at 1000 × *g* for 15 minutes at 4°C. Next, 12 mL of the supernatant was subject to filtration using a 0.2 µm rapid-flow Nalgene filter (Thermo Fisher Scientific). Next, 9 mL of the filtrate was aliquoted in tubes and placed in a fixed-angle Ti-70 rotor (Beckman Coulter, Inc., Brea, CA, USA) before being subject to ultracentrifugation at 52,000 rpm for 90 min at 4°C. The resulting pellet was resuspended in 200 µl of filtered 1X Phosphate-Buffered Saline (1XPBS) and stored at -80°C.

Nanoparticle tracking analysis

An NS300 machine equipped with TA 3.4 Build 3.4.4 Software (Malvern; UK) was used to determine the size and concentration of urinary EVs (prepared at a 1:1000 dilution in filtered 1XPBS). The samples were fed though the system using an automatic infusion pump and the infusion rate was 65.

Western blotting

A bicinchoninic acid (BCA) protein assay (ThermoFisher Scientific) was performed to determine the concentration of protein in each soluble and membrane fraction sample. For soluble fractions, fifty micrograms of total protein were loaded onto 4-20% gradient SDS PAGE gels (ThermoFisher Scientific). For membrane fractions, twenty micrograms of total protein were loaded onto these gels. The proteins were resolved by electrophoresis for 1 hour using a Criterion apparatus (BioRad). The proteins were transferred onto nitrocellulose membranes (ThermoFisher Scientific) using a Criterion apparatus (BioRad). The membranes were blocked in a solution of 5% milk 1X Tris-Buffered Saline (1XTBS) for 1 hour and then incubated with primary antibody (**Table 1**) prepared in a solution of 5% bovine serum albumin (BSA) 1XTBS overnight at 4°C. After a series of washes the membranes were incubated in secondary antibody solution (1:3000 dilution of goat anti-rabbit antibody (BioRad) prepared in blocking solution). The blots were incubated with enhanced chemiluminescence (ECL) reagent (BioRad) for 5 minutes and then imaged on a BioRad imager.

Data analysis and statistics

All results are presented as mean ± standard error of the mean (SEM). Statistical differences between the three groups were determined after performing a one-way ANOVA followed by a post hoc Tukey test using SigmaPlot software (Jandel Scientific, San Rafael, CA, USA). Differences with a p-value of <0.05 were considered significant.

Results

Tempol mitigates high blood pressure from salt-loading in diabetic db/db mice

Our group previously showed that diabetic db/db mice develop profound hypertension after

Table 2. Systolic blood pressure, osmolality, and urinary sodium concentrations in wild-type (WT) mice and db/db mice treated with vehicle or tempol

test	*Groups*	*Mean ± SEM*	*P*
Systolic blood pressure (mmHg)	WT[a]	106.25 ± 6.58	0.132
	db/db VEH[b]	162.75 ± 2.016	<0.001
	db/db Tempol[a]	121.50 ± 5.204	
Osmolality (mOsm/kg)	WT	1300.50 ± 51.09	0.593
	db/db VEH	1442.75 ± 98.76	
	db/db Tempol	1480.50 ± 190.64	
Urinary sodium (mmol/L/24 hours)	WT	137.75 ± 2.529	0.195
	db/db VEH	131.250 ± 4.715	
	Db/db Tempol	140.500 ± 2.398	

VEH refers to vehicle. Different letters (i.e. a and b) indicate that there was statistically significant differences in the means.

Table 3. Cathepsin levels in 24 urinary samples from wild-type (WT) mice and db/db mice treated with vehicle or tempol

test	*Groups*	*Mean ± SEM (ng/mL)*	*P*
Cataphsin *B*	WT	22918 ± 4558	0.901
	db/db Veh	24433 ± 1685	
	db/db Tempol	24594 ± 810	
Cataphsin S	WT	24046 ± 6679	0.133
	db/db VEH	34196 ± 1343	
	db/db Tempol	35833 ± 1255	

VEH refers to vehicle.

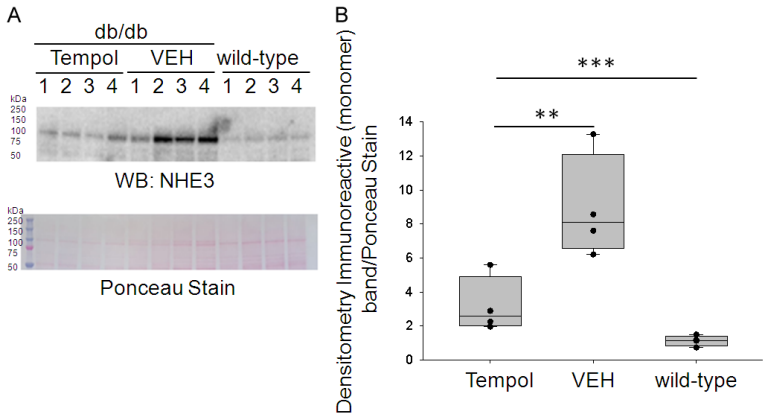

Figure 1. Western blot and densitometric analysis of sodium hydrogen exchanger 3 (NHE3) protein in kidney cortex lysates from hypertensive diabetic db/db mice treated with vehicle or tempol and from healthy wild-type mice. A. Western blot of NHE3 protein. Ponceau staining was used to assess lane loading. B. Densitometric analysis of the immunoreactive band corresponding to 100 kDa indicated by the arrow. ** represents a *p*-value <0.01. *** represents a *p*-value of <0.001. VEH represents vehicle. n=4 mice per group.

salt-loading [11-13]. After inducing hypertension in adult diabetic db/db mice by salt-loading, urinary sodium, urine osmolality, and systolic blood pressure were measured. Although an n=4 mice in each group was not enough to observe a statistically significant difference in urinary sodium or urine osmolality between the groups, tempol significantly reduced systolic blood pressure in salt-loaded hypertensive diabetic db/db mice compared to mice that received vehicle (**Table 2**).

Analysis of protease activity in wild-type mice and diabetic db/db mice treated with vehicle or tempol

Since cathepsin B was previously shown to contribute to the pathogenesis of hypertension in nephrotic syndrome [14], and cathepsins play a role in the development of diabetic nephropathy [15], we investigated whether there were differences in the basal levels of various members of the cathepsin family in the diabetic db/db kidney treated with tempol compared to vehicle. As shown in **Table 3**, there was a modest upward trend for cathepsin B and cathepsin S activities in the diabetic kidney of db/db mice treated with vehicle compared to the healthy kidney of wild-type mice.

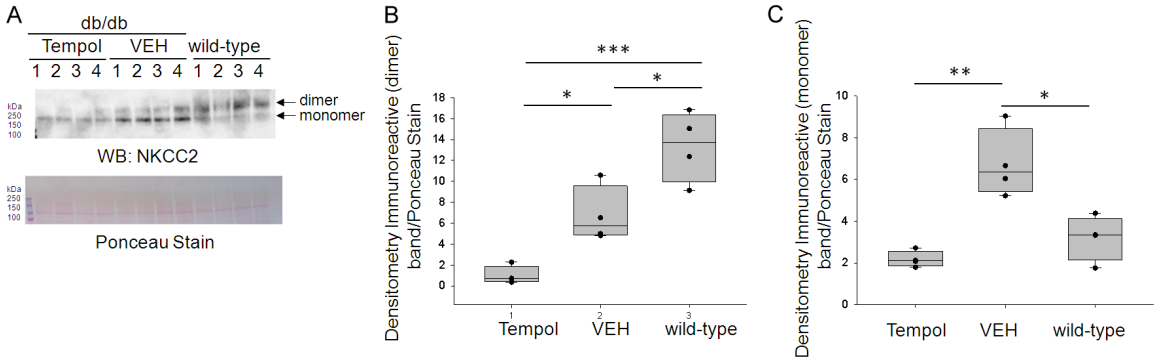

Figure 2. Western blot and densitometric analysis of sodium potassium chloride cotransporter (NKCC2) protein in kidney cortex lysates from hypertensive diabetic db/db mice treated with vehicle or tempol and from healthy wild-type mice. A. Western blot of NKCC2 protein. Ponceau staining was used to assess lane loading. B. Densitometric analysis of the immunoreactive band corresponding to the dimer of NKCC2 indicated by the arrow. C. Densitometric analysis of the immunoreactive band corresponding to the monomer of NKCC2 indicated by the arrow. * represents a *p*-value <0.05. *** represents a *p*-value of <0.001. VEH represents vehicle. n=4 mice per group.

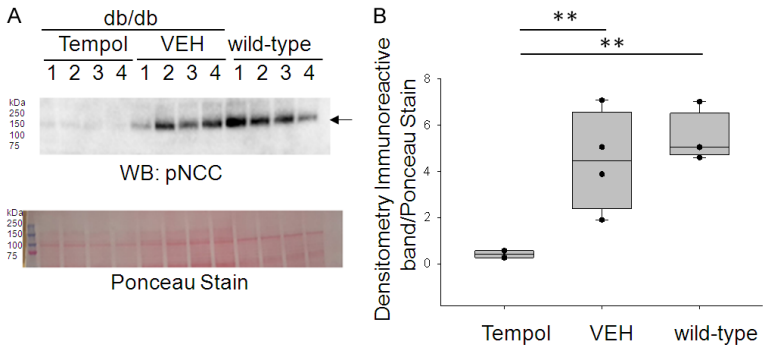

Figure 3. Western blot and densitometric analysis of the phosphorylated and active form of the sodium potassium cotransporter (pNCC) protein in kidney cortex lysates from hypertensive diabetic db/db mice treated with vehicle or tempol and from healthy wild-type mice. A. Western blot of pNCC protein. Ponceau staining was used to assess lane loading. B. Densitometric analysis of the immunoreactive band corresponding to pNCC indicated by the arrow. ** represents a *p*-value of <0.01. VEH represents vehicle. n=4 mice per group.

Tempol decreases sodium-hydrogen exchanger (NHE3) protein expression in the hypertensive diabetic db/db kidney

Although tempol was previously shown to normalize NHE3 activity after augmentation by angiotensin [16], the ability of tempol to reduce elevated membrane protein expression of NHE3 in the kidney of hypertensive diabetic db/db mice has not been investigated. Compared to membrane protein expression in the kidney of wild-type mice, NHE3 membrane protein expression was greater in the kidney of diabetic db/db mice (**Figure 1**). Importantly, tempol treatment normalized the protein expression levels of NHE3 in the hypertensive diabetic db/db kidney (**Figure 1**).

Tempol decreases sodium-potassium-chloride co-transporter (NKCC2) protein expression in the hypertensive diabetic db/db kidney

Haque and Ortiz showed that tempol blocks free radical superoxide (O^-_2) stimulated surface expression of the monomer form of the NKCC2 at 160 kDa in thick ascending limb (TAL) suspensions from kidneys of male Sprague-Dawley rats [17]. Mariappan et al. showed that increased nuclear factor kappa B (NF-κB) activity in diabetic db/db mice is associated with increased oxidative stress as demonstrated by increased reactive oxygen species (ROS) and superoxide [18]. Thus, here we investigated whether NKCC2 membrane protein expression is increased in the hypertensive diabetic db/db mouse kidney compared to the healthy wild-type kidney, and whether tempol treatment mitigates this increased expression in the hypertensive diabetic kidney. First, our Western blot and densitometric analysis showed that membrane expression of the 160 kDa monomer form of NKCC2 in the hypertensive diabetic db/db mouse kidney is augmented compared to its expression in the healthy wild-type kidney, and tempol treatment reduced the membrane protein expression of NKCC2 (**Figure 2**). Additionally, these studies showed that the dimer form of NKCC2 in membrane

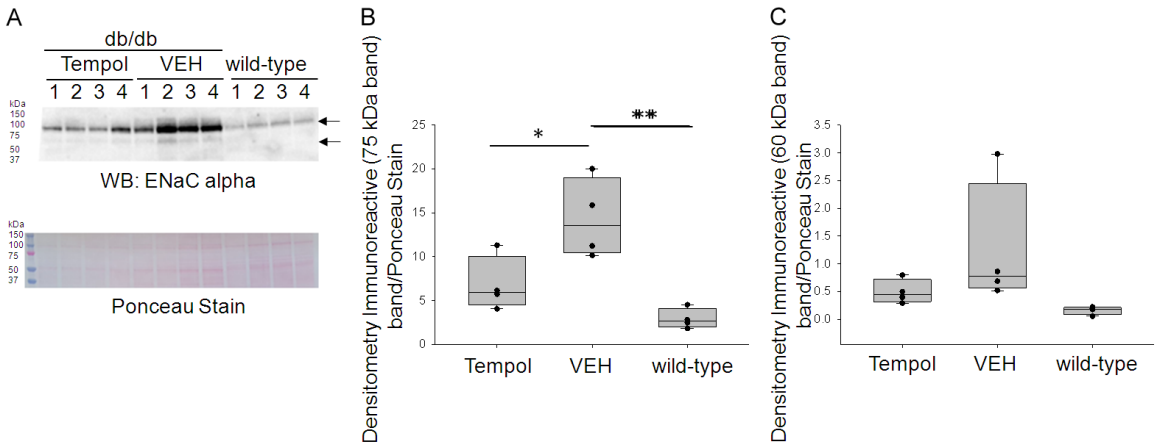

Figure 4. Western blot and densitometric analysis of epithelial sodium channel (ENaC) alpha protein in kidney cortex lysates from hypertensive diabetic db/db mice treated with vehicle or tempol and from healthy wild-type mice. A. Western blot of ENaC alpha protein. Ponceau staining was used to assess lane loading. B. Densitometric analysis of the immunoreactive band corresponding to the 75 kDa form of ENaC alpha indicated by the top arrow. C. Densitometric analysis of the immunoreactive band corresponding to 60 kDa form of ENaC alpha indicated by the bottom arrow. * represents a *p*-value <0.05. ** represents a *p*-value <0.01. VEH represents vehicle. n=4 mice per group.

fractions is greater in the healthy wild-type kidney compared to the hypertensive diabetic db/db kidney, and tempol treatment causes a further reduction in the membrane expression of the NKCC2 dimer (**Figure 2**).

Tempol decreases sodium-chloride co-transporter (NCC) protein expression in the hypertensive diabetic db/db kidney

Previous studies suggested that the NCC is increased in the kidney of STZ induced type 1 diabetic rats [19] and in type 2 diabetic db/db mice [20]. Here we investigated whether tempol treatment could attenuate elevated amounts of the active form of NCC, phospho NCC (pNCC), in salt-loaded hypertensive diabetic db/db mice. Although the amounts of pNCC in the hypertensive diabetic db/db kidney and healthy wild-type mice were comparable, the amount of pNCC in tempol treated mice was significantly reduced compared to vehicle treatment (**Figure 3**).

Tempol decreases ENaC protein expression in the hypertensive diabetic db/db kidney

Our group previously showed that tempol treatment ameliorated systolic blood pressure in salt-loaded hypertensive 129Sv mice presumably by reducing renal ENaC alpha protein expression [9]. Here, we investigated whether tempol could normalize systolic blood pressure and reduce elevated levels of ENaC alpha subunit protein expression in the hypertensive diabetic db/db kidney. Indeed, tempol was able to reduce both systolic blood pressure (**Table 2**) and membrane protein expression of ENaC alpha subunit (**Figure 4**).

Tempol attenuates PKC protein expression in the hypertensive diabetic kidney

PKC is known to be upregulated and activated in the diabetic kidney and this may contribute to the development of diabetic nephropathy [21]. Here we investigated whether the administration of tempol in hypertensive diabetic db/db mice could reduce protein expression of some of the most prominent isoforms of PKC expressed in the hypertensive diabetic kidney. Consistent with published studies, PKC protein expression in the hypertensive diabetic kidney was augmented compared to its expression in the healthy kidney (**Figure 5**). Importantly, there was a significant reduction in PKC epsilon protein expression in the kidney of hypertensive diabetic db/db mice that were infused with tempol compared to vehicle (**Figure 5**).

The serine/threonine protein kinase G (PKG) protein expression is reduced in the hypertensive diabetic db/db kidney compared to the healthy kidney

PKG has been previously shown to play a role in the regulation of filtration barrier permeability and in the regulation of the constriction apparatus in podocytes [22]. Here we investigated

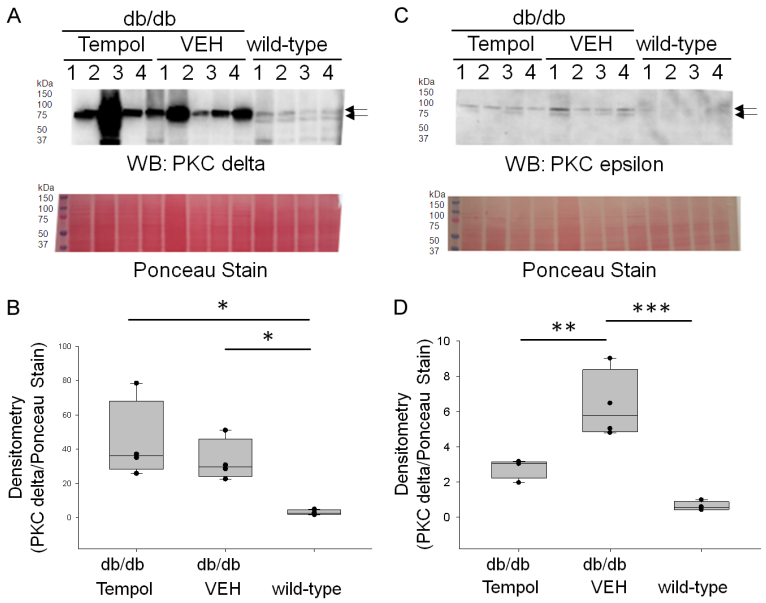

Figure 5. Western blot and densitometric analysis of various isoforms of protein kinase C (PKC) protein in kidney cortex lysates from hypertensive diabetic db/db mice treated with vehicle or tempol and from healthy wild-type mice. A. Western blot of PKC delta. Ponceau staining was used to assess lane loading. B. Densitometric analysis of the immunoreactive band corresponding to PKC delta indicated by the arrows. C. Western blot for PKC epsilon. Ponceau staining was used to assess lane loading. D. Densitometric analysis of the immunoreactive band corresponding to PKC epsilon indicated by the arrows. * represents a *p*-value <0.05. ** represents a *p*-value <0.01. *** represents a *p*-value of <0.001. VEH represents vehicle. n=4 mice per group.

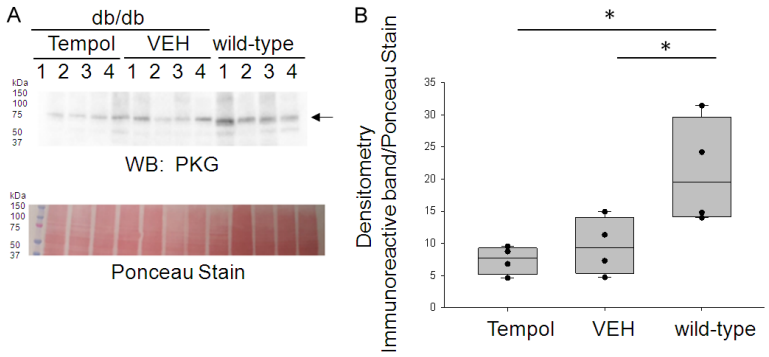

Figure 6. Western blot and densitometric analysis of protein kinase G (PKG) protein in kidney cortex lysates from hypertensive diabetic db/db mice treated with vehicle or tempol and from healthy wild-type mice. A. Western blot of PKG protein expression. Ponceau staining was used to assess lane loading. B. Densitometric analysis of the immunoreactive band corresponding to PKG indicated by the arrow. * represents a *p*-value <0.05. VEH represents vehicle. n=4 mice per group.

PKG protein expression in the diabetic kidney from db/db mice treated with tempol or vehicle compared to its expression in the kidney of healthy wild-type mice. Western blot and densitometric analysis showed that the protein expression of PKG was lower in the kidney of hypertensive diabetic db/db mice compared to its expression in the kidney of healthy wild-type mice (**Figure 6**). PKG protein expression was comparable between the vehicle treated and tempol treated db/db groups (**Figure 6**).

Tempol does not alter urinary EV size or excretion in hypertensive diabetic db/db kidney

Urinary EVs (uEVs) represent a mixed population of EVs primarily released from renal epithelial cells. Since uEVs have been previously shown to be enriched in various membrane transporters and ion channels of the kidney, we investigated whether tempol treatment could alter the overall size and amount of EVs released into the urine. As shown in **Figure 7**, the size and concentration of EVs isolated from the urine of hypertensive diabetic db/db mice were comparable to those isolated from the urine of healthy wild-type mice and comparable between hypertensive diabetic db/db mice treated with vehicle or tempol.

Discussion

The results from this study are consistent with published studies showing beneficial effects of tempol in the kidney of hypertensive diabetic rodents. Oba et al. showed that tempol treatment for 8 weeks reduced mesangial fibrosis in diabetic db/db mice [23]. Another study showed that chronic tempol treatment mitigated the dysregulation of potassium channel dependent control of afferent arteriole tone in streptozotocin (STZ) induced type 1 diabetic male Sprague-Dawley rats [24]. Here we show for the first time tempol

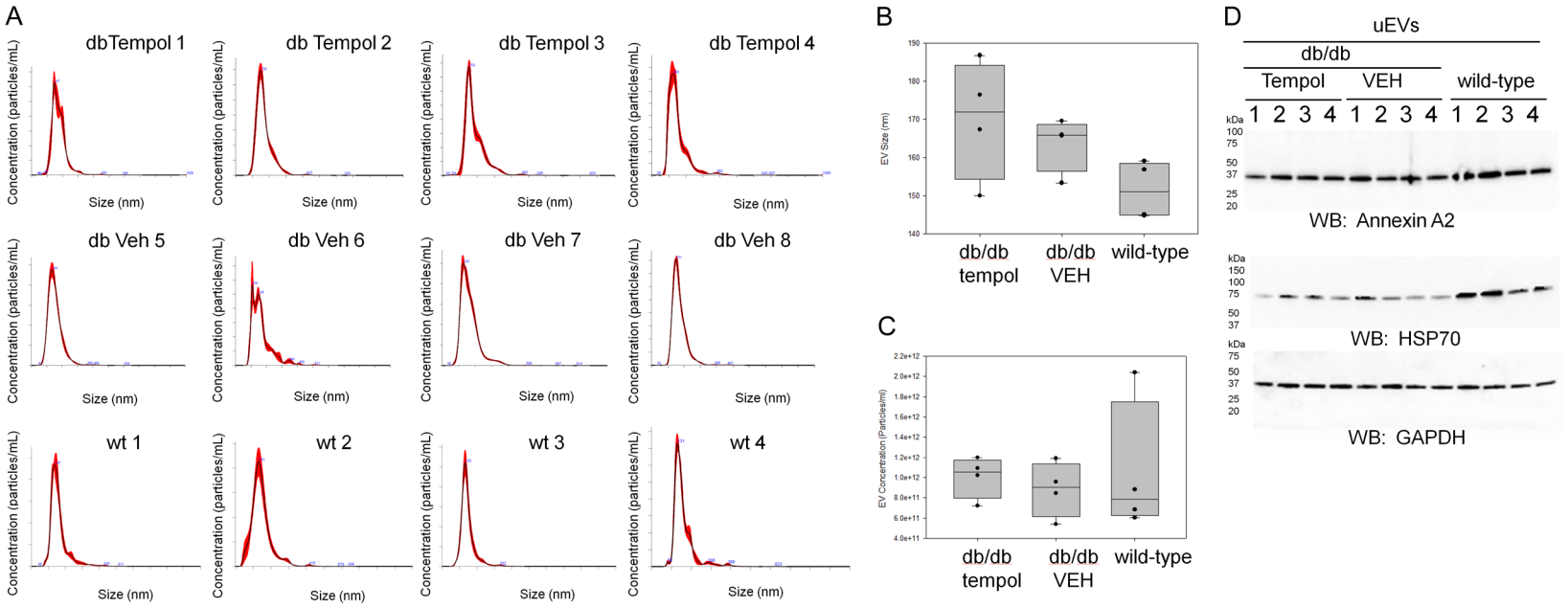

Figure 7. Characterization of urinary extracellular vesicles (uEVs) from hypertensive diabetic db/db mice treated with vehicle or tempol and from healthy wild-type mice. A. NanoParticle tracking analysis peak profiles showing size distribution and particle concentration of uEVs isolated from diabetic db/db mice treated with vehicle or tempol and from healthy wild-type mice. B. Summary plot of EV size between the three groups. C. Summary plot of EV concentration between the three groups. D. Western blot analysis of the EV markers annexin A2, heat shock protein 70 (HSP70), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enriched in uEVs from each group. VEH represents vehicle. n=4 mice per group.

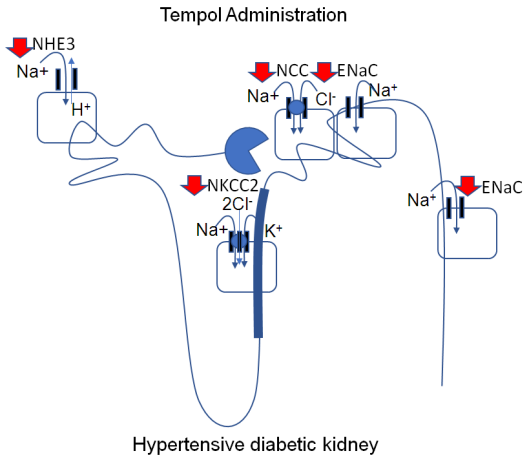

Figure 8. Schematic diagram showing differences in the density of epithelial transport proteins in the kidney of hypertensive diabetic db/db mice treated with tempol compared to mice treated with vehicle. Tempol reduces protein expression of the sodium hydrogen exchanger 3 (NHE3), sodium potassium chloride cotransporter (NKCC2), sodium chloride cotransporter (NCC), and epithelial sodium channel (ENaC) in the kidneys of salt-loaded hypertensive diabetic db/db mice.

treatment normalized blood pressure in the salt-loaded hypertensive diabetic db/db kidney while reducing membrane protein expression of epithelial transport proteins in different segments of the nephron.

Since previous studies showed that tempol does not affect blood glucose levels in diabetic rats we did not investigate blood glucose levels in db/db mice given tempol or vehicle [24]. However, tempol has been shown to increase the excretion of sodium and alleviate high blood pressure in animals [25, 26]. This is not surprising because ROS is known to contribute to the pathogenesis of hypertension in a mechanism involving salt and water retention [27].

The blood pressure lowering ability of tempol in salt-loaded hypertensive diabetic db/db mice that we observed in this study is not surprising since previous studies have shown that tempol can normalize blood pressure in hypertensive animals given a high salt diet. Welch et al. showed that tempol prevented a rise in mean arterial pressure (MAP) with high salt intake in spontaneously hypertensive rats [28]. A previous study by our group showed that tempol reduced systolic blood pressure in salt-loaded hypertensive 129Sv mice [9].

Western blot and densitometric analysis showed that epithelial transport proteins in multiple segments of the nephron are augmented in the salt-loaded hypertensive diabetic db/db kidney compared to the healthy wild-type kidney. Tempol infusion reduced the elevated protein expression of NHE3, NKCC2, NCC, and ENaC in membrane fractions of the kidney cortex. Also, protein expression of PKC epsilon was significantly reduced after tempol infusion. The normalization of PKC protein expression and activity may reduce the risk of diabetic nephropathy. Since PKC is known to regulate the integrity of the actin cytoskeleton and proteins that associate with it, the mechanism by which tempol reduces membrane expression of epithelial transport proteins may at least in part involve the attenuation of this kinase.

This study provides important insight into the ability of tempol to normalize membrane protein expression of multiple epithelial transport proteins in the luminal plasma membrane of the hypertensive diabetic kidney. But, one limitation of this study is that we did not elucidate the exact mechanism by which tempol downregulates epithelial transport mechanisms in the hypertensive diabetic kidney. Second, many of the epithelial transport proteins that were found to be sensitive to tempol treatment are regulated by circadian rhythms. Future studies should be performed to determine if these effects mediated by tempol treatment occur in a time-of-day dependent manner. Third, we had a relatively small sample size for each of the three animal groups that was likely not enough to observe a statistically significant difference in some of the biochemical and physiological measures including urinary electrolyte concentrations. However, this sample size was enough to observe differences in the density of epithelial transport proteins in each segment of the nephron (**Figure 8**), PKC epsilon protein expression, and systolic blood pressure.

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Disclosure of conflict of interest

None.

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