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Mirandy Schiphen Li April 11, 2016

NADPH Oxidases Mediate Zinc Deficiency-Induced Oxidative Stress in Mouse Kidneys

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Abstract

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Zn^{2+} deficiency (ZnD) is comorbid with Chronic Kidney Disease (CKD). Experimental studies show that ZnD worsens kidney complications. Oxidative stress caused by increased reactive oxygen species (ROS) plays a role in the harmful effects of ZnD. However, the sources of oxidative stress continue to be identified. In the kidney, NADPH oxidases (Nox) are enzymes that promote oxidative stress. The objective of this study is to determine the role of Nox in ZnD-induced oxidative stress. We hypothesize that ZnD upregulates Nox resulting in sustained ROS levels and kidney damage. WT mice were pair-fed a ZnD- or control-diet for 6 weeks. After collecting kidneys and urine, kidney damage (urinary albumin:creatinine ratios), ROS levels (Amplex Red and 2',7'-dichlorofluorescein diacetate) and Nox expression (qRT-PCR and Western blot) were examined. To further investigate the effects of Zn^{2+} bioavailability on Nox, mouse tubular epithelial cells (mTECs) were exposed to the Zn^{2+} chelator N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) or vehicle (DMSO) for 24 hours ± Zn^{2+} supplementation for an additional 24 hours. Mice fed a ZnD-diet develop microalbuminuria that is accompanied by elevated renal Nox2 expression and ROS levels compared to control mice. In mTECs, Nox inhibition with diphenyleneiodonium (DPI) blocks TPEN-induced ROS levels. Furthermore, TPEN-induced Nox2 upregulation is attenuated with Zn^{2+} supplementation. Nox2 via si-RNA inhibits Zn^{2+} depletion-induced H_2O_2 generation. These findings identify Nox2 as a mediator of ZnD-induced oxidative stress and kidney damage. Understanding the mechanisms by which ZnD contributes to kidney injury may have important impact on the treatment of chronic kidney disease.

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INTRODUCTION

Chronic Kidney Disease: Chronic Kidney Disease (CKD) affects over 19 million people in the United States and the global prevalence of CKD is rapidly increasing (1). Furthermore, CKD is a precursor to End-Stage Renal Disease (ESRD), and 70% of CKD patients above the age of 50 also have diabetes, hypertension or both (2). Diabetes is the leading cause of CKD, accounting for over 40% of new CKD patients (2). In the United States, over 400,000 ESRD patients require long-term dialysis (3). Early stages of CKD are often asymptomatic and reversible (4). While genetic and environmental factors contribute to the pathogenesis of CKD, experimental and clinical studies have shown that proteinuria is an in important factor in CKD progression (6). In addition, microalbuminuria is an early sign of CKD patients with and without diabetes (7).

Zn^{2+} Deficiency in Chronic Kidney Disease: Clinical studies reveal that CKD, regardless of etiology, is accompanied by Zn^{2+} deficiency (ZnD). Furthermore, 40-78% of hemodialysis patients are ZnD (8). Experimental studies have shown that ZnD exacerbates complications in the diabetic kidney and is one cause of kidney damage in diabetic patients (12). Studies have shown that diabetes patients are ZnD due to enhanced renal excretion and impaired gastrointestinal absorption of Zn^{2+} (10). ZnD can also occur due to increased expression of intracellular metallothioneins following oxidative stress (11). Symptoms of chronic renal failure parallel symptoms of ZnD, in particular: impaired wound healing, growth retardation, and hypogonadism (13). Therefore, it is believed that some symptoms of chronic renal failure can be attributed to or are exacerbated by ZnD.

Oxidative Stress in Chronic Kidney Disease: Oxidative stress is one of the etiologies that contribute to increased mortality and poor nutritional status of patients with CKD. As CKD

progresses, markers of oxidative stress increase and correlate with levels of renal dysfunction (5). Mounting evidence demonstrates that sustained reactive oxygen species (ROS), which lead to oxidative stress, play a critical role in the detrimental effects associated with ZnD (4). Two forms of ROS are superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). $O_2^{\bullet-}$ is short-lived due to its high reactivity, and is converted into H_2O_2 via superoxide dismutase (SOD). Furthermore, most of signaling that occurs as a result of $O_2^{\bullet-}$ is mediated by its more stable dismutation product, H_2O_2 (14).

Sources of Oxidative Stress: Zn^{2+} is an essential trace metal involved in various cellular functions. Many enzymes and transcription factors are Zn^{2+} regulated (9). These molecules are essential for proper cell function and are an essential product of cellular respiration under normal cell conditions. However, increased ROS production can lead to oxidative stress-induced cell and tissue damage if anti-oxidant defense mechanisms are unable to neutralize ROS accumulation in the cell. Zn^{2+} is known to be a cofactor of anti-oxidant enzymes. For example, Zn^{2+} is a cofactor in both intracellular and extracellular copper-zinc superoxide dismutase (CuZn-SOD), which catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2. Furthermore, it has been shown that ZnD-induced oxidative stress can trigger these protective responses by regulating CuZn-SOD and MnSOD activity (15). In addition to decreased neutralization of ROS, oxidative stress can also be caused by increased ROS production from *pro*-oxidant sources. The mitochondrial electron transport chain, xanthine oxidase, uncoupled endothelial nitric oxide synthase, and NADPH oxidases are important sources of renal ROS (16).

NADPH oxidase in Oxidative Stress: In the kidney, NADPH oxidases (**Nox**) are primary sources of oxidative stress, leading to elevated generation of ROS and subsequent kidney damage (17). Nox enzymes are comprised of several subunits that include catalytic, membrane-bound

components such as Nox1-5, Duox1/2 and $p22^{phox}$, as well as regulatory cytosolic components such as $p47^{phox}$, $p67^{phox}$, NoxA1, NoxO1, rac-1 and Poldip2 (5). It is known that various stimuli, including hyperglycemia, oxidatively modified lipoproteins, and advanced glycation end products induce Nox enzymes by stimulating the assembly of a functional complex (ref). Currently, it is unclear whether the Nox2 or Nox4 isoform is responsible for ROS-dependent tissue damage in diabetic nephropathy (18). The activation of Nox2 requires assembly of its cytosolic regulatory units, $p47^{phox}$ and $p67^{phox}$, to the membrane. Once assembled, the Nox2 complex generates $O_2^{\bullet-}$ by transferring an electron from NADPH to oxygen (18). Nox4 is highly expressed in the kidney, particularly in renal tubular cells (19). Unlike Nox2, Nox4 does not require assembly of cytosolic subunits for its activity, and is thought to be constitutively active (20). In contrast to other Nox proteins, Nox4 produces H_2O_2 constitutively (21).

Study Objective: The role of Nox enzymes in ZnD-induced oxidative stress and renal injury has not been investigated. Therefore, the objectives of this study are to determine if these Nox2 and Nox4 are 1) regulated by Zn^{2+} bioavailability and 2) sources of ZnD-induced renal oxidative stress. In this study, we investigated the role of Nox enzymes in ZnD-induced oxidative stress *in vivo* and *in vitro* models of ZnD. These data reveal that Nox2 is a Zn^{2+}-regulated enzyme that mediates ZnD-induced oxidative stress.

MATERIALS AND METHODS

Zn^{2+} Deficiency Models

***In vivo*:** To induce Zn^{2+} deficiency (ZnD), male and female WT mice (8-12 weeks old) received a Zn^{2+} deficient diet (1ppm, Harlan Teklad, Madision, WI) for 6 weeks. Control mice received a pair-matched Zn^{2+} adequate (ZnA) diet (50ppm, Harlan Teklad). A Zn^{2+}-free environment was maintained by providing mice with deionized drinking H_2O in Zn^{2+}-free containers along with daily cage changes. After the experimental period, urine and kidneys were collected for analysis.

***In vitro*:** To reduce intracellular Zn^{2+} levels, mouse tubular epithelial cells (mTECs) were cultured in N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN)- or vehicle (DMSO)-containing media for 24 hours. To replete intracellular Zn^{2+}, TPEN-exposed mTECs were cultured in Zn^{2+} supplemented media for 24 hours. To determine the role of NADPH oxidases in ROS generation, select monolayers were pre-treated with diphenyleneiodonium (DPI), a general Nox enzyme inhibitor, or vehicle. To identify the specific Nox enzyme subunits involved in ROS generation, mDCT cells were transfected with scrambled oligonucleotides or oligonucleotides targeting Nox2 or Nox4.

Zn^{2+} Assessment

FluoZin3: To assess changes in intracellular Zn^{2+} levels, confluent cell monolayers were loaded with 500 μg/ml Fluozin3, a fluorescent Zn^{2+} probe, for 1 h at 37°C in Krebs-Ringer phosphate buffer (KRPG; 145 mM NaCl, 5.7 mM KH_2PO_4, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, and 5.5 mM glucose pH 7.35). FluoZin3 fluorescence was monitored by confocal microscopy (Olympus) at excitation and emission wavelengths of 494 and 516 nm, respectively.

Zip10 mRNA expression: To examine Zn^{2+} bioavailability, Zip10 mRNA expression was measured. Briefly, total RNA was isolated from cells with TRIzol according to the manufacturer's protocol (Invitrogen). cDNA was generated and amplified using One-Step SYBR Green (Bio-Rad). All data were normalized to the 9s content of the same sample, and Zip10 mRNA expression was calculated using the $\Delta\Delta Ct$ method.

Kidney Injury Assessment

Proteinuria: To assess kidney injury, total urinary protein was measured using a bicinchoninic (BCA) protein assay (Pierce). Protein concentrations were calculated by extrapolating values from a protein standard curve. Urinary protein was normalized to 24-hour urine volume.

Microalbuminuria: To measure urinary albumin, albumin and creatinine were measured in spot urine samples by ELISA assay (EXOCELL, Inc.). Microalbuminuria was determined by calculating albumin/creatinine ratios.

Hypertrophy: To assess renal changes, whole kidney hypertrophy was examined by total kidney weight to body weight ratios. To assess cellular hypertrophy, protein to DNA ratios were determined. Briefly, cells were incubated with 1 μm Hoechst in Krebs-Ringer phosphate buffer (KRPG; 145 mm NaCl, 5.7 mm KH_2PO_4, 4.86 mm KCl, 0.54 mm $CaCl_2$, 1.22 mm $MgSO_4$, and 5.5 mm glucose, pH 7.35) for 30 min at 37 °C to detect DNA content. Cells were washed with 1× phosphate-buffered saline (PBS), and fluorescence was measured at 350-nm excitation/460-nm emission. Finally, cells were lysed and the protein content was quantified using a bicinchoninic acid (BCA) assay (Pierce). Protein concentrations for each sample were normalized by respective Hoechst fluorescence intensity.

Reactive Oxygen Species Measurement

Amplex Red: H_2O_2 was measured using horseradish peroxidase-catalyzed oxidation of the nonfluorescent molecule *N*-acetyl-3,7-dihydroxyphenoxazine into the highly fluorescent molecule resorufin (Amplex Red Assay, Invitrogen). Cells were incubated in Krebs-Ringer phosphate buffer (KRPG; 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl2, 1.22 mM MgSO4, 5.5 mM glucose, pH 7.35) containing 100µL/mL Amplex Red and 0.2 units/mL horseradish peroxidase for 1 hour at 37ºC. Resorufin fluorescence was measured at the excitation and emission wavelengths of 540 and 590 nm, respectively. Sample fluorescence was compared with that generated by a H_2O_2 standard curve to calculate the concentrations of H_2O_2. H_2O_2 concentrations were normalized to total protein.

DCF: $O_2^{\bullet-}$ was detected using the fluorescent probe 2′,7′-dihydrodichlorofluorescein diacetate (DCF-DA; Invitrogen). Confluent mTECs monolayers were loaded with 25 µg/ml DCF-DA for 1 h at 37°C in Krebs-Ringer phosphate buffer (KRPG; 145 mM NaCl, 5.7 mM KH_2PO_4, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, and 5.5 mM glucose pH 7.35). For quantification, DCF fluorescence intensity was measured on a Victor plate reader (PerkinElmer, Waltham, MA) at excitation and emission wavelengths of 488 and 520 nm, respectively.

NADPH Oxidase Expression

qRT-PCR: To measure Nox mRNA, qRT-PCR was performed. Total RNA was isolated from cells with TRIzol according to the manufacturer's protocol (Invitrogen). cDNA was generated and amplified using One-Step SYBR Green (Bio-Rad). All data were normalized to the 9s content of the same sample, and Nox2 and Nox4 mRNA expression was calculated using the $\Delta\Delta Ct$ method.

Western blot: To measure Nox proteins, Western blot analysis was performed. Briefly, cells were lysed using RIPA lysis buffer. 50µg of protein was separated by 12% SDS-PAGE and then transferred onto a PVDF membrane. The membrane was incubated in 1% bovine serum albumin in phosphate buffered saline (PBS) and then immunoblotted with appropriate dilutions of primary antibodies specific for Nox1 (Santa Cruz Biotechnology, Inc.), Nox2, Nox4 (Abcam, Cambridge, MA), or GAPDH (Cell Signaling Technology, Danvers, MA). After incubating in rabbit secondary antibody, immunoreactive bands were detected using the G:BOX (Gel box) Imaging System.

NADPH oxidase Activation

Co-immunoprecipitation: To determine Nox subunit association, co-immunoprecipitation assays were performed. Briefly, cells were lysed using RIPA lysis buffer. Proteins were immunoprecipitated with p67phox or Poldip2 conjugated protein-A beads. Bound Nox2 and Nox4 proteins were measured by Western blot analysis.

Statistical Analysis

GraphPad: For all experiments, graphing and statistical analyses were performed using GraphPad software (Prism, San Diego, CA). For all experiments comparing only two groups, statistical analysis was performed by Student's t-test. When more than two groups were analyzed, two-way ANOVA (samples varied by genotype and oxygen exposure) followed by post-hoc analysis.

RESULTS

Dietary Zn^{2+} restriction promotes kidney injury in mice. To investigate renal consequences of reduced Zn^{2+} bioavailability, WT mice were pair fed a ZnD- or ZnA-diet. Consistent with ZnD retarding growth, body weights are significantly reduced in mice fed a Zn^{2+}-restricted diet compared to controls (Table 1). To access changes in kidney function, urinary proteins were measured. After 6 weeks of dietary Zn^{2+} restriction, total urinary proteins are significantly increased (Figure 1A). Additionally, urinary albumin levels are elevated with a ZnD diet (Figure 1B). Consistent with changes in function, kidneys of Zn^{2+} deficient mice undergo hypertrophy (Table 1). Collectively, these results indicate that ZnD promotes kidney injury.

Dietary Zn^{2+} restriction stimulates renal reactive oxygen species generation and NADPH oxidase upregulation in mice. To examine effects of reduced Zn^{2+} bioavailability on renal ROS generation, WT mice were fed a ZnD- or ZnA-diet. To examine changes in ROS generation, H_2O_2 levels were measured. Findings show that renal (Figure 2A) and urinary (Figure 2B) H_2O_2 levels are significantly increased in mice with a dietary Zn^{2+} restriction. To examine alterations in NADPH oxidases, Nox subunit expression was assessed. The results show that elevated ROS generation is accompanied by increased Nox2 mRNA (Figure 2C) and protein (Figure 2D) expression. However, Nox4 expressions are unaltered. These findings reveal that ZnD induces oxidative stress and Nox2 upregulation in kidneys.

Intracellular Zn^{2+} modulates reactive oxygen species generation in mTECs. To manipulate intracellular Zn^{2+} bioavailability, mTECs were treated with the Zn^{2+} chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) ± Zn^{2+} supplementation. Figure 3A demonstrates that TPEN reduces intracellular Zn^{2+} levels compared to vehicle-treated cells. Furthermore, Zn^{2+} supplementation restores intracellular Zn^{2+} to control levels. Consistent with

these findings, intracellular Zn^{2+} reduction by TPEN promotes mRNA upregulation of the Zn^{2+}-influx transporter ZRT/IRT-like protein 10 (Zip10) (Figure 3B). Additionally, restoration of intracellular Zn^{2+} levels by Zn^{2+} supplementation reduces Zip10 mRNA back to control levels. These results indicate that TPEN reduces intracellular Zn^{2+} levels and Zn^{2+} supplementation reverses TPEN-induced Zn^{2+} deficiency in mTECs.

To investigate a possible role of Zn^{2+} in reactive oxygen species (ROS) generation, H_2O_2 and $O_2^{\bullet-}$ levels were measured in mTECs exposed to TPEN ± Zn^{2+} supplementation. Amplex Red findings show that TPEN-induced reduction of intracellular Zn^{2+} stimulates H_2O_2 generation compared to vehicle-treated cells (Figure 2C). However, Zn^{2+} repletion significantly reduces TPEN-induced H_2O_2 generation to control levels. Unexpectedly, $O_2^{\bullet-}$ is reduced in TPEN-treated mTEC, as assessed by two independent methods dichlorodihydrofluorescein (DCF) (Figure 2D) and dihydroethidium (DHE) (data not shown). Furthermore, similar to TPEN-treated cells, decreased $O_2^{\bullet-}$ levels are observed with Zn^{2+} repletion. However, $O_2^{\bullet-}$ generation is significantly increased in cells treated with the ROS inducer tert-butyl hydroperoxide (TBHP). Collectively, these results demonstrate that Zn^{2+} plays a role in ROS generation.

NADPH oxidases mediate Zn^{2+} deficiency-induced oxidative stress in mTECs. To investigate a possible role of NADPH oxidases in ZnD-induced ROS generation, mTECs were pre-treated with diphenyleneiodonium (DPI), a general NADPH oxidase inhibitor, prior to TPEN exposure. ROS generation was examined by measuring H_2O_2 and $O_2^{\bullet-}$ levels. Figure 4A shows that TPEN-induced H_2O_2 generation is significantly blunted with DPI treatment. Additionally, basal $O_2^{\bullet-}$ generation is reduced in DPI-treated mTECs (Figure 4B). These findings indicate that NADPH oxidases play a role in ZnD-induced oxidative stress.

Intracellular Zn^{2+} modulates NADPH oxidases in mTECs. To investigate the effects of Zn^{2+} bioavailability on NADPH oxidases, Nox expression was examined in TPEN-exposed mTECs ± Zn^{2+} supplementation. Findings show that Nox2 and Nox4 mRNA expressions are upregulated with TPEN-induced intracellular Zn^{2+} depletion compared to vehicle-treated cells (Figure 5A). Furthermore, Nox2 mRNA expression is significantly reduced with intracellular Zn^{2+} repletion. However, Western blot analysis shows that Nox2 protein expression is increased, but Nox4 protein expression is significantly reduced with TPEN treatment and TPEN ± Zn^{2+} supplementation (Figure 5B). These results demonstrate that Nox2 is regulated by intracellular Zn^{2+}.

Nox2- and Nox4-containing NADPH oxidases mediate Zn^{2+} deficiency-induced oxidative stress and cellular hypertrophy in mTECs. To investigate the role of Zn^{2+} in NADPH oxidase activation, subunits association was examined in mTECs exposed to TPEN ± Zn^{2+} supplementation. Data show increased Nox2 and $p67^{phox}$ association in Zn^{2+} depleted cells (Figure 6A). Furthermore, Zn^{2+} repletion reverses enhanced Nox2 and $p67^{phox}$ association. These results demonstrate that Nox2 activation is regulated by intracellular Zn^{2+}.

Furthermore, Figure 6B shows decreased Nox4 association with Poldip2 in Zn^{2+} depleted cells. This is not reversed with Zn^{2+} repletion. These results demonstrate that Nox4 activation is regulated by intracellular Zn^{2+}.

To identify the specific Nox subunit involved in ZnD-induced oxidative stress, mTECs were transfected with Nox2- and/or Nox4-targeting oligonucleotides to knockdown Nox expression. Reduced Nox expression is accompanied with lower basal H_2O_2 (Figure 6C) levels.

These results reveal that Nox2- and Nox4-containing NADPH oxidases mediate ZnD-induced oxidative stress.

To investigate the role of NADPH oxidases in ZnD-induced renal hypertrophy, si-Nox transfected cells were exposed to TPEN. Figure 6D shows that TPEN induced cellular hypertrophy. However, Additionally, TPEN induced cellular hypertrophy that is prevented with knock-down of Nox2 and Nox4. (Figure 6D). Together, these findings demonstrate that Nox2- and Nox4-containing NADPH oxidases mediate ZnD-induced cellular hypertrophy.

FIGURES

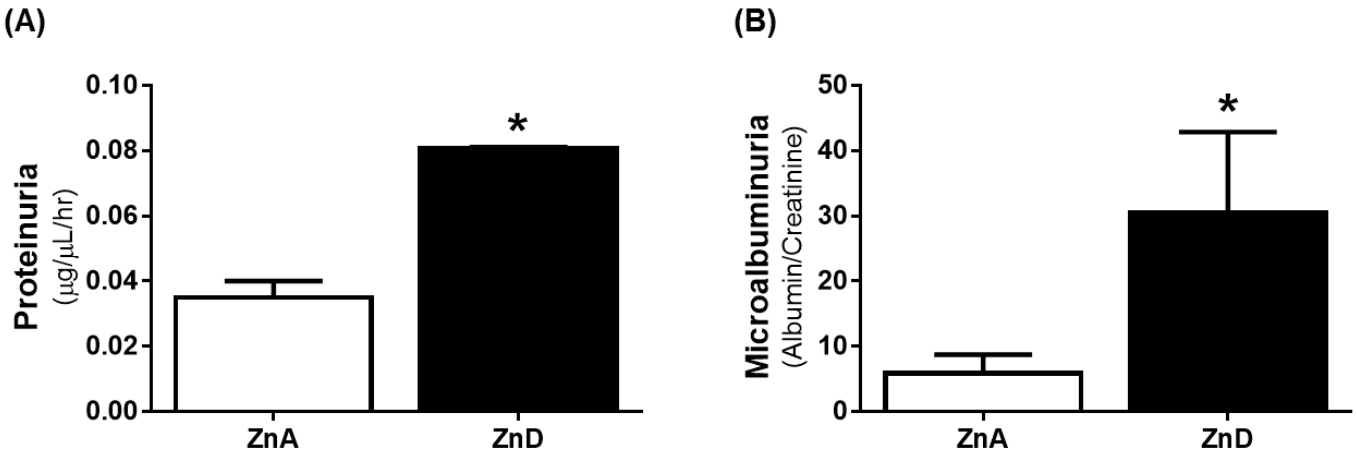

Figure 1. Dietary Zn^{2+} restriction promotes kidney injury in mice. To examine renal consequences of reduced Zn^{2+} bioavailability, WT mice were pair-fed a Zn^{2+} deficient (ZnD)- or Zn^{2+} adequate (ZnA)-diet. To assess changes in kidney function, (**A**) proteinuria and (**B**) microalbuminuria were calculated. n = 5-6 mice per group. *$p<0.05$ vs ZnA diet.

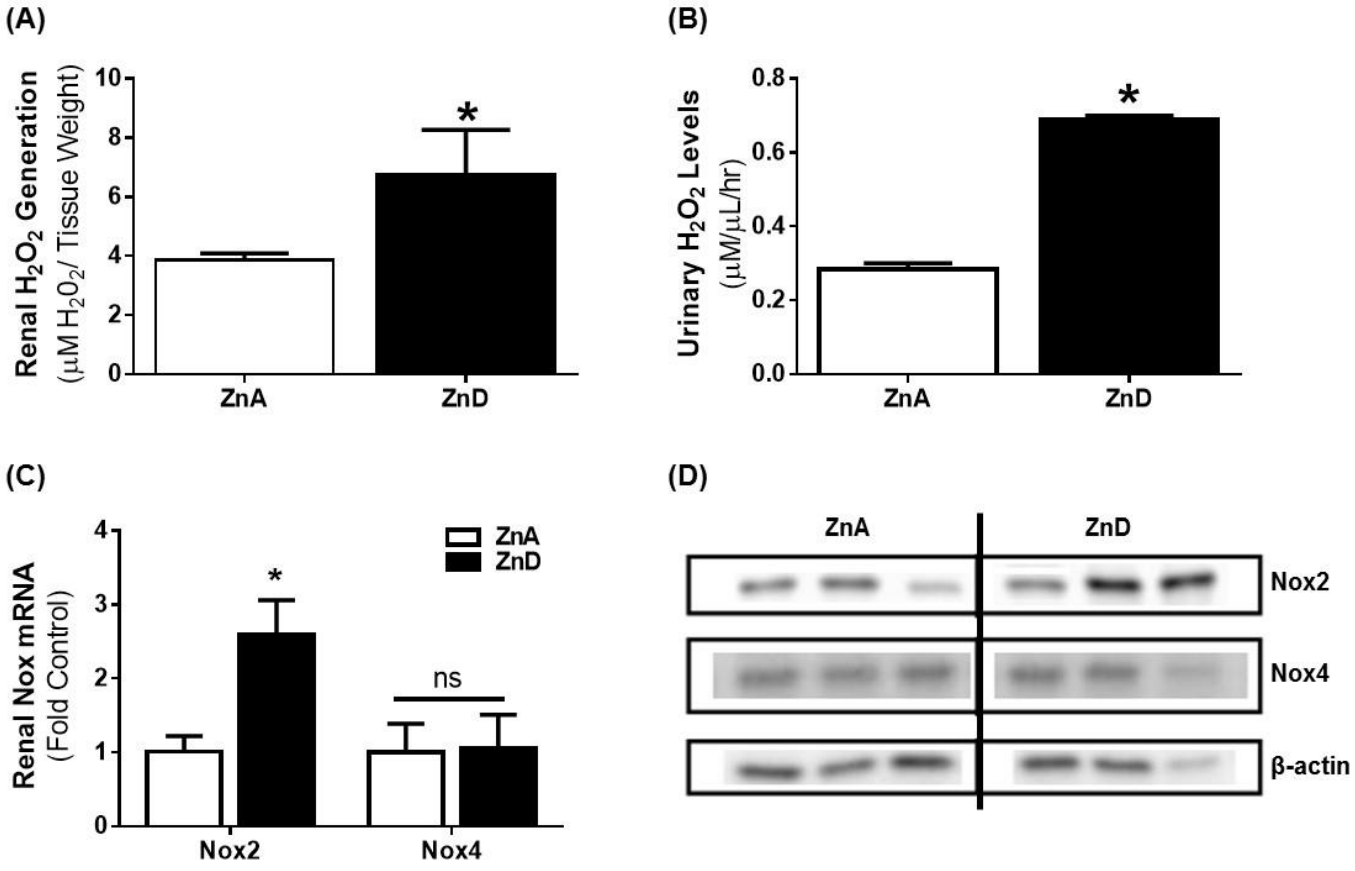

Figure 2. Dietary Zn^{2+} restriction stimulates renal reactive oxygen species generation and NADPH oxidase upregulation in mice. To examine effects of reduced Zn^{2+} bioavailability on renal reactive oxygen species (ROS) generation, WT mice were pair-fed a Zn^{2+} deficient (ZnD)- or adequate (ZnA)-diet. To examine changes in ROS generation, (**A**) renal and (**B**) urinary H_2O_2 levels were measured. Alterations in NADPH oxidases were assessed by examining Nox subunit (**C**) mRNA and (**D**) protein expressions. n = 5-6 mice per group. *$p<0.05$ vs ZnA diet.

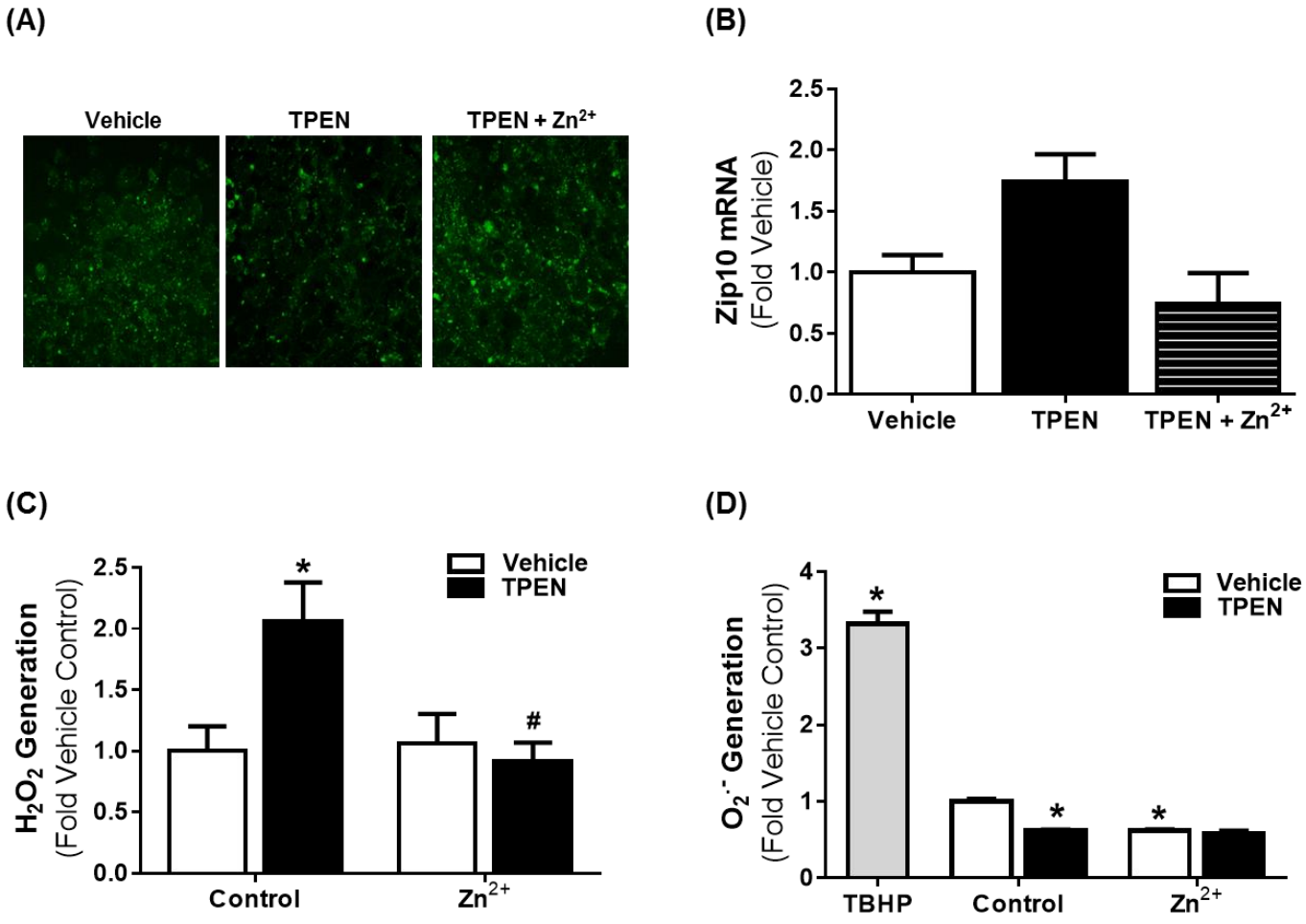

Figure 3. Intracellular Zn^{2+} modulates reactive oxygen species generation in mTECs. To examine reactive oxygen species (ROS) generation, mTECs were exposed to TPEN (intracellular Zn^{2+} chelator) ± Zn^{2+} supplementation. To assess Zn^{2+} bioavailability, (**A**) intracellular Zn^{2+} and (**B**) Zip10 mRNA levels were examined. Changes in ROS generation were assessed by examining (**C**) H_2O_2 and (**D**) $O_2^{\bullet-}$ levels. n = 3 independent experiments performed in replicates. Representative images shown. *$p<0.05$ vs control vehicle. $^{\#}p<0.05$ vs control TPEN.

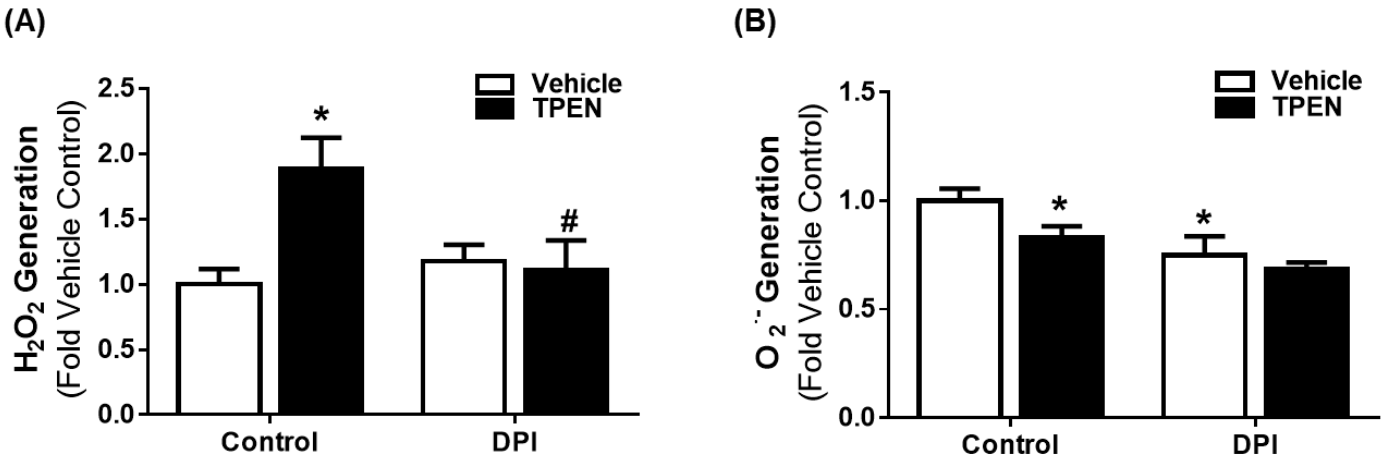

Figure 4. NADPH oxidases mediate Zn^{2+} deficiency-induced oxidative stress in mTECs. To investigate the role of NADPH oxidases in Zn^{2+} deficiency-induced ROS generation, mTECs were exposed to TPEN (intracellular Zn^{2+} chelator) ± DPI (NADPH oxidase inhibitor). To assess ROS generation, (**A**) H_2O_2 and (**B**) $O_2^{\bullet-}$ levels were examined. n = 3 independent experiments performed in replicates. *$p<0.05$ vs control vehicle. $^{\#}p<0.05$ vs control TPEN.

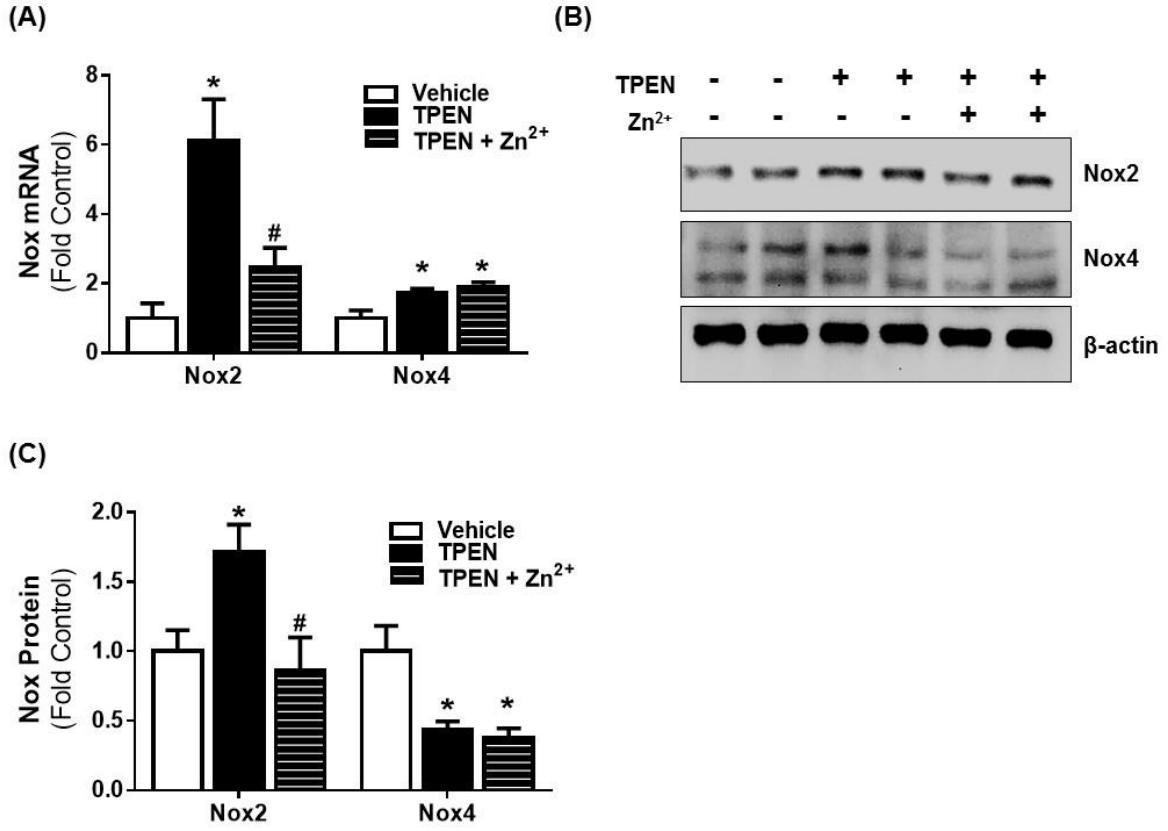

Figure 5. Intracellular Zn^{2+} modulates NADPH oxidases in mTECs. To examine the effects of Zn^{2+} bioavailability on NADPH oxidases, Nox subunit expression was examined in mTECs exposed to TPEN (intracellular Zn^{2+} chelator) ± Zn^{2+} supplementation. To assess changes in NADPH oxidases, Nox subunit (**A**) mRNA and (**B** and **C**) protein expressions were examined. n = 3 independent experiments performed in replicates. Representative images shown. *$p<0.05$ vs vehicle. $^{\#}p<0.05$ vs TPEN.

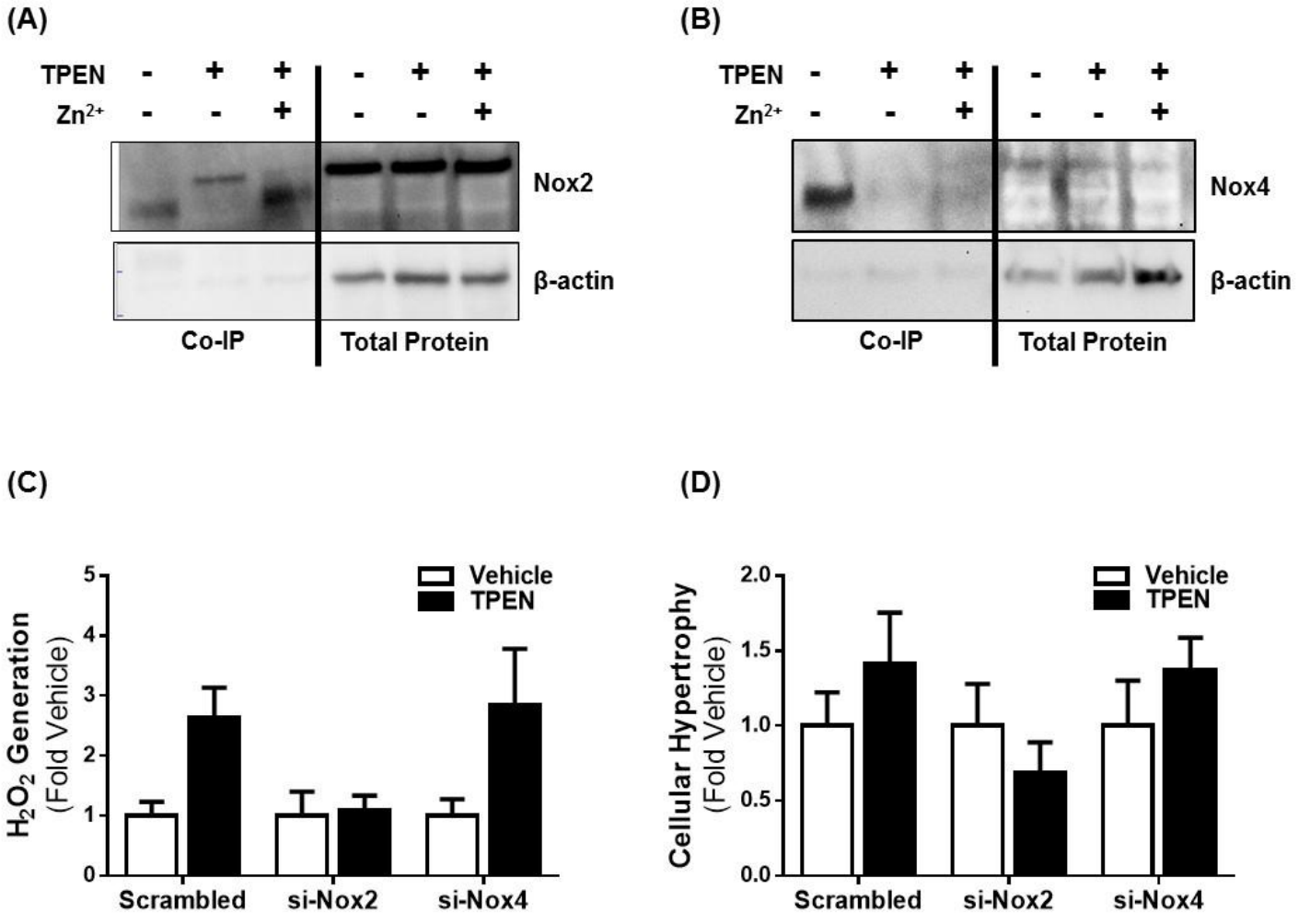

Figure 6. Nox2- and Nox4-containing NADPH oxidases mediate Zn^{2+} deficiency-induced oxidative stress and kidney cellular hypertrophy in mTECs. To identify the Nox subunit involved in Zn^{2+} deficiency-induced renal effects, mTECs were transfected with si-Nox or scrambled oligonucleotides prior to TPEN exposure. To identify the active Nox-containing NADPH oxidase, (**A**) Nox2 co-immunoprecipitation (co-IP) with $p67^{phox}$ and (**B**) Nox4 co-IP with Poldip2 was assessed. To confirm knock-down of Nox subunit, (**C**) ROS generation was examined by measuring H_2O_2 generation. (**D**) Cellular hypertrophy was assessed by calculating protein/DNA ratios.

	ZnA	ZnD
Body Weights (g)	26.60 ± 1.652	22.49 $\pm 1.678^{*}$
Kidney Weights (mg)	294.2 ± 31.73	301.7 ± 27.01
Kidney/Body Weights (mg/g)	10.65 ± 0.4429	14.07 ± 0.8885*

Table 1. Kidney and body weights of ZnA and ZnD mice.

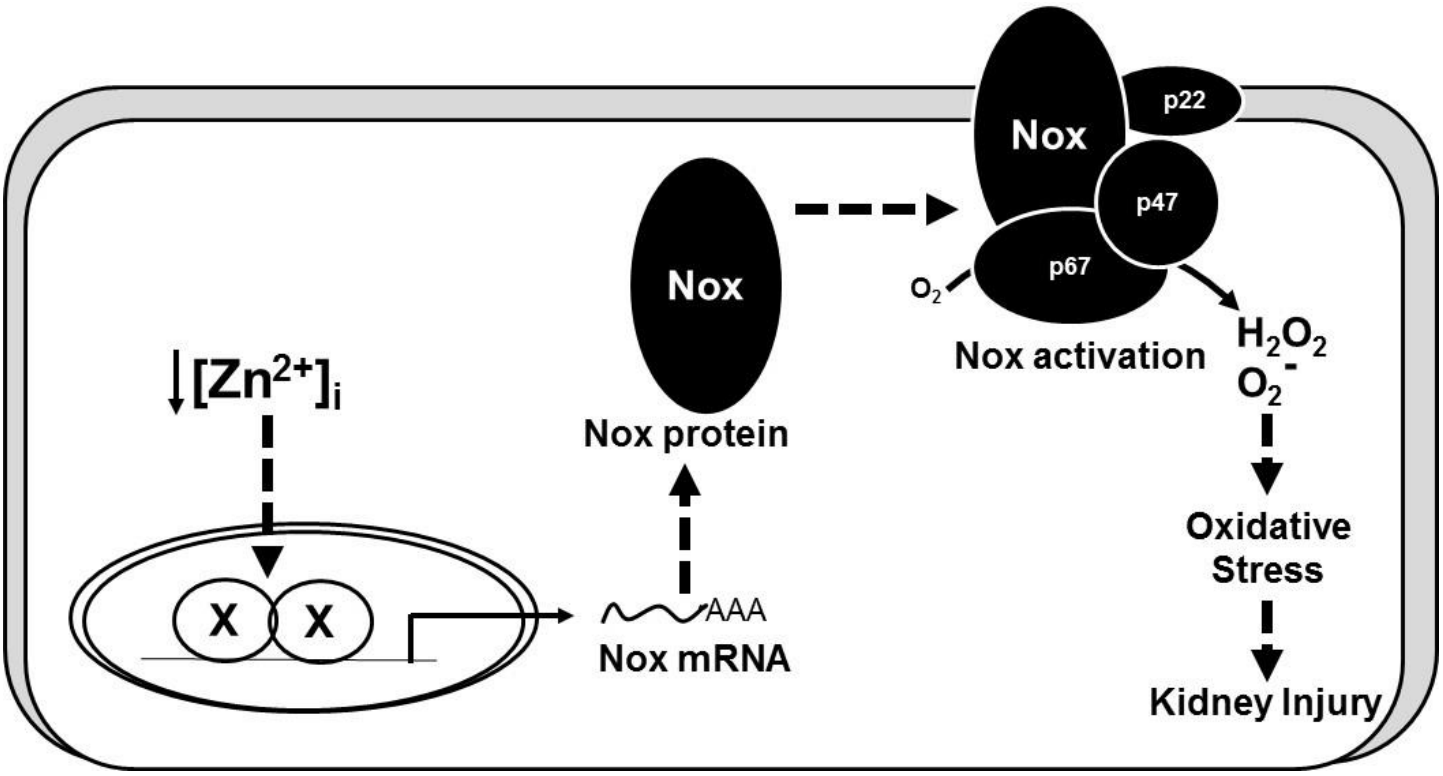

Figure 7. Proposed Schema. NADPH oxidases are Zn^{2+} regulated enzymes that mediate Zn^{2+} deficiency-induced oxidative stress and subsequent kidney injury.

DISCUSSION

Clinical and experimental studies reveal that chronic kidney disease, regardless of etiology, is accompanied by ZnD. It is well established that ZnD exacerbates diabetic kidney damage, in part, by promoting oxidative stress (5). However, the renal effects of ZnD alone have not been investigated. Previous studies have determined that antioxidant pathways are down-regulated in response to ZnD, thereby contributing to oxidative stress (22). However, the effects of ZnD on pro-oxidant pathways continue to be defined. Since NADPH oxidases are the primary sources of ROS generation in the kidney, this study tested the hypothesis that reduced Zn^{2+} bioavailability induces NADPH oxidase upregulation, contributing to elevated ROS levels and subsequent kidney injury. Using *in vivo* and *in vitro* models of ZnD, our novel findings demonstrate that Nox2 containing NADPH oxidase is a Zn^{2+}-regulated enzyme that plays a central role in ZnD-induced oxidative stress and early kidney injury (Figure 7).

Studies have shown that in cardiomyocytes, ischema and reperfusion injury is attributed, in part, to ZnD (23). In this model, Nox2 expression and $p47^{phox}$ phosphorylation are enhanced. These changes are prevented in cells with Zn^{2+}supplementation (23) Furthermore, increased $p67^{phox}$ activation is observed in models of Zn^{2+} toxicity (24). Specifically, Matsunaga et al. demonstrated $p67^{phox}$ translocation in response to toxic Zn^{2+} levels in renal proximal tubular cells (24). Taken together, these findings suggest that NADPH oxidases are modulated by Zn^{2+}. However, direct effects of Zn^{2+} on NADPH oxidase regulation had not been investigated. Using *in vivo* and *in vitro* models of Zn^{2+} depletion and repletion, this study directly demonstrates that NADPH oxidases are modulated by intracellular Zn^{2+} bioavailability. *In vivo* findings demonstrate that ZnD is accompanied by enhanced Nox2 expression and ROS generation (Figure 2A-D). To further investigate the role of Zn^{2+} bioavailability on Nox regulation, tubular epithelial cells were treated with the intracellular Zn^{2+} chelator TPEN. Consistent with *in vivo* results, *in vitro* findings

reveal that intracellular Zn^{2+} depletion promotes Nox2 upregulation and activity, which is reversed with Zn^{2+} repletion (Figure 5A and 6A). These data are the first to directly demonstrate that Nox2-containing NADPH oxidase is a Zn^{2+}-regulated enzyme.

Oxidative stress is one of the etiologies that contribute to increased mortality and poor nutritional status of patients with CKD. CKD patients are often ZnD. Using *in vivo* and *in vitro* models of Zn^{2+} depletion and repletion, this study directly demonstrates that ROS levels are modulated by intracellular Zn^{2+} bioavailability. *In vivo* findings demonstrate that ZnD is accompanied by increased H_2O_2 in kidneys and urine (Figure 2A-B). Consistent with our *in vivo* findings, *in vitro* results demonstrate that intracellular Zn^{2+} depletion stimulates H_2O_2 generation (Figure 3C). Interestingly, levels of $O_2^{\bullet-}$ were decreased with TPEN treatment and remained decreased with Zn^{2+} supplementation or DPI treatment (Figure 3D). One reason why changes in $O_2^{\bullet-}$ are not seen could be because $O_2^{\bullet-}$ is short-lived due to its high reactivity, and is converted into H_2O_2 via superoxide dismutase. Furthermore, most of signaling that occurs as a result of $O_2^{\bullet-}$ is mediated by its more stable dismutation product, H_2O_2 (14). This is consistent with increases in H_2O_2 that are seen with TPEN treatment.

Previous studies have shown that chronic renal failure is associated with elevated Nox expression and increased ROS generation (25). Currently, it is unclear whether the Nox2 or Nox4 isoform is responsible for ROS-dependent tissue damage (18). Using a pharmacological and molecular approach, this study demonstrates that Nox2-containingNADPH oxidase mediates ZnD-induced oxidative stress. *In vitro* findings demonstrate that Nox inhibition via DPI attenuates Zn^{2+} depletion-induced H_2O_2 generation but not $O_2^{\bullet-}$ (Figure 4A-B). Moreover, knockdown of Nox2 via si-RNA inhibits H_2O_2 generation (Figure 6C). These data indicate that Nox2 is the isoform responsible for ROS generation.

Microalbuminuria is an early derangement in CKD patients (7). *In vivo* findings reveal that ZnD is accompanied by proteinuria and microalbuminuria, markers of kidney injury (Figure 1A-B). Furthermore, ZnD is also accompanied by kidney hypertrophy (Table 1), which is an early event that occurs in kidney injury that predicts later loss of kidney function (26). Consistent with these results, *in vitro* findings reveal that si-RNA knockdown of Nox2 reverses Zn^{2+} depletion-induced cellular hypertrophy (Figure 6D). These data are the first to directly demonstrate that Nox2-containing NADPH oxidase mediates ZnD-induced kidney injury.

Our findings reveal that Nox2-containing NADPH oxidase is a Zn^{2+}-regulated enzyme that plays a central role in Zn^{2+} deficiency-induced oxidative stress (Figure 7). This study is the first to identify Nox enzymes as a source of ZnD-induced oxidative stress and early kidney injury. Using a molecular approach, we demonstrated that Nox2 mediates ZnD-induced sustained ROS generation and kidney injury. Taken together, these results reveal that NADPH oxidases are Zn^{2+}-regulated enzymes that play a critical role in ZnD-induced oxidative stress and kidney injury. Our findings show that targeting Nox2-containing NADPH oxidase may be an important intervention in CKD damage.

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