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Chronic NF- κ B blockade reduces cytosolic and mitochondrial oxidative stress and attenuates renal injury and hypertension in SHR

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Elks CM, Mariappan N, Haque M, Guggilam A, Majid DS, Francis J. Chronic NF- κ B blockade reduces cytosolic and mitochondrial oxidative stress and attenuates renal injury and hypertension in SHR. *Am J Physiol Renal Physiol* 296: F298–F305, 2009. First published December 10, 2008; doi:10.1152/ajprenal.90628.2008.—Nuclear factor- κ B (NF- κ B) plays an important role in hypertensive renal injury; however, its roles in perpetuating mitochondrial oxidative stress and renal dysfunction remain unclear. In this study, we assessed the effects of chronic NF- κ B blockade with pyrrolidine dithiocarbamate (PDTC) on renal dysfunction and mitochondrial redox status in spontaneously hypertensive rats (SHR). PDTC (150 mg·kg body wt⁻¹·day⁻¹) or vehicle was administered orally to 8-wk-old SHR and their respective controls for 15 wk. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography at the start of and at every third week throughout the study. After 15 wk of treatment, anesthetized rats underwent acute renal experiments to determine renal blood flow and glomerular filtration rate using PAH and inulin clearance techniques, respectively. Following renal experiments, kidneys were excised from killed rats, and cortical mitochondria were isolated for reactive oxygen species (ROS) measurements using electron paramagnetic resonance. Tissue mRNA and protein levels of NF- κ B and oxidative stress genes were determined using real-time PCR and immunofluorescence or Western blotting, respectively. PDTC treatment partially attenuated the increase in SBP (196.4 ± 9.76 vs. 151.4 ± 2.12; *P* < 0.05) and normalized renal hemodynamic and excretory parameters and ATP production rates in SHR. PDTC treatment also attenuated the higher levels of cytosolic and mitochondrial ROS generation and tissue mRNA and protein expression levels of NF- κ B and oxidative stress genes in SHR without any comparable responses in control rats. These findings suggest that NF- κ B activation by ROS induces the cytosolic and mitochondrial oxidative stress and tissue injury that contribute to renal dysfunction observed in SHR.

glomerular filtration rate; mitochondria; reactive oxygen species

HYPERTENSION-INDUCED KIDNEY disease is a significant cause of morbidity and mortality in hypertensive patients (15). Current antihypertensive treatments are mostly effective in reducing the severity of hypertensive renal disease; however, the progressive clinical course of the disease underscores the need for additional novel therapies. The progression of hypertensive kidney disease depends not only on neurohormones, such as norepinephrine and aldosterone, but also on increased proinflammatory cytokine (PIC) and reactive oxygen species (ROS) production, and on nuclear factor- κ B (NF- κ B) activation (11,

39). Increased production of ROS, which include superoxide and hydrogen peroxide, is a particularly detrimental aspect of renal disease progression (39). The major producers of these ROS include plasma membrane-bound NAD(P)H oxidases and mitochondria.

Mitochondria are critical modulators of ATP generation and redox-dependent intracellular signaling. The mitochondrial respiratory chain continuously releases ROS during oxidative phosphorylation. Approximately 90% of the cellular oxidative burden is attributed to mitochondrial ROS, thus signifying the role of mitochondria in cellular ROS production (1). In normal physiological conditions, small amounts of ROS are needed for critical cellular processes; however, excessive ROS production causes oxidative damage and is associated with hypertension (27, 28) and other diseases. The contributions of ROS to the regulation of intracellular signaling pathways, including NF- κ B activation, are already known. Excess ROS activate the redox-sensitive transcription factor NF- κ B, causing increases in its activity and expression (13, 33). Increased activity and expression of NF- κ B induces gene transcription for PIC, such as TNF- α , IL-1 β , and IL-6, to increase their production (13, 32). Increased levels of PIC, along with adhesion molecules, lead to macrophage infiltration of the tubulointerstitium and inflammation of renal tissue (30, 37). However, the roles of NF- κ B and of ROS in modulating renal function and tissue injury in hypertensive renal damage have not yet been examined.

Evidence from our laboratory indicates that peripheral TNF- α administration increases ROS production in rat myocardial tissue and mitochondria (23). Findings from other laboratories also indicate that TNF- α augments ROS production in liver mitochondria and endothelial cells (4, 8). IL-6-dependent ROS production has been noted in fibroblasts and in endothelial cells (18, 36); and fibroblasts have previously been shown to release ROS in response to IL-1 β and TNF- α (24). Taken together, these data support a role for NF- κ B-regulated PIC in cytosolic and mitochondrial ROS production in a variety of tissues. Therefore, it is plausible to suggest that NF- κ B blockade (and, therefore, blockade of PIC gene transcription) may improve the redox status of hypertensive renal cortical tissue and mitochondria.

Renal inflammation is thought to be a key mediator in the development and progression of hypertension, and compelling evidence suggests that ROS overproduction and NF- κ B activation promote glomerular and tubulointerstitial inflammation

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in rat models of hypertension (22, 40). Furthermore, blockade of NF- κ B or of ROS has demonstrated both antihypertensive and anti-inflammatory effects in rats (25, 31, 38). However, the effects of NF- κ B blockade on mitochondrial ROS and the roles of these ROS in modulating renal function and tissue injury in hypertension are unknown. The aim of this study was to examine the effects of long-term NF- κ B blockade on cortical cytosolic and mitochondrial ROS production and on renal function during hypertension and to investigate the contributions of these ROS to hypertensive renal injury. We hypothesized that long-term NF- κ B blockade with pyrrolidine dithiocarbamate (PDTC) would decrease both cytosolic and mitochondrial ROS production in the kidney cortex, thereby protecting the kidneys from damage, and resulting in improved renal function parameters in spontaneously hypertensive rats (SHR).

MATERIALS AND METHODS

An expanded materials and methods section can be found in the accompanying data supplement (all supplementary material for this article is available at the journal website).

All experimental procedures with animals were approved by the Louisiana State University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Experimental Protocol

Eight-week-old male Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR), obtained from Harlan (Indianapolis, IN), with initial body weights of between 200 and 250 g, were used in this study. Animals were housed in temperature ($23 \pm 2^\circ\text{C}$)- and light-controlled (12:12-h light-dark cycle) animal quarters. Four groups of six rats each were used: WKY, WKY+PDTC, SHR, and SHR+PDTC. All animals were allowed ad libitum access to standard rodent chow (LabDiet; Purina Mills, St. Louis, MO). Control animals were given access to tap water ad libitum; PDTC-treated rats were allowed ad libitum access to tap water with PDTC added. Beginning at 8 wk of age, the appropriate groups of rats were treated with PDTC dissolved daily in drinking water for 15 wk. At the end of *week 13*, rats were placed into metabolic cages for a 1-wk acclimatization period. Immediately following acclimatization, daily urine output was measured for 1 wk. Animals were killed at the end of *week 15*, after completion of renal clearance experiments. Plasma and kidney tissues were collected for later analyses.

Blood Pressure Measurement

Blood pressures for all animals were measured by tail-cuff plethysmography as previously described (35).

Renal Clearance Experiments

At the end of 15-wk treatment, acute clearance experiments were performed to determine renal function in anesthetized (thiobutobarbital, 50 mg/kg ip) rats, as described previously (17).

Biochemical Assays for Urine and Plasma

Albumin in urine was quantified with a Nephrot II ELISA kit (Exocell, Philadelphia, PA) and TNF- α , IL-6, and IL-1 β were quantified in plasma samples with ELISA kits from Biosource/Invitrogen (Carlsbad, CA). Creatinine and urea were quantified in plasma and urine with QuantiChrom Creatinine and Urea Assay Kits (BioAssay Systems, Hayward, CA).

Isolation of Mitochondria and Measurement of Mitochondrial Permeability Transition

Kidney mitochondria were isolated by differential centrifugation of renal cortical homogenates, and mitochondrial swelling was measured as described previously (23). Mitochondrial purity was determined by Western blotting with an anti-voltage-dependent anion channel (VDAC; a mitochondrial marker) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and by transmission electron microscopy (Fig. 1, A and B).

Measurement of NAD(P)H-Dependent Superoxide Anion Production

Lucigenin-enhanced chemiluminescence was used to measure NAD(P)H oxidase activity in kidney tissues according to the method of Li et al. (20).

Determination of Catalase Activity

Catalase activity was measured by the method of Beers and Sizer, as previously described (3, 23).

Determination of Glutathione Peroxidase Concentrations

Glutathione peroxidase concentrations were determined in cortex homogenates and in isolated mitochondria by use of a commercially available kit, according to the manufacturer's protocol (Cayman Chemicals, Ann Arbor, MI).

Measurement of ATP Production

Rates of ATP production were quantified in cortical mitochondria using a commercially available kit (BioVision, Mountain View, CA).

Western Blotting and EMSA

Protein expression in kidney cortical tissue was analyzed by Western blotting as previously described (35), using anti-p65, anti-p50,

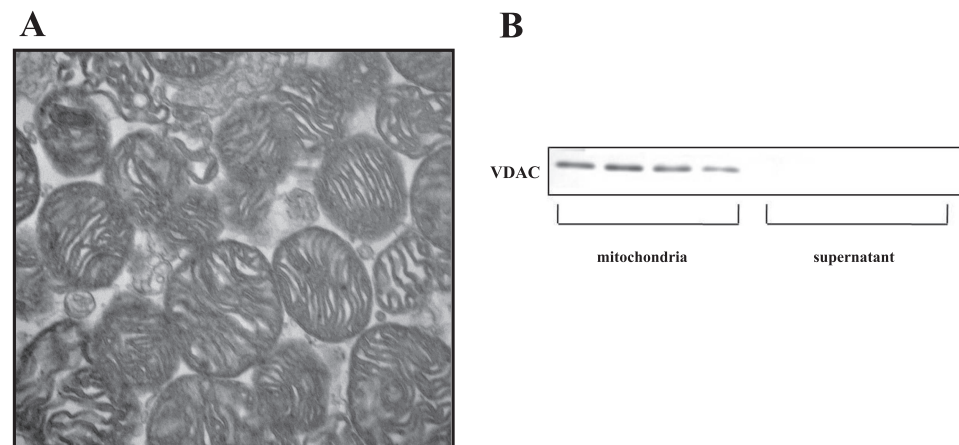


Fig. 1. Mitochondrial purity as determined by transmission electron microscopy (A) and Western blotting (B) for the mitochondrial marker voltage-dependent anion channel (VDAC). A representative electron micrograph is shown in A ($\times 40,000$).

Table 1. Mean final body weights and mean baseline and ending arterial and systolic pressures (MAP and SBP, respectively) of study groups

Group (n = 6 each)	Final Body Weight, g	MAP Baseline, mmHg	MAP Week 15, mmHg	SBP Baseline, mmHg	SBP Week 15, mmHg
WKY	384.7 \pm 6.08	99.5 \pm 1.96*†	94.5 \pm 2.84*	121.8 \pm 4.62*†	120.2 \pm 10.14*
WKY+PDTC	392.3 \pm 5.99	99.0 \pm 3.40*†	99.9 \pm 0.83*	112.8 \pm 3.42*†	119.5 \pm 6.65*
SHR	375.3 \pm 5.95	141.2 \pm 6.16	168.9 \pm 7.94†	160.4 \pm 8.23	196.4 \pm 9.76†
SHR+PDTC	387.5 \pm 9.26	132.7 \pm 10.12*	105.0 \pm 1.13*	161.4 \pm 8.30	151.4 \pm 2.12*

Values are means \pm SE; n = no. of animals. MAP, mean arterial pressure; SBP, systolic blood pressure. WKY, Wistar-Kyoto; PDTC, pyrrolidine dithiocarbamate; SHR, spontaneously hypertensive rat. Blood pressures were measured at baseline and every third week throughout the study using tail-cuff plethysmography. Pressures were measured at week 15 (study end) using a pressure transducer, at the time of acute renal experiments. Body weights were measured weekly for the duration of the study. * $P < 0.05$ vs. SHR. † $P < 0.05$ vs. SHR+PDTC.

anti-I κ B α , anti-TNF- α , anti-IL-6, and anti-IL-1 β antibodies (Santa Cruz Biotechnology). NF- κ B p65 DNA binding activity was assessed by EMSA, as previously described (21). Densitometric analyses were performed with Image J software.

RNA Isolation and Real-Time RT-PCR

Total RNA extraction, cDNA synthesis, and real-time RT-PCR were performed as previously described (35). Rat primers used appear in the data supplement (Table).

Immunofluorescence

Immunofluorescence detection of desmin, TNF- α , NOX2, and NOX4 was performed as previously described (16), with minor modifications (see data supplement).

Electron Paramagnetic Resonance Studies

All electron paramagnetic resonance (EPR) measurements were performed with a BenchTop EPR spectrophotometer e-scan R (Noxygen Science Transfer and Diagnostics, Elzach, Germany). Three different spin probes were used for EPR studies. 1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) was used to measure total tissue ROS and superoxide ($O_2^{\cdot-}$), and mitochondrial total ROS and $O_2^{\cdot-}$; 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH) was used for measurement of tissue peroxynitrite (OONO $^-$); and 1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine (PPH) was used for mitochondrial hydrogen peroxide (H_2O_2) studies.

Cortical tissue total ROS, $O_2^{\cdot-}$, and OONO $^-$ production. Pieces of kidney cortex were incubated at 37°C with 6.6 μ l of CMH (200 μ M) for 30 min for ROS measurement; CMH for 30 min, then 1.5 μ l of PEG-SOD (50 U/ μ l) for an additional 30 min for $O_2^{\cdot-}$ measurement; or 30 μ l of CPH (500 μ M) for 30 min for OONO $^-$ measurement. Aliquots of incubated probe media were then taken in 50- μ l disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics) for determination of total cortical ROS, $O_2^{\cdot-}$, or OONO $^-$ production, under the EPR settings that appear in the data supplement.

Mitochondrial total ROS, $O_2^{\cdot-}$, and H_2O_2 production. Aliquots of renal cortical mitochondria (~4–6 μ g protein) from each animal were probed with CMH for mitochondrial total ROS and $O_2^{\cdot-}$ measurements and with PPH (500 μ M) and catalase (50 U/ml) for H_2O_2 measurement. Superoxide detection in mitochondria was confirmed by inhibition of the $O_2^{\cdot-}$ signal with 50 U/ml SOD. After adequate mixing, 50 μ l mitochondria were taken in disposable glass capillary tubes. Mitochondrial total ROS, $O_2^{\cdot-}$, or H_2O_2 production was detected using EPR under the settings that appear in the data supplement.

Statistical Analyses

Data were analyzed with GraphPad Prism 5 software. All data are presented as means \pm SE. One-way repeated measures ANOVA with a post hoc Bonferroni procedure was used to compare differences in blood pressure measurements; one-way ANOVA with a Bonferroni

procedure was used to assess renal parameters, EPR measurements, antioxidant activities, and ATP production. Two-way ANOVA with a Bonferroni procedure was used in comparisons of NAD(P)H oxidase-dependent superoxide production rates and mitochondrial swelling assays. Results were considered significant when $P < 0.05$.

RESULTS

Body Weight, Mean Arterial Pressure, and SBP

No significant differences in body weights, food intake, or water intake were noted among animal groups while in standard housing or while in metabolic cages during this study. The average daily delivered dose of PDTC was ~85–105 $mg \cdot kg^{-1} \cdot day^{-1}$ in both the WKY and SHR treatment groups. WKY rats had significantly lower MAP and SBP than SHR at baseline and study end (Table 1). SHR+PDTC animals had lower SBP than SHR by week 6, and these values remained significantly lower than the values in SHR for the remainder of the study. Mean values for each time point appear in Fig. 2.

Glomerular Filtration Rate, Renal Blood Flow, Urine and Plasma Creatinine, and Urine Albumin

Significant decreases in glomerular filtration rate (GFR) and renal blood flow (RBF) were noted in SHR compared with other groups. Creatinine clearances (C_{Cr}), which were calculated to verify inulin clearance data, followed the same trend. Additionally, urine albumin, plasma creatinine, and blood urea nitrogen (BUN) were all significantly higher in SHR than in other groups. Mean values for each parameter appear in Table 2.

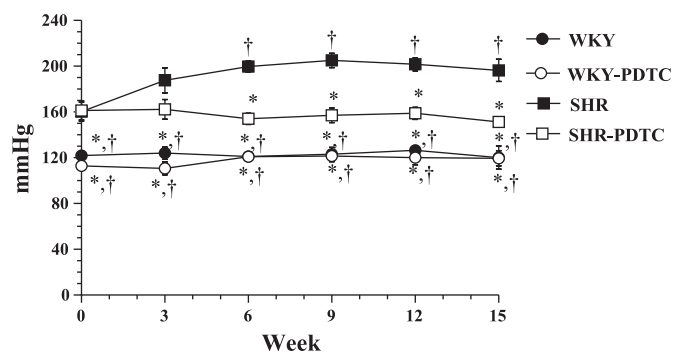


Fig. 2. Systolic blood pressure trends for each study group. WKY, Wistar-Kyoto; PDTC, pyrrolidine dithiocarbamate; SHR, spontaneously hypertensive rat. * $P < 0.05$ vs. SHR. † $P < 0.05$ vs. SHR+PDTC.

Table 2. Mean values of selected urine and plasma parameters for each study group

Groups (n = 6)	GFR, ml·min ⁻¹ ·g KW ⁻¹	RBF, ml·min ⁻¹ ·g KW ⁻¹	Plasma Cr, mmol/l	BUN, mmol/l	UAE, mmol·l ⁻¹ ·24 h ⁻¹	Alb/Cr
WKY	0.972±0.104*	9.70±0.791*	0.72±0.04*	16.74±1.13*	441.2±35.7*	0.320±0.022*
WKY+PDTC	1.020±0.049*	11.03±0.610*	0.49±0.13*	11.41±2.21*	345.2±25.7*	0.341±0.006*
SHR	0.641±0.043†	3.83±0.589†	1.71±0.24†	25.18±1.15†	925.6±33.3†	0.511±0.033†
SHR+PDTC	0.938±0.063*	7.70±0.804*	0.51±0.09*	16.78±0.86*	358.7±31.2*	0.334±0.029*

Values represented are means ± SE; n = no. of animals. KW, kidney weight; GFR, glomerular filtration rate; RBF, renal blood flow; Cr, creatinine; BUN, blood urea nitrogen; UAE, urine albumin excretion rate; Alb, albumin. *P < 0.05 vs. SHR. †P < 0.05 vs. SHR+PDTC.

Cortical Tissue and Mitochondrial Total ROS, O₂^{•-}, and OONO⁻ Production

Total ROS, O₂^{•-}, and OONO⁻ production rates in tissue and total ROS, O₂^{•-}, and H₂O₂ production rates in mitochondria, as determined by EPR, were all significantly higher in cortical tissues of untreated SHR than in WKY, WKY+PDTC, and SHR+PDTC animals (Table 3). NAD(P)H-dependent O₂^{•-} production, as measured by lucigenin assay, peaked in tissues from all groups at minute 2 of measurement (Supplemental Fig. 1). Peak values were significantly higher in the SHR group than in other groups.

Immunofluorescence, Western Blotting, mRNA, and EMSA

Protein expression levels of desmin (Fig. 3A), NOX4 (Fig. 3B), and NOX2 (gp91^{phox}; Fig. 3C), as determined by densitometric analysis, were all significantly lower in WKY, WKY+PDTC, and SHR+PDTC rats than in SHR (Fig. 3D); mRNA expression followed a similar trend (Table 4). Expression levels of NF-κB p65, p50, and IκBα followed similar trends (Figs. 4, A and B). SHR also exhibited significantly higher DNA binding activity of NF-κB p65 (Fig. 4C).

Cytokine Levels, Antioxidant Enzymes, Mitochondrial Membrane Integrity, and ATP Production

Plasma levels of IL-6 and TNF-α, but not IL-1β, were significantly higher in SHR than in WKY, WKY+PDTC, and SHR+PDTC animals (Fig. 5A). Protein and mRNA expression of these cytokines followed a similar trend (Table 4; Fig. 5, B–D). SHR also exhibited significantly lower activities of tissue catalase and tissue and mitochondrial glutathione peroxidase, along with significantly decreased ATP production [indicative of electron transport chain (ETC) damage] (Supplementary Fig. 2). Increased mitochondrial swelling (indicative of membrane damage) was also observed in SHR (Supplementary Fig. 3).

DISCUSSION

In this study, we examined the effects of chronic NF-κB blockade with PDTC on cortical tissue and mitochondrial ROS production in the hypertensive kidney. The salient findings of the present study are 1) cytosolic and mitochondrial oxidative stress, caused by upregulation of NF-κB and NF-κB-induced PIC, contribute to renal damage and hypertension in SHR; and 2) NF-κB blockade partially attenuates blood pressure, and normalizes renal function parameters and cytosolic and mitochondrial redox status in SHR. These data suggest that NF-κB plays a role in hypertensive injury in renal cortical tissue and mitochondria by increasing production of PIC and ROS and that long-term NF-κB blockade can ameliorate these detrimental effects.

We found significant decreases in MAP and SBP in SHR+PDTC rats compared with untreated SHR, and saw no change in MAP or SBP in WKY treated with PDTC. PDTC treatment also improved GFR, RBF, plasma creatinine and levels, BUN, and urinary albumin levels in SHR. Other researchers have used PDTC in hypertensive rat models and have found similar improvements in blood pressure and end-organ damage (12, 25, 29).

Renal and vascular oxidative stress are known to accompany hypertension in the SHR (19). Increased ROS production, the exact cause of which remains unknown, is thought to be both a cause and a consequence of hypertension (40). A number of mediators of this oxidative stress have been identified, including PIC (such as TNF-α and IL-6) and angiotensin II (ANG II); both can degrade IκBα to cause NF-κB activation and further increase ROS production. PDTC is believed to exert its inhibitory effects on NF-κB by directly impeding IκBα degradation (5).

PDTC treatment in SHR attenuated the upregulation of protein and mRNA expression of desmin (a marker of glomerular injury). Desmin expression in most rat strains is

Table 3. Mean production values for tissue total reactive oxygen species, superoxide, and peroxynitrite and for mitochondrial total ROS, O₂^{•-}, and hydrogen peroxide as measured by electron paramagnetic resonance

	Tissue, μmol·mg protein ⁻¹ ·min ⁻¹			Mitochondria, nmol·mg protein ⁻¹ ·min ⁻¹		
	Total ROS	O ₂ ^{•-}	OONO ⁻	Total ROS	O ₂ ^{•-}	H ₂ O ₂
WKY	0.2132±0.0375*	0.0103±0.0019*	0.00524±0.0101*	0.4386±0.0305*	0.0411±0.014*	0.0032±0.0001*
WKY+PDTC	0.1314±0.0329*	0.0206±0.0041*	0.00510±0.0157*	0.4981±0.0699*	0.0337±0.0124*	0.0031±0.0002*
SHR	0.4615±0.0779†	0.0925±0.0188†	0.01974±0.0392†	1.100±0.1246†	0.2505±0.0649†	0.0063±0.0005†
SHR+PDTC	0.2013±0.0318*	0.0193±0.0043*	0.00641±0.0444*	0.6385±0.0789*	0.0605±0.0166*	0.0041±0.0006*

Values represented are means ± SE; n = 6 animals/group. ROS, reactive oxygen species; O₂^{•-}, superoxide; OONO⁻, peroxynitrite; H₂O₂, hydrogen peroxide. *P < 0.05 vs. SHR. †P < 0.05 vs. SHR+PDTC.

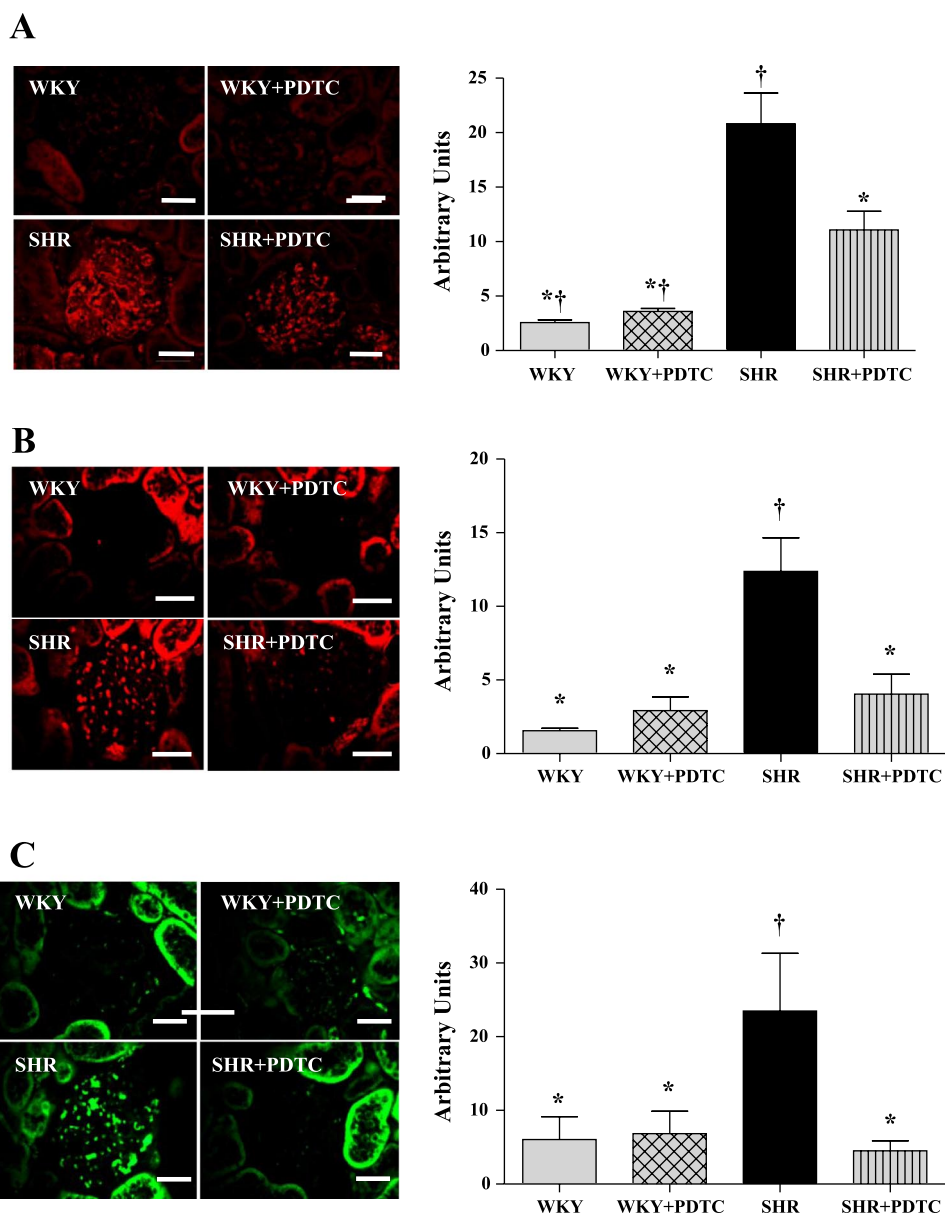


Fig. 3. Immunofluorescence staining and luminometric analysis for glomerular desmin (A), NAD(P)H oxidase (NOX)2 (gp91^{phox}, B), and NOX4 (C). Scale bars = 50 μ m. * P < 0.05 vs. SHR. † P < 0.05 vs. SHR+PDTC.

confined to mesangial cells; podocytes only express desmin following injury (14). ROS are known to alter several signaling cascades in podocytes (26). Furthermore, NF- κ B activation has been shown to upregulate ROS-induced inflammation in mouse podocytes (10). This evidence, along with our current findings, suggests a critical role for NF- κ B in glomerular epithelial injury. NAD(P)H oxidase is the predominant source of ROS production in the renal cortex, and the predominant NOX isoform expressed in the kidney cortex is NOX4 (9). PDTC administration also attenuated protein and mRNA expression of NOX2 (gp91^{phox}) and NOX4 in glomeruli. NAD(P)H-dependent O₂^{•-} production was also decreased in the cortical tissues of PDTC-treated SHR. Taken together, these results suggest that NF- κ B-mediated activation of NAD(P)H oxidases, and of desmin, contributes at both the transcriptional and translational levels to the renal damage seen in SHR.

Both mitochondria and NAD(P)H oxidases are important sources of ROS in cells. NAD(P)H oxidases are activated and upregulated in SHR before the onset of hypertension in this model (30). Although O₂^{•-} from NAD(P)H oxidases is considered the major player in glomerular injury, the possible contributory role of mitochondrial ROS in perpetuating renal dysfunction in hypertension cannot be ignored. Recently, Doughan et al. (7) demonstrated that the full enzymatic activity of NAD(P)H oxidase was required for ANG II-induced mitochondrial damage. Also in that study, NAD(P)H oxidase blockade with apocynin was shown to attenuate ANG II-induced mitochondrial damage in endothelial cells (7). The same effects on mitochondrial dysfunction were also seen in a study by De Cavanaugh et al. (6), which demonstrated decreased mitochondrial oxidant production and improved mitochondrial membrane potential in SHR with ANG II receptor blockade. These results suggest

Table 4. Gene expression values of desmin, NOX homologs, cytokines, I κ B α , and NF- κ B p65 in cortical tissues of WKY and SHR animals after 15 wk of PDTC treatment

Gene	WKY + PDTC	SHR	SHR + PDTC
Desmin	0.839 \pm 0.258*	1.737 \pm 0.111 \dagger	0.333 \pm 0.223*
NOX2	0.716 \pm 0.024*	3.428 \pm 0.693 \dagger	0.251 \pm 0.666*
NOX4	0.814 \pm 0.356*	2.970 \pm 0.291 \dagger	0.494 \pm 0.151*
TNF- α	0.695 \pm 0.050*	2.901 \pm 0.985 \dagger	1.038 \pm 0.147*
IL-1 β	0.893 \pm 0.025	1.091 \pm 0.125	0.873 \pm 0.416
IL-6	0.403 \pm 0.014* \dagger	1.834 \pm 0.183 \dagger	0.638 \pm 0.030*
I κ B α	1.261 \pm 0.080*	0.372 \pm 0.080 \dagger	1.381 \pm 0.197*
NF- κ B p65	0.785 \pm 0.148* \dagger	1.451 \pm 0.084 \dagger	0.112 \pm 0.022*

Values are means \pm SE expressed as fold-change ($2^{-\Delta\Delta Ct}$) vs. WKY control. NOX, NAD(P)H oxidase. * P < 0.05 vs. SHR. $\dagger P$ < 0.05 vs. SHR+PDTC.

that NAD(P)H oxidase-dependent $O_2^{\bullet-}$ can act as an upstream signal to cause increased mitochondrial $O_2^{\bullet-}$ production. This mitochondrial $O_2^{\bullet-}$, along with H_2O_2 which can diffuse out of the mitochondrion, can then act to further stimulate NAD(P)H activation in a feed-forward mechanism. Furthermore, $O_2^{\bullet-}$ produced from NAD(P)H oxidases can activate NF- κ B either directly, or indirectly through an increase in mitochondrial ROS production; however, further studies are needed to elucidate the degree of involvement of both mitochondria and NAD(P)H oxidases in the activation of this ROS-dependent transcription factor.

Until now, no studies have examined the effect of NF- κ B blockade on mitochondrial functionality in the hypertensive kidney. We employed EPR, a reliable and sensitive method of measuring and quantifying ROS production, to analyze production of various ROS in both tissue and isolated

mitochondria of experimental animals. As expected, PDTC treatment decreased production of total ROS, $O_2^{\bullet-}$, and $OONO^-$ as determined by EPR in the renal cortical tissue of SHR, thereby signifying the role of NF- κ B in tissue ROS production.

Mitochondrial total ROS, $O_2^{\bullet-}$, and H_2O_2 production rates were all significantly lower in PDTC-treated SHR. Mitochondrial membrane integrity and ATP production rates were also significantly improved in PDTC-treated SHR. Hypertension is associated with mitochondrial dysfunction in several tissues, including the heart and kidney (6, 27). ROS generated by the mitochondrial ETC may act as second messengers to the activation of NF- κ B by cytokines such as TNF- α (2). Also, cells lacking functional mitochondrial ETC show significant downregulation of NF- κ B activation (34), thus reinforcing a role for mitochondrial ROS in activation of NF- κ B. However, some caution must be used in interpreting these results, as isolated mitochondria were used. Further studies should employ in vivo mitochondrial blockade and ROS measurement; results from these procedures would allow us to provide more representative data in regard to the function of mitochondrial ROS in the hypertensive kidney as a whole.

NAD(P)H-dependent $O_2^{\bullet-}$ and other cytoplasmic ROS can also activate NF- κ B, causing a further increase in ROS production, which leads to increased mitochondrial ROS production and further perpetuates this vicious positive feedback cycle. In this study, increased activity and expression of NF- κ B and the cytokines TNF- α and IL-6 were associated with increased tissue ROS production (especially that of $O_2^{\bullet-}$), as determined by EPR and lucigenin assay. Increased NF- κ B and PIC expression were also associated

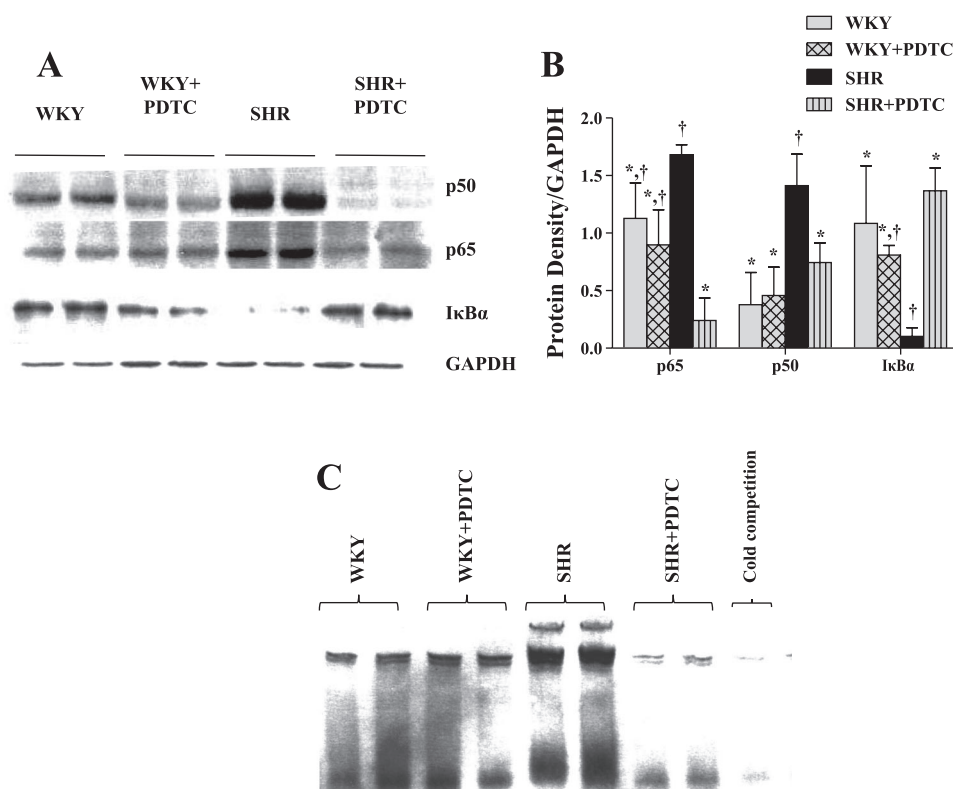


Fig. 4. A: representative Western blots for NF- κ B p65, p50, I κ B α , and GAPDH (house-keeping) in cortical tissues. B: densitometric analysis of expression levels of proteins in Fig. 3A expressed as protein density/GAPDH density ratio. C: EMSA for NF- κ B p65 DNA binding activity. * P < 0.05 vs. SHR; $\dagger P$ < 0.05 vs. SHR+PDTC.

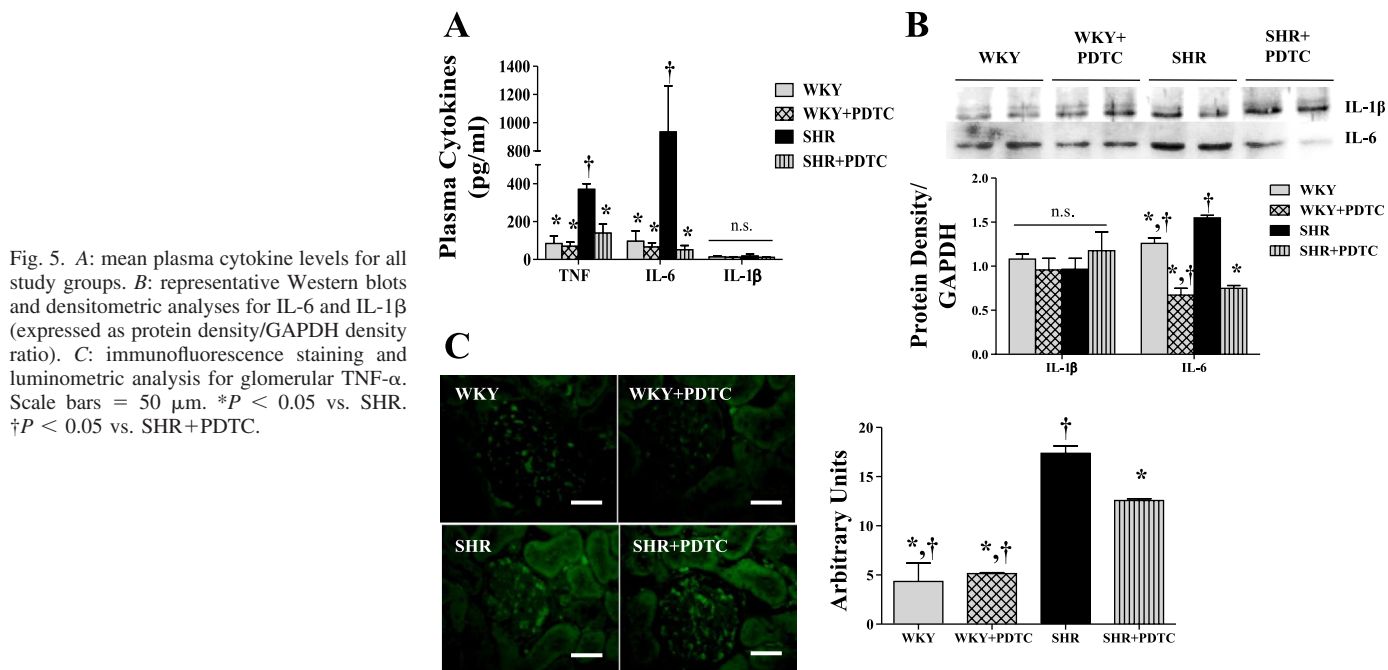


Fig. 5. *A*: mean plasma cytokine levels for all study groups. *B*: representative Western blots and densitometric analyses for IL-6 and IL-1 β (expressed as protein density/GAPDH density ratio). *C*: immunofluorescence staining and luminometric analysis for glomerular TNF- α . Scale bars = 50 μ m. * P < 0.05 vs. SHR. † P < 0.05 vs. SHR+PDTC.

with increased mitochondrial ROS production and decreased ATP production, both of which suggest a contributory role for mitochondrial dysfunction in hypertensive renal injury. Decreased ATP production is an indicator of ETC dysfunction, as is increased mitochondrial ROS production; damage to the ETC results in free radical leakage, thereby perpetuating mitochondrial damage and ROS production. Chronic NF- κ B blockade with PDTC may have attenuated increases in ROS by partially inhibiting the positive feedback between mitochondrial and cytosolic ROS, NF- κ B, and NF- κ B-regulated PIC in the hypertensive kidney.

Superoxide generated by the mitochondrial ETC can be converted to H₂O₂ in the mitochondrial matrix or in the intermembrane space. H₂O₂ can be detoxified to water by mitochondrial glutathione peroxidase, or to water and oxygen by catalase. These enzymes comprise a complex mitochondrial defense system that is critical in ROS detoxification. In this study, SHR exhibited lower activities of catalase and glutathione peroxidase, and an increased H₂O₂ production rate, as measured by EPR, indicating the impairment of the mitochondrial antioxidant defense system in the presence of increased NF- κ B activity. Long-term NF- κ B blockade with PDTC restored the activities of these antioxidant enzymes to near control levels in SHR. These results suggest that impairment of mitochondrial antioxidants, combined with overproduction of mitochondrial ROS, could contribute to the mitochondrial damage seen in hypertensive renal injury.

This study provides a first glance at the role of NF- κ B in mitochondrial ROS production in the hypertensive rat kidney. Although this study is the first to quantify mitochondrial ROS in the kidney cortex in the presence or absence of NF- κ B blockade, some limitations exist. First, our results suggest an obvious role for mitochondrial ROS in renal injury, but they do not allow us to determine the exact

source of mitochondrial ROS overproduction, or the degree of involvement of mitochondrial ROS, since mitochondrial inhibitors were not used. Second, we cannot exclude the possibility that the antioxidant properties of PDTC are also involved in the reduction of ROS production in our study. Last, there was a significant decrease in SBP (196.4 ± 9.76 vs. 151.4 ± 2.12 mmHg) with PDTC treatment; this also could have altered the antioxidant/oxidant parameters measured in this study, thereby contributing to the beneficial effects seen with PDTC. However, the EMSA results and mRNA and protein expression data showing decreased I κ B α and increased p65 and p50 in SHR kidney cortex suggest that direct inhibition of NF- κ B is responsible, at least in part, for the beneficial effects seen in this study. In an extension of this study that is currently ongoing, we are using mitochondrial inhibitors; this will allow us to better define the role of the mitochondrion in contributing to renal abnormalities in SHR.

In conclusion, the results of the present investigation support a possible role for mitochondrial ROS in hypertensive renal injury (in addition to cytosolic ROS) and suggest that NF- κ B-induced PIC negatively affect mitochondrial and tissue ROS production in the hypertensive renal cortex. Successful prevention of renal damage should therefore involve therapies that not only inhibit cytokine-induced NF- κ B activation but also offer mitochondrial protection from NF- κ B-induced PIC and ROS production. Future research should focus on the precise signaling mechanisms by which NF- κ B-induced PIC and mitochondrial ROS interact in the kidney in the setting of essential hypertension.

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REFERENCES

- Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell* 120: 483–495, 2005.
- Ballinger SW. Mitochondrial dysfunction in cardiovascular disease. *Free Radic Biol Med* 38: 1278–1295, 2005.
- Beers RF Jr, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* 195: 133–140, 1952.
- Corda S, Laplace C, Vicaut E, Duranteau J. Rapid reactive oxygen species production by mitochondria in endothelial cells exposed to tumor necrosis factor- α is mediated by ceramide. *Am J Respir Cell Mol Biol* 24: 762–768, 2001.
- Cuzzocrea S, Chatterjee PK, Mazzon E, Dugo L, Serraino I, Britti D, Mazzullo G, Caputi AP, Thiemermann C. Pyrrolidine dithiocarbamate attenuates the development of acute and chronic inflammation. *Br J Pharmacol* 135: 496–510, 2002.
- de Cavanagh EMV, Toblli JE, Ferder L, Piotrkowski B, Stella I, Inserra F. Renal mitochondrial dysfunction in spontaneously hypertensive rats is attenuated by losartan but not by amlodipine. *Am J Physiol Regul Integr Comp Physiol* 290: R1616–R1625, 2006.
- Doughan AK, Harrison DG, Dikalov SI. Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circ Res* 102: 488–496, 2008.
- García-Ruiz I, Rodríguez-Juan C, Díaz-Sanjuan T, del Hoyo P, Colina F, Muñoz-Yagüe T, Solís-Herruzo J. Uric acid and anti-TNF antibody improve mitochondrial dysfunction in ob/ob mice. *Hepatology* 44: 581–591, 2006.
- Gill PS, Wilcox CS. NADPH oxidases in the kidney. *Antioxid Redox Signal* 8: 1597–1607, 2006.
- Greiber S, Muller B, Daemisch P, Pavenstadt H. Reactive oxygen species alter gene expression in podocytes: induction of granulocyte macrophage-colony-stimulating factor. *J Am Soc Nephrol* 13: 86–95, 2002.
- Guijarro C, Egido J. Transcription factor- κ B (NF- κ B) and renal disease. *Kidney Int* 59: 415–424, 2001.
- Gupta S, Young D, Sen S. Inhibition of NF- κ B induces regression of cardiac hypertrophy, independent of blood pressure control, in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol* 289: H20–H29, 2005.
- Janssen-Heininger YMW, Poynter ME, Baeuerle PA. Recent advances towards understanding redox mechanisms in the activation of nuclear factor κ B. *Free Radic Biol Med* 28: 1317–1327, 2000.
- Johnson RJ, Iida H, Alpers CE, Majesky MW, Schwartz SM, Pritzl P, Gordon K, Gown AM. Expression of smooth-muscle phenotype by rat mesangial cells in immune-complex nephritis— α -smooth muscle actin is a marker of mesangial cell proliferation. *J Clin Invest* 87: 847–858, 1991.
- Kannel WB. Review of recent Framingham study hypertension research. *Curr Hypertens Rep* 2: 239–240, 2000.
- Khaleduzzaman M, Francis J, Corbin ME, McIlwain E, Boudreaux M, Du M, Morgan TW, Peterson KE. Infection of cardiomyocytes and induction of left ventricle dysfunction by neurovirulent polytropic murine retrovirus. *J Virol* 81: 12307–12315, 2007.
- Kopkan L, Castillo A, Navar LG, Majid DSA. Enhanced superoxide generation modulates renal function in ANG II-induced hypertensive rats. *Am J Physiol Renal Physiol* 290: F80–F86, 2006.
- Koren R, Rucker D, Kotestiano O, Liberman UA, Ravid A. Synergistic anticancer activity of 1,25-dihydroxyvitamin D₃ and immune cytokines: the involvement of reactive oxygen species. *J Steroid Biochem Mol Biol* 73: 105–112, 2000.
- Lazaro A, Gallego-Delgado J, Justo P, Esteban V, Osende J, Mezzano S, Ortiz A, Egido J. Long-term blood pressure control prevents oxidative renal injury. *Antioxid Redox Signal* 7: 1285–1293, 2005.
- Li YL, Gao L, Zucker IH, Schultz HD. NADPH oxidase-derived superoxide anion mediates angiotensin II-enhanced carotid body chemoreceptor sensitivity in heart failure rabbits. *Cardiovasc Res* 75: 546–554, 2007.
- Liu D, Gao L, Roy SK, Cornish KG, Zucker IH. Neuronal angiotensin II type 1 receptor upregulation in heart failure: activation of activator protein 1 and Jun N-terminal kinase. *Circ Res* 99: 1004–1011, 2006.
- Liu SF, Ye X, Malik AB. Inhibition of NF- κ B activation by pyrrolidine dithiocarbamate prevents in vivo expression of proinflammatory genes. *Circulation* 100: 1330–1337, 1999.
- Mariappan N, Soorappan RN, Haque M, Sriramula S, Francis J. TNF- α -induced mitochondrial oxidative stress and cardiac dysfunction: restoration by superoxide dismutase mimetic Tempol. *Am J Physiol Heart Circ Physiol* 293: H2726–H2737, 2007.
- Meier B, Radeke HH, Selle S, Younes M, Sies H, Resch K, Habermehl GG. Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor- α . *Biochem J* 263: 539–545, 1989.
- Muller DN, Dechend R, Mervaala EMA, Park JK, Schmidt F, Fiebeler A, Theuer J, Breu V, Ganten D, Haller H, Luft FC. NF- κ B inhibition ameliorates angiotensin II-induced inflammatory damage in rats. *Hypertension* 35: 193–201, 2000.
- Pavenstadt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* 83: 253–307, 2003.
- Piotrkowski B, Fraga CG, de Cavanagh EMV. Mitochondrial function and nitric oxide metabolism are modified by enalapril treatment in rat kidney. *Am J Physiol Regul Integr Comp Physiol* 292: R1494–R1501, 2007.
- Puddu P, Puddu GM, Cravero E, De Pascalis S, Muscari A. The putative role of mitochondrial dysfunction in hypertension. *Clin Exp Hypertens* 29: 427–434, 2007.
- Rodríguez-Iturbe B, Ferrebuz A, Vanegas V, Quiroz Y, Mezzano S, Vaziri ND. Early and sustained inhibition of nuclear factor- κ B prevents hypertension in spontaneously hypertensive rats. *J Pharmacol Exp Ther* 315: 51–57, 2005.
- Rodríguez-Iturbe B, Quiroz Y, Ferrebuz A, Parra G, Vaziri ND. Evolution of renal interstitial inflammation and NF- κ B activation in spontaneously hypertensive rats. *Am J Nephrol* 24: 587–594, 2004.
- Rodríguez-Iturbe B, Quiroz Y, Nava M, Bonet L, Chavez M, Herrera-Acosta J, Johnson RJ, Pons HA. Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats. *Am J Physiol Renal Physiol* 282: F191–F201, 2002.
- Sanlioglu S, Williams CM, Samavati L, Butler NS, Wang G, McCray PB Jr, Ritchie TC, Hunninghake GW, Zandi E, Engelhardt JF. Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor- α secretion through IKK regulation of NF- κ B. *J Biol Chem* 276: 30188–30198, 2001.
- Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF- κ B transcription factor and HIV-1. *EMBO J* 10: 2247–2258, 1991.
- Schulze-Osthoff K, Beyaert R, Vandevoorde V, Haegeman G, Fiers W. Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO J* 12: 3095–3104, 1993.
- Sriramula S, Haque M, Majid DSA, Francis J. Involvement of tumor necrosis factor- α in angiotensin II-mediated effects on salt appetite, hypertension, and cardiac hypertrophy. *Hypertension* 51: 1345–1351, 2008.
- Sung JY, Hong JH, Kang HS, Choi I, Lim SD, Lee JK, Seok JH, Lee JH, Hur GM. Methotrexate suppresses the interleukin-6 induced generation of reactive oxygen species in the synoviocytes of rheumatoid arthritis. *Immunopharmacology* 47: 35–44, 2000.
- Vaziri ND. Causal link between oxidative stress, inflammation, and hypertension. *Iran J Kidney Dis* 2: 1–10, 2008.
- Welch WJ, Blau J, Xie H, Chabrashvili T, Wilcox CS. Angiotensin-induced defects in renal oxygenation: role of oxidative stress. *Am J Physiol Heart Circ Physiol* 288: H22–H28, 2005.
- Wilcox CS. Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? *Am J Physiol Regul Integr Comp Physiol* 289: R913–R935, 2005.
- Wilcox CS, Welch WJ. Oxidative stress: cause or consequence of hypertension. *Exp Biol Med* 226: 619–620, 2001.