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RESEARCH ARTICLE

The synthetic triterpenoid RTA dh404 (CDDO-dhTFEA) restores Nrf2 activity and attenuates oxidative stress, inflammation, and fibrosis in rats with chronic kidney disease

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Abstract

1. Chronic oxidative stress and inflammation are major mediators of chronic kidney disease (CKD) and result in impaired activation of the cytoprotective transcription factor Nrf2. Given the role of oxidative stress and inflammation in CKD pathogenesis, strategies aimed at restoring Nrf2 activity may attenuate CKD progression.
2. The present study investigated whether the synthetic triterpenoid RTA dh404 (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid-9,11-dihydro-trifluoroethyl amide or CDDO-dhTFEA) would afford renal protection in a 5/6 nephrectomized rat model of CKD. RTA dh404 (2 mg/kg/day) was orally administered once daily for 12 weeks after 5/6 nephrectomy surgery.
3. The remnant kidneys from the vehicle-treated CKD rats showed activation of nuclear factor kappaB (NF-κB), upregulation of NAD(P)H oxidase, glomerulosclerosis, interstitial fibrosis and inflammation, as well as marked reductions in Nrf2 and its target gene products (i.e. catalase, heme oxygenase-1, thioredoxin 1, thioredoxin reductase 1 and peroxiredoxin 1). The functional and structural deficits in the kidney were associated with increased (~30%) mean arterial pressure (MAP). Treatment with RTA dh404 restored MAP, increased Nrf2 and expression of its target genes, attenuated activation of NF-κB and transforming growth factor-β pathways, and reduced glomerulosclerosis, interstitial fibrosis and inflammation in the CKD rats.
4. Thus, chronic treatment with RTA dh404 was effective in restoring Nrf2 activity and slowing CKD progression in rats following 5/6 nephrectomy.

Keywords

Keap1, NF-κB, nephrectomy, reactive oxygen species

History

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Introduction

Oxidative stress and inflammation play a critical role in the pathogenesis and progression of chronic kidney disease (CKD) (Fujihara et al., 2007; Manning et al., 2005; Quiroz et al., 2008; Remuzzi et al., 2006; Vaziri et al., 1998). Under physiological conditions, oxidative and electrophilic stress elicit upregulation of endogenous antioxidant and cytoprotective enzymes and proteins, which all work in concert to prevent and limit inflammation, tissue injury and dysfunction, a process controlled primarily by activation of the transcription factor nuclear factor-erythroid-2-related factor 2 (Nrf2) (Li et al., 2008; Wakabayashi et al., 2010).

Unfortunately, Nrf2 activity and expression of its target gene products are markedly reduced in the kidney in different models of CKD, including Imai rats with spontaneous focal segmental glomerulosclerosis (Kim et al., 2011), rats with

CKD induced by 5/6 nephrectomy (Kim & Vaziri, 2010) and adenine-induced chronic interstitial nephropathy (Aminzadeh et al., 2013). Given the central role of Nrf2 in the regulation of cellular antioxidant and anti-inflammatory machinery (Yoh et al., 2001, 2008), strategies aimed at restoring Nrf2 activity are effective in preventing or hindering CKD progression (Ruiz et al., 2013). In fact, a number of studies have shown salutary effects of natural compounds with weak Nrf2 activating properties such as curcumin, the green tea polyphenol (–)-epigallocatechin-3-gallate, resveratrol, anthraquinone and ankaflavin, as well as the synthetic antioxidant tertbutylhydroquinone, in experimental animals with CKD of diverse etiologies (Ghosh et al., 2009; Lee et al., 2012; Li et al., 2011; Palsamy & Subramanian, 2011; Peng et al., 2011; Soetikno et al., 2013; Tapia et al., 2012; Tsai et al., 2011). As the 5/6 nephrectomy model is the one that produces pressure overload, it is worthy to note that blood pressure was lowered in the above studies which included it as a measured parameter (Ghosh et al., 2009; Soetikno et al., 2013).

Bardoxolone methyl and its analogs are oleanolic acid-derived synthetic triterpenoid compounds which potently

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activate the Nrf2-Keap1 pathway (Dinkova-Kostova et al., 2005; Liby et al., 2007). Via interaction with the Nrf2 repressor molecule, Keap1, bardoxolone methyl and its analogs promote translocation of Nrf2 to the nucleus, where it binds to antioxidant response elements in the promoter region of its target genes, leading to induction of many antioxidant and cytoprotective enzymes and related proteins (Ichikawa et al., 2009; Sporn et al., 2011; Yates et al., 2007). Bardoxolone methyl and its analogs are also potent inhibitors of the nuclear factor kappaB (NF- κ B) inflammatory pathway through both direct (i.e. inhibition of IKK β kinase activity) and indirect mechanisms (i.e. detoxification of reactive oxygen species) (Liby & Sporn, 2012). Previous studies have shown that bardoxolone methyl and its analog, RTA 405 (or CDDO-ethyl amide), ameliorate murine ischemic acute kidney injury and attenuate renal interstitial inflammation and fibrosis in mice with protein overload proteinuria (Wu et al., 2011). It is now recognized that rodent-specific adverse metabolite formation precludes chronic dosing of bardoxolone methyl in rodents, and therefore, analogs where such adverse metabolite formation does not occur must be used for longer term studies (Reisman et al., 2012, 2013). Additionally, in a phase 2, double-blind, randomized, placebo-controlled trial enrolling patients with type 2 diabetes and mild to moderate CKD, bardoxolone methyl-treatment for 52 weeks resulted in a significant increase in the estimated glomerular filtration rate (Pergola et al., 2011). However, due to unforeseen complications, the nature of which is currently under investigation, the BEACON trial (de Zeeuw et al., 2013), a large randomized, placebo-controlled, phase 3 study which had enrolled patients with type 2 diabetes and stage 4 CKD was terminated.

The specific reason for the early termination of the BEACON trial has not yet been publicly communicated. Notably, many previously conducted rodent studies with bardoxolone methyl analogs and a one-year non-human primate study demonstrated tolerability and efficacy at markedly higher doses than used in the BEACON trial (20 mg/day) (Chin et al., 2013; Reisman et al., 2012, 2013; Xing et al., 2012). Moreover, the maximum-tolerated dose described in a phase 1 oncology trial with bardoxolone methyl was 900 mg/day (Hong et al., 2012). Non-clinically, there are conflicting reports of tolerability to bardoxolone methyl analogs in Zucker diabetic fatty (ZDF) rats, where one study by Zoja et al. (2013) demonstrated poor tolerability characterized by kidney and liver injury. In a separate report by Chin et al. (2013), chronic administration of either RTA dh404 or RTA 405 was well-tolerated by ZDF rats, streptozotocin-induced type I diabetic rats, and high fat diet-induced obese mice, while also producing salutary effects. With the data presented by Chin et al. strongly questioning the interpretation and relative importance of the results published by Zoja et al., further studies investigating the tolerability of bardoxolone methyl analogs in non-clinical models are warranted. Therefore, the present study was undertaken to determine the effects of long-term (12 weeks) administration of RTA dh404 on kidney structure and function, as well as oxidative, inflammatory, fibrosis and Nrf2 pathways in Sprague-Dawley rats with CKD induced by 5/6 nephrectomy.

Methods

Materials

All reagents, unless otherwise specified, were purchased from Sigma Aldrich (St. Louis, MO). RTA dh404 was synthesized and provided by Reata Pharmaceuticals, Inc. (Irving, TX). The chemical name for RTA dh404 is CDDO-9,11-dihydro-trifluoroethyl amide (CDDO-dhTFEA).

Animals

Male Sprague-Dawley rats, weighing 225–250 g, were purchased from Harlan Laboratories (Indianapolis, IN). They were housed in a climate-controlled and light-regulated facility with 12:12-h day-night cycles. The animals were fed regular rat chow (Purina Mills, Brentwood, MO) and water *ad libitum* and randomly assigned to the CKD or normal control groups. The animals assigned to the CKD group were subjected to 5/6 nephrectomy by surgical resection using dorsal incisions, as described previously (Vaziri et al., 2007). The animals assigned to the control group were subjected to sham operation ($n = 6$). All surgical procedures were carried out, while the animals were under general anesthesia (ketamine/xylazine, 100/5 mg/kg, i.p.). Rats with CKD were orally administered RTA dh404 (2 mg/kg) or vehicle (sesame oil) once daily for 12 weeks ($n = 9$ /group) starting immediately prior to surgery. The selection of the 2 mg/kg/day dose was based on a previous study where similar oral doses of the RTA dh404 analog CDDO-Im produced significant induction of Nrf2 targets in kidneys of mice and afforded significant protection from cisplatin-induced nephrotoxicity (Aleksunes et al., 2010). At the conclusion of the observation period, the animals were placed in metabolic cages for a 24-h urine collection. They were then anesthetized (ketamine/xylazine, 100/5 mg/kg, i.p.) and euthanized by exsanguination using cardiac puncture. The kidneys were immediately removed and processed for histological evaluation, according to standard techniques for using hematoxylin and eosin (H&E) and periodic acid Schiff (PAS)-staining, as well as western blot analyses. Serum creatinine, urine protein and hematocrit were determined using standard laboratory procedures. Blood pressure was determined three days prior to sacrifice by tail plethysmography as described previously (Vaziri et al., 2002). Plasma malondialdehyde (MDA) was measured by the method of Naito & Yamanaka (1978). All experiments were approved by the University of California-Irvine Institutional Committee for the Use and Care of Experimental Animals.

Western blot analyses

Cytoplasmic and nuclear extracts were prepared as previously described (Kim et al., 2011). Target proteins in the cytoplasmic and nuclear fractions of the kidney tissue were measured by western blot analysis using the following antibodies: rabbit antibodies against rat NF- κ B p65, Nrf2, Keap1, modulatory and catalytic subunits of glutamate-cysteine ligase (Gclm and Gclc), cyclooxygenase-2 (COX-2) and heme oxygenase-1 (HO-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated I κ B- α (p-I κ B- α ; Cell Signaling Technology, Denver, CO), catalase (EMD Chemicals, Gibbstown, NJ), inducible nitric oxide

Table 1. General parameters.

	Control ^a	CKD ^a	CKD + RTA dh404 ^a
Body weight (g)	417 ± 4.2	391 ± 14*	397 ± 14.5*
Serum creatinine (mg/dL)	0.455 ± 0.033	0.794 ± 0.059*	0.743 ± 0.035*
Urine protein (mg/24 h)	10.2 ± 4.5	75.1 ± 13.4*	53.7 ± 4.8#
Urine volume (mL/24 h)	11.0 ± 1.5	20.1 ± 2.0*	21.2 ± 3.0*
Hematocrit (%)	43 ± 1	39 ± 1*	43 ± 1#
Mean arterial pressure (mmHg)	113 ± 5	146 ± 2*	119 ± 3#
Plasma MDA (μmol/L)	0.7 ± 0.1	1.4 ± 0.1*	0.9 ± 0.1#

^aValues are presented as mean ± standard error of the mean ($n = 5-7$ in each group).

* $p < 0.05$ versus control.

$p < 0.05$ versus CKD.

synthase (iNOS), 3-Nitrotyrosine (NT) (Abcam, Cambridge, MA), α -smooth muscle actin (α -SMA), transforming growth factor- β (TGF- β), SMAD7, phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich, St. Louis, MO), P22^{Phox}, gp91^{phox} and Rac1 (BD Bioscience, San Jose, CA) were purchased from the cited sources. Antibodies to histone H1 (Santa Cruz Biotechnology) and GAPDH were used for measurements of the housekeeping proteins for nuclear and cytosolic target proteins, respectively.

Briefly, aliquots containing 50 μ g proteins were fractionated on 8 and 4–20% Tris-glycine gel (Novex, San Diego, CA) at 120 V for 2 h and transferred to a Hybond-ECL membrane (Amersham Life Science, Arlington Heights, IL). The membrane was incubated for 1 h in blocking buffer

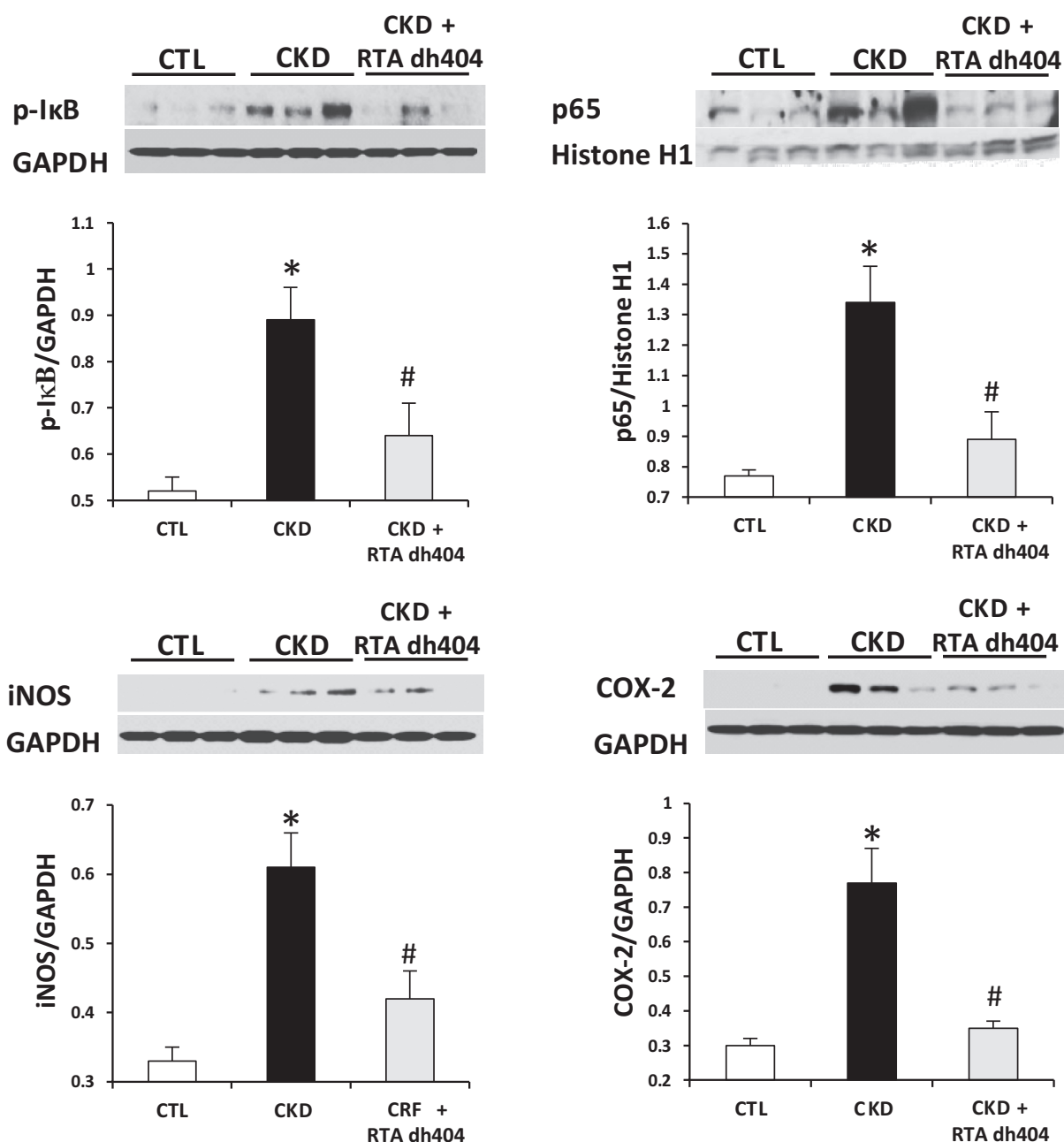


Figure 1. Representative western blots and group data depicting protein abundance of phospho-IκB and nuclear contents of p65 active subunit of NF-κB, iNOS and COX-2 in the renal tissues of sham-operated control (CTL; $n = 6$) and a 5/6 nephrectomized rat CKD treated with vehicle (CKD; $n = 9$) or RTA dh404 (CKD + RTA dh404; $n = 9$); * $p < 0.05$ versus CTL, # $p < 0.05$ versus CKD.

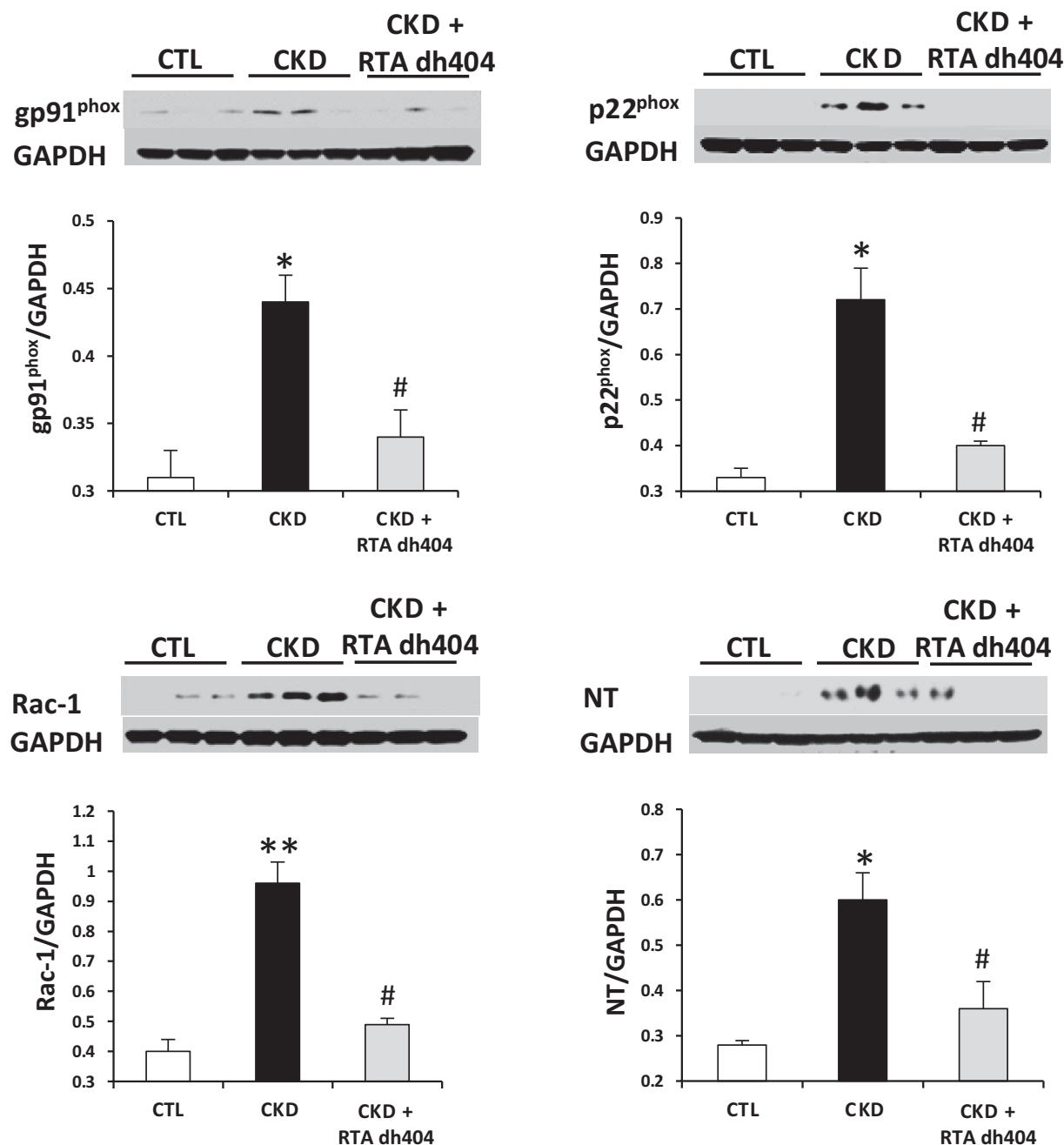


Figure 2. Representative western blots and group data depicting protein abundance of NAD(P)H oxidase subunits (p22^{phox}, gp91^{phox} and rac1) and NT in the renal tissues of sham-operated control (CTL; $n = 6$) and a 5/6 nephrectomized rat CKD treated with vehicle (CKD; $n = 9$) or RTA dh404 (CKD + RTA dh404; $n = 9$); * $p < 0.05$ versus CTL, ** $p < 0.01$ versus CTL, # $p < 0.05$ versus CKD.

(1 × Tris-buffered saline (TBS), 0.05% Tween-20 and 5% non-fat milk) and then overnight in the same buffer containing the given antibodies. The membrane was washed three times for 5 min in 1 × TBS, 0.05% Tween-20 before a 2-h incubation in a buffer (1 × TBS, 0.05% Tween-20 and 3% non-fat milk) containing horseradish peroxidase-linked anti-rabbit IgG and anti-mouse IgG (Amersham Life Science) at 1:1000 dilution. The membrane was washed four times and developed by autoluminography using ECL chemiluminescent reagents (Amersham Life Science).

Messenger RNA analyses

Messenger RNA was quantified, as previously described (Reisman et al., 2009), using the QuantigeneTM Plex 2.0

assay from Affymetrix (Santa Clara, CA). A modified panel (Catalog #31177) with targets designed against the rat genome was used. Descriptions of the panels with accession numbers can be found at <http://www.panomics.com>. Thioredoxin reductase 1 (Txnrd1), thioredoxin 1 (Txn1) and peroxiredoxin 1 (Prdx1) were quantified. The mRNA expression data were standardized to the internal control ribosomal protein 119 (Rpl19) and presented as fold the mean vehicle control.

Statistical analysis

Data were analyzed using SPSS software version 17.0 (SPSS, Chicago, IL) to perform a one-way analysis of variance, followed by a Bonferroni *post-hoc* test with significance set at

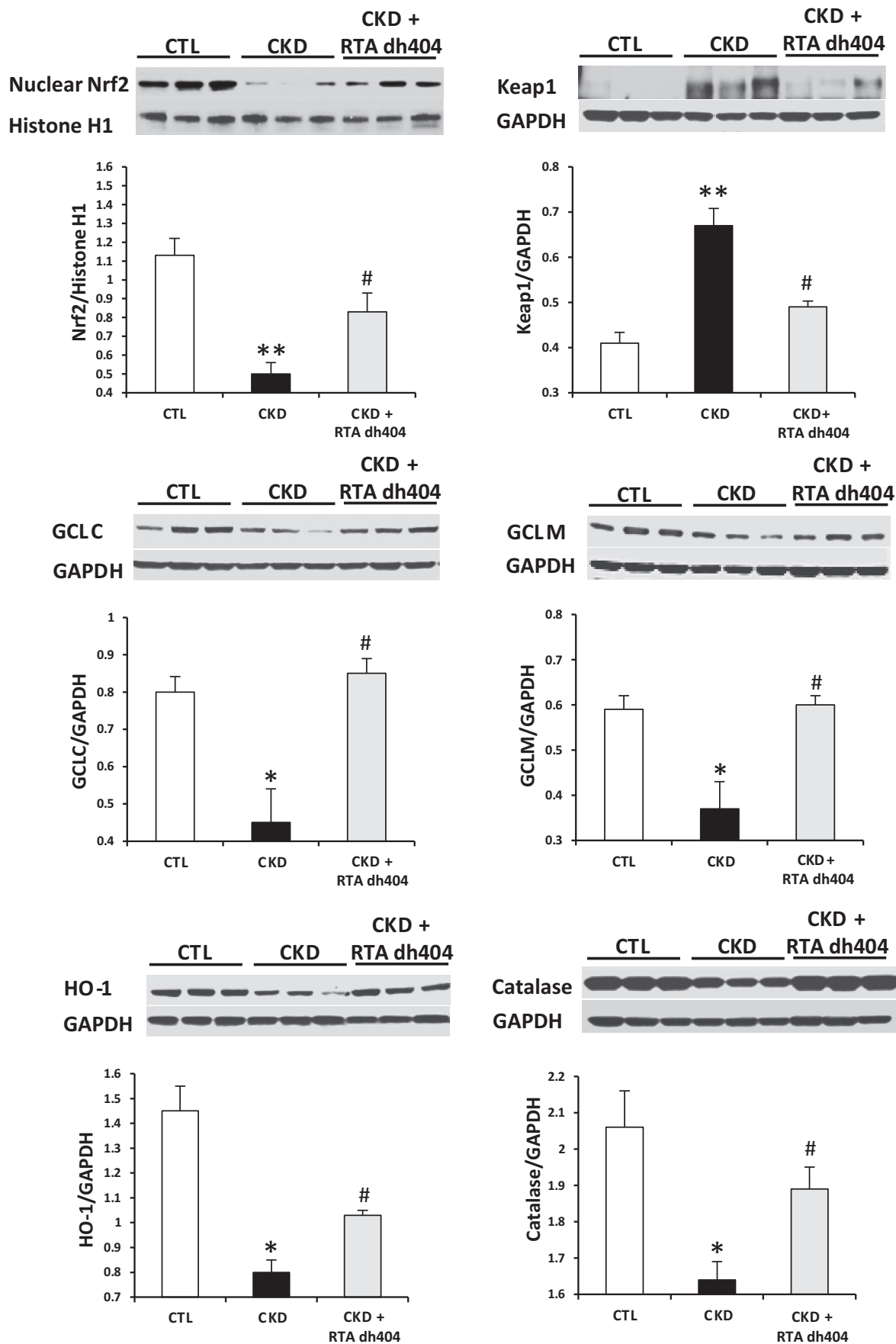


Figure 3. Representative western blots and group data depicting protein abundance of Nrf2, Keap1 and Nrf2 downstream gene products: catalase, HO-1 and catalytic (GCLC) and modulatory (GCLM) subunits of glutamate-cysteine ligase in the renal tissues of sham-operated control (CTL; $n = 6$) and a 5/6 nephrectomized rat CKD treated with vehicle (CKD; $n = 9$) or RTA dh404 (CKD + RTA dh404; $n = 9$); * $p < 0.05$ versus CTL, ** $p < 0.01$ versus CTL, # $p < 0.05$ versus CKD.

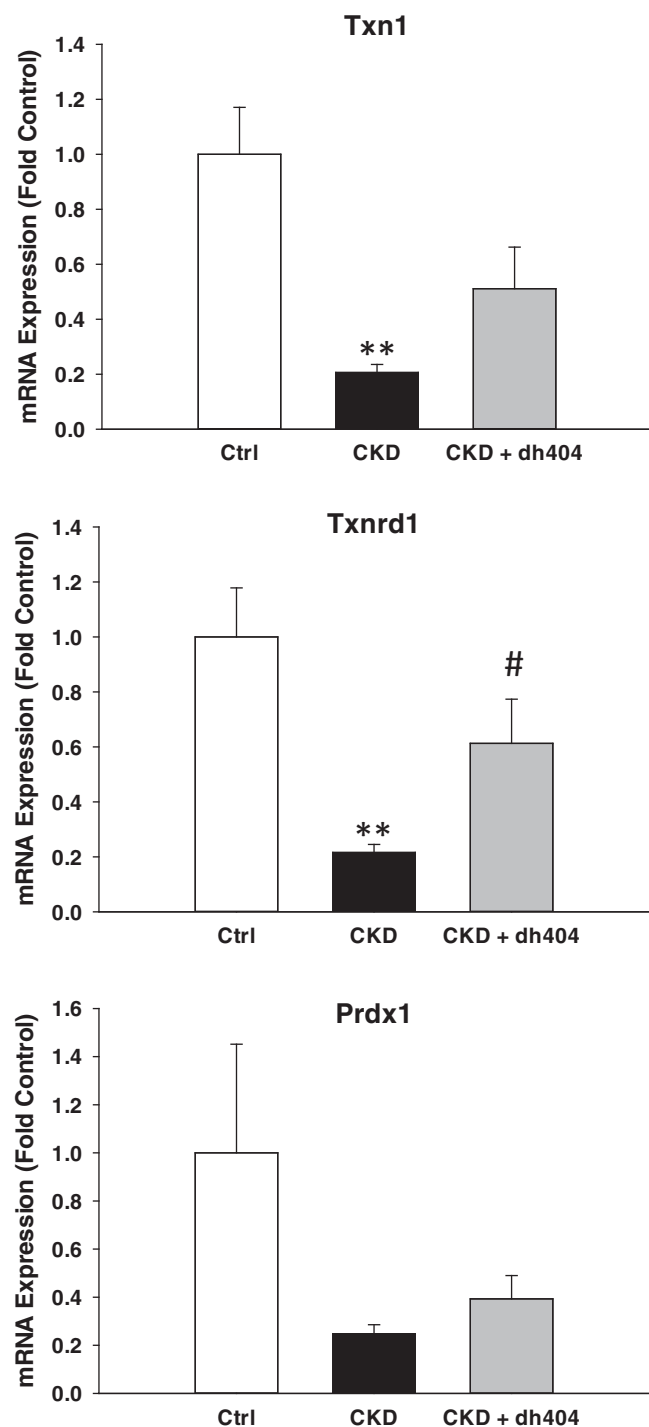


Figure 4. Messenger RNA expression of peroxiredoxin 1 (Prdx1), thioredoxin 1 (Txn1) and thioredoxin reductase 1 (Txnrd1) in the renal tissues of sham-operated control (CTL; $n = 6$) and a 5/6 nephrectomized rat CKD treated with vehicle (CKD; $n = 9$) or RTA dh404 (CKD + RTA dh404; $n = 9$); ** $p < 0.01$ versus CTL, # $p < 0.05$ versus CKD.

$p < 0.05$. Data are expressed as mean \pm standard error of the mean (SEM).

Results

General data

Data are summarized in Table 1. Compared with the sham control group, the vehicle-treated CKD group exhibited weight loss, proteinuria, polyuria, hypertension (evaluated

as MAP) and anemia. Administration of RTA dh404 at 2 mg/kg/day resulted in significant reduction of urinary protein excretion, and amelioration of hypertension and anemia in the treated CKD animals. This was associated with significant reduction in plasma lipid peroxidation product, MDA, denoting amelioration of oxidative stress. No significant differences were observed in body weight, serum creatinine or urine volume between the vehicle-treated CKD rats and the RTA dh404-treated CKD rats. Furthermore, RTA dh404 appeared to be well-tolerated with no apparent adverse effects (e.g. clinical or macroscopic) observed (data not shown).

Oxidative and inflammatory pathways

Data are shown in Figures 1 and 2. Compared with the control group, the vehicle-treated CKD group showed a significant increase in phosphorylated I κ B and elevated nuclear content of p65 subunit, indicating activation of NF- κ B in kidney. This was accompanied by significant upregulation of the NADPH oxidase subunits (i.e. gp91^{phox}, p22^{phox} and Rac1), COX-2, and iNOS, and accumulation of NT, a biomarker of oxidative/nitrosative stress. Administration of RTA dh404 was associated with attenuated NF- κ B activity, reduced expression of NADPH oxidase subunits, COX-2, and iNOS, and lowered kidney tissue NT abundance in rats with CKD.

Nrf2/Keap1 pathway

Data are shown in Figures 3 and 4. In confirmation of our earlier studies (Kim & Vaziri, 2010; Kim et al., 2011), the nuclear Nrf2 content was markedly reduced and Keap1 abundance was significantly increased in the kidneys of the vehicle-treated CKD rats, as compared with those found in the control animals. This was associated with significant down-regulation of Nrf2 target gene products, including protein expression of catalase, HO-1, Gclc, and Gclm and mRNA expression of Txn1, Txnrd1 and Prdx1 ($p < 0.1$) in kidneys of vehicle-treated CKD rats, indicating impaired Nrf2 activation in this model. Administration of RTA dh404 to rats with CKD was associated with increased nuclear Nrf2 abundance, decreased Keap1 abundance and increased protein expression of catalase, HO-1, Gclc and Gclm. The mRNA expression of Txnrd1 was significantly increased, while Txn1 and Prdx1 tended to be increased in kidneys of CKD rats treated with RTA dh404 compared with CKD rats treated with vehicle.

TGF- β , α -SMA, SMAD7 and ERK1/2 protein expression

Data are illustrated in Figure 5. Compared with the control rats, the vehicle-treated CKD group showed marked increase in TGF- β , ERK1/2 and α -SMA abundance, and a significant decrease in the SMAD7 abundance in the remnant kidney. These abnormalities were mitigated by RTA dh404 administration to rats with CKD.

Histological data

Representative photomicrographs of kidney histology are presented in Figures 6 and 7, where similar results extended to all animals in each group. As expected the vehicle-treated CKD rats exhibited marked interstitial fibrosis and leukocyte

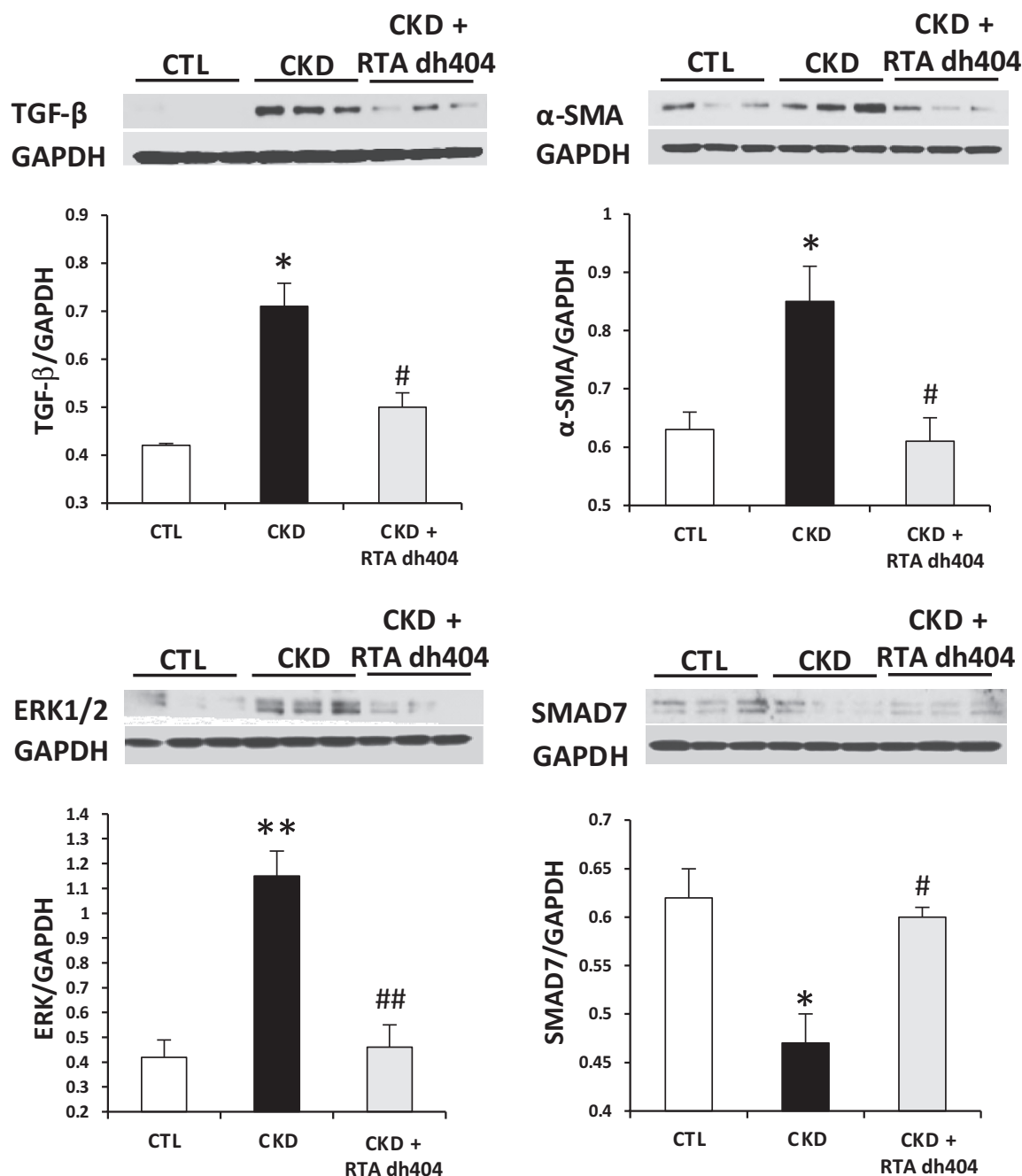


Figure 5. Representative western blots and group data depicting protein abundance of TGF- β , α -SMA, extracellular signal-regulated kinase 1/2 (ERK 1/2) and SMAD7 in the renal tissues of sham-operated control (CTL; $n=6$) and a 5/6 nephrectomized rat CKD treated with vehicle (CKD; $n=9$) or RTA dh404 (CKD + RTA dh404; $n=9$); * $p<0.05$ versus CTL, ** $p<0.01$ versus CTL, # $p<0.05$ versus CKD, ## $p<0.01$ versus CKD.

accumulation, tubular atrophy and dilatation, and glomerular sclerosis. Long-term therapy with RTA dh404 attenuated interstitial inflammation and fibrosis and glomerulosclerosis in rats with CKD.

Discussion

As expected, the vehicle-treated CKD rats employed in the present study exhibited heavy interstitial infiltration of inflammatory cells, interstitial fibrosis, tubular atrophy and dilatation, and glomerulosclerosis. This was accompanied by activation of NF- κ B, the master regulator of the inflammatory cascade, upregulation of NAD(P)H oxidase, COX-2, and

iNOS, and accumulation of NT, pointing to oxidative stress and inflammation in the remnant kidney tissue. Interstitial fibrosis and glomerulosclerosis in the vehicle-treated CKD rats were associated with upregulation of pro-fibrotic mediators, TGF- β and α -SMA, and down-regulation of anti-fibrosis factor, SMAD7. These cellular and structural deficits resulted in increased serum creatinine (SCr), proteinuria and MAP.

In confirmation of previous findings (Kim & Vaziri, 2010), oxidative stress and inflammation in the remnant kidney tissue was associated with impaired activation of Nrf2 and depressed expression of its key target gene products in the remnant kidneys of the vehicle-treated rats with CKD.

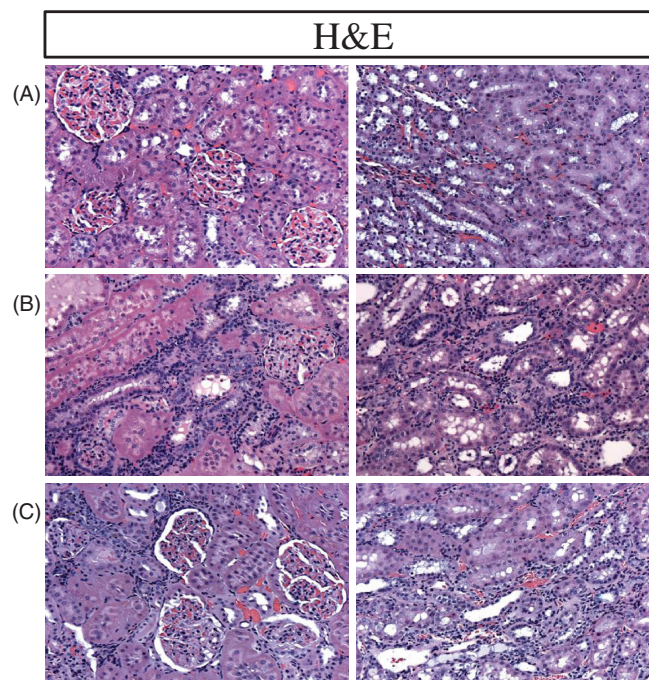


Figure 6. Representative photomicrographs of the H&E stained renal tissue (20X) in a sham-operated control (A) and a 5/6 nephrectomized rat (CKD) treated with vehicle (B) or RTA dh404 (C). The remnant kidney in the CKD animals (B) exhibited significant glomerulosclerosis, tubulo-interstitial injury and heavy inflammatory cell infiltration. RTA dh404 treatment-reduced inflammatory cell infiltration and glomerular and tubulo-interstitial injury (C).

In fact, nuclear Nrf2 content was markedly reduced, tissue abundance of Nrf2 repressor molecule, Keap1, was significantly elevated, and expression of the Nrf2 target gene products, catalase, HO-1, Gclc Gclm, Txn1, Txnrd1 and Prdx1 was reduced in the vehicle-treated rats with CKD. These findings support the hypothesis that defective Nrf2 function contributes to the pathogenesis of oxidative stress and inflammation and progression of kidney disease. This supposition is also supported by the occurrence of renal disease in Nrf2-deficient mice and the reported salutary action of natural compounds with Nrf2 activating properties on progression of kidney disease of various etiologies in experimental animals (Ghosh et al., 2009; Lee et al., 2012; Li et al., 2011; Palsamy & Subramanian, 2011; Peng et al., 2011; Ruiz et al., 2013; Soetikno et al., 2013; Tapia et al., 2012; Tsai et al., 2011).

The synthetic triterpenoid, bardoxolone methyl, is a potent activator of the Nrf2-Keap1 pathway (Liby et al., 2007; Sporn et al., 2011; Yates et al., 2007). Via interaction with Keap1, this compound and its analogs interfere with binding of Nrf2 by Keap1, thereby enhancing nuclear translocation of Nrf2 and up-regulation of its target genes encoding antioxidant and cytoprotective molecules (Dinkova-Kostova et al., 2005; Liby et al., 2007; Sporn et al., 2011). Short-term administration of bardoxolone methyl attenuates ischemia-reperfusion-induced acute kidney injury in rats and reduces renal interstitial inflammation and fibrosis in mice with protein overload proteinuria (Wu et al., 2011). Given the role of Nrf2 dysfunction in the pathogenesis of oxidative stress and inflammation, which are the driving force behind progression

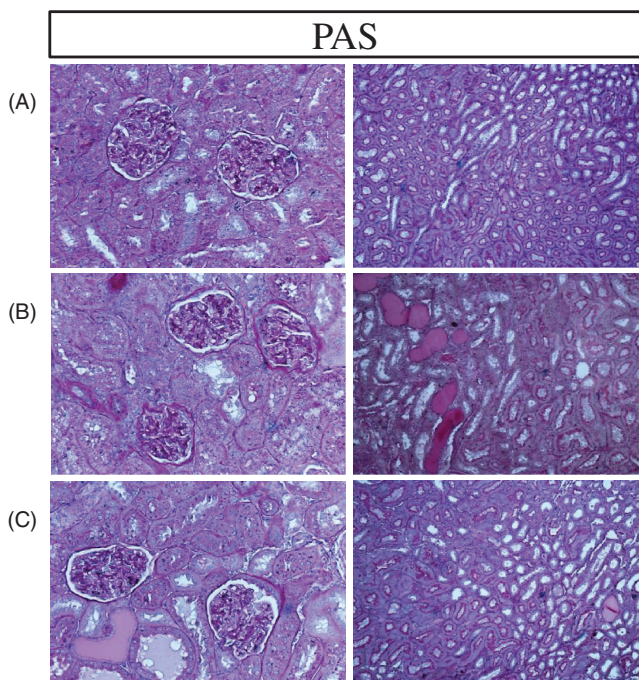


Figure 7. Representative photomicrographs of the PAS-stained renal tissue in a sham-operated control (A) and a 5/6 nephrectomized rat (CKD) treated with vehicle (B) or RTA dh404 (C). The remnant kidney in the CKD animals exhibited significant fibrosis (B) which was reduced with RTA dh404 treatment (C). 20 × magnification.

of CKD, it seemed plausible that long-term administration of the potent Nrf2 activator and bardoxolone methyl analog RTA dh404 might prevent or retard progression of kidney disease by restoring Nrf2 activity.

Treatment of CKD rats with RTA dh404 restored Nrf2 protein expression, which was associated with Keap1 and target gene expression more similar to controls that underwent sham surgery only. RTA dh404 also attenuated activation of pro-inflammatory NF- κ B and pro-fibrotic TGF- β pathways, which is consistent with the known inhibitory effects of bardoxolone methyl and its analogs on NF- κ B (Liby & Sporn, 2012). Collectively, restoration of Nrf2 function and inhibition of NF- κ B activation was associated with reduced glomerulosclerosis, interstitial fibrosis and inflammation in the CKD rats.

Overall, this study revealed that long-term administration of the RTA dh404, at a dose of 2 mg/kg/day, yielded salutary effects in the 5/6 nephrectomy rat model. In summary, long-term administration of the bardoxolone methyl analog, RTA dh404, resulted in attenuation of oxidative stress, inflammation and fibrosis in the remnant kidneys of rats with CKD induced by 5/6 nephrectomy, which was accompanied by an increase or a rescue of Nrf2 and its target genes, and decreased activation and expression of NF- κ B and its target genes.

Declaration of interest

S.A. Reisman and C.J. Meyer are employed by and have a financial interest in Reata Pharmaceuticals, Inc. M.A. Aminzadeh, N.D. Vaziri, J. Yuan and M. Khazaeli have received an unrestricted research grant from Reata Pharmaceuticals, Inc.

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