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Original Article

Uremia induces upregulation of cerebral tissue oxidative/inflammatory cascade, down-regulation of Nrf2 pathway and disruption of blood brain barrier

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Abstract: Chronic kidney disease (CKD) results in various central nervous systems (CNS) disorders including cognitive dysfunction, depression, anxiety, movement disorders, seizures and encephalopathy. Uremic retention products, oxidative stress, inflammation and impaired blood-brain barrier have been implicated as the major mediators of CKD-induced CNS disorders. However, mechanisms of CKD-induced cerebral tissue oxidative stress, inflammation and impaired blood brain barrier have not been fully elucidated and were explored. Male Sprague Dawley rats underwent sham operation or 5/6 nephrectomy and were observed for 10 weeks. Arterial pressure, body weight, and renal function were monitored. Under general anesthesia the animals' cerebral cortex was harvested. Nuclear translocations of NF-κB and Nrf2 and their key target gene products, neuronal, endothelial and inducible NO synthase (NOS) isoforms, markers of oxidative, nitrosative and myeloperoxidase reactions, fibrosis mediators and key protein constituents of capillary endothelial junctional complex were determined by Western blot analysis. The CKD rats exhibited reduced body weight, hypertension, elevated serum urea and creatinine concentrations. Compared to control group cerebral cortex of the CKD group showed activation (increased nuclear translocation) of NF-κB, elevation of pro-oxidant and pro-inflammatory molecules, diminished nuclear translocation of Nrf2 and expression of cytoprotective antioxidant molecules and depletion of the key protein constituents of endothelial junctional complex. In conclusion CKD results in the cerebral tissue activation of inflammatory and oxidative pathways, inhibition of antioxidant and cytoprotective system and erosion of cerebral capillary junctional complex, events that contribute to CNS dysfunction and impaired blood brain barrier.

Keywords: Chronic kidney disease, uremic encephalitis, cerebral oxidative stress, impaired blood-brain barrier, uremic encephalopathy

Introduction

Prevalence of chronic kidney disease (CKD) has risen dramatically during the past two decades and has emerged as a major public health problem worldwide [1]. CKD results in a wide array of neurological complications affecting the central and peripheral nervous systems [2]. The central nervous system (CNS) complications of CKD include stroke, seizures, movement disorders, cognitive dysfunction, encephalopathy, depression and anxiety [3-5]. The CKD-associated peripheral nervous system complications include uremic polyneuropathy, autonomic neuropathy, restless legs syndrome, and myopathy [6-9]. The neurological complications contribute to morbidity and mortality in this population [10].

Studies conducted in experimental animal have shown that CKD causes depression, anxiety and reduced exploratory and locomotor activities [11-13]. Using the adenine-induced CKD rats Ali et al found significant psychomotor and behavioral abnormalities, reduced motor activity and depression marked by increased immobility time in forced swimming test [11]. In addition, both chronic and acute renal injury have been shown to cause psychomotor behavior abnormalities and disruption of blood brain barrier (BBB) in experimental animals [14, 15].

Table 1. General Data in the CTL and CKD groups

	CTL (N = 6)	CKD (N = 10)
Body weight change (g)	187.6 ± 28.8	143.3 ± 35.0*
SBP (mmHg)	90.0 ± 1.5	143.1 ± 2.1*
Serum urea (mg/dL)	8.6 ± 0.2	53.8 ± 1.8*
Serum Creatinine (mg/dL)	0.34 ± 0.02	1.11 ± 0.10*
24 h Urine Protein (mg/dL)	4.6 ± 0.5	20.5 ± 2.3*

SBP = systolic tail blood pressure; Values are mean ± SEM. *P < 0.05.

The factors contributing to the pathogenesis of CNS disorders in CKD can be subdivided into vascular and nonvascular disorders. The vascular causes of CNS complications in CKD may be linked to the presence of traditional risk factors including hypertension, diabetes, dyslipidemia, blood coagulation disorders, etc. and non-traditional risk factors such as inflammation, oxidative stress, vascular calcification, endothelial dysfunction and uremic toxins [11]. Among the nonvascular factors, uremic retention products including indoxyl sulphate, uric acid, p-cresyl sulphate, inflammatory mediators including interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-a (TNF-α) [12], and oxidative stress [16], have long been considered as likely mediators of CNS injury and dysfunction in CKD.

CKD results in oxidative stress and systemic inflammation which play an important role in the pathogenesis and progression of CKD and its cardiovascular and numerous other complications [17]. Oxidative stress, inflammation and fibrosis in the diseased kidney and cardiovascular tissues in animal models of CKD are mediated by: a-activations of nuclear factor kappa B (NF-κB), the master regulator of the genes encoding inflammatory mediators, b-upregulation of enzymes involved in reactive oxygen production, chlorine and nitrogen species, c-downregulation of Nuclear factor erythroid-derived 2-like 2 (Nrf2), the master regulator of genes encoding over 200 antioxidant and cytoprotective proteins, and d-upregulation of transforming growth factor beta (TGF-β), the master regulator of pro-fibrotic proteins [17, 18]. Although inflammation and oxidative stress have been implicated in the pathogenesis of CKD-induced CNS complications, their presence and their underlying mechanisms have not been fully demonstrated. The present study was designed to evaluate the impact of CKD on oxidative, inflammatory and Nrf-2 pathways in the brain tissue. In addition, to explore the mechanism of CKD-induced impairment of blood brain barrier, we sought to determine the impact of CKD on the cerebral endothelial tight junction proteins.

Material and methods

Study groups

Male Sprague-Dawley rats (~250 g) (Charles River Labs, Raleigh, NC, USA) were used in this study. Animals were housed in a climate-controlled vivarium with 12 h day/night cycles and provided with food and water *ad libitum*. The animals were randomly assigned to the CKD and sham-operated control groups. The CKD group underwent 5/6 nephrectomy by surgical resection of the upper and lower thirds of the left kidney followed by right nephrectomy 7 days later. The control group underwent sham operation. For surgical procedures and euthanasia, the animals were placed into a sealed anesthesia induction chamber under 5% isoflurane (Piramal Clinical Care, Bethlehem, PA, USA)/oxygen gaseous mixture to induce sedation and maintained at 2-4%. Before the start of 5/6 nephrectomy procedures, all animals received 0.05 mg/kg of Buprenex (Reckitt Benckiser Pharmaceutical Inc., Richmond, VA, USA) for pain relief. At the beginning and the final week (week 10) of the study, the animals were placed in metabolic cages for a 24 h urine collection and systolic blood pressure (SBP) was measured by tail plethysmography as described previously [19]. At the end of the study period, the animals were euthanized by exsanguination using cardiac puncture and the brain tissues were immediately removed and processed for Western blot analyses.

Serum urea was measured by QuantiChrom™ Urea Assay Kit (BioAssay Systems, Hayward, CA, USA). Serum and urinary creatinine were measured using QuantiChrom™ Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA) and urinary protein was determined by Rat Urinary Protein Assay Kit (Chondrex Inc. Redmond, WA). This study was performed in compliance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The institutional Animal Care and Use Committee at University of California Irvine approved this project.

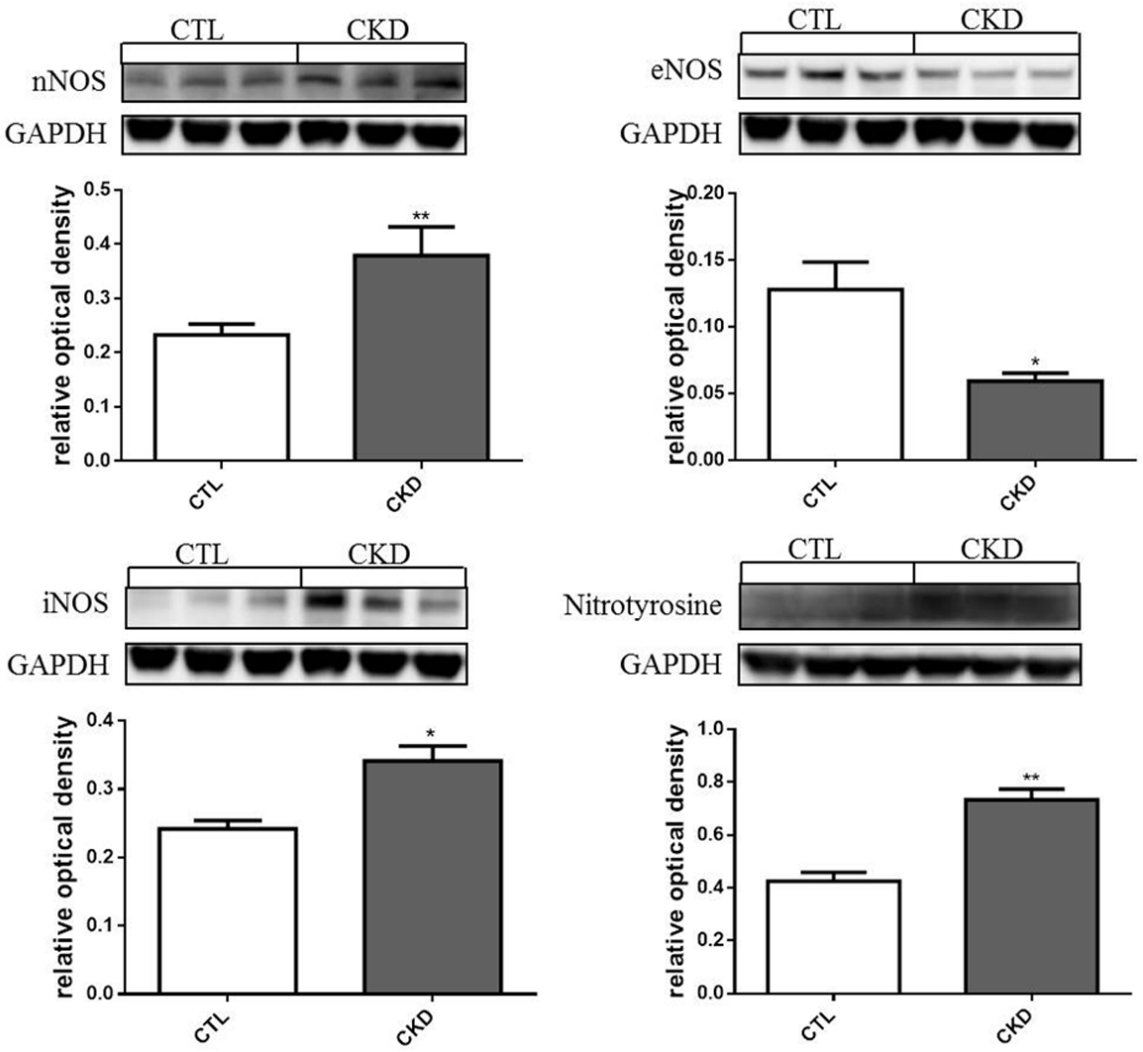

Figure 1. Representative immunoblots and group data depicting nNOS, eNOS, iNOS, and nitrotyrosine abundance in the cerebral cortex of the control and CKD rats. *P < 0.05; **P < 0.01 vs. control group. Data represent the mean ± SEM (n = 6 rats/group). GAPDH was used as the loading control.

Western blot analyses

Cytoplasmic and nuclear extracts of the brain cortex were prepared as described previously [20]. The target proteins in the cytoplasmic and nuclear fractions of the brain tissue were measured by Western blot analysis as previously described [18, 21] using the following antibodies: Rabbit antibodies against rat zonula occludens 1 (ZO-1, Cat # ab190085), junctional adhesion molecule-1 (JAM-1, Cat # ab52647), p65 subunit NFκB (NF-κB p65, Cat # ab16502), monocyte chemotactic protein 1 (MCP-1, Cat # ab25124), inducible nitric oxide synthase (iNOS, Cat # ab3526), neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS, Cat # ab95254), NADPH-oxidase 4 (NOX4, Cat # ab133303), $Gp91^{phox}$ (Cat # ab129068), nitrotyrosine (Cat # ab183391), Nrf-2 (Cat # ab31163), Keap1 (Cat # ab139729), copper-zinc superoxide dismutase (Cu/Zn-SOD, Cat # ab13498), cyclooxygenase-2 (COX-2, Cat # 15191), heme oxygenase-1 (HO-1, Cat # ab13248), catalase (Cat # ab676110) and myeloperoxidase (MPO, Cat # ab65871) were purchased from Abcam (Cambridge, MA). Antibody against claudin-5 (Cat # SAB4502981), occluding (Cat # SAB4200489), and glutathione peroxidase (GPX, Cat # AF3798) were obtained from Sigma-Aldrich (St. Louis, MO), Invitrogen, and R&D Systems (Minneapolis, MN), respectively. Mouse antibodies against

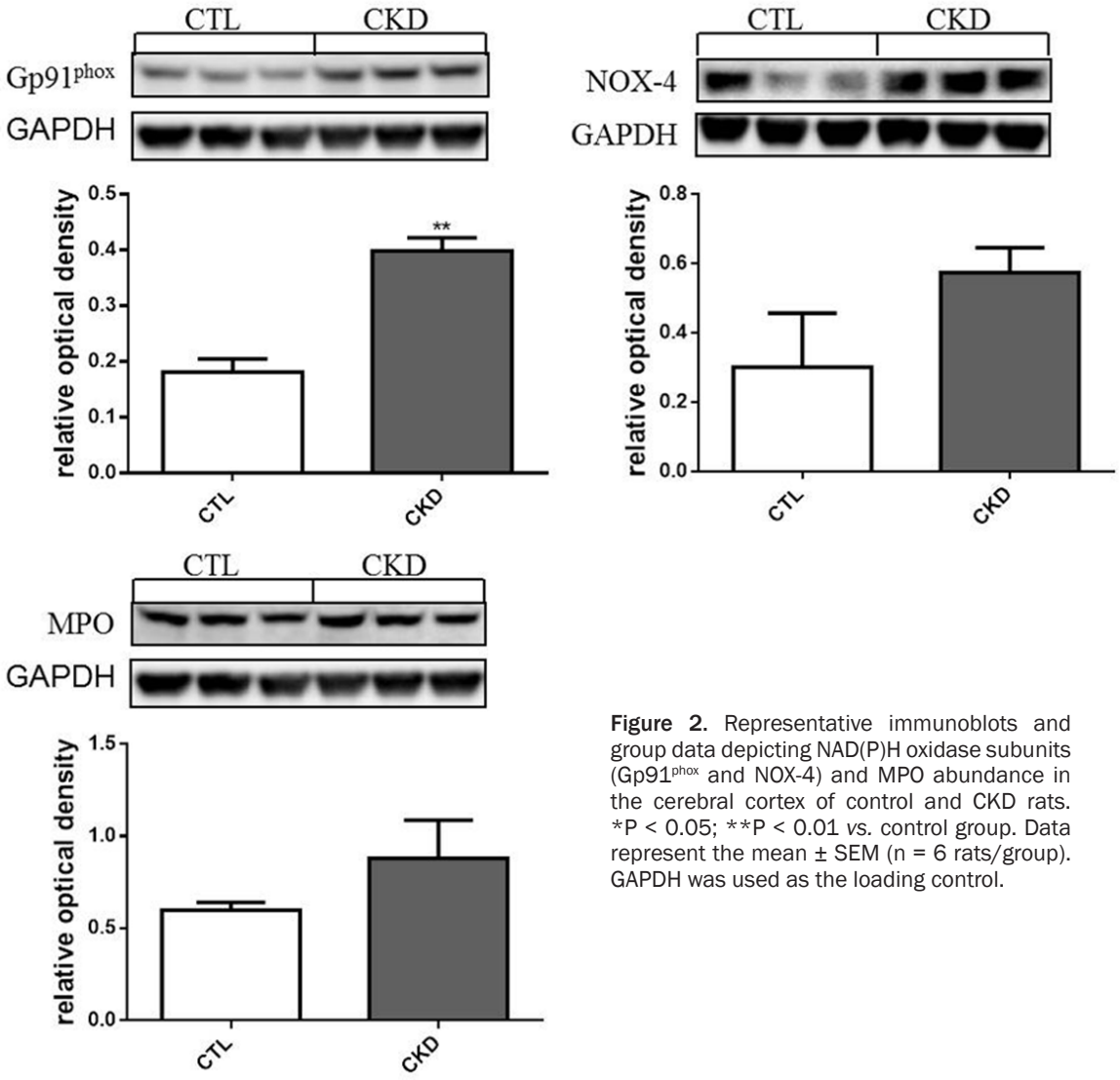

Figure 2. Representative immunoblots and group data depicting NAD(P)H oxidase subunits ($Gp91^{phox}$ and NOX-4) and MPO abundance in the cerebral cortex of control and CKD rats. *P < 0.05; **P < 0.01 *vs.* control group. Data represent the mean ± SEM (n = 6 rats/group). GAPDH was used as the loading control.

histone H3 (Cat # ab32356) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cat # ab8245) purchased from Abcam (Cambridge, MA) were used for measurements of nuclear and cytosolic housekeeping proteins, respectively.

Statistical analysis

Data are presented as mean ± SEM. Student's *t*-test was used in statistical evaluation of the data. *P* values less than 0.05 were considered significant.

Results

General data

As expected, compared with the sham-operated control group, the CKD group showed significant reduction in body weight gain and significant increase in arterial pressure, serum urea and creatinine concentrations and urinary protein excretion (**Table 1**).

Inflammatory and oxidative pathway data

The brain cortex tissues from the CKD rats showed a significant accumulation of nitrotyrosine, pointing to presence of oxidative stress and generation of reactive nitrogen species (**Figure 1**). This was accompanied by significant upregulations of $gp91^{phox}$ subunit of NAD(P)H oxidase, insignificant upregulations of NOX-4 and MPO pointing to increased production of reactive oxygen and halogen species in the cerebral cortex of the CKD rats (**Figure 2**). Furthermore, the cerebral cortex of the CKD rats showed a significant increase in nuclear

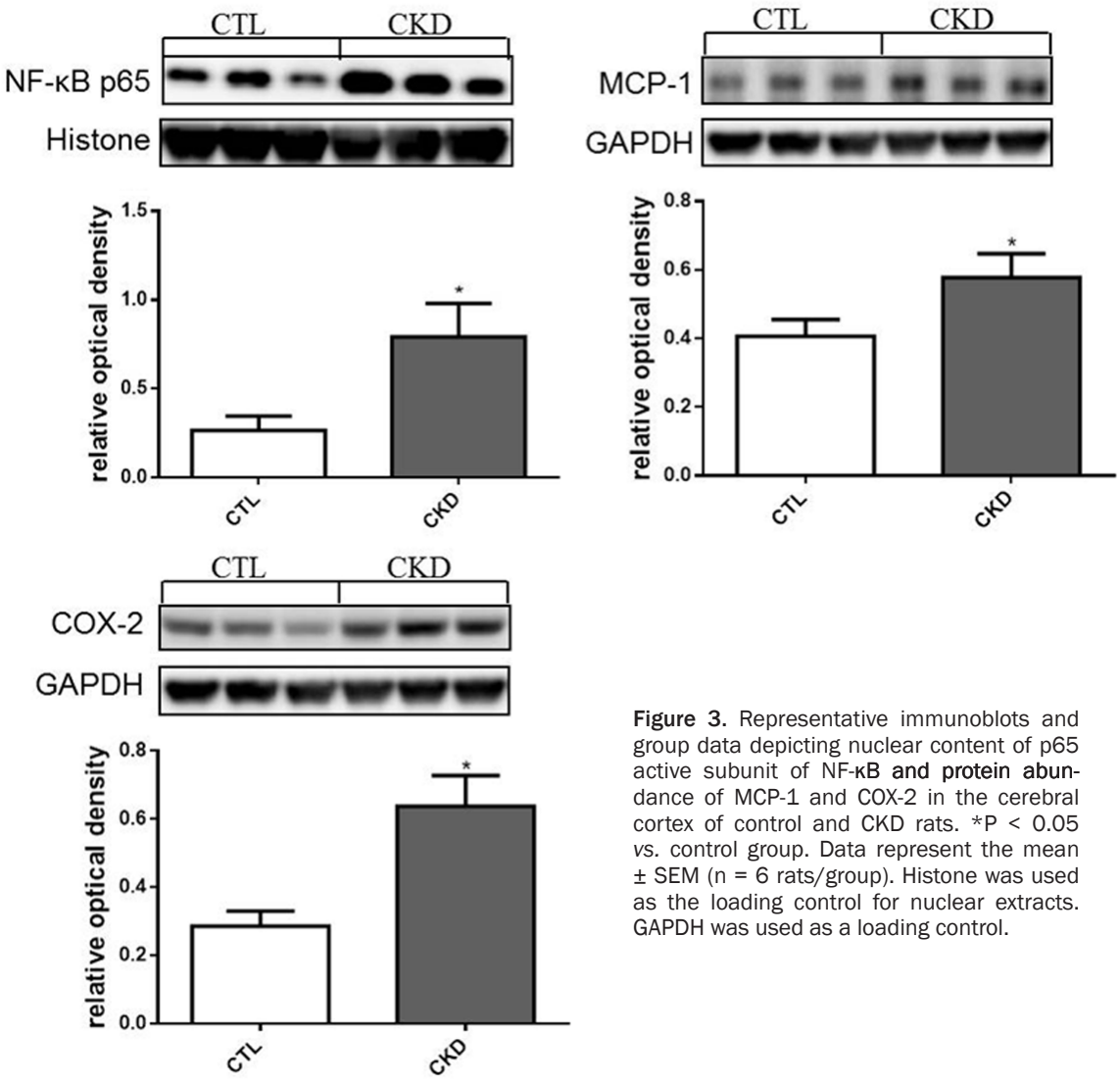

Figure 3. Representative immunoblots and group data depicting nuclear content of p65 active subunit of NF-κB and protein abundance of MCP-1 and COX-2 in the cerebral cortex of control and CKD rats. *P < 0.05 *vs.* control group. Data represent the mean ± SEM (n = 6 rats/group). Histone was used as the loading control for nuclear extracts. GAPDH was used as a loading control.

translocation of p65, pointing to activation of NF-κB. This was accompanied by significant upregulation of pro-inflammatory molecules including MCP-1, cyclooxygenase-2 (COX-2) and iNOS (**Figures 1** and **3**).

Nrf2 pathway data

The cerebral cortex tissues from the CKD animals showed significant increase in cytoplasmic abundance of Nrf2 inhibitor, Keap1, a significant reduction in nuclear translocation of Nrf2, and significant down-regulation of the key target gene products including glutathione peroxidase (GPx), catalase, heme oxygenase-1 (HO-1), and eNOS (**Figures 1** and **4**). These results point to impairment of the Nrf-2 pathway which makes a major contribution to the prevailing oxidative stress and inflammation in CKD.

Cerebral tissue NO related data

nNOS is the major isomer of NO synthase (NOS) enzymes in the central nervous system. Nitrotyrosine is identified as an indicator of cell damage, inflammation and NO production. In the current study, nNOS and iNOS expressions and nitrotyrosine abundance were upregulated whereas eNOS expression was downregulated in the brain cortex tissues of the CKD rats (**Figure 1**).

Brain cortex endothelial tight junction data

The brain cortex tissues from the CKD rats showed marked reductions of ZO-1, occludin

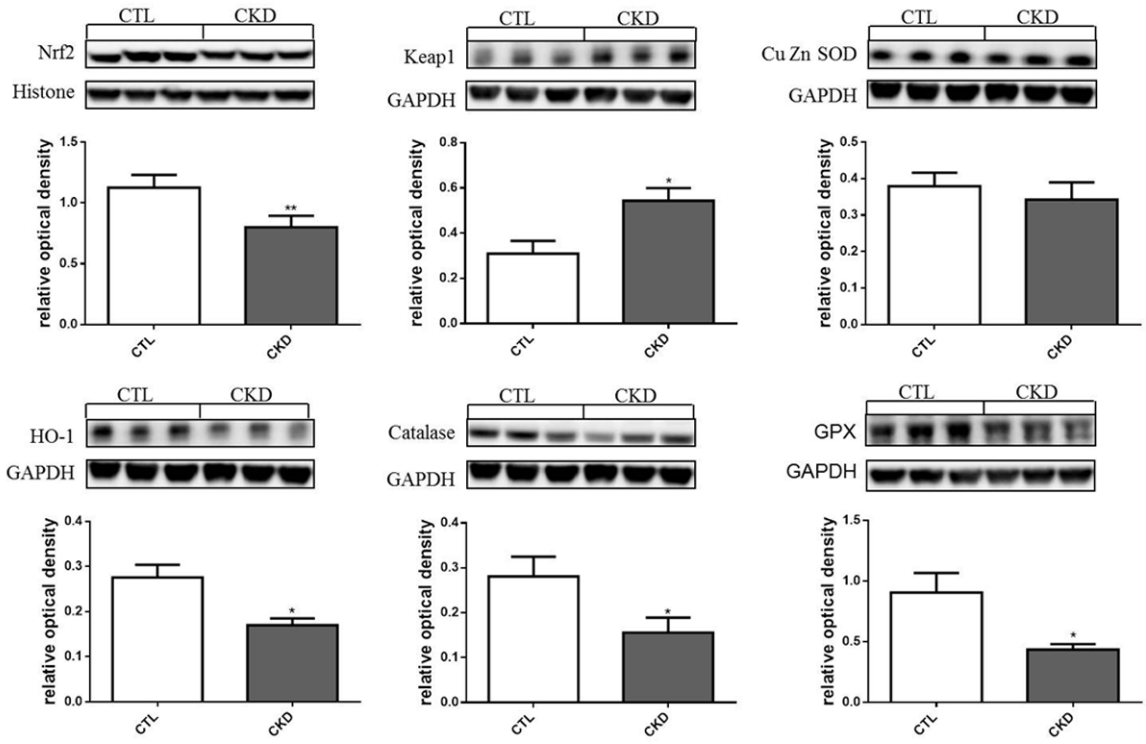

Figure 4. Representative immunoblots and group data depicting nuclear translocation of Nrf2 and protein abundance of its repressor molecule, Keap1, and downstream gene products, Cu/Zn-SOD, HO-1, catalase, and GPX in the cerebral cortex of control and CKD rats. *P < 0.05; **P < 0.01 *vs.* control group. Data represent the mean ± SEM (n = 6 rats/group). Histone was used as the loading control for nuclear extracts. GAPDH was used as the loading control for cytoplasmic proteins.

and JAM-1 levels. However, no significant difference was found in claudin-5 protein abundance between the CKD and control rats (**Figure 5**).

Discussion

NF-κB is the master regulator of genes encoding inflammatory cytokines and chemokines. The cerebral cortex of the CKD rats showed significant increase in the nuclear NF-κB content and elevation of its measured target gene products, MCP1, COX-2 and iNOS, denoting activation of inflammatory pathway. This was accompanied by upregulation of superoxide generating ($GP91^{phox}$) and reactive nitrogen species generating (iNOS) enzymes in the cerebral cortex of the CKD animals. Under physiological condition, the rise in reactive oxygen species provokes activation (nuclear translocation) of Nrf2, the transcription factor which encodes numerous antioxidant and cytoprotective enzymes and related proteins. However, increased production of reactive oxygen species in the cerebral cortex of CKD rats was paradoxically accompanied by impaired Nrf2 activity and significant reduction of its down-stream target gene products including key cytoprotective antioxidant enzymes. Earlier studies have demonstrated impairment of Nrf2 cascade despite presence of oxidative stress in the remnant kidney, cardiovascular tissue and intestine of CKD rats [18, 22-25] and the circulating leukocytes of patients with end-stage renal disease [26]. Together, the results of the present study of the brain tissue and previous studies of the remnant kidney, cardiovascular and intestinal tissues point to the systemic nature of the inflammation and oxidative stress in CKD.

Upregulation of oxidative and inflammatory pathways shown here identified the underlying mechanism of previously demonstrated cerebral atrophy and oxidative stress in CKD animals [27] and cerebral atrophy in CKD patients [16, 28].

Compared to the control group, the CKD rats showed a significant upregulation of nNOS and

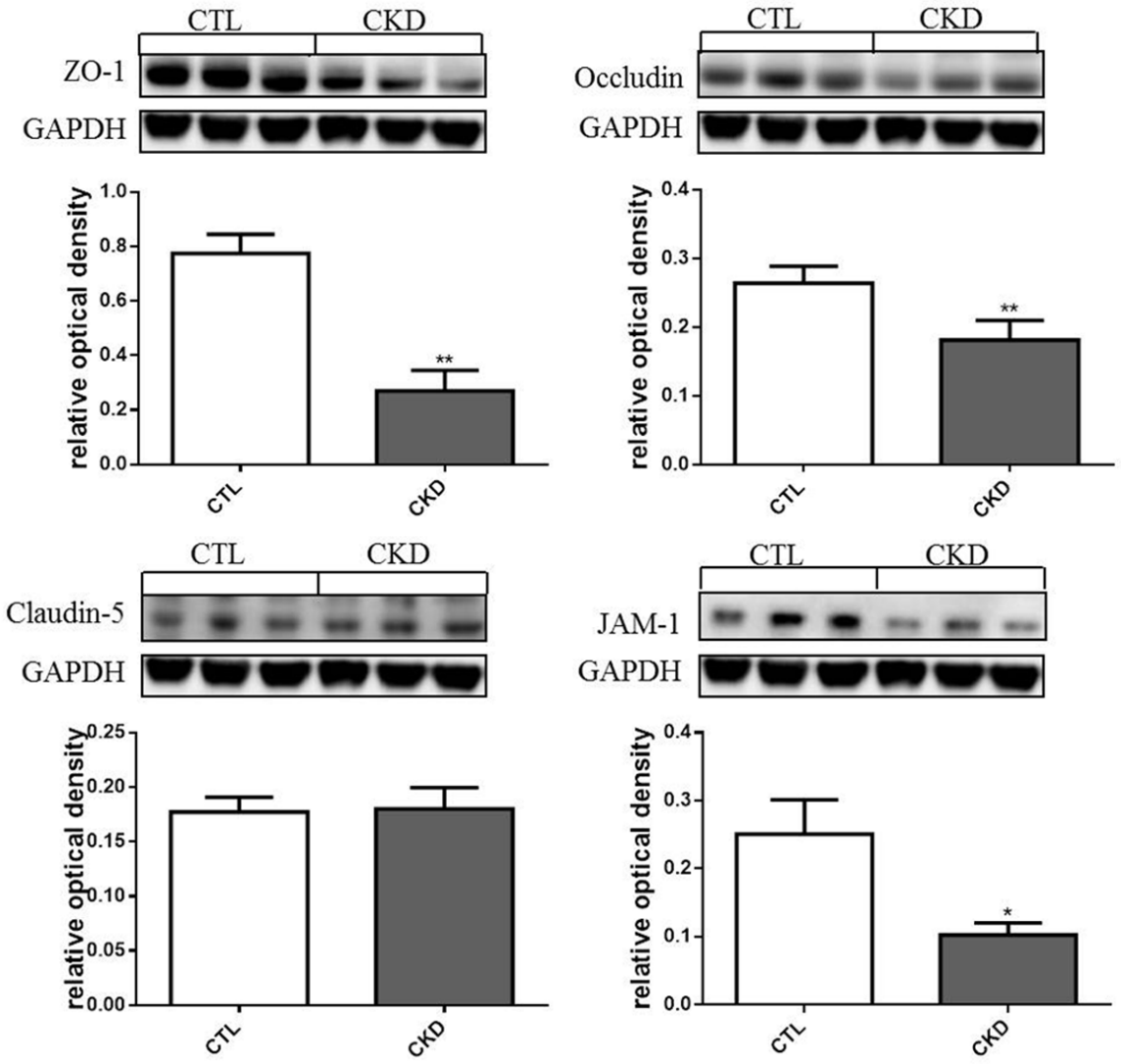

Figure 5. Representative immunoblots and group data depicting ZO-1, Occludin, Claudin-5, and JAM-1 abundance in the cerebral cortex of control and CKD rats. *P < 0.05; **P < 0.01 *vs.* control group. Data represent the mean ± SEM (n = 6 rats/group). GAPDH was used as the loading control.

increased nitrotyrosine abundance in the cerebral cortex. The neuronal NOS (nNOS) is constitutively expressed in the brain tissue where it produces NO which serves as a major neurotransmitter involved in the neural signaling, synaptic plasticity, regulation of autonomic and somatic functions as well as learning and memory [29, 30]. Oxidative stress leads to inactivation of NO, compensatory upregulation of nNOS, and formation of peroxynitrite ($^{\bullet}O_{-2}$ + NO → $^{\bullet}$ONOO) which is a highly cytotoxic reactive nitrogen species. By attacking the cellular and extracellular molecular targets, peroxynitrite inflicts cellular and tissue damage. One of the targets of attack by peroxynitrite is tyrosine which leads to formation nitrotyrosine, a well-known marker of tissue damage and inflammation. Therefore, the observed upregulation of nNOS and marked increase in nitrotyrosine abundance in the cerebral cortex of CKD rats must contribute to the reported CKD-associated CNS dysfunction. In fact, upregulation of nNOS and increased production of NO and peroxynitrite have been shown to contribute to inflammation and neural damage in several neurological diseases including patients with epilepsy and animals exposed to prolonged stress-induced anxiety [31-33]. Moreover, upregulation of the brain tissue nNOS is observed in different diseases that affect CNS including

Alzheimer's disease, stress-related mental illnesses, hypoxic-ischemic brain damage, diabetes and obesity [31, 33-38].

Using the Evans blue dye extravasation method in an earlier study, Mazumder et al demonstrated marked impairment of blood-brain barrier in the CKD mice [15]. The blood-brain barrier which separates the brain tissue from the circulating blood consists of the capillary endothelial cells and the tight junction proteins which seal the gap between the adjacent endothelial cells [39]. The tight junction complex consists of several adhesive branched trans-membrane proteins which are anchored to an intracellular protein complex consisting of ZO-1, actin and myosin [40]. The tight junction's trans-membrane proteins include occludin, claudin-5 and junctional adhesion molecules (JAMs) which via formation of disulfide bonds on their extracellular limbs, tightly link the plasma membranes of the adjacent cells together, thereby forming the barrier to the paracellular diffusion of fluids and solutes between the circulating blood and extracellular compartment [39, 41]. The intracellular arms of these proteins are attached to their anchor, ZO-1 protein [42]. ZO-1 is, in turn, linked to actin and myosin which represent the cytoskeleton of the endothelial cells [42, 43]. Conditions that cause dissociation of the disulfide bonds in the extracellular domains of the transmembrane proteins or contraction of their intracellular domains via actin and myosin result in a rapid endocytosis, degradation and depletion of these proteins [42]. This results in the opening of the tight junction apparatus and disruption of the blood-brain barrier [42]. To determine the underlying mechanisms of CKD-induced disruption of the blood-brain barrier, in the present study we determine the abundance of cerebral tissue endothelial tight junction proteins, ZO-1, claudin-5, occludin, and JAM-1. Compared to the control group the cerebral cortex of our CKD rats showed significant reductions of the ZO-1, occludin and JAM-1 abundance which are the constituents of blood brain barrier. Depletion of these key components of the cerebral capillary tight junction proteins accounts for the previously demonstrated impairment of blood brain barrier in CKD using the Evans blue dye extravasation method [15].

The gastrointestinal epithelial tight junction complex plays a major role in preventing the entry of the gut's harmful luminal contents into the intestinal wall and systemic circulation. Earlier studies have demonstrated marked depletion of the colonic epithelial tight junction proteins and its role in the pathogenesis of endotoxemia and systemic inflammation commonly observed in patients and animals with advanced CKD [44-47]. Together these findings demonstrate the disruptive impact of uremia on both intestinal epithelial barrier and blood brain barrier.

The underlying cause of CKD-induced disruption of blood-brain barrier observed in previous study in CKD mice and the present study in CKD rats is not clear. However, inflammation and elevated urea concentration can contribute to this phenomenon. First, cerebral tissue inflammation as shown by NF-κB activation can lead to disruption of endothelial tight junction by mediating endocytosis and degradation of tight junction proteins as shown in the gut epithelial barrier in CKD rats [17]. Second, by dissociating the disulfide bonds which connect the extracellular portions of the tight junction proteins of the neighboring epithelial and endothelial cells, high urea concentration results the breakdown of the barrier [48, 49].

In conclusion, CKD results in the cerebral tissue activation of inflammatory and oxidative pathways, inhibition of antioxidant and cytoprotective system and erosion of cerebral capillary junctional complex, events that contribute to CNS dysfunction and impaired blood brain barrier.

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Disclosure of conflict of interest

None.

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