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### Permalink

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### Journal

Journal of Nephrology, 28(4)

### ISSN

1121-8428

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### Publication Date

2015-08-01

### DOI

10.1007/s40620-014-0162-0

Peer reviewed

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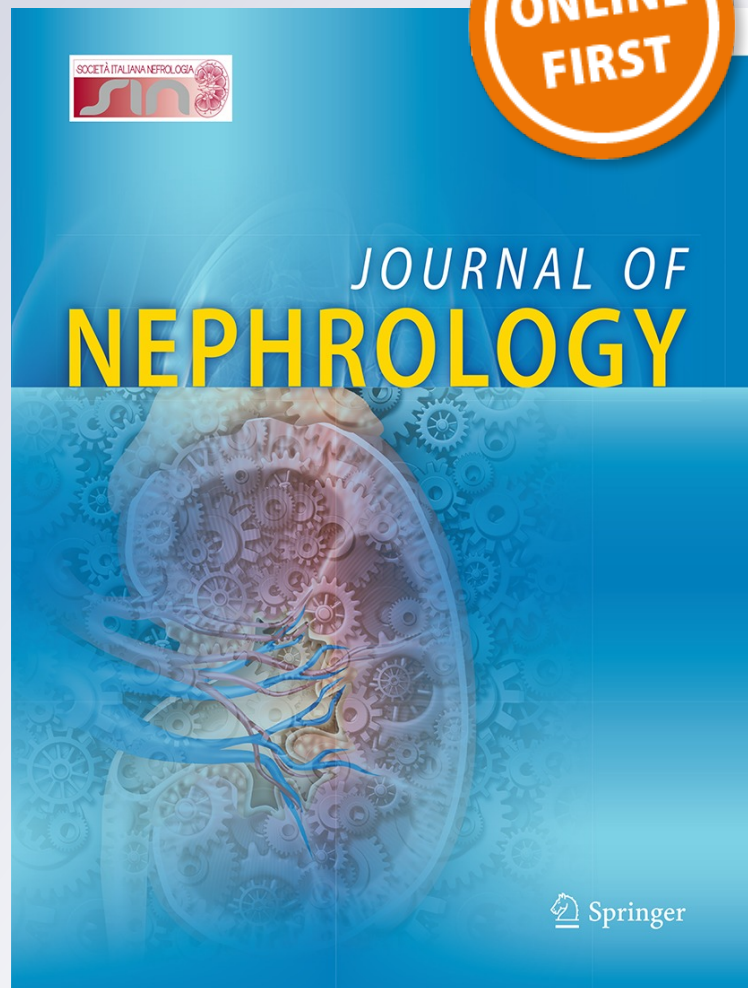
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**Journal of Nephrology**

ISSN 1121-8428

J Nephrol

DOI 10.1007/s40620-014-0162-0



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## Systemic inflammation and oxidative stress in hemodialysis patients are associated with down-regulation of Nrf2

Liliana M. Pedruzzi · Ludmila F. M. F. Cardozo · Julio B. Daleprane · Milena B. Stockler-Pinto · Elisa B. Monteiro · Maurilo Leite Jr · Nosratola D. Vaziri · Denise Mafra

Received: 3 July 2014 / Accepted: 18 November 2014  
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### Abstract

**Background** Oxidative stress and inflammation are common features and the main mediators of progression of chronic kidney disease (CKD) and its cardiovascular complications. Under normal conditions, oxidative stress activates the transcription factor, nuclear factor E2-related factor 2 (Nrf2), which is the master regulator of genes encoding antioxidant and detoxifying enzymes and related proteins. The available data on expression of Nrf2 and its key target gene products in CKD patients is limited. We therefore investigated this topic in a group of CKD patients on hemodialysis.

**Methods** Twenty adult hemodialysis (HD) patients (aged  $54.9 \pm 15.2$  years) and 11 healthy individuals (aged  $50.9 \pm 8.0$  years) were enrolled. Peripheral blood

mononuclear cells (PBMC) were isolated and processed for expression of nuclear factor- $\kappa$ B (NF- $\kappa$ B), Nrf2, heme oxygenase-1 and NADPH: quinoneoxidoreductase 1 (NQO1) by quantitative real-time polymerase chain reaction and western blot analysis. Plasma malondialdehyde (MDA) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels were measured.

**Results** Peripheral blood mononuclear cells from HD patients had significantly lower NQO1 and Nrf2 mRNA expressions ( $0.58 \pm 0.35$  vs.  $1.13 \pm 0.64$ ,  $p = 0.005$ ), and significantly higher NF- $\kappa$ B expression ( $2.18 \pm 0.8$  vs.  $1.04 \pm 0.22$ ,  $p = 0.0001$ ) compared to the healthy individuals. The NF- $\kappa$ B expression was inversely correlated with Nrf2 levels ( $r = -0.54$ ,  $p < 0.01$ ) in CKD patients. Plasma MDA and TNF- $\alpha$  levels were significantly higher in CKD patients than in the healthy individuals.

**Conclusions** Up-regulation of NF $\kappa$ B in the CKD patients' PBMC is coupled to down-regulation of Nrf2 and NQO1 expression. These observations are consistent with recent findings in CKD animals and point to the contribution of the impaired Nrf2 system in the pathogenesis of oxidative stress and inflammation in hemodialysis patients.

**Keywords** Chronic kidney disease · Inflammation · NF- $\kappa$ B · Nrf2 · Oxidative stress

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### Introduction

Inflammation and oxidative stress are common features of chronic kidney disease (CKD) and the major mediators of progression of renal disease and the associated cardiovascular disease, cachexia, anemia, and many other complications in CKD patients, particularly those requiring hemodialysis [1–4]. Oxidative stress causes inflammation

by activating nuclear factor- $\kappa$ B (NF- $\kappa$ B), a transcription factor that regulates transcription of several genes encoding pro-inflammatory cytokines, chemokines, and leukocyte adhesion molecules [5, 6]. The NF- $\kappa$ B system components are highly expressed in CKD patients, playing a pathogenic role in mediating chronic inflammation [7]. Oxidative stress in CKD is due to increased production of reactive oxygen species (ROS) and deficiency of the endogenous antioxidant capacity. The latter is caused by impaired activation of the nuclear factor E2-related factor 2 (Nrf2) [8, 9] which is the master regulator of numerous antioxidant and phase-2 detoxifying enzymes and related proteins. Nrf2 is inhibited by its cytosolic repressor protein, Kelch-like ECH-associated protein 1 (Keap1), an adapter component of Cullin 3 (Cul3)-based E3 ubiquitin ligase complex, that ubiquitinates and promotes proteasomal degradation of Nrf2. Via interaction with the sulfhydryl groups of certain cysteine residues in the Keap1 molecules, electrophiles or ROS prevent its ability to bind Nrf2 and thereby facilitate accumulation and nuclear translocation of Nrf2. In the nucleus, Nrf2 interacts with small musculoaponeuroticfibrosarcoma (Maf) and co-activator proteins. It then activates transcription of the target genes [10, 11] by binding to the antioxidant response elements (ARE) in their promoter regions. This leads to up-regulation of antioxidant and phase 2 detoxifying enzymes, including NADPH: quinoneoxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) which play a crucial role in cellular defense against oxidative stress and inflammation [12, 13].

Recent studies have documented the role of impaired Nrf2 activity in the pathogenesis of oxidative stress and inflammation in animals with CKD of diverse etiologies [14–16]. However, data on the effect of CKD on the Nrf2 system in humans is limited. We, therefore, designed this study to investigate mRNA expression of Nrf2, HO-1, NQO1 and NF- $\kappa$ B in a group of CKD patients on hemodialysis (HD) and compared the results with those obtained in healthy individuals.

## Materials and methods

### Subjects

Twenty HD patients (13 men and 7 women, mean age  $55.0 \pm 15.2$  years, average time on dialysis  $76.5 \pm 46.3$  months) attending the Clínica Nefrológica, Niterói, Rio de Janeiro, Brazil were enrolled. Inclusion criteria were age  $>18$  years, and HD treatment for at least 6 months. Patients with infection, cancer, acquired immune deficiency syndrome (AIDS) and autoimmune disease, as well as smokers and patients using shunts or central catheters as blood access for HD, were excluded.

Patients received hemodialysis treatment using hollow fiber cellulose acetate dialyzers for 3–4.5 h three times/week with blood flow  $>250$  ml/min, and dialysate flow of 500 ml/min. The patients studied were compared to eleven healthy individuals [5 men and 6 women, mean age  $50.9 \pm 8.0$  years, body mass index (BMI)  $23.8 \pm 1.9$  kg/m<sup>2</sup>]. This control group was composed of individuals without any disease and not on any medication. The healthy individuals were mainly the staff members of the dialysis unit and presented similar age and BMI compared to the HD patients. The study protocol was reviewed and approved by the Ethics Committee of the Universidade Federal Fluminense School of Medicine (Niterói, Brazil). All patients and controls signed the informed consent.

### Analytic procedures and sample processing

Blood samples were drawn from each subject in the morning, after overnight fasting and before the dialysis session, using a syringe containing 1.0 mg/ml ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Plasma was separated (15 min,  $3,000 \times g$ , 4 °C), and stored at  $-80$  °C until analysis.

To obtain the peripheral blood mononuclear cells (PBMC), blood samples with EDTA were diluted in phosphate buffered saline (PBS), and cells were separated in 5 ml Ficoll gradient (lymphocyte isolation solution, Axis-Shield, Oslo, Norway) by centrifugation at 800g for 20 min. PBMC were collected and washed twice with cold PBS and separated into two fractions; one of them re-suspended in buffer A [10 mM N-(2-hydroxyethyl) piperazine-N 0 -(2-ethanesulfonic acid) (HEPES); pH 7.5, 10 mM KCl, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] for protein analysis, and the second fraction was re-suspended and stored ( $-80$  °C) with 1 ml of recovery cell culture freezing (Invitrogen, Life Technologies, Waltham, MA, USA) for RNA isolation.

### Markers of oxidative stress, inflammation and clinical biochemical analysis

Serum urea nitrogen, phosphorus, potassium, calcium, albumin, creatinine and parathormone (PTH), and blood hemoglobin and hematocrit levels were determined through Bioclin<sup>®</sup> kits by automatic biochemical analyzer (Bioclin BS-120 Chemistry Analyzer, Mindray, Nanshan, Shenzhen, China). BMI was calculated as body weight divided by height squared. Dialysis dose (Kt/V) was calculated from values of blood urea nitrogen, pre- and post-dialysis, body weight, and dialysis duration using the standard formula [17].



Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was measured by enzyme-linked immunosorbent assay (ELISA) using DY210<sup>®</sup> duoset kits (R&D Systems, Minneapolis, MN, USA). Malondialdehyde (MDA) levels were measured by reaction with thiobarbituric acid. Samples were diluted with thiobarbituric acid (0.6 % m/v), heated to 95°, and the supernatant separated and read at 532 nm.

#### Real-time quantitative polymerase chain reaction (PCR) analysis

Nuclear factor E2-related factor 2, NF- $\kappa$ B, NQO1 and HO-1 mRNA levels were assessed in PBMC using real-time quantitative PCR (qPCR). PBMC were isolated from blood, and RNA was extracted with an SV Total RNA Isolation System (Promega Corp., Madison, WI, USA). The cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Waltham, MA, USA). TaqMan Gene Expression Assays (Applied Biosystems) were used to detect Nrf2 (Hs00975961\_g1), NF- $\kappa$ B (Hs00765730\_m1), NQO1 (Hs00168547\_m1), and HO-1 (Hs02512143\_s1) mRNA and the control gene, ABL1 (Hs00245445\_m1). PCR amplification was carried out using the ABI Prism 7,500 Sequence Detection System (Applied Biosystems) and standard cycling conditions. The amount of NQO1 transcript was normalized to corresponding ABL1 levels. The expression of Nrf2, NF- $\kappa$ B, NQO1 and HO-1 mRNA was normalized against ABL1, and the expression level was calculated using the  $\Delta\Delta$ CT (delta delta threshold cycle) method.

#### Western blot analysis

To evaluate the protein expression in PBMC we performed western blotting assay. PBMC were washed twice in ice-cold PBS and lysed on ice with lysis solution (1 % Triton X-100, 150 mM NaCl, 20 mM Tris pH 8 and protease inhibitors). The protein concentration of the samples was determined by the protein assay Quick Start<sup>®</sup> (Bio-Rad, Hercules, CA, USA). Cell lysates were boiled at 100 °C for 5 min and later separated on 10 % Bis-Tris gels. The proteins were electrotransferred to nitrocellulose membranes (Pierce<sup>®</sup>, Thermo Scientific, Waltham, MA, USA). The filters were blocked for 1 h at room temperature in 5 % bovine serum albumin (BSA) (Invitrogen, USA) dissolved in tris-buffered saline tween (TBS-T), and then incubated with specific antibodies against p-NF kappa B p65 (Santa Cruz, dilution 1:250), NF kappa B p65 (Santa Cruz, dilution 1:500), Nrf2 (Santa Cruz, dilution 1:500) overnight at 4 °C.  $\beta$ -actin (Santa Cruz, 1:2,000) was also determined in each filter in order to normalize slight variations in protein loading. Blots were then incubated

with antibodies conjugated to horseradish peroxidase (HRP) (1:3,000 in 1 % milk/TTBS) for 1 h followed by enhanced chemiluminescence detection. Band intensities were quantified by densitometric analysis using ImageJ 1.3 software (NIH).

#### Statistical analysis

Normal distribution of the data was determined using the Shapiro–Wilk test. Data are expressed as mean  $\pm$  standard deviation (SD). Differences between continuous data were analyzed by two-tailed unpaired Student's *t* test. Pearson's correlation coefficient was calculated to examine the relation between variables. Statistical significance was accepted at  $p < 0.05$ . Statistical analyzes were performed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

## Results

General data are summarized in Table 1. As expected, serum urea nitrogen, creatinine, and PTH levels were high and hematocrit and hemoglobin were low in the HD patients. Considering the criteria established by the World Health Organization (WHO) for the classification of nutritional status according to BMI [18], no patient showed malnutrition or obesity (BMI  $<18.5$  kg/m<sup>2</sup> and BMI  $\geq 30.0$  kg/m<sup>2</sup>, respectively). Seven patients presented overweight (BMI 25.0–29.9 kg/m<sup>2</sup>).

The mRNA expression of the P65 subunit of NF- $\kappa$ B in PBMC was significantly higher in HD patients than in the healthy individuals (Table 2; Fig. 1). This was associated with a significant increase in phosphorylated P65 and an elevated phosphorylated: non-phosphorylated P65 ratio in the HD group, denoting activation of NF- $\kappa$ B which is the master regulator of many proinflammatory, cytokine and other mediators (Fig. 1). In contrast, Nrf2 mRNA and protein expressions were markedly reduced in the PBMC of HD patients compared to healthy individuals. This was associated with a significant reduction of mRNA expression of NQO1 (one of the Nrf2 target gene products) in the HD patients compared to healthy individuals. Moreover, the Nrf2:NF- $\kappa$ B mRNA ratio was significantly lower in the PBMC of HD patients than in the healthy individuals (Table 2). The Nrf2 mRNA was inversely correlated with NF- $\kappa$ B mRNA ( $r = -0.54$ ,  $p = 0.014$ ,  $n = 20$ ) in HD patients, and directly in healthy individuals ( $r = 0.77$ ,  $p = 0.006$ ,  $n = 11$ ). These findings point to dysregulation of the Nrf2 pathway in HD patients (Fig. 2).

Plasma MDA and TNF- $\alpha$  levels, markers of oxidative stress and inflammation, respectively, were significantly higher in HD patients compared to healthy individuals. No significant difference was found in the HO-1 mRNA

**Table 1** Biochemical and clinical profile of the HD patients

Parameters	Values	Normal values
BMI (kg/m <sup>2</sup> )	23.6 ± 3.0	18.5–24.9
Urea nitrogen (mg/dl)	142.1 ± 27.7	<18.0
Creatinine (mg/dl)	8.4 ± 2.6	<1.0
Potassium (mg/dl)	5.4 ± 0.5	3.5–5.5
Phosphorus (mg/dl)	5.2 ± 1.2	3.5–5.5
Calcium (mg/dl)	8.9 ± 0.5	8.4–9.5
Albumin (g/dl)	3.9 ± 0.6	>3.8
Hematocrit (%)	31.5 ± 6.0	35–45
Hemoglobin (g/dl)	10.6 ± 1.9	>12
PTH (pg/dl)	401 ± 380	150–300
Kt/V	1.57 ± 0.4	Not applicable

Values are mean ± SD (n = 20)

HD hemodialysis, BMI body mass index, PTH parathormone, Kt/V dialysis dose

**Table 2** The mRNA expression of NF-κB, Nrf2 and antioxidants enzymes, lipid peroxidation and inflammation markers levels in healthy individuals and HD patients

Parameters	HD patients (n = 20)	Healthy individuals (n = 11)
Nrf2	0.58 ± 0.35*	1.13 ± 0.64
NF-κB	2.18 ± 0.8*	1.04 ± 0.22
Nrf2/NF-κB ratio	1.0 ± 0.4*	0.3 ± 0.3
HO-1	1.7 ± 0.7	1.5 ± 1.4
NOQ1	0.47 ± 0.26*	1.04 ± 0.25
TNF-α (pg/ml)	46.0 ± 11.8**	36.1 ± 10.1
MDA (nmol/ml)	13.2 ± 5.3*	5.1 ± 2.7

Nrf2, NF-κB, HO-1 and NOQ1 mRNA expression was assessed in PBMC by real-time quantitative PCR. TNF-α was measured by ELISA. MDA levels were measured by reaction with thiobarbituric acid

HD hemodialysis, Nrf2 nuclear factor E2-related factor 2, NF-κB nuclear factor-κB, HO-1 heme oxygenase-1, NOQ1 quinone oxidoreductase 1, TNF-α tumor necrosis factor-alpha, MDA malondialdehyde, PCR polymerase chain reaction, ELISA enzyme-linked immunoassorbent assay

\* p < 0.01; \*\* p < 0.05

expression between the HD patients and healthy individuals (Table 2).

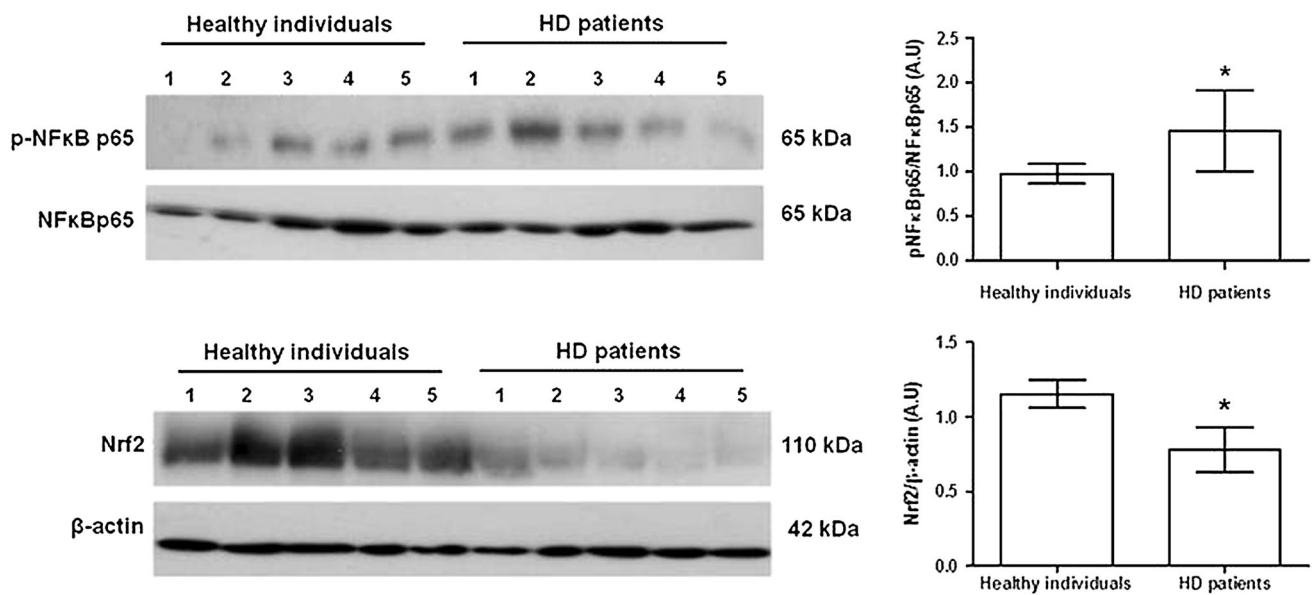
## Discussion

The main finding of this study was the marked reduction of Nrf2 mRNA and protein expressions in the PBMC of end-stage renal disease (ESRD) patients compared to healthy individuals. This was associated with a significant

reduction in expression of NOQ1, which is a major Nrf2 target gene product. The reduction in Nrf2 expression was accompanied by a significant elevation of plasma MDA concentration denoting presence of systemic oxidative stress in the ESRD group. In addition, the PBMC from ESRD patients showed marked increases in P65 mRNA, P65 phosphorylation, and plasma TNF-α concentration, pointing to up-regulation and activation of NF-κB and systemic inflammation. These findings are consistent with in-depth studies in animal models of CKD which demonstrated marked impairment of the Nrf2 pathway and down-regulation of its key target gene products in the renal and vascular tissues together with activation of NF-κB, systemic oxidative stress and inflammation [14–16].

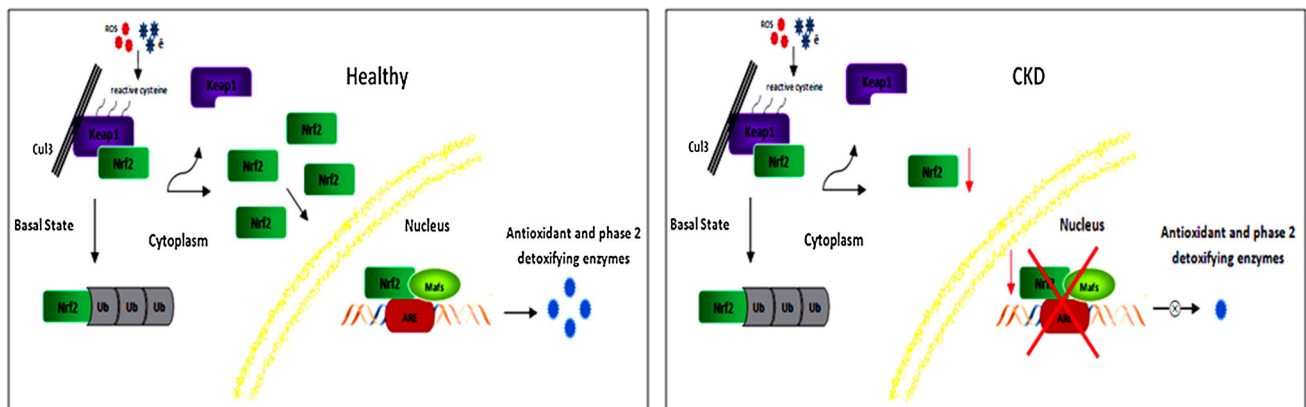
Nuclear E2-related factor 2 plays a central role in constitutive expression and coordinated induction of over 250 genes [19], encoding phase II detoxifying and antioxidant enzymes including NQO1, HO-1, glutathione S-transferase, glutathione peroxidase, glutamate cysteine ligase, catalase, superoxide dismutase, UDP-glucuronosyl-transferase, thioredoxin and peroxiredoxins, and others. These enzymes play a crucial role in cellular defense by removal of ROS and toxic products [12, 13, 20]. Thus, Nrf2 is essential in cellular protection against oxidative stress and inflammation [5, 21–23]. In fact, Nrf2(-/-) mice with streptozotocin-induced diabetic nephropathy exhibit greater oxidative stress, DNA damage, and renal injury than the wild-type mice [24]. In addition Nrf2 knockout mice develop a lupus-like autoimmune nephritis [25] and show far more severe acute kidney injury and dysfunction, and higher mortality when exposed to ischemic or nephrotoxic insults compared to the wild-type mice [26].

The contribution of the impaired Nrf2 pathway to the pathogenesis of oxidative stress, inflammation and renal and cardiovascular disorders in CKD is further illustrated by the salutary effect of treatment with various Nrf2 inducers in experimental animal models of acute and chronic kidney diseases. For example, long-term administration of the synthetic Nrf2 inducer, dh404, at 2 mg/kg/day has been shown to restore Nrf2 activity, attenuate oxidative stress and inflammation and improve renal lesion and endothelial function in CKD rats [27, 28]. Likewise sulforaphane, which is an organosulfur compound found in vegetables, improves nephropathy in animals with streptozotocin-induced diabetes [29], and epigallocatechin-3-gallate, the polyphenol found in green tea, attenuates renal lesions in animals with systemic lupus erythematosus [30], in the mouse model of rapidly progressive glomerulonephritis [31], and in animals with cisplatin-induced acute kidney injury [32]. Similarly curcumin, a natural Nrf2 inducer isolated from turmeric, protects against chromium-induced nephrotoxicity in experimental animals [33].



**Fig. 1** Representative western blots and group data depicting p65NF-κB, NF-κB (a and b) and Nrf2 (c and d) protein expressions in PBMCs of HD patients ( $n = 20$ ) and healthy individuals ( $n = 11$ ). Compared to the healthy individuals, the PBMC in HD patients had showed a significant increase in phosphorylated NF-κBp65 (pNF-

κBp65), an increased phosphorylated/:non-phosphorylated NF-κBp65 ratio, and a significant reduction in Nrf2 protein expression. Data are mean  $\pm$  SD. \* $p < 0.5$ . *PMBC* peripheral blood mononucleated cells, *HD* hemodialysis



**Fig. 2** In the healthy individuals, in response to oxidative stress, the Nrf2 is targeted to the nucleus where it binds to the antioxidant response element. In contrast, it seems that in CKD patients there is a down-regulation of Nrf2

While the association of CKD with Nrf2 deficiency and its contribution to systemic oxidative stress and inflammation is reasonably clear, the mechanism by which CKD results in Nrf2 deficiency and dysfunction is not known. However, a likely candidate is systemic inflammation, which is invariably present in patients and animals with advanced CKD. This is based on the observation that the active subunits of NF-κB, i.e. P65 and P53, can interfere with the dissociation of Nrf2 from its repressor molecule, Keap1, in the cell cytoplasm and with binding of Nrf2 to the antioxidant response elements of the target genes in the cell nucleus [34, 35]. This accounts for the presence of

impaired Nrf2 activity in various disorders which are associated with chronic inflammation, including chronic granulomatous disease [36], asthma [37], and chronic kidney disease [15, 16]. In fact, in a recent study using human proximal tubular cells and rat kidneys, Bolati et al. [38] found that indoxyl sulfate, a uremic toxin, down-regulates expression of Nrf2 through activation of NF-κB, with consequent decrease in HO-1 and NQO1 and increase in production of ROS. They further demonstrated that the inhibition of NF-κB attenuates indoxyl sulfate-induced decrease of Nrf2 expression. It should be noted that the Nrf2 gene has an antioxidant response sequence and as such



is positively regulated by Nrf2. Consequently inflammation-induced impairment of Nrf2 activation contributes to its depressed expression, as seen in the present study. It is of interest that, in a recent study, Zaza et al. [39] found a significant increase in Nrf2 and SOD2 expressions in the PBMC of CKD patients maintained on peritoneal dialysis, contrasting the results found in hemodialysis patients. This may reflect the higher burden of inflammation in hemodialysis patients caused by intermittent blood exposure to the extracorporeal circuit and the dramatic rise and fall in body fluid volume during the inter- and intra-dialytic intervals. Further studies are needed to address these issues.

Given the contribution of Nrf2 dysfunction in the pathogenesis of oxidative stress and inflammation, which play a central role in promoting cardiovascular disease and numerous other morbidities, interventions aimed at restoring Nrf2 expression and activity in CKD patients—maybe highly beneficial. In our recent review we proposed that nutrients containing plant-based Nrf2 inducers may help to improve the Nrf2-Keap1 system in this population [40]. This is particularly relevant since consumption of fruits, vegetables and nuts which contain natural Nrf2 activators are generally restricted in patients with advanced CKD in an attempt to prevent hyperkalemia and fluid overload.

In conclusion, the present study revealed down-regulation of Nrf2 and up-regulation of NF- $\kappa$ B in the PBMC of CKD patients maintained on hemodialysis, confirming the results observed in experimental animal models of CKD. These findings point to an impaired Nrf2 pathway in the pathogenesis of oxidative stress and inflammation in CKD patients.

**Acknowledgments** We wish to thank the Clínica Nefrológica, Niterói-Rio de Janeiro, for allowing their patients to participate in this study. We also thank Conselho Nacional de Pesquisa (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), for the funding.

**Conflict of interest** The authors declare there are no conflicts of interest.

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