Toxico-pharmacological perspective of the Nrf2-Keap1 defense system against oxidative stress in kidney diseases

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Abbreviations: ADMA, asymmetric dimethylarginine; AGE, advanced glycation end product; AKI, acute kidney injury; CKD, chronic kidney disease; CVD, cardiovascular disease; ERK, extracellular signal-regulated kinase; GLC, glutamate cysteine ligase; GSH, glutathione; GST, GSH S-transferase; HO-1, heme oxygenase-1; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; MCP-1, monocyte chemoattractant protein-1; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NF-κB, nuclear factor-κB; NQO1, NADPH quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; PPAR-γ, peroxisome proliferator-activated receptor-γ; ROS, reactive oxygen species; SOD, superoxide dismutase.

ABSTRACT

Oxidative stress, including the generation of reactive oxygen species (ROS), appears to be responsible for the high incidence of cardiovascular events in patients with chronic kidney disease (CKD), and for the progression of CKD to end-stage renal disease. The processes for oxidative stress include increased generation and decreased elimination of ROS that could be caused by an impaired antioxidant defense system. Nuclear factor-erythroid-2-related factor 2 (Nrf2) helps protect the kidney against oxidative stress by playing a pivotal role in the cooperative induction of genes that encode antioxidant and detoxifying enzymes. Nrf2 is confined to the cytoplasm as an inactive complex bound to a repressor Kelch-like ECH-associated protein 1 (Keap1), which facilitates ubiquitination of Nrf2. Studies using CKD model animals showed that despite stimulated oxidative stress the nuclear Nrf2 level was suppressed, which led to downregulation of the antioxidant enzymes. Hence, deterioration in Nrf2-Keap1 signaling could contribute to the severity of oxidative stress and the progression of CKD. By contrast, acute kidney injury (AKI) induces activation of renal Nrf2. Nrf2 activators or its proteasomal degradation inhibitors enhance nuclear Nrf2 translocation, inducing potential renoprotective actions against CKD and AKI. In both chronic and acute kidney diseases, sulfate-conjugated uremic toxins appear to enhance ROS production when accumulated in renal cells. An intestinal indole adsorbent ameliorates the progression of CKD by decreasing accumulation of indoxyl sulfate. Therapeutic approaches to prevent oxidative stress *via* activation of the Nrf2-Keap1 signaling and/or suppression of uremic toxin-induced ROS production could be effective strategies for maintaining kidney function.

Keywords: Nrf2, chronic kidney disease, acute kidney injury, uremic toxins, renal tubules.

1. Introduction

The incidence of chronic kidney disease (CKD) is increasing in both developed and developing nations. It is generally recognized that many patients with CKD are likely to die of cardiovascular disease (CVD) rather than kidney dysfunction [1]. A cohort study comprising >13,000 elderly patients revealed that an increase in the incidence of cardiovascular events could, in part, be related to the fact that patients with kidney disease are less likely to receive preventive treatments against CVD [2]. However, the mechanisms for the enhanced susceptibility to CVD in CKD patients are not fully clarified. The injured and/or dysfunctional kidney-specific risk factors such as endothelial dysfunction, inflammation, oxidative stress, anemia, proteinuria and changes in vitamin D metabolism have been suggested to play a pathophysiological role not only in CVD but also in further progression of CKD [1]. Among these factors, oxidative stress has attracted a great deal of interest from researchers. Oxidative stress appears to increase in the serum of CKD patients because of increased oxidant activity as well as a reduced antioxidant defense system, which is accompanied by kidney dysfunction and/or severe cardiorenal syndrome [3-6].

A transcription nuclear factor erythroid 2-related factor 2 (Nrf2) is characterized as “an oxidative stress-sensing guarding regulator” of more than 200 cytoprotective genes encoding proteins that neutralize or detoxify both endogenous metabolites and environmental toxins [7-9]. Nrf2 appears to function when released from its repressive redox-sensitive companion protein Keap1 (Kelch-like ECH-associated protein 1) by sensing cytoplasmic oxidative stress or some chemical agents [8-10] (Fig.1). After translocation into the nucleus, Nrf2 stimulates transcription of genes encoding detoxifying and antioxidant enzymes, such as NADPH (nicotinamide adenine dinucleotide phosphate) quinone oxidoreductase1 (NQO1), GSH S-transferase (GST),

heme oxygenase-1 (HO-1), glutamate cysteine ligase (GLC) and peroxiredoxin I, GSH peroxidase, which contribute to cellular protection by removing reactive oxygen species (ROS) including superoxide anions, hydrogen peroxide and hydroxyl radicals [11]. Although the principal role of the Nrf2-Keap1 defense system in renal ROS production has been well characterized, its toxico-pharmacological role and regulation in "oxidative stress management" of CKD situation are not fully elucidated. Alternatively, ischemic acute kidney injury (AKI) remains a major frequent clinical problem, as AKI aggravates acute mortality and results in permanent and progressive kidney disease, i.e., CKD. In ischemia-reperfusion-induced AKI model animals, ROS appeared to enhance both endothelial and renal tubular injuries [12]. In murine models of AKI, bardoxolone methyl, an orally-available first-in-class synthetic triterpenoid (also known as "RTA 402" or "CDDO-methyl ester), alleviated functional and structural kidney injuries in association with activation of Nrf2 in glomerular endothelium, cortical peritubular capillaries and renal tubules [13]. Therefore, the Nrf2-Keap1 defense system has been suggested to play a pivotal guardian role in protection of kidneys against diverse oxidative stress generated in both chronic and acute kidney injuries through activating potent antioxidant tools. In this commentary, possible strategic approaches and perspectives focusing on oxidative stress and the Nrf2-Keap1 defense system to prevent the progression of CKD and CVD are discussed.

2. Oxidative stress and the role of Nrf2 in CKD

2.1. Oxidative stress in CKD

The role of oxidative stress has attracted an increasing attention in the field of CKD, cardiorenal syndrome and their preventive strategies [14,15]. Oxidative stress is

provoked by excessive production of free radicals, low antioxidant defense or a combination of these two factors. The consequence of oxidative stress is chemical modifications of biomolecules, resulting in structural and/or functional changes. Oxidative stress is defined as the tissue damage resulting from an imbalance between an excessive generation of oxidant species and insufficient antioxidant defense mechanisms. Several processes appear to be involved in ROS production, including mitochondrial respiration, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidases and uncoupled nitric oxide (NO) synthesis. Oxidative stress and inflammation are common features found in patients with CKD in different disease stages, particularly in patients requiring hemodialysis [14,15]. ROS are considered to be major mediators of cardiovascular events and numerous other physiological complications, in addition to playing a critical role in the progression of CKD. Indeed, CKD patients who generate excessive ROS have an increased risk of morbidity and mortality [15]. Several lines of evidence suggest that CKD is a pro-oxidant state. Specifically, (i) oxidation markers of lipid, protein and DNA are increased in the serum of CKD patients; (ii) oxidative markers, such as hypochlorous acid (HOCl)-modified lipoproteins and advanced glycation end products (AGEs), are accumulated in atherosclerotic lesions of CKD patients; (iii) there are numerous defects in the antioxidant defense mechanism, resulting in a decreased elimination clearance of ROS [15]. The defects can be used as indirect markers for oxidative stress, e.g. an increased oxidized to reduced plasma ratio of vitamin C and red blood cell GSH level. Increases in the circulating levels of oxidative markers have been documented in patients with CKD [4,5,14]. Thus, oxidative stress is considered to occur in the early stages of renal failure. Dialysis treatment appears to be ineffective in correcting this oxidative stress. However, the prevalence of oxidative stress in CKD patients remains to be determined because population-based clinical studies have

not been conducted.

2.2 ROS production

Oxidative stress occurs under a state of imbalance between free radical production and degradation by antioxidant defense systems, resulting in increased accumulation of free radical species [16,17]. ROS appear to be typical examples of free radicals[17]. Over 90% of ROS formation occurs “accidentally” in mitochondria during metabolism of oxygen when some of electrons passing “down” the electron transport chain leak away from the main path, and go directly to reduce oxygen molecules to generate the superoxide anion [16]. Multiple enzyme systems also synthesize ROS in cells of the vascular endothelial wall and various other tissues including kidneys [18]. When ROS are produced in excess, they can react with various molecules such as lipids, carbohydrates, proteins and DNA, thereby altering their structures and functions in association with cellular damage that leads to pathological processes including enhanced formation of atherosclerosis in blood vessels. These potentially harmful reactions are surveyed by cytoprotective defense systems made up of enzymatic and nonenzymatic antioxidants, which eliminate pro-oxidants and scavenge-free radicals [19]. Superoxide dismutase (SOD), GSH peroxidase, GSH reductase and catalase are considered to be the predominant enzymatic antioxidant tools. GSH, thiols, ascorbic acid, α-tocopherol (vitamin E), mixed carotenoids and bioflavonoids are recognized as the nonenzymatic antioxidant tools in many cells.

2.3. Nrf2-Keap1 activation and its downstream events

Nrf2 is a transcription factor of the cap “n” collar basic region leucine zipper (cnc bZip) family, controlling expression of various cytoprotective antioxidant enzymes

[9,10,20,21]. This transcription factor is found in ubiquitous tissues, but is activated in response to a wide range of oxidative and electrophilic stimulation, including ROS and some chemicals agents. Given that Nrf2 manages a broad sweep of cellular antioxidant defense mechanisms, this pathway may also contribute to the multi-factorial phenotype associated with the aging process. Recent studies demonstrate that Nrf2 modulates the expression of antioxidant genes through interaction with antioxidant stress element, ARE [20]. Under normal physiological conditions and a low oxidative stress environment, Nrf2 is confined to the cytoplasm associated with the suppressor protein Keap1, and is degraded by the ubiquitin proteasome pathway. Oxidative and electrophilic stress factors stimulate dissociation of the Nrf2-Keap1 complex, thereby promoting the release and translocation of Nrf2 into the nucleus to upregulate expression of Nrf2/ARE-linked antioxidant genes as described above [9,21]. An issue to be addressed is the toxico-pathological role of the Nrf2-Keap1 1 defense system and its products in the kidney of patients with CKD. A recent study by Kim and Vaziri [22], using renal tissue from subtotal nephrectomized kidney of CKD model rats, showed impairment in the activation of Nrf2, thereby leading to consequent derangement of regulation of downstream antioxidant enzymes. The nephrectomized rats also exhibited increased lipid peroxidation, GSH depletion, NF-kB activation, mononuclear cell infiltration, and upregulation of monocyte chemoattractant protein-1, NADPH oxidase, cyclooxygenase-2, and 12-lipoxygenase in the remnant kidney, enhancing oxidative stress and inflammation. Nrf2 activity in the remnant kidney, i.e., nuclear translocation, was markedly reduced at 12 weeks, whereas the Nrf2 repressor Keap1 was upregulated and the Nrf2 target gene proteins, including catalase, superoxide dismutase, GSH-Px, HO-1, NQO1 and glutamate-cysteine ligase, were significantly decreased at 12 weeks. They concluded that despite severe oxidative stress and inflammation, which should have induced Nrf2 activation and consequent upregulation of

antioxidant and detoxifying enzymes, the remnant kidney in CKD animals exhibited "paradoxical reduction" of Nrf2 activation and its downstream antioxidant molecules [22]. Therefore, it is possible that impaired activation of the Nrf2-Keap1 system aggravates further oxidative stress generation and inflammation in CKD, leading to progression of the disease [23]. In such a way, therapeutic treatment enhancing Nrf2 activation and its regulated antioxidants could be an attractive strategy for alleviating the effects of oxidative stress and inflammation in the progression of CKD. Possible antioxidant interventions in therapeutic treatment of CKD are discussed in the later section of this review.

3. Oxidative stress and Nrf2 in ischemic acute kidney injury (AKI)

3.1. ROS production in AKI

AKI is observed in 5–20% of patients in the intensive care unit, resulting in a risk of death that is independent of other complications or co-existing diseases. Despite this situation, there is as yet no effective pharmacological intervention or treatment to improve outcome in patients with AKI. Renal ischemia-reperfusion injury, which could occur in clinical settings such as renal transplantation, shock and vascular surgery, is a major cause of AKI. Clinical and experimental studies have demonstrated that renal tissue damage following ischemia-reperfusion, especially during reperfusion, is due in part to the local production of ROS [12,13,18]. The critical role of ROS in the pathophysiology of ischemia-reperfusion injury is evident by the increased formation of lipid hydroperoxides and other toxic products that occurs in accordance with kidney injury [24]. The previous study demonstrated that treatment with N-acetylcysteine, a compound with antioxidant activity as a scavenger, ameliorated the decline in GFR and reduced hyperkalemia on day 1, lowered plasma creatinine levels on days 1 and 3, and

decreased renal interstitial inflammation on day 7, after ischemia-reperfusion of the kidney [25]. Renal tissue glutathione (GSH) levels were decreased significantly in the group of ischemia-reperfusion with saline in response to ischemia, but were completely restored in the group of ischemia-reperfusion with N-acetylcysteine. Groups with ischemia-reperfusion-induced AKI showed increased plasma ascorbyl radical levels, and elevated urinary 8-iso-prostaglandin F2α excretion, compared with the control group. N-acetylcysteine treatment reduced plasma ascorbyl concentrations 24 h after renal ischemia-reperfusion, but had no effect on the rate of urinary 8-iso-prostaglandin $F_{2\alpha}$ excretion. Moreover, N-acetylcysteine improved kidney function and reduced renal interstitial inflammation in rats following ischemia-reperfusion of the kidney [25].

3.2. Nrf2 activators

Sulforaphane, 1-isothiocyanate-(4R)-(methylsulfinyl)butane, is a dietary isothiocyanate produced by myrosinase activity on glucopharanin, a 4-methylsulfinylbutyl glucosinolate contained in cruciferous vegetables of the genus Brassica such as broccoli, Brussels sprouts and cabbage, and is known to activate the Nrf2 signaling pathway [9]. Histological studies suggested that pretreatment with sulforaphane prevented morphological damage to the rat kidney following ischemia-reperfusion, but resulted in slight swelling of the tubular epithelium and a slight loss of brush border [26]. In HK2 cells, which originate from human renal tubular epithelium, the cytoplasmic suppressing protein, Keap1 is bound directly to Nrf2 under normal quiescent conditions, thereby repressing its nuclear translocation. However, exposure of HK2 cells to sulforaphane for 12 h decreased the cytoplasmic level of Keap1 protein and increased the expression level of nuclear Nrf2 [26]. Sulforaphane effectively reduces renal dysfunction or injury caused by ischemia-reperfusion of the kidney. The

renoprotective mechanism of sulforaphane is thought to be mediated by preconditioning of the kidney by activation of Nrf2 and the resultant induction of phase 2 enzymes such as heme oxygenase-1, NADPH: quinone oxidoreductase 1, GSH reductase and GSH peroxidase [26].

An earlier report suggested that bardoxolone methyl ameliorated ischemic marine AKI as evaluated by both renal function and pathology [13]. Bardoxolone methyl may exert its renopreventive action by increasing expression of genes previously shown to protect against ischemic AKI, including Nrf2, peroxisome proliferator-activated receptor-γ (PPAR-γ) and HO-1. Indeed, treatment with bardoxolone methyl or ischemia-reperfusion increased expression of these genes. However, an even greater increase in expression occurred after combined treatment with ischemia-reperfusion and bardoxolone methyl. Furthermore, bardoxolone methyl increased the level of Nrf2 mRNA in renal tissue. This observation suggests a separate mechanism for the increased level of Nrf2 protein must exist in addition to the direct activation and/or increased half-life of Nrf2 [13].

3.3. Low molecular-weight uremic toxins in CKD

Among the various metabolites that accumulate in the plasma of CKD patients, uremic solutes are thought to be candidates for specific pathogenic events such as inducing oxidative stress, endothelial dysfunction and injury (Table 1) [27]. It was shown that indoxyl sulfate, a typical sulfate-conjugated uremic solute, induces endothelial dysfunction by producing ROS that modify the balance between pro- and antioxidant mechanisms in vitro and in patients with CKD [28-31]. Impairment of renal functions leads to the extensive retention of a large number of compounds which, under normal physiological conditions, are excreted mostly into urine. Owing to this accumulation, the plasma retained

molecules are called uremic retention solutes or, as adopted by the EUTOX (the European Uremic TOXins) Work group, uremic toxins [32]. It is noteworthy that most of these uremic toxins possess biological and/or biochemical activities. The uremic toxins are categorized based on their molecular weight (MW) and their protein-binding ability; the most convenient classification being (1) low MW water-soluble compounds; (2) protein-bound compounds; and (3) high MW compounds. Undesirable effects can be induced by toxins that are difficult to remove by dialysis. This is the case for the second group, namely the protein-bound uremic toxins, which are poorly removed by common dialysis tools [32]. Two typical protein-bound uremic retention solutes, *p*-cresyl sulfate and indoxyl sulfate have been extensively studied. The serum accumulation of these two compounds has been shown to be elevated in advanced stages of CKD and correlate with a decline of glomerular filtration rate [33].

In addition to classical Framingham risk factors, uremic toxins can be considered as nontraditional risk factors in patients with CKD developing CVD. Previous clinical studies obtained in different CKD patient cohorts (i.e., both in hemodialysis and in predialysis patients) identified indoxyl sulfate and *p*-cresyl sulfate as emerging mortality risk factors [34,35]. Both uremic toxins are thought to be actively excreted by the kidneys *via* proximal tubular secretion through the basolateral organic anion transporters OAT1 and OAT3 [36,37] (Fig. 1). As a consequence, the toxins accumulate in the serum of patients with both acute and chronic impaired renal functions. In addition, both toxins cannot be efficiently removed by conventional hemodialysis due to their high binding affinity for albumin. A cohort study involving patients in different stages of CKD demonstrated that serum levels of total and free indoxyl sulfate and total and free *p*-cresyl sulfate were increased in the advanced stages of the disease [34]. Moreover, the serum levels of indoxyl sulfate and *p*-cresyl sulfate correlated with glomerular filtration rate in pre-dialysis

patients.

3.4. Indoxyl sulfate and ROS production

Indoxyl sulfate is one of the uremic toxins that induces free radicals. Tubulointerstitial injury evoked by indoxyl sulfate has been explained by oxidative stress in renal tubules. Induction of intracellular superoxide production *via* NADPH oxidase was also shown in cultured rat mesangial cells when treated with indoxyl sulfate [38]. In hemodialyzed patients, serum concentrations of indoxyl sulfate were associated with levels of pentosidine, a marker of carbonyl and oxidative stress [39]. *In vitro* studies suggested that indoxyl sulfate increased ROS production in renal tubular cells, thereby upregulating the expression of MCP-1 (monocyte chemoattractant protein-1) and activating NF-κB, p53, ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinases) in association with tubulointerstitial injury [40].

A portion of dietary protein-derived tryptophan is metabolized into indole in the intestine by tryptophanase of intestinal microflora. Indole is absorbed from the intestine into the bloodstream and then metabolized by cytochrome P450 (CYP) and sulfotransferase in the liver to indoxyl sulfate [41]. In patients with CKD a decrease in the renal clearance of indoxyl sulfate leads to its increased serum accumulation. AST-120, an oral carbon adsorbent, has been shown to reduce the levels of indoxyl sulfate in the serum and urine of CKD model rats and patients with CKD [42-45]. This observed decrease in the level of indoxyl sulfate occurs because AST-120 adsorbs indole in the intestine and thereby stimulates its excretion in the feces. Administration of indoxyl sulfate and its precursor, indole, to subtotal nephrectomized rats enhanced glomerular sclerosis in the remnant kidney with declined renal function. Furthermore, indoxyl sulfate stimulated transcription of genes related to renal fibrosis, such as transforming growth factor-β1

(TGF-β1) [42]. The nephrotoxic activity of indoxyl sulfate is mediated by organic anion transporters, OAT1 and OAT3, localized at the basolateral membrane of renal proximal tubular cells [36,46,47]. Indoxyl sulfate suppresses superoxide scavenging activity in the kidneys of normal and uremic rats [48]. Thus the nephrotoxicity of indoxyl sulfate could be developed by both stimulating ROS production and impairing the antioxidative system in the kidney. Despite the fact that indoxyl sulfate produces ROS when accumulated in renal tubular cells and endothelial cells, there is no available data regarding the relationship between indoxyl sulfate and Nrf2 activation.

4. Antioxidant interventions and Nrf2 activation

4.1. Clinical antioxidant therapy in CKD

Several clinical studies have been performed to examine the efficacy of antioxidant interventions on oxidative stress markers in patients with CKD [4,5,15,49,50]. Unfortunately, there are only a few randomized controlled clinical trials to study the impact of antioxidant interventions on CVD outcomes in patients with CKD. SPACE (Secondary Prevention with Antioxidants of Cardiovascular Disease in End-Stage Renal Disease) was a clinical trial involving 196 patients with ESRD who were randomized to either receive 800 IU of α-tocopherol (vitamin E) per day or matching placebo [51]. During a median follow-up period of 519 days, statistically significant reduction in the primary composite outcome, consisting of myocardial infarction, ischemic stroke, peripheral vascular disease and unstable angina, was found in patients receiving vitamin E supplementation. In the second randomized controlled trial, N-acetylcysteine at an oral dose of 600 mg was given twice daily over a period of 15 months [52]. This regimen was found to significantly suppress primary outcomes of cardiac events including fatal and

nonfatal myocardial infarction, CVD-related death, need for coronary angioplasty or coronary bypass surgery, ischemic stroke, peripheral vascular disease with amputation, or need for angioplasty. However, no beneficial effects of vitamin E or N-acetylcysteine administration were observed on all-cause mortality, suggesting that additional strategies are required to improve overall survival in dialysis patients [51,52]. Subgroup analysis of some lipid-lowering trials, which included CKD patients, suggested that statin treatments may also reduce the serum inflammatory and oxidative markers. Additional studies addressing the clinical impact of a novel class of antioxidants, including endogenous antioxidants such as HO-1, for reducing oxidative stress are needed in CKD patients [53].

4.2. Bardoxolone methyl in CKD patients with diabetes mellitus

Bardoxolone methyl has been described as a drug that protected cells from radiation-induced damage *via* Nrf2-dependent pathways [54]. Bardoxolone methyl promotes activation of Nrf2, which is released from the suppressor Keap1 and translocates into the nucleus where it regulates transcription of antioxidant genes containing ARE sequences in their promoter region. These phase 2 genes are involved in the elimination and/or removal of ROS and inhibition of NF-κB. Thus, bardoxolone methyl may protect renal cells by both antioxidant and anti-inflammatory effects (i.e., promoting the activity of Nrf2 and suppressing the activity of NF-κB) [49,55].

In humans, the potential anticancer activity of bardoxolone methyl was evaluated. During phase I trials involving patients with cancer, bardoxolone methyl unexpectedly improved renal function, assessed in terms of serum creatinine and creatinine clearance, especially in patients who had previously suffered from CKD [55]. These observations suggested that bardoxolone methyl induces potential renoprotective

actions in patients with CKD and type 2 diabetes mellitus. This was initially noted in an exploratory phase II open-label trial and then in a larger randomized clinical trial. In the first trial, 20 patients with moderate-severe diabetic CKD were evaluated after 8 weeks of bardoxolone methyl treatment [56]. Notably, there was a significant increase in estimated GFR at 4 weeks (+2.8 mL/min/1.73 m^2) using a dosage of 25 mg/day and at 8 weeks (+7.2 mL/min/1.73 m^2) using a dosage of 75 mg/day. Serum creatinine and BUN decreased and creatinine clearance increased, without any changes in total excretion or tubular secretion of creatinine. Notably, a slight, but significant albuminuria was observed in patients with CKD in association with bardoxolone methyl administration. Adverse events were generally manageable and mild to moderate in severity. In the phase II, double-blind, randomized, placebo-controlled trial (BEAM study), bardoxolone methyl was associated with improvement in the estimated GFR in patients with advanced CKD and type 2 diabetes at 24 weeks [57]. The improvement persisted at 52 weeks, suggesting that bardoxolone methyl may have promise for the treatment of CKD. Adverse events were generally manageable and mild to moderate in severity. The most frequently reported adverse event in the bardoxolone methyl group was muscle spasm. A multinational, double-blind, placebo-controlled Phase III outcomes study (BEACON) was started in June 2011, testing bardoxolone methyl's impact on progression to ESRD or cardiovascular death in 1,600 patients with Stage 4 CKD (eGFR 15-30 ml/min/1.73m²) and type 2 diabetes. This phase 3 trail was halted in October 2012 because of adverse effects. This decision of discontinuance was made based upon a recommendation of the Independent Data Monitoring Committee to stop the trial for safety concerns due to excess serious adverse events and mortality in the bardoxolone methyl arm. Further information about why these safety problems arose, the magnitude of the problems, or the next steps with the drug have not been released.

Recently, Resimann et al. [58] investigated whether the bardoxolone methyl-induced albuminuria may result from the downregulation of megalin, a protein involved in the tubular reabsorption of albumin and lipid-bound proteins. Administration of bardoxolone methyl to cynomolgus monkeys significantly decreased the protein expression of megalin in renal tubules, which inversely correlated with the urine albumin-to-creatinine ratio. Moreover, daily oral administration of bardoxolone methyl to monkeys for 1 year did not lead to any adverse effects on renal histopathological findings, but reduced serum creatinine and BUN, as observed in patients with CKD. The bardoxolone methyl-induced decrease in megalin corresponded with pharmacological induction of renal Nrf2 targets, including NADPH:quinone oxidoreductase 1 enzyme activity and GSH content. These findings suggest that the increase in albuminuria that accompanies bardoxolone methyl administration may result, at least in part, from reduced expression of megalin, which seems to occur without adverse effects and with strong induction of Nrf2 target genes [58].

4.3. Antioxidants in drug-induced AKI and hyperglycemia

Several chemicals, including polyphenol compounds, are thought to protect kidney function through their antioxidant effects. Curcumin, a typical phytochemical polyphenol, attenuated subtotal nephrectomy-induced proteinuria, systemic and glomerular hypertension, hyperfiltration, glomerular sclerosis, interstitial fibrosis, interstitial inflammation, and increase in plasma creatinine and blood urea nitrogen in CKD model rats [59]. This protective effect appeared to be associated with enhanced nuclear translocation of Nrf2 and with prevention of subtotal nephrectomy-induced oxidant stress.

Sulforaphane is considered an indirect antioxidant, as this product can induce several

cytoprotective proteins, including antioxidant enzymes, *via* activation of the Nrf2-antioxidant response pathway. Cisplatin, cis-diamminedichloroplatinum II, is a platinum chemotherapeutic agent that induces nephrotoxicity associated in part with oxidative stress. Moreover, cisplatin exerts various cellular toxic events, including cytotoxicity *via* ROS production, thereby activating mitogen-activated protein kinases, inducing apoptosis and stimulating inflammation and fibrosis [60]. The nephrotoxicity of cisplatin in animal models can be attenuated by free radical scavenging agents, suggesting that oxidative stress-related injury is predominantly involved in the pathogenesis of cisplatin-induced AKI, with features of lipid peroxidation, increased urine volume and serum creatinine levels and decreased urine osmolarity [61].

Preincubation of LLC-PK_1 cells, derived from pig renal tubular epithelial cells, with 0.5-5 μM sulforaphane can prevent cisplatin-induced cell death in a concentration-dependent fashion [62]. Immunofluorescent staining revealed the nuclear translocation of Nrf2 after treatment with sulforaphane. In the *in vivo* studies, cisplatin was given to rats as i.p. injection at a dose of 7.5 mg/kg. Sulforaphane (500 μg/kg i.v.) was given to the rats two times, i.e., 24 h before and 24 after cisplatin treatment. Sulforaphane attenuated cisplatin-induced renal impairment, structural damage, oxidative stress, GSH depletion, enhanced urinary hydrogen peroxide excretion and the decreased levels of antioxidant enzymes. The renal protective effect of sulforaphane on cisplatin-induced nephrotoxicity was accompanied by alleviation of oxidative stress and the preservation of antioxidant enzymes.

Previously, Morisaki et al [63] reported that administration of AST-120 significantly decreased IS accumulation in the serum and kidney, and also ameliorated cisplatin-induced AKI. It was suggested that indoxyl sulfate accelerated cisplatin-induced AKI as a key mediator, and that AST-120 treatment may be useful in preventing the

progression of renal dysfunction by decreasing indoxyl sulfate accumulation in the kidney. Therefore, indoxyl sulfate could serve as a cytotoxic metabolite mediating enhanced ROS production under cisplatin-induced AKI. Additionally, Kusumoto et al. [64] reported that oral administration of polyphenols, especially quercetin, showed a preventive effect on cisplatin-induced AKI in rats. Moreover, these effects were accompanied by suppression of serum and renal accumulations of IS. Agents, including phytochemical polyphenols with a potential inhibitory effect on hepatic indoxyl sulfate production, could be useful for preventing the progression of cisplatin-induced AKI.

Hyperglycemia-induced oxidative stress plays a pivotal role in the progression of diabetic nephropathy. Oral administration of resveratrol to diabetic rats resulted in a significant normalization in the levels of creatinine clearance, plasma adiponectin, C-peptide and renal superoxide anions, hydroxyl radicals, nitric oxide, TNF-α, IL-1β, IL-6 and NF-κB p65 subunit and activities of renal aspartate transaminase, alanine transaminase and alkaline phosphatase by comparison to diabetic rats [65]. Moreover, administration of resveratrol resulted in a significant improvement in superoxide dismutase, catalase, GSH peroxidase, GSH-S-transferase and GSH reductase activities and vitamins C and E, and reduced levels of GSH, with a significant decline in lipid peroxides, hydroperoxides and protein carbonyls levels in diabetic kidneys. Resveratrol treatment appeared to normalize the renal expression of Nrf2-Keap1 complex and its downstream regulatory proteins in the diabetic group of rats. Histological and ultrastructural observations supported the proposal that resveratrol protects the kidneys from hyperglycemia-induced oxidative injury. These findings demonstrated the renal protective action of resveratrol by attenuating markers of oxidative stress in renal tissues of diabetic rats through activation of the Nrf2-Keap1 signaling pathway 6[5].

5. Nrf2 Enhancers: a novel therapeutic approach

Over the last couple of years, several natural and synthetic compounds that activate Nrf2 have been characterized [9]. Chemical enhancers of Nrf2 have been organized into 10 classes according to their chemical structure. Several agents of the more commonly used Nrf2 activators and their effects *in vivo* were examined. Some compounds have been investigated in human clinical trials and appear to be promising therapeutic agents for the treatment of several diseases including multiple sclerosis, muscular dystrophy, skin cancer and CKD. For example, in the phase II clinical trial, BG-12, dimethyl fumarate was administered to patients with relapsing–remitting multiple sclerosis [66]. The treatment showed to reduce brain magnetic resonance imaging activity and lesions associated with multiple sclerosis. In addition, Protandim (LifeVantage) was suggested to be an effective treatment for several diseases . This compound is a dietary supplement of particular interest as it consists of five low-dose natural Nrf2 activators that activate Nrf2 and induce HO-1 through multiple kinase pathways, including PI-3, p38, mitogen-activated protein kinase and protein kinase Cδ, in a mouse β-cell line MIN6 and a human neuroblastoma cell line [67]. These components are considered difficult to administer orally and are toxic at therapeutic doses. The synergistic effect of these five components has been shown in a human clinical trial to yield significant elevations in the levels of antioxidant enzymes, including superoxide dismutase 1 and catalase, as well as a decline in levels of lipid peroxidation markers, resulting in an overall reduction in oxidative stress. As new pharmacological Nrf2 activators emerge, so do concerns regarding their use in a complex biological system. The first concern regarding Nrf2 activators is the specificity of the compounds being developed. Understanding the downstream effects of the targeted pathways is essential for predicting other potential

beneficial or adverse effects associated with the therapeutic use of these Nrf2 activators and/or enhancers.

More recently, representatives from a class of proteasome inhibitors have been shown to activate Nrf2. Administration of proteasome inhibitors promises to be an effective method for increasing Nrf2 activity. MG132 is a potent, reversible and cell-permeable proteasome inhibitor, which reduces degradation of ubiquitin-conjugated protein in mammalian cells. Non-toxic concentrations of MG132 are reported to inhibit Nrf2 proteasomal degradation and stimulate the translocation of Nrf2 into the nucleus. A recent study in rats made diabetic with a single dose of streptozotocin indicated that MG132 was also protective against diabetic nephropathy [68]. Shortly after inducing diabetes, the rats were treated with MG132 at a daily dosage of 10 μg/kg for 3 months. This regimen produced renal protection as indicated by reductions in proteinuria, basement membrane thickening and glomerular mesangial expansion. MG132 also reduced kidney markers of oxidative stress and increased protein levels of Nrf2 and several antioxidant enzymes. These results suggest the beneficial effects of the proteasomal inhibitor MG132 are due to elevated Nrf2 protein content, which induces increased expression of multiple downstream antioxidants. Furthermore, Cui et al. [69] reported that administration of a low dose of the proteasomal inhibitor MG132 could provide a therapeutic effect for diabetes-induced nephropathy using a transgenic type 1 diabetic OVE26 mouse model. The authors concluded that MG132 induced the upregulation of Nrf2 function *via* inhibiting the increased proteasomal activity in diabetes and elicited a therapeutic effect by protecting the kidney against oxidative damage, inflammation, fibrosis and eventually dysfunction. Therefore, MG132 has significant potential as a novel therapeutic agent for diabetic patients including those with diabetic nephropathy.

6. Conclusions and perspectives

Nrf2 activators and/or enhancers are a promising novel class of candidate therapeutic agents for the treatment of chronic and acute kidney injury and diseases, including CKD, ischemia- or chemical-induced AKI as well as diabetic-induced nephropathy. However, the mechanisms responsible for Nrf2 activation-dependent prevention or attenuation of these diseases should be different. The suppressive effects of Nrf2 activation on the inflammatory reaction *via* NF-κB inhibition are likely to be much more important for preventing the progression of CKD with fibrogenesis than for more severe disease states. In contrast, the cytoprotective effects of Nrf2 activation on emergent elimination of toxins and ROS are likely to be much important in AKI. Considering the versatile functions of the Nrf2-Keap1 defense signaling pathway, its real "toxico-pharmacological roles in kidney diseases" should be further examined for the development or discovery of novel therapeutics targeting the Nrf2 system, because the mechanistic regulation and its effect in terms of renal protection have not been fully elucidated. Additionally, further biochemical and pharmacological information regarding the transcriptional regulatory cofactors accompanied with Nrf2 are required for understanding the entire system in kidney diseases.

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Footnotes

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Figure legend

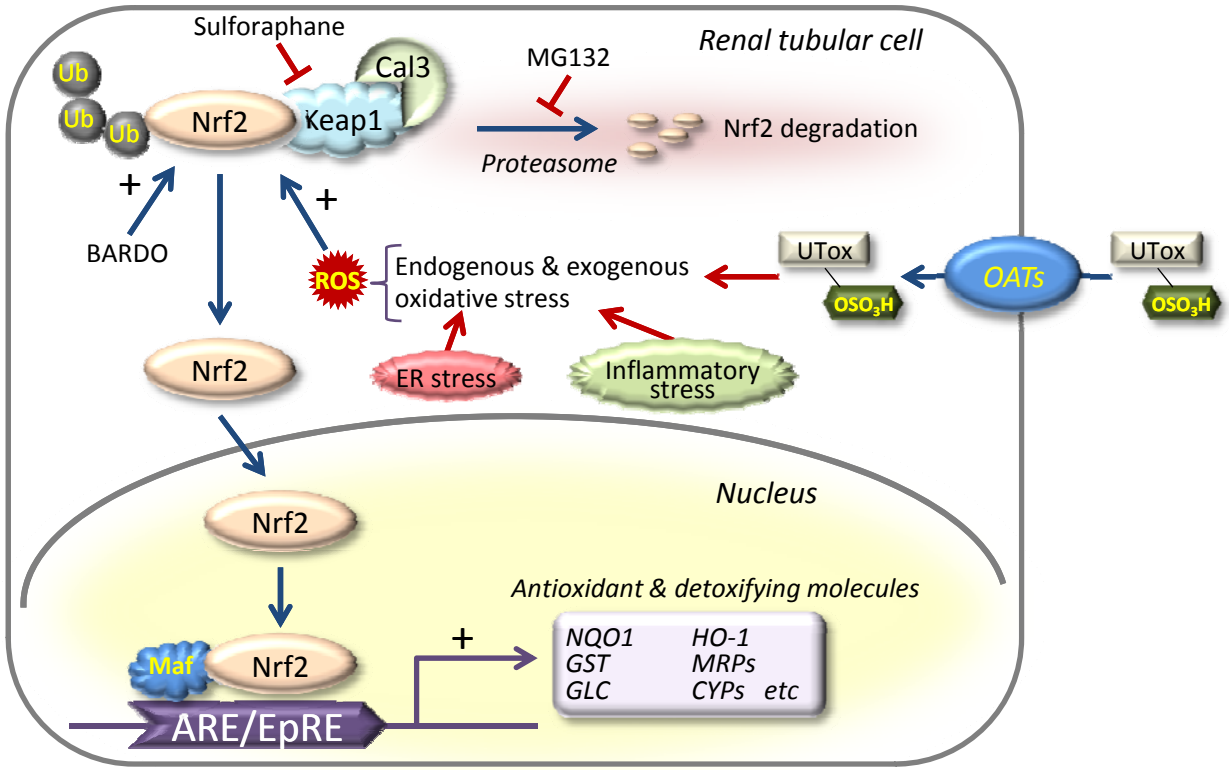
Figure 1. Schematic representation of the Nrf2-Keap 1 defense pathway in renal tubular cells. Steady state level of Nrf2 in cytoplasm is regulated primarily by modulation of its continuous proteasome degradation following ubiquitination (Ub). Non-toxic concentrations of MG132 inhibit proteasomal degradation of Nrf2 and stimulate the translocation of Nrf2 into the nucleus. Sulforaphane potently activates Nrf2 by modifying Keap1 cysteine residues. Under stress-free conditions, Nrf2 is stably bound in the cytoplasm to Keap1, an E3 ubiquitin ligase substrate adaptor targeting Nrf2 for degradation. Under stressful conditions with endogenous or exogenous ROS evoked by ER (endoplasmic reticulum) stress, inflammatory stress and uremic toxins, conformational changes in Keap1 release Nrf2, increasing half-life of Nrf2 to translocate into the nucleus where it binds to ARE/EpRE (Antioxidant／Electrophile Responsive Element with a modulator Maf. After binding to ARE, Nrf2 induces expression of a multitude of antioxidant and detoxifying molecules including NQO1, GST, GLC, HO-1, and MRPs. Bardoxolone methyl (BARDO) activates Nrf2, thereby inducing the transcription of genes that reduce oxidative stress. Sulfate-conjugated uremic toxins (Utox-OSO_3H), such as indoxyl sulfate and p-cresyl sulfate, are accumulated in the cytoplasm via OATs, basolateral membrane organic anion transporters OAT1 and OAT3, inducing production of oxidative stress.

Table 1.

Typical list of the low molecular-weight uremic toxins involved in vascular dysfunction (partially modified from Ref. 27).

Uremic toxins	Effects
Indoxyl sulfate	Reduction of endothelial proliferation Stimulation of monocyte chemoattracant protein 1, intercellular adhesion molecule 1 and E-selectin expression on endothelial cells Stimulation of the production of ROS by endothelial cells Stimulation of circulating endothelial microparticles Association with mortality
p-Cresyl sulfate	Leukocyte activation Stimulation of circulating endothelial microparticles Association with mortality
Guanidine compounds	Leukocyte activation Decrease in endothelial proliferation
AGE	Leukocyte activation Stimulation of the production of ROS Stimulation of peroxynitrite generation Inhibition of NO synthase activity Reduction of the digestibility of collagen and matrix proteins
ADMA	Inhibition of NO synthase activity
Indole-3-acetic acid	Associated with low levels of endothelial progenitor cells

Fig. 1.


Sulforaphane
Renal tubular cell
Cal3
MG132
Ub
Ub
Ub
Nrf2
Keap1
Nrf2 degradation
Proteasome
+
+
BARDO
ROS
Endogenous & exogenous
oxidative stress
UTox
OATs
UTox
OSO3H
OSO3H
Nrf2
ER stress
Inflammatory
stress
Nucleus
Nrf2
Antioxidant & detoxifying molecules
Maf
Nrf2
+
NQO1
GST
GLC
HO-1
MRPs
CYPs etc
ARE/EpRE