The Nrf2 pathway in the progression of renal disease

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ABSTRACT

The Nrf2/Keap1 system regulates the transcription of antioxidant and cytoprotective genes through direct Nrf2 binding to responsive elements in the promoter region of target genes or via Keap1-induced NF-kB inhibition. The association between oxidative stress and inflammation with progression of chronic kidney diseases (CKDs) directed attention towards bardoxolone methyl and its analogues, potent Nrf2/Keap1 inducers, as a potential modality of renoprotective intervention. In a phase II clinical trial (BEAM), bardoxolone methyl was shown to increase the estimated glomerular filtration rate (eGFR) in patients with CKD associated with type 2 diabetes. The study generated great interest but raised concerns as well, on the adverse event profile of the drug. Experiments in rats with type 2 diabetic nephropathy treated with bardoxolone methyl analogues reproduced some drawbacks of bardoxolone methyl therapy in humans. Despite these warnings, a long-term phase III trial (BEACON) was started that was prematurely terminated because of an excess serious adverse events and mortality. Lessons from the above studies suggest that before jumping into use in clinical practice, adequately designed experiments in animal models are needed to provide insights into pathogenetic mechanisms as well as unexpected side effects.

Keywords: bardoxolone, diabetic nephropathy, Keap1

Nrf2-Keap1 SIGNALLING PATHWAY

The Nrf2-Keap1 (nuclear factor erythroid 2-related factor 2-Kelch-like ECH-associated protein 1) system is one of the most critical cytoprotective mechanisms acquired in vertebrates over the course of evolution. Nrf2 is a basic-region leucine zipper transcription factor that regulates basal activity and inducible expression of a battery of environmental stress response genes [1, 2]. Nrf2 is normally maintained in the cytoplasm by interaction with the cytosolic repressor protein Keap1, an adaptor component of Cullin 3-based ubiquitin E3 (Cul-E3) ligase complex, which promotes ubiquitination and proteosomal degradation of Nrf2 [3, 4]. Keap1 contains several reactive cysteine residues that serve as sensors of intracellular redox state, and acts as a negative regulator of Nrf2 [5]. Exposure to both endogenous and exogenous molecules as reactive oxygen species, 15-deoxy-delta12,14-prostaglandin J2, dithiolethiones, triterpenoids, isothiocyanates, can modify reactive cysteins in Keap1 [6] and lead to dissociation of Nrf2 from the Keap1/Nrf2 complex, or induce conformational changes in Keap1 rescuing Nrf2 from proteosomal degradation [7]. Besides the cysteine modification of Keap1, phosphorylation of specific serine or threonine residues present in Nrf2 by upstream kinases, including protein kinase C, mitogen-activated protein kinases (MAPK), phosphatidylinositol-3-kinase/Akt may also facilitate the nuclear localization of Nrf2 [8]. In the nucleus, Nrf2 heterodimerizes with other transcription factors such as small Maf protein, leading to the binding of Nrf2 to the cis-regulatory, antioxidant response element (ARE) or electrophile response element located in the promoter region of Nrf2 target genes, thereby activating their transcription. A series of proteins have been identified that may modulate Nrf2 transcription activation, thereby showing the influence of multiple signalling pathways on Nrf2 responses [9]. Nrf2 transcriptional activity has been enhanced by the nuclear transcription coactivator CREB-binding protein (CBP), phosphatidylinositol-3-kinase/Akt may also facilitate the nuclear localization of Nrf2 [8]. In the nucleus, Nrf2 heterodimerizes with other transcription factors such as small Maf protein, leading to the binding of Nrf2 to the cis-regulatory, antioxidant response element (ARE) or electrophile response element located in the promoter region of Nrf2 target genes, thereby activating their transcription. A series of proteins have been identified that may modulate Nrf2 transcription activation, thereby showing the influence of multiple signalling pathways on Nrf2 responses [9]. Nrf2 transcriptional activity has been enhanced by the nuclear transcription coactivator CREB-binding protein (CBP) following the activation by upstream MAPK signalling pathways [10]. Instead, CCAAT enhancer-binding protein alpha [11] and p53 [12] have been found to suppress Nrf2 transactivation of antioxidant response genes.

Nrf2 regulates the transcription of a plethora of cytoprotective genes including those encoding antioxidant and phase II-
detoxifying enzymes such as catalase, superoxide dismutase, haeme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1, glutathione peroxidase-2 and glutathione S-transferase [1, 2]. Since Nrf2 is ubiquitously expressed, it plays a critical role in protecting many cell types and organ systems from oxidative stress and an array of toxic insults [13]. Its implication under disease conditions has been proved through the use of Nrf2-deficient mice in different models of disease [13]. Nrf2-deficient mice have enhanced susceptibility to pulmonary inflammation [14], aceterminophen hepatotoxicity [15] and neurodegenerative disease [16]. Moreover, ablation of the Nrf2 gene in mice has been shown to cause lupus-like autoimmune nephritis, and worsen diabetes-induced oxidative stress, inflammation and renal injury [17, 18]. Nrf2 knockout mice were more susceptible either to ischaemia reperfusion injury or to cisplatin-induced nephrotoxicity than wild-type mice, and their treatment with antioxidants limited renal dysfunction [19]. On the other hand, using Keap1-deficient mice with a protective role of Nrf2 activation has been demonstrated, as in the case of a cholestatic liver injury model where disruption of Keap1 resulted in sustained activation of hepatic Nrf2-regulated detoxifying enzymes and antioxidative stress genes, with attenuation of liver injury [20].

**CROSS-TALK BETWEEN Nrf2/Keap1 AND NF-kB**

There is compelling evidence showing that Nrf2 is involved in cross-talk with other signalling pathways affecting cell survival and functions, which include p53, Notch1 and NF-kB (reviewed by [21]). The NF-kB/Rel family that comprises homodimeric or heterodimeric complexes designated as p50, p52, p65, c-rel and RelB participates in the regulation of inflammation, apoptosis, cell growth and immune response [22]. The prototype NF-kB is composed of p50–p56 subunits. NF-kB proteins normally exist in the cytoplasm bound to the inhibitory protein IkBα. Upon cell activation by different stimuli, such as cytokines, viruses and oxidants, IkBα is phosphorylated by the IkB kinase (IKKβ) complex, ubiquitinated and degraded, allowing NF-kB translocation into the nucleus for binding to DNA motifs in gene promoters [23]. That Nrf2 and NF-kB signalling pathways together orchestrate the transcription of relevant genes and regulate the function of downstream target proteins was initially suggested by the observation that anti-inflammatory or anti-carcinogenetic phytochemicals suppressed NF-kB signalling, while activating Nrf2-ARE pathways [21, 24, 25]. Experiments provided the evidence that the NF-kB p65 subunit and Nrf2 both bind to the same domain of CBP, a well-established coactivator of Nrf2, and that p65 after phosphorylation at Ser276, through a competitive mechanism, suppresses transcription of ARE-dependent genes by depleting CBP from Nrf2 [26]. The Nrf2/Keap1 pathway has been involved in the control of NF-kB through reduction of IkBα phosphorylation, thereby favouring NF-kB degradation. Thus, Nrf2-deficient mouse embryonic fibroblasts (MEFs) after stimulation with lipopolysaccharide or TNF-α had higher levels of phosphorylated IkBα and greater IKK kinase activity than Nrf2+/+ MEFs [27]. In addition, Nrf2 knockout mice showed enhanced NF-kB activity and expression of inflammatory genes in response to different insults than the corresponding wild-type mice [28, 29]. Although Nrf2 is a well-known substrate for the Keap1-Cul3-E3 ligase complex, Keap1 may also bind IKKβ [30]. Under basal conditions, a Keap1-Cul3-E3 ligase complex leads to IKKβ ubiquitination and proteosomal degradation; under stress conditions, the Keap1 complex undergoes conformational change to release IKKβ which then phosphorylates the negative inhibitory protein IkBα causing its degradation. At the same time, IKKβ also phosphorylates p65, thereby enhancing its transactivation potential. Depletion of Keap1 led to the accumulation and stabilization of IKKβ and to upregulation of NF-kB-dependent genes [31]. A recent review by Wakabayashi et al. [21] has pointed out the complexity of the interactions between downstream targets of Nrf2 and NF-kB that may lead to modulation of transcription factor activity. For example, induction of Nrf2-dependent antioxidative proteins such as HO-1 was able to limit NF-kB activity by inhibiting IkBα degradation, while inhibition of HO-1 increased p65 activity. HO-1 exhibits in fact anti-inflammatory effects that complement the antioxidant properties. An NF-kB target such as COX-2 was found to cause a reduction of transcription of Nrf2 and antioxidiant genes [21].

**MODULATION OF Nrf2 ACTIVITY IN EXPERIMENTAL RENAL DISEASE**

Oxidative stress and inflammation are critical mediators in the pathogenesis and progression of chronic kidney disease (CKD), acting in a self-perpetuating vicious circuit in which oxidative stress causes inflammation by several mechanisms including the activation of NF-kB. Inflammation in turn causes oxidative stress via production of reactive oxygen, nitrogen and halogen species by activated leukocytes and resident cells (reviewed by [32]). The constitutive Nrf2 activity is crucial in maintaining redox balance under normal conditions, and its induction in response to oxidative stress with consequent transcription of cytoprotective genes represents an important defense system against oxidative stress [32]. However, studies in experimental models of CKD like rats with 5/6 nephrectomy [4] and Imai rats, a model of spontaneous focal glomerulosclerosis [33], have shown that despite the presence of oxidative stress and inflammation, which should have induced Nrf2 activation, the diseased kidneys paradoxically had impaired Nrf2 activity and reduced expression of its target gene products. Inability to limit oxidative stress because of Nrf2 deficiency possibly contributed to enhance NF-kB activation and inflammation in the diseased kidney of the CKD rats [4]. Recently, in a rat model of chronic tubulo interstitial nephropathy induced by feeding animals with an adenine-containing diet, impaired activation of Nrf2 and down-regulation of catalase, HO-1 and glutamate–cysteine ligase have been also found to contribute to the pathogenesis of oxidative stress and inflammation and to amplification of their damaging effects on the kidney [34].
Conversely, a renal protective role of Nrf2 is supported by the finding that dietary Nrf2 activators such as sulforaphane or cinnamic aldehyde administered to rats with streptozotocin-induced diabetes limited albuminuria and protected against renal oxidative damage [35]. Interestingly, besides antioxidant function, Nrf2 activation negatively regulated TGF-β, extracellular matrix and p21 expression as shown both in the diabetic kidney and in an in vitro model of mesangial cells [35].

Synthetic triterpenoid bardoxolone methyl (CDDO-Methyl ester) and its analogues are the most potent inducers of the Nrf2/Keap1 pathway [36–38] (Figure 1). Bardoxolone methyl directly interacts with Keap1, allowing Nrf2 to translocate to the nucleus where it upregulates antioxidant and cytoprotective genes. The structure and activity profile of bardoxolone methyl resemble those of the cyclopentenone prostaglandins, the endogenous activators of Nrf2, which favour the resolution of inflammation [39]. Similarly to cyclopentenone prostaglandins, bardoxolone methyl has anti-inflammatory activity by inhibiting the IKKβ/NF-κB signalling pathway [40]. Bardoxolone methyl, unless administered acutely at low doses, is not well tolerated in rodents due to species-specific CYP metabolism. There are other molecules, including the triterpenoid CDDO-ethyl amide or RTA 405 [41], with a similar target-based activity and the same site relative to bardoxolone methyl, but devoid of the major adverse rodent metabolism. Bardoxolone methyl and related analogues have demonstrated efficacy in models of acute kidney injury such as cisplatin-induced nephrotoxicity [42] and ischemia-reperfusion by increasing the expression of Nrf2, PPARγ and HO-1 protective genes [43]. Treatment with RTA 405 attenuated blood pressure increases and endothelial dysfunction in a 5/6 nephrectomy model of pressure overload [44]. In a mouse model of protein overload proteinuria, we found that early administration of RTA 405 for a short time period (i.e. 23 days) limited interstitial inflammation and fibrosis and reduced oxidative stress in the kidney [45]. These molecules also effectively improved glucose control, lowered plasma triglyceride and free-fatty acid levels, reduced hepatic lipid accumulation and inflammation in both high fat diet and genetically induced mouse models of obesity and diabetes [46, 47].

**Figure 1:** Schematic representation of antioxidant and anti-inflammatory actions of Nrf2/Keap1 inducers bardoxolone methyl and analogues.

### The Case of Bardoxolone Methyl

The association between inflammation and progression of diabetic nephropathy [48, 49] directed attention towards bardoxolone methyl as a potential modality of intervention for patients with CKD and diabetes [50, 51]. The rationale for looking at type 2 diabetes as a possible ideal target rests on the following: (i) type 2 diabetes, which accounts for 90–95% of all diagnosed cases of diabetes, is dramatically increasing worldwide and is emerging as a global health care problem with devastating human, social and economic impact in both developed and developing countries [52]. (ii) About one-third of diabetic patients develop diabetic nephropathy which accounts for >40% of new cases of end-stage renal disease [53]. Type 2 diabetic patients have a 2- to 4-fold higher risk for cardiovascular events than for the general population, and cardiovascular diseases are responsible for ~60% of early mortality [54]. (iii) Therapy with renin–angiotensin system inhibitors which normally delays progression of non-diabetic nephropathy is effective in type 2 diabetes only when administered in the early microalbuminuric phase, whereas when patients are treated later on ACE inhibitors or angiotensin II type I receptor blockers provided imperfect protection [55, 56]. Moreover, multimodal interventions to afford renoprotection in overt type 2 diabetic nephropathy have given disappointing results, as recently shown, among others, by the ORIENT study of olmesartan [57] and the ALTITUDE study of aliskiren [58], raising the need for alternative therapeutic strategies.

The phase II, double-blind, randomized, placebo-controlled trial study known as the BEAM study involving 227 subjects showed that treatment with bardoxolone methyl increased in a dose-dependent manner the estimated glomerular filtration rate (eGFR) in patients with advanced CKD and type 2 diabetes at 24 weeks [51]. The increase in the eGFR persisted
for 52 weeks. The study generated great interest and, as underlined in a recent editorial, a 'dramatically high level of enthusiasm about the miraculous effect of the drug' [59]. However, a number of questions and concerns had raised as well [60–63]. In particular, criticism emerged that the effect on the eGFR could have been mediated by a potentially deleterious increase in intraglomerular pressure, which could also explain the increase in albuminuria observed in patients receiving bardoxolone methyl [61–63]. The chemical structure of bardoxolone methyl is similar to that of cyclopenitone prostaglandins shown to cause renal vasodilation [63]. It is conceivable that bardoxolone may have increased the eGFR by causing afferent arteriolar dilatation and increasing intraglomerular pressure [59, 63]. This haemodynamic effect may result in short-term hyperfiltration which predisposes to accelerated renal function loss and progression of nephropathy in the long term [64]. Also raising concern was the increased frequency of adverse events including massive weight loss, muscle spasm, hypomagnesaemia, elevations in alanine aminotransferase (ALT) levels and gastrointestinal symptoms, among others [60–63]. All these potential adverse effects of bardoxolone methyl on CKD patients of the BEAM study have been critically reviewed by Tayek and Kalantar-Zadeh in a recent editorial [59]. These authors also pointed out that in the rush to move forward with the phase III trial, the investigators did not consider the massive weight loss, the paradoxical increase in proteinuria, nor any of the other side effects [61–63]. Instead, in June 2011, the Bardoxolone Methyl Evaluation in Patients with Chronic Kidney Disease and Type 2 Diabetes: the Occurrence of Renal Events (BEACON) trial, a multinational, multicentre, double-blind, randomized, placebo-controlled phase III study began. It was aimed at determining whether long-term administration of bardoxolone methyl, on a background of standard therapy, including RAAS inhibitors, safely reduced renal and cardiac morbidity and mortality [65]. Approximately 2500 patients were recruited. BEACON would have been the first event-driven trial to evaluate the effect of an oral antioxidant and anti-inflammatory drug in advanced CKD. Unfortunately, the trial was terminated prematurely in October 2012 based on the recommendation of the Independent Data Monitoring Committee 'for safety concerns due to excess serious adverse events and mortality'. Fifty-seven out of the 2185 patients who have been randomized either to bardoxolone methyl or to placebo died at that time for reasons that have not yet been disclosed by Reata Pharmaceuticals.

No published studies on the effects of bardoxolone in animal models of type 2 diabetic nephropathy have been available before the BEAM trial [51]. To investigate potential therapeutic mechanisms of bardoxolone in diabetic nephropathy, we took advantage of the Zucker diabetic fatty (ZDF) rats, a model of type 2 diabetes characterized by hyperlipidaemia, progressive renal disease and cardiac abnormalities [66–69]. The animals received a 3-month treatment with two dosages of the bardoxolone methyl analogue RTA 405 alone or in combination with the ACE inhibitor ramipril starting at a phase of overt nephropathy [70]. The results were unexpected. RTA 405 caused adverse changes in the physical status of ZDF rats as early as 1 month after starting the treatment. Acute reductions in food intake and diuresis with decline in body weight, worsening of dyslipidaemia and increase in blood pressure were recorded. Early elevation in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels suggested acute toxicity of the molecule. Some similar trends were reported in the BEAM study [51]. Transient increases in ALT levels were seen in the majority of bardoxolone methyl-treated patients that generally resolved while the patients continued to receive the drug. Total bilirubin levels were unchanged, and signs and symptoms of hepatic injury were not observed. There is evidence that Nrf2 transcriptionally upregulates both the ALT and AST genes, and that Nrf2-mediated transaminase induction is associated with increased hepatic glutathione levels and hepatoprotection in animal models of liver injury [71]. In ZDF rats, the increase in transaminase levels after Nrf2 activation by RTA 405 was instead followed by increases in liver weight in association with remarkable histological changes, as documented by severe and diffuse hepatocyte vacuolization, swelling and degeneration. Another disappointing result was that RTA 405 worsened proteinuria, glomerulosclerosis and tubular damage. While ramipril afforded renoprotection, when administered in combination with RTA 405 the favourable effect of the ACE inhibitor in this model could not be detected any longer. Post-hoc analysis by Reata Pharmaceuticals revealed the presence of both unknown impurities and structurally identified drug-related peaks (i.e. RTA 401, a key intermediate during the synthesis of RTA 405, and its 1,2-dihydro derivative), in the drug substance they had supplied for the study. The concentrations of these impurities were very low, suggesting that they were either extremely toxic or were not the cause of the deleterious effects. Unfortunately, the company was not able to provide a ‘pure’ RTA 405 for further experiments. They instead offered to test in ZDF rats a variant of bardoxolone methyl and RTA 405, the novel synthetic triterpenoid derivative dihydro-CDDO-trifluoroethyl amide (dh404) [72] said to be well tolerated by rodents. In this study, dh404 did not show any beneficial effects on renal disease of ZDF rats, rather it caused a trend towards an increase of proteinuria, glomerulosclerosis, tubular casts and interstitial inflammation. A trend towards an increase in GFR, measured by iohexol clearance, was found in intraglomerular pressure which predisposes to accelerated renal function loss and progression of nephropathy in the long term [64].

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short-term study with RTA 405 and dh404 in ZDF rats [73]. The results showed increases in the urinary protein-to-creatinine ratio with both compounds, while there were no adverse effects on the liver. Inconsistencies with our study [70] might derive from the shorter period of treatment of diabetic rats in the Reata study.

What can be learned from the above experiments is that carefully performed studies in experimental animals are needed to provide important insights not only into pathogenic mechanisms but also into unexpected side effects [74]. The use of animal models of human disease still remains a mandatory step for the investigation of new drugs before jumping into use in clinical practice. Finally, it has to be reminded that ‘animal experimentation is essential not only for its clinical application to human and animal health, but because it is usually the only feasible way to examine basic questions of structure, function, development, behavior and even welfare. It is significantly more difficult to do this effectively if we are required to treat animal experimentation as a moral imperfection to be eliminated’ [75].

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CONFLICT OF INTEREST STATEMENT

G.R. is a member of the Reata Bardoxolone Methyl CKD Steering Committee. He does not accept any personal remuneration, and compensation is paid to his institution for research and educational activities outside the Nephrology division.

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