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Review

Keap1/Nrf2/ARE signaling unfolds therapeutic targets for redox imbalanced-mediated diseases and diabetic nephropathy

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ABSTRACT

Hyperglycemia/oxidative stress has been implicated in the initiation and progression of diabetic complications while the components of Keap1/Nrf2/ARE signaling are being exploited as therapeutic targets for the treatment/management of these pathologies. Antioxidant agents like drugs, nutraceuticals and pure compounds that target the proteins of this pathway and their downstream genes hold the therapeutic strength to put the progression of this disease at bay. Here, we elucidate how the modulation of Keap1/Nrf2/ARE had been exploited for the treatment/management of end-stage diabetic kidney complication (diabetic nephropathy) by looking into (1) Nrf2 nuclear translocation and phosphorylation by some protein kinases at specific amino acid sequences and (2) Keap1 downregulation/Keap1-Nrf2 protein-protein inhibition (PPI) as potential therapeutic mechanisms exploited by Nrf2 activators for the modulation of diabetic nephropathy biomarkers (Collagen IV, Laminin, TGF-β1 and Fibronectin) that ultimately lead to the amelioration of this disease progression. Furthermore, we brought to limelight the relationship between diabetic nephropathy and Keap1/Nrf2/ARE and finally elucidate how the modulation of this signaling pathway could be further explored to create novel therapeutic milestones.

Abbreviations: Keap1, Kelch-like ECH associated-protein 1; Nrf2, nuclear factor erythroid 2–related factor 2; DN, diabetic nephropathy; PPI, protein protein interaction; DM, diabetic mellitus; TGF-β1, transforming growth factor beta 1; ESRD, end stage renal disease; RAAS, renin angiotensin aldosterone system; DKD, diabetic kidney disease; GFR, glomerular filteration rate; Maf, musculoaponeurotic fibrosarcoma; ARE, antioxidant response element; IVR, intervening region; DNA, deoxyribonucleic acid; TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase; RNA, ribonucleic acid; Neh Region, Nrf2-ECH homology domain; CNC, cap n collar; ECH, erythroid cell-derived protein with CNC homology; bZip, basic leucine zipper; NTR, N-terminal region; BTB, broad complex, tramtrack bric-A-bric; CTR, carboxyl terminal region; DRG, double glycine repeats; ROS, reactive oxygen species; PKC, protein kinase C; NADPH Oxidase, reduced nicotinamide adenine dinucelotide phosphate oxidase; ACE, angiotensin converting enzyme; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; AGEs, advance glycation end products; SGLT2, sodium glucose transporter 2; VEGF, vascular endothelial growth factor; NQO-1, NADPH oxidase 1; HMOX1, heme oxygenase 1; GLCL, glutamate cysteine ligase; GSTs, gluthatione S transferases; AKI, acute kidney injury; SIRT1, sirtuin 1; FN, fibronectin; ECM, extracellular matrix; CPDT, 5, 6-dihydrocyclopentane-1, 2-dithiole-3-thione; SF, sulforaphane; CGA, chlorogenic acid (3-(3, 4-dihydroxycinnamoyl)-quinic acid); HBZY, rat glomerular mesangial cell lines; NaB, sodium bromide; WT Mice, wild type mice; HDAC, histone deacetylase; STZ, streptozotocin; SOD1, superoxide dismutase 1; HG, high glucose; ICAM, intercellular adhesion molecules; Crm1, chromosome region maintenance 1; Fyn, proto-oncogene tyrosine-protein kinase; SRC, cellular sarcoma kinase; GSK-3β, glycogen synthase kinase 3; MAPK, mitogen activated protein kinase; LPS, lipopolysacharide; PI3K, phosphoinositide-3-kinase; Akt, protein kinase B (PKB also known as Akt); ARPE-19 Cells, adult retinal pigment epithelial cell line-19; GSH, reduced glutathione; LY294002, morpholine-containing and non-specific inhibitor of PI3K; CDK, cyclin dependent kinase; JNK, c-Jun N-terminal kinase; P38, a class of mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; PKA, protein kinase A; DGR, double glycine repeat; NRK-52E, normal rat kidney 52E cell line; LLCPK1 Cells, lilly laboratories cell-porcine kidney 1; HepG2 Cells, immortalized human hepatic G2 cancer cell lines; Hepa1c1c7 Cells, murine *(*Hepa1c1c7*)* hepatoma cells; KO WT Mice, knockout wide type mice; miR-200a, micro-ribonucleic acid 200a; MDA, malondialdehyde; DAG, diacyl glycerol; ETC, electron transport chain; HK-2, immortalized human proximal tubule epithelial cell lines; GFR, glomerular filtration rate; RAS, renin angiotensin system; IGP, intraglomerular pressure; ARB, angiotensin receptor blockers; ACEI, angiotensin converting enzyme inhibitor; RAGE, receptor for advanced glycation end products; GBM, glomerular basement membrane; CTGF, connective tissue growth factor; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of matrix metalloproteinases; IRI, ischemia reperfusion injury; CKD, chronic kidney disease; MPO, myeloperoxidase; TNF-α, tissue necrotic factor-alpha; ZnPP, zinc protoporphyrin; HEK 293T Cells, human embryonic kidney 293T cells; IκB, inhibitor of κB; NFκB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; CPT1-β, carnitine palmitoyltransferase 1β; GSK-3β, glycogen synthase kinase 3β; β-TrCP, β-transducin repeat containing protein; SNP, sodium nitroprusside; CNC, Cap 'N' Collar; ERK, extracellular signal-regulated kinases SRC – non-receptor tyrosine kinase; FDA, Food and Drug Administration; scb-eGFR, serum creatinine-based estimated Glomerular Filtration Rate

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1. Introduction

In this review, we focused on the relationship between Keap1/Nrf2/ARE signaling and diabetic nephropathy progression in order to point out some therapeutic standpoints that could be exploited in the development of drugs with better therapeutic efficacy. We established the central role of redox imbalance in the pathogenesis of DN and also elucidate Nrf2-dependent genes that have been associated with DN. Moreover, we discuss Nrf2 nuclear translocation, Nrf2 phosphorylation and emphasize the inhibition of keap1-Nrf2 Protein-Protein Interaction (PPI) as molecular therapeutic targets for oxidative stress-related diseases and diabetic nephropathy. Furthermore, we link Nrf2 transcription factor to some biological markers of DN and illuminate some molecular therapeutic means that could be of better anti-diabetic nephropathy efficacy.

Type 1 diabetes mellitus was the platform used to shed light on the natural history of diabetic nephropathy which serves as the secondary complication to diabetes mellitus and presents a spectrum of progressive renal lesions which results to glomerular hyperfiltration and embraces a poorly regulated hypertension which later set the stage for end-stage kidney failure. 30 % of diabetes type 1 patients have been found to have a high propensity to fall victim of this disease, whereas the period of onset for type 2 patients has not yet been speculated due to some confounding comorbidities like hypertension and obesity which make the progress of this disease unclear. Progressive deterioration of renal function and structures has been reported in more than one-third of DM patients with developed diabetic nephropathy. This disease is not only the most serious microvascular complication of diabetes but also the largest single cause of ESRD which usually leads to renal replacement therapy [1]. Defining DN with respect to changes in renal structures and functions; it involves mesangial expansion, glomerular and tubular basement membrane thickening and glomerular sclerosis. While the conventional pathogenesis of diabetic kidney disease defines renal fibrosis as the final common end status orchestrated by kidney hemodynamic alterations, ischemia and increased oxidative stress associated glucose metabolic perturbations and inflammatory reactions with extremely active Renin Angiotensin Aldosterone System (RAAS) [2], novel pathogenesis elucidates genetic and epigenetic modulation, podocyte autophagy and mitochondrial dysfunction as specific therapeutic targets that could present better DKD (Diabetic Kidney Disease) treatment. Renin angiotensin receptor blockers/angiotensin-converting enzyme inhibition coupled with blood pressure and glucose control could help prevent the progression of DN aggravation. Recently, novel therapeutic hypoglycemic agents, paricalcitol (structural analogue of vitamin D), pyridoxine, ruboxistaurin, sulodexide, janus kinase inhibitors, and nonsteroidal mineralocorticoids receptor antagonist are explored for their anti-DKD potentials [3]. Since the morbidity and mortality rates surrounding the global prevalence of diabetes and its chronic kidney complications are not reduced by the available therapeutic measures, urgent understanding of mechanisms of DN might reveal some therapeutic positions that could be exploited for better treatment. Although, one of the worldwide growing public health problems is the high risks of intense microvascular and macrovascular complications of diabetes while its downstream kidney complication diabetic nephropathy has been emphasized as a major burden with 30 % of diabetic patients standing the high risk of developing it. Key factors associated with DN include hyperglycemia, hypertension, dyslipidemia, obesity, ethnicity as well as familial and genetic predisposition [4–6]. Early interventions through long-term intensified glucose control in type 1 and type 2 diabetes coupled with Rennin-Angiotensin System (RAS) inhibition could only help prevent its progression partially and therefore, the need for novel

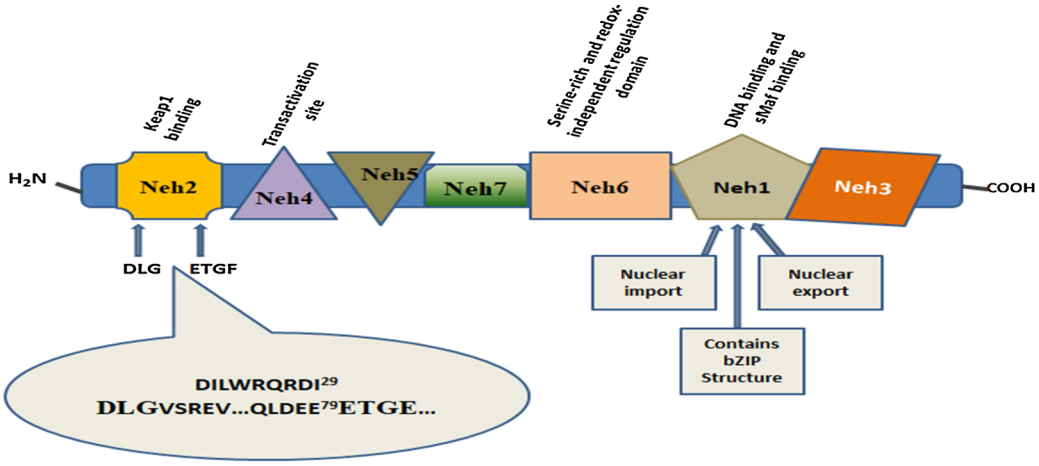

Fig i

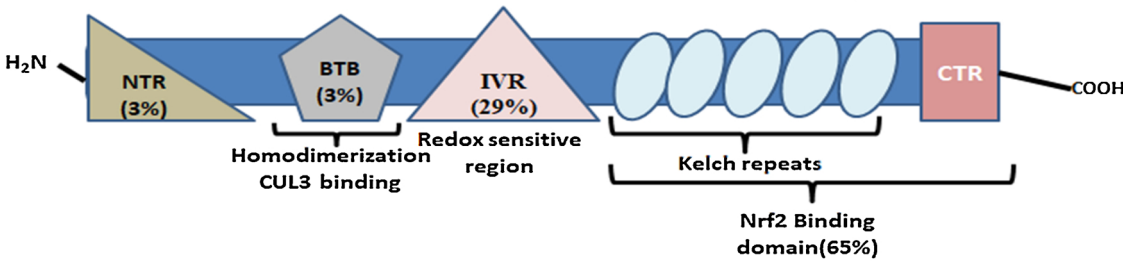

Fig ii

Figs. 1 and 2. Components and functions of Nrf2 and Keap1 respectively.

therapeutic measures of better efficacy is of great importance [6–10]. Diabetic nephropathy has its microalbuminuria threshold around 30 − 300 mg/24 h and if left untreated, could progress to overt albuminuria with Urinary Albumin Excretion (UAE) rate of > 300 mg/24 h with significant renal function decline including increase in serum creatinine (ranging from 1 mg/dL to 5 mg/dL from microalbuminuria to overt type) and elevated blood pressure which synergistically could erupt decline in glomerular filtration rate (GFR) in association with cardiovascular complications to induce end-stage renal failure [11,12].

Nrf2 (Fig. 1) is a seven (7) highly conserved domains (Neh 1-7) 66KDa cap "n" collar (CNC) transcription factor protein, with a basic leucine zipper (bZip) DNA binding motif which is crucial for maintaining cellular homeostasis [13]. These domains are responsible for dimerization with other small proteins (Maf) and binding to DNA, nuclear translocation, Nrf2 negative regulation, the formation of Keap1-Nrf2 complex and transactivation of ARE genes which collectively define its detoxifying and cytoprotective mechanisms of action [14–17]. The cysteine-rich Nrf2 repressor keap1 (Fig. 2) is a 625 amino acid residues protein which consists of five distinct domains namely N-terminal region (NTR), broad complex, tramtrack and bric-a-bric domain (BTB), intervening region (IVR), double glycine repeat or kelch domain (DGR) and C-terminal region (CTR) [18,19] while the 16 nucleotide electrophile response element/antioxidant response element (ARE) is an enhancer sequence or cis-regulatory element found at the promoter region of many genes encoding antioxidation, cytoprotection and detoxification [20]. These three components (Nrf2, Keap1 and ARE) represent the most important cytoprotective defense signaling pathway (Keap1/Nrf2/ARE) with a mechanism strong enough to eradicate cellular oxidative insults. Keap1/Nrf2/ARE signaling pathway is the most crucial regulator of cytoprotective defense to electrophilic and oxidative stress caused by reactive oxygen species and electrophiles and the transcription factor Nrf2 has been seen as a critical therapeutic target for the prevention of oxidative stress-driven diseases, diabetes and its end-stage diabetic nephropathy [21,22]. Diverse molecular targets involving the modulation of proteins/enzymes and signaling pathway components associated with DN have been discovered to present therapeutic efficacy for diabetic kidney complications through the studies of molecular mechanisms surrounding the disease [23,24].

2. Pathophysiology and biochemistry of diabetic nephropathy

As described in Fig. 3, three pertinent factors to the initiation and progression of diabetic nephropathy are hyperglycemia, hypertension and oxidative stress [25]. Hyperglycemia, through some complex pathways involving AGE (Advanced Glycation End-products), PKC (Protein Kinase C) activation, increase ROS (Reactive Oxygen Species) and increase DAG (Diacylglycerol) induce injury to the glomerular vessels. Evidence supporting the central role of ROS in the initiation and progression of diabetic nephropathy [26] proved that NADPH Oxidase (Nox) (Nicotinamide Adenine Dinucleotide Phosphate Oxidase) might be responsible for this excessive ROS production. Although antioxidant remedy in animal studies was provided; clinical evidence is not yet confirmed. Diabetes induces PKC-α translocation in renal membranes and this incidence is related to increased NADPH-dependent superoxide production and elevated renal, serum and urinary VEGF (Vascular Endothelial Growth Factor) concentrations [27].

Derivatives of molecular oxygen and itself (O^{2-}), hydroxyl radical (HO-), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), nitric oxide (NO) and lipid radicals are called ROS while some of them that possess free electrons are referred to as free radicals [26,28–30]. In hyperglycemia, some of these ROS are generated and their production increases as hyperglycemia worsen. The involvement of this ROS production when oxidative stress (a condition where there are more ROS than antioxidants) is established is a factor that links hyperglycemia with DN vascular complications and this can be described in two perspectives [26,31]. The first mechanism is the involvement of metabolic modulations of target tissue molecules while the second is the alterations in renal hemodynamics. Robust clinical and experimental evidence proved that metabolic modulations of target tissue molecules and renal hemodynamic alterations have synergistic adverse effects on the target tissues [26]. Glomerular mesangial cells are one of the target cells for diabetic nephropathy that respond to hyperglycemia-induced ROS generation by upregulating oxidative phosphorylation due to the altered electron transport chain (ETC) protein complexes (complex I and the junction between Q and complex III during mitochondrial dysfunction state of ETC) and excessive O^{2-} generation which therefore subject them to extreme ROS generation in diabetic condition in a reaction involving NADPH oxidase [32–35]. Various cells of the kidneys (mesangial cells, proximal tubule epithelial cells e.g. HK-2 and vascular smooth muscle cells) have been described

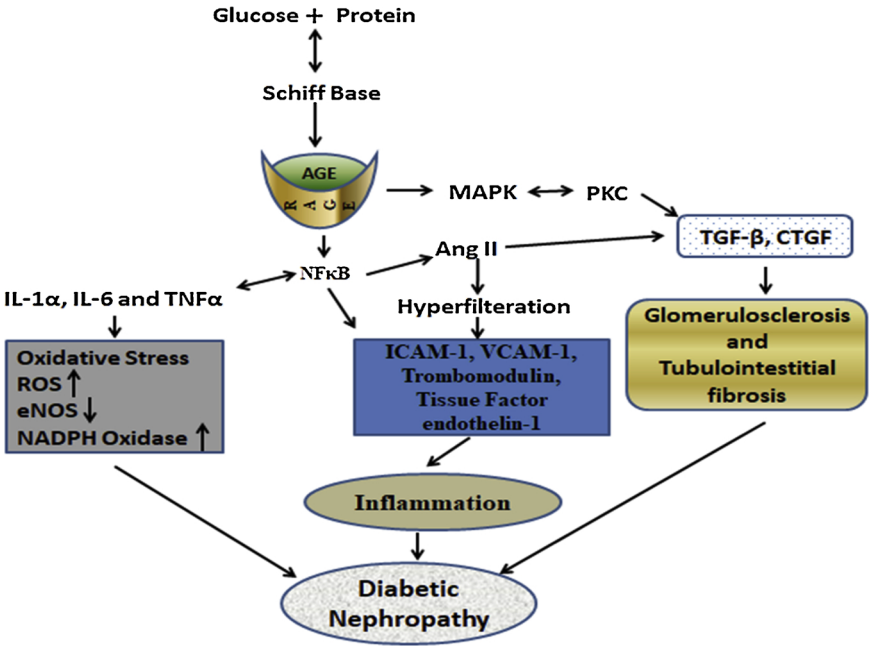

Fig. 3. Schematic diagram illustrating the signaling pathways and components involve in hyperglycemia and Oxidative stress-induced diabetic nephropathy condition. In the hyperglycemic state, glucose could interact with proteins in a reaction that produces AGEs and the response generated when these AGEs dock their receptors (RAGE) in kidney cells could modulate several kinases and transcription factors including MAPK, PKC, and NFκB. The downstream effect of these protein modulations involves the release of cytokines that provokes series of reactions like cellular hypertrophy, oxidative stress, inflammation; glomerulosclerosis and tubulointerstitial fibrosis that finally cause end-stage kidney failure (diabetic nephropathy).

to express NADPH oxidase [36–40]. These ROS, at the basal level, serves as intracellular second messengers that trigger redox-sensitive signaling transduction cascade while in pathological conditions, activated Nox-driven ROS overproduction causes kidney homeostatic imbalance initiated through ligand (cytokines/growth factors/agonistic G protein-coupled receptor)-receptor complex interactions [41,42]. Out of all the ligands, high blood pressure-linked angiotensin II (Ang II); a prominent vasoconstrictor of RAS (Renin Angiotensin System) had been reported as the most effective Nox modulator. It then means that the binding of Ang II to its ATIR (Angiotensin Type 1 receptor) will stimulate more intracellular ROS generation because Nox is been activated through transcriptional activation of its subunits and signaling pathways which include c-Src, PKC and phospholipase A_2/D [36]. Furthermore, metabolic factors like hyperglycemia and AGEs could also increase Nox activation thereby, aggravating intracellular ROS production [43,44]. The several intracellular Nox-mediated ROS overproduction downstream effects (involving several signaling pathways and transcription factors) in glomerular mesangial cells include inflammation and proliferation which causes mesangium expansion (hypertrophy of mesangial cells), extracellular matrix protein (collagen IV, fibronectin and laminin) accumulation and glomerular atrophy [45–47].

In a longitudinal cohort studies to investigate the renal efficacy and safety of ACEIs (captopril, enalapril, fosinopril and perindopril) and ARBs (irbesartan, losartan and valsartan) in diabetic nephropathy patients, 3,316 ARB and 3,799 ACEI monotherapy users facing the fate of end-stage renal disease and renal transplantation with a secondary mortality fate were compared. It was reported that ARBs might be inferior to ACEIs due to the poor renoprotective anti-proteinuria therapeutic effectiveness of ARBs nevertheless; the hyperkalemic side effect of these two drugs should be attended to [48,49].

The two major functions of mesangium include the creation of a mesh-like network of collagen fibers that hold glomerulus integrity thereby, controlling the constriction and dilation of the glomerular fenestrae to control ultrafiltration and GFR (Glomerular Filtration Rate) while the other function involves the release of cytokines in response to several kinds of cellular injuries or xenobiotics. In hyperglycemia condition, there is an automatic regulation/adjustment of IGP (Intraglomerular Pressure) and this involves several mechanisms which include an increase in renal plasma flow (RPF) and GFR. This same hyperglycemia could induce RAS that causes angiotensin II-induced vasoconstriction of the efferent arteriole thereby, contributing to intraglomerular pressure-mediated hypertension which consequently causes an increase in capillary pressure. This effect could be inhibited by RAS inhibitors which could either be ACE (Angiotensin Converting Enzyme) inhibitors or ARB (Angiotensin II Receptor Blockers) [48,49].

The untreated diabetic condition that results in kidney disease could be early-marked with glomerular hyperfiltration through the estimation of GFR. In diabetes and its complications, theautoregulatory mechanisms maintaining the glomerular capillary pressure is impaired, therefore, there is high IGP due to increased glomerular capillary pressure [50,51]. Another factor to be considered is the L-type Ca^{2+}-gated channels impairment due to its inability to influx Ca^{2+}because there is no depolarization thereby, impairing afferent arterioles from executing theirbasal myogenic responsive influx of blood in diabetes condition [51]. Therefore, the synergistic effect of afferent arteriole impairment with the constriction of efferent arteriole stimulated by angiotensin II and hyperglycemia or both would provoke serious glomerular lesion experienced in diabetic nephropathy. These amounts to hypertrophy of glomerular mesangium and mesangial cells secrete excess ECM proteins through the induction of fibrogenic cytokine TGF-β that has been associated with DN glomerulosclerosis [52,53]. This glomerulosclerotic plague includes some ECM proteins which are collagen IV, laminin and fibronectin. The fact that some glomerular cells including mesangial cells and podocytes have been seen to express Angiotensin II and its AT1R (Angiotensin I Receptors) is a reason enough to suggest that glomerular hyperglycemia/ROS-induced RAS activation could be provoked in the kidneys and it might invoke mechanical damage perpetrated by the intraglomerular pressure causing serious injury to the functional units of the kidney [54,55]. Therefore, the crosstalk signaling between IGP-induced hemodynamic alterations and RAS-induced oxidative stress development could probably express a weighty impact on DN progression [56,57].

In Maillard reaction, the non-enzymatic reaction of glucose with the amino group of proteins produces schiff base amadori compound that later undergoes irreversible dehydration andcondensation reactions to yield AGEs. So, in chronic hyperglycemia, these AGEs accumulate in capillaries and tissues therefore, resulting in diabetic vascular complications [58]. Glyoxal, methylglyoxal, and 2-deoxyglucosone or glyceraldehyde and glycolaldehyde are dicarbonyl products of glucose autoxidation and dehydration that could also produce AGEs [59,60]. Diabetic patients with kidney complications' podocytes and mesangial cells have been reported to express RAGE (Advanced Glycation End Products Receptor) while innumerable animal studies have supported the crucial impact that RAGE contributes to the development and progression of DN. When diabetic mice were genetically engineered to overexpress RAGE, glomerulosclerotic renal dysfunction was on the rise when compared with diabetic mice without RAGE transgene [61–63]. Furthermore, when RAGE was knocked out in diabetic mice, ECM failed to accumulate and there wasn't any GBM thickening. In addition to this, it was also reported that RAGE activation in podocytes contributed to VEGF (Vascular Endothelial Growth Factor) expression and inflammation in diabetic glomeruli thereby provoking albuminuria and glomerulosclerosis [64]. Glomerular hypertrophy, GBM thickening, mesangial cell hypertrophy, overexpression of CTGF (Connective Tissue Growth Factor) and NFκB activation are inhibited by the administration ofanti-RAGE antibody in db/db or STZ-induced diabetic nephropathy mice [65,66]. These robust shreds of evidence suggest that AGE-RAGE axis could be involved in DN pathogenesis and therefore, signal transduction induced through the binding of AGE to RAGE could promote inflammatory reactions expressed by glomerular cells (Podocytes and mesangial cells) [61–64]. AGE-RAGE complex promotes an inflammatory fibrogenic response in DN where ROS overexpression stimulates TGF-β and CTGF through MAPK (Mitogen-Activated Protein Kinase), NFκB and PKC pathways in mesangial and renotubulointestitial cells in DN condition [28,67–70]. Therefore, the inhibition/suppression of TGF-β and its downstream CTGF might present therapeutic measure for the treatment of DN because it has been proven that CTGF plasma level is elevated in DN patients and in the glomeruli of diabetic animals while aminoguanidine (a potentAGEs inhibitor) inhibited the production of CTGF and fibronectin in experimental DN mice [71,72]. As shown in Fig. 3, High glucose-induced mesangial cells could stimulate Ang II generation in association with TGF-β upregulation [73] while this Ang II could cause podocytes DNA denaturation and consequent detachment [74]. When AGE binds to V domain of RAGE, receptor-dependent signaling perpetrated cellular activation provokes inflammation. Other ligands that could stimulate AGE include amphoterin, β-amyloids and fibrillary proteins which are all proinflammatory cytokines. Since this AGE-RAGE complex could increase more intracellular ROS generation in renal cells, it may stimulate more AGEs and further promote the AGE-RAGE system in DN thereby, promoting DN progression [75–80]. Besides the innumerable evidence thatestablished the pathophysiological crosstalking between RAS and AGE-RAGE axis in DN, it has been proven that AGEs could activate TGFβ-Smad pathway through ROS-induced Ang II stimulation [81]. Therefore, a wealth of researches that investigated the inhibition of RAS in DN condition reported several instances that suggest the critical involvement and beneficial effect of RAS inhibition to DN and also confirmed that this effect could be attributed to the inhibition of AGE-RAGE-mediated oxidative stress [78,76–80,82]. Some researchers also reported thatrosiglitazone-induced STAT (Signal Transducer and Activator of Transcription) inactivation could attenuate AGE-induced IL-8

and ICAM-1 (Intercellular Adhesion Molecule-1) produced by proximal tubular cells. It was further reported that this same rosiglitazone could inhibit ECM accumulation thereby inhibiting AGE-mediated proteinuria in DN mice [83].

Therefore, the inhibition of both hyperglycemia-related signaling pathway and RAS-mediated signaling might present better therapeutic efficacy when compared to any monotherapy pathway since DN complications had been linked with these two mechanisms. Furthermore, inhibition of some factors central to oxidative stress, inflammation, glomerulosclerosis and tubulointerstitial fibrosis like NFκB and TGF-β might also present significant therapeutic remedy.

3. Oxidative damage and diabetic nephropathy

As represented in Fig. 3, through various mechanisms involving glucose metabolic upregulation and ROS production, hyperglycemia could induce oxidative stress which has been implicated in the pathogenesis of diabetes and its complications including diabetic nephropathy [84–87]. Therefore, the relationship between hyperglycemia, oxidative stress and diabetes with the accumulative effect could provoke glomerular dysfunction, mesangial cell contraction, glomerular fibrosis and resultant kidney failure [87,88]. While hyperglycemia could cause non-enzymatic glycation of proteins, activation of Protein kinase C (PKC), activation of aldose reductase and polyol pathway which ultimately aggravate cellular prevalence of reactive oxygen species [89–92], some of these reactive oxygen species like the ones generated from glucose metabolism act as signaling molecules for glucose-stimulated pancreatic beta-cells insulin secretion. As this might enlighten researchers on the paradoxical challenges of ROS in beta-cells functions, it also suggests the link between ROS, antioxidants, diabetes and its complications including diabetic nephropathy [84,93].

The link between oxidative damage and diabetic nephropathy could be seen through the elevated ROS generation, abnormal glucose metabolism perpetrated by hyperglycemia, which ultimately provokes oxidative stress. Renin-Angiotensin System (RAS) has also been associated with DN progression. Living organisms produce ROS as a result of normal cellular metabolic status which is responsible for cell proliferation, differentiation and degradation of abnormal tertiary structurally synthesized proteins (misfolded proteins) through the ubiquitin 26S proteasome [94,95]. On the other hand, the imbalance between the antioxidant system and oxidants/ROS/oxidative stress generated by electron transport chain components, endoplasmicreticular oxidases, intracellular cytosolic xanthine oxidase, and the plasma NADPH oxidase could impair cellular components including DNA, proteins, and lipids [96]. Agents of oxidative stress which include superoxide, hydrogen peroxide and nitric oxide though are responsible for normal physiological development, but in many disease states, they are also responsible for the mediation of cellular damages [97]. This cellular damage with further aggravation of ROS production results in tissue damage while excessive renal ROS production is been induced by diabetes mellitus and other metabolic syndromes [98].

In hyperglycemia, there are many junctions in DN-associated pathways where ROS are been generated and the pathways include the Mitochondrial Electron Transport Chain (ETC), Pentose Phosphate Pathway/Hexose Monophosphate Shunt (HMP Shunt) and Polyol/Sorbitol Pathway. Although substrate-level phosphorylation (glycolysis and Krebs Cycle) and oxidative phosphorylation are both responsible for the synthesis of cellular ATP, it is well known that mitochondrial oxidative phosphorylation synthesizes most ATP and the system responsible for this involve the protein complexes (Complex I-Complex IV). After the entry of glucose into the cell, substrate-level phosphorylation through the glycolytic generation of pyruvate and Krebs cycle produced ATP, NADH, and $FADH_2$ which are all being transported through malate aspartate/glycerol phosphate shunt where they serve as electron donors for oxidative phosphorylation complexes. Electrons from either NADH or $FADH_2$ are transferred to O_2 in the Complex I-Complex IV to synthesize ATP catalyzed by F_oF_1 ATPase. In a normal physiological state, almost 99 % of this O_2 is reduced to H_2O while less than 1 % is converted to O^{2-}. Under hyperglycemia, there is mitochondrial dysfunction therefore, a large number of electrons leak out at two critical junctions as mentioned above. So, it therefore infer that diabetic cells including glomerular mesangial cells will be unable to regulate intracellular glucose concentrations and this situation would cause the over-activation of oxidative phosphorylation complexes and a consequent release of ROS at these two junctions leading to ROS-orchestrated oxidative stress, denaturing the single/double-stranded DNA, exposing mtDNA to oxidative stress and thereby aggravating mitochondrial electron transportation leading to mitochondria-mediated ROS overload that causes the denaturation of mitochondrial enzymes, inhibiting ATP synthesis, altering mitochondrial permeability transition, cytochrome C translocation, therefore, activating caspase-driven apoptosis or necrosis [26,99–102]. Hexose monophosphate shunt rate-limiting enzyme G6PDH (Glucose 6 Phosphate Dehydrogenase) utilizes NAD(P)H to shuttle G6P from glycolytic pathway into the pentose pathway. In oxidative stress-mediated disease status, there is upregulation of G6PDH which commands the activation of NAD(P)H which also activates NAD(P)H oxidase-catalyzed ROS generation thereby leaving the cell in a state of oxidation because the synthesis of GSH from GSSG requires NAD(P)H. So, as the usage of glutathione reductase cofactor NAD(P)H H^+ is being depleted, GSH level reduced and there is a prevalence of oxidative stress [103,104].

In a normal physiological state, little amount of glucose is being diverted to polyol pathway where sorbitol is being produced in a reaction catalyzed by the intracellular NADPH-dependent aldose reductase enzyme after which NAD-dependent sorbitol dehydrogenase catalyzes the formation of fructose from sorbitol; however, in hyperglycemic state, more than 10 times glucose is channeled into aldose reductase rate-limiting enzyme-catalyzed polyol/sorbitol pathway. The metabolites which include F-3-P and 3-DG causes the formation of AGEs and just as we already discussed above, AGE:RAGE complex invokes downstream ROS-mediated oxidative insults. Besides this AGE:RAGE-mediated ROS induction, NADPH is used up as aldose reductase cofactor thereby, inhibiting the synthesis of GSH from GSSG in a reaction catalyzed by glutathione reductase. As this reaction reduces cellular GSH level, antioxidant status is being disturbed. In addition, the impermeability of the cellular membrane to the accumulated sorbitol enforces osmotic pressure that is controlled by ROS-generated oxidative stress [104,105].

The fact that angiotensinogen, renin, angiotensin-converting enzyme (ACE), angiotensin II, angiotensin I and II receptors (AT1R and AT2R) at both protein and mRNA levels have been expressed in the kidneys showed that this system might contribute to the progression of DN [106]. In renal proximal convoluted tubular cells, angiotensinogen is converted to inactive angiotensin I by rennin after which angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II. Angiotensin II is the key driving factor of DN which acts through the G-protein-coupled cell membrane type 1 receptor and due to high AT2Rs affinity of the renal tubular mitochondria, its hypertensive downstream effect is well pronounced [107]. In diabetes, there is intrarenal RAS gene expression which further implicates RAS in the progression of DN and therefore means that hypertension together with the already established diabetes condition could provoke the microalbuminuria to the overt type thereby increasing kidney damage. Therefore, it is safe to conclude that hyperglycemia, glucose metabolic dysregulation, reactive oxygen species, and rennin angiotensin system could elevate oxidative stress that has been witnessed in kidney tissues in diabetic condition. So, in order to maintain oxidants/antioxidant balance to shield cellular environs against damage, Nrf2, a crucial regulating cytoprotective gene when being activated, binds to the antioxidant response element at the promoter region of its target gene to express genes that neutralize and rescue biological system from these harmful toxic xenobiotic substances.

4. Keap1/Nrf2/ARE signaling and diabetic nephropathy

Transcription factors are well known for their typical gene-expressing regulation through the binding of cis-acting regulatory enhancer, thereby recruiting other coactivators to target and initiate downstream transcription of genes meant to express their essence [1,108,109]. This ubiquitously expressed nuclear factor erythroid 2-related factor 2 (Nrf2) regulates cytoprotective genes and detoxifying enzymes which express the strength to shield the cellular biological system against oxidants and xenobiotics [110]. Exogenous and endogenous antioxidants that could activate Nrf2 have been reported in several articles to arrest the oxidative entropy caused by oxidants and xenobiotics in the biological system through the activation of a pathway called Keap1/Nrf2/ARE – A powerful pathway that resuscitates cells from the ashes of oxidative stress and downstream complications [111,112]. Nrf2 has been recognized in the world of antioxidant research as the major regulator and initiator of cytoprotective genes transcription, engineered by Nrf2 activator and stress-oriented oxidative insults [113].

Nrf2, just like its contemporaries, has spatial and temporal regulation which involves its response to its stimuli by switching on and off when the stimuli are present or absent respectively. Basally (without oxidative insults or Nrf2 activators), normal function of Nrf2 is kept intact in the cytoplasm through its covalent binding to Keap1 which prepares it for proteasomal degradation. Under this same homeostatic condition, the binding of this actin-bound keap-1 protein to Nrf2 had been found to be responsible for the suppression of Keap1/Nrf2 pathway which leads to the sequestration and degradation of cytoplasmic Nrf2. The binding product, which is the adaptor-like complex formed between the Neh2 domain (Fig. 1) of Nrf2 and the region between the BTB and keap1 ketch repeat (Fig. 2) serves as a binding signal for cullin3/ring box1E3 ubiquitin ligase complex which promotes ubiquitin-driven Nrf2 degradation by 26S proteasome [114,115]. During stressed condition or presence of Nrf2 activators, Nrf2 dissociates from its repressor keap1, translocates into the nucleus where it heterodimerizes with small maf (musculoaponeurotic fibrosarcoma) proteins which facilitates its binding to the Antioxidant Response Element (ARE) that has been described as a cis-acting enhancer at the upstream promoter region of its target gene to initiate the transcription of genes that mastermind the cytoprotection of cells from oxidative stress and xenobiotics [116,117]. These genes had been reported to include phase II detoxifying, metabolizing and antioxidant genes that do not only reduce the effects of electrophiles, free radicals and reactive oxygen species to nontoxic/less toxic intermediates, but also fortify the cells against the aftermath of oxidative damages. They include NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HMOX1), glutamate cysteine ligase (GLC) and glutamate S transferase (GSTs).

The capacity of the Keap1/Nrf2/ARE signaling pathway to regulate energy metabolic processes reveals its therapeutic function against metabolic diseases including diabetes. Nrf2 protein levels are low in both pre-diabetics and diabetic patients when compared with non-diabetics, this infers that Nrf2 upregulation might reverse the condition and that the lowered Nrf2 expression might be responsible for the oxidative damage and oxidative stress-induced complications experienced in diabetes [118]. Oxidative stress induces transient Nrf2 activation due to the accumulation of reactive oxygen species in the kidneys of animals induced with acute kidney injury and the renal tubular pharmacological enhancement of this transcription factor significantly ameliorates damages related to AKI through the reduction of ROS and its resultant oxidative stress. Therefore, the Keap1/Nrf2 signaling system could be seen as a potential target for the treatment of oxidative stress-related kidney dysfunctions [119]. Although the link between biological hallmarks of diabetic nephropathy and Keap1/Nrf2/ARE signaling has not been directly elucidated, it was reported that advanced glycation end products could initiate accumulation of oxidative stress and oxidative stress had been proven to be a potent reprobate in the pathology of diabetic nephropathy. It was also established that SIRT1, through the activation of Keap1/Nrf2/ARE significantly ameliorated diabetic nephropathy by downregulating fibronectin (FN) and TGF-β1 protein expressions which are prominent biomarkers of extracellular matrix production and oxidative stress in diabetic nephropathy respectively. In this research, it was further noticed that SIRT1 downregulates keap1 thereby, promoting Nrf2 nuclear translocation, Nrf2 ARE-binding potential and HO-1 expression which is responsible for inhibiting ROS overproduction and ultimately, the downregulation of FN and TGF-β1 in AGE-induced rats glomerular mesangial cells. SiRNA-mediated Nrf2 (Nrf2-siRNA) and Sirt1 (Sirt1-siRNA) knockdown were used to explain the mechanism here. It was found that Sirt1 was downregulated in Nrf2-siRNA while Keap1/Nrf2/ARE was downregulated in Sirt1-siRNA and AGE-induced fibrotic rat's glomerular mesangial cells after which tBHQ-mediated-Nrf2 activation reduced fibronectin and TGF-β1 protein levels [120]. The result of this research exposes the mechanism that might be exploited by keap1 downregulating small molecule compounds in the amelioration of diabetes and its complications including diabetic nephropathy and besides, it also suggests that Keap1/Nrf2/ARE-Sirt1 might be a novel pathway to be more explored in the course of DN therapeutic findings.

Matrix metalloproteinases are proteolytic enzymes of the zinc-containing endopeptidases housing the hydrolyzing strength to degrade the

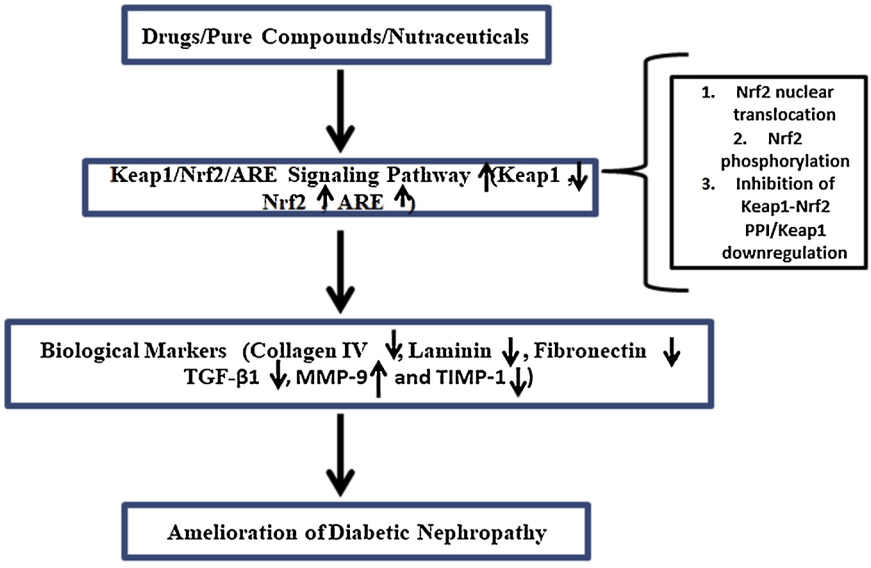

Fig. 4. This schematic diagram is illustrating how Nrf2 activating agents could ameliorate diabetic nephropathy. The mechanisms could be through Nrf2 nuclear translocation, Nrf2 phosphorylation, and inhibition of Keap1-Nrf2 complex dissociation. These might lead to the modulation of the proteins and enzymes that dictate the integrity of ECM in DN condition.

components of extracellular matrix (ECM) which has been defined as the morphological hallmark of diabetic nephropathy when accumulated and, this role critically signifies the reason behind their importance in renal disease amelioration. Recently, it was evidenced that MMP expression and DN progression are correlated in renal disease patients with DN in both humans and experimental animal models and this expression is being perpetrated by multiple factors including high glucose, advanced glycation end (AGEs) products, transcription factors and oxidative stress [121]. The integrity of glomerular structure and function are being driven by ECM turnover and MMPs may play a significant role in the regulation of ECM synthesis and degradation balance through several mechanisms. Therefore, abnormal ECM regulation could cause dysregulation of matrix protein synthesis and degradation balance and this might lead to altered glomerular cells function – a critical hallmark of DN [122,123]. Although MMP had been long linked with DN, TIMP (Tissue Inhibitor of Metalloproteinase) on the other hand had been proven to work together in regulation of ECM turnover in the progression of DN. This TIMP as their name implies are the inhibitor of MMPs which infer that the balance in the turnover of these two proteins might be significant in the regulation of ECM synthesis and degradation in DN.

We therefore propose as illustrated in Fig. 4 that if diabetic nephropathy through Keap1/Nrf2/ARE signaling could be ameliorated by downregulating Fibronectin (a critical biomarker of diabetic nephropathy) and TGF-β1 (transforming growth factor-β1) [120], it is absolutely reasonable to investigate if this same disease could be ameliorated by investigating other biological markers including extracellular matrix proteins (collagen IV and laminin), matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinase (TIMP). Therefore, studies of the relationship between Nrf2 signaling, extracellular matrix proteins and RNA-mediated silencing of Nrf2 in high glucose-induced kidney cells (e.g. SV40 glomerular mesangial cells and HK-2 cells) might reveal the functions of Nrf2 in diabetic conditions and also give insight on the regulatory functions of Nrf2 on MMPs and TIMPs on some extracellular biological markers including collagen IV, laminin and fibronectin and therefore, might expose some therapeutic measures that could be specifically directed to any of these proteins towards the treatment of DN.

Through the silencing/knocking down of Nrf2 expression and its upstream repressor Keap1, Nrf2-dependent genes associated with DN have been reported to play a significant role in DN pathophysiology, therefore; therapeutic strategies targeting these genes have been established. The transcriptional activation of Nrf2 through its binding to ARE region of its target DNA promotes antioxidant-mediated gene expression which include NQO-1, SRXN1 (Sulfiredoxin 1), TXNRD1 (Thioredoxin reductase 1), HO-1, SOD, CAT and GSH; NADPH synthetic-induced genes which include G6PD, PGD and ME1; glutathione metabolism (GCLC, GCLM and GSTs) and mitochondrial dysfunction-mediated genes (CPT1 (Carnitine palmitoyltransferase I) and NRF1) [118,119]. When genetically modified mice including Nrf2-KO, Keap1-KD and Keap1-CKO (Keap1-Conditional Knockout) were used to investigate the protective function of Nrf2 in kidney disease, it was noticed that Nrf2 blocks the progression of kidney disease through the inhibition of kidney disease-induced oxidative stress. Besides the fact that oxidative stress is central to kidney disease progression, its accumulation is regarded as the final common pathway to many kidney disease model in mice [124–129]. In addition to this genetic modification method, orally administered Nrf2 activator CDDO imidazole was used to investigate the role of Nrf2 in kidney disease prevention in rodents. It was observed that CDDO-imidazole-mediated Nrf2 upregulation is critical to the prevention of kidney damage progression at the early stage. This was because the administration of CDDO-imidazole on the 1, 3 and 5 days after unilateral IRI treatment shows mild kidney injury on the 14th day when compared with vehicle-treated group whereas CDDO-imidazole administration on days 7–13 was ineffective. In another similar work, it was observed that keap1 inhibitors attenuated oxidative stress and protected cells from damages in various disease models using rodents. [130,131]. CDDO-9, 11-dihydrotrifluoroethyl amide (CDDO dhTFEA, also referred to as dh404) which is a potent keap1 inhibitor promoted kidney fibrosis in dh404 high dosage induced CKD model through the activation of NFκB-mediated inflammatory response. In this research, the inhibition of Keap1 which is the upstream repressor of Nrf2 could inhibit the antioxidant-regulatory mechanism of Nrf2 which therefore promoted NFκB-propagated inflammation [132,133]. Maybe the downregulation of Keap1 released Nrf2, thereby increasing Nrf2 availability for nuclear translocation and subsequent binding to its DNA target to promote the transcription of its antioxidant downstream genes, however; high dosage of Keap1 inhibitor might pose some inflammatory threats probably through NFκB activation. When the nephroprotective effect of HO-1 was investigated in STZ-induced DN mice models with or without hemin (HO-inducer) and ZnPP (HO-1 inhibitor), hyperglycemia with hyperlipidemia was experienced with renal impairment in DN mice due to the distortion assessed in renal histopathological architecture and kidney function. Increased MDA, nitric oxide and myeloperoxidase (MPO) with decreased glutathione, superoxide dismutase and catalase were all reported due to renal oxidative and nitrosative stress. Besides all these observations, it was also noticed that DN group exhibited high renal proinflammatory cytokine expression and increased TNF-α assessed by immunoblotting assay while renal HO-1 protein expression and activity were increased in DN rats when compared to control. Hemin (HM) administration to DN rats improved kidney function, histopathologic features, lipid profile and TNF-α expression with no hypoglycemic effect. It is noteworthy that the increased HO-1 by HM administration and decreased HO-1 in ZnPP administration does not affect HO-1 activity or renal oxidative capacity of non-diabetic rats while there was HO-1 upregulation in both HM and ZnPP (Zinc Protoporphyrin) in DN rats. This infers that the mechanism behind HM-mediated renal damage amelioration in STZ-induced DN mice might be through antioxidation, antinitrosative and antiinflammatory potential perpetrated by its HO-1 upregulating potential [134,135]. Therefore, with this evidence, we could infer that HO-1 expression is not strictly dependent on Nrf2 transcriptional activities; NFκB could also induce HO-1 expression during inflammation. It has been found that a small GTPase RAC1 is being induced through NFκB upregulation to promote Nrf2 expression and therefore, a new mechanism that involves the modulation of RAC1 inflammatory pathway has been discovered to occur through a crosstalk between NFκB and Nrf2 transcription factors. This conclusion emanated from the fact that both dominant negative mutant of IκBα that leads to NFκB degradation or the use of p65-NFκB-deficient HEK-293 T cells exhibited Nrf2 downregulation at the protein levels and impaired Nrf2 functions in the control group. Therefore, the mechanism underlying the initiation and promotion of inflammation in diabetic nephropathy might have its explanation hidden in this system [136]. The anti-inflammatory mechanism of action of a synthetic chalcone 3′, 4′, 5′, 3, 4, 5-hexamethoxy chalcone (CH) in RAW 264.7 cells was reported to be driven probably by its potential to simultaneously induce HO-1 cytoprotective response and downregulate NFκB without antioxidation. The details of this research revealed that CH inhibited NO production through the inhibition of NO synthase protein expression, prevents LPS (lipopolysaccharide)-stimulated NO overproduction in RAW 264.7 macrophage through NFκB inhibition, inhibits NFκB-IκB complex which leads to the blockage of NFκB nuclear translocation thereby, inhibiting DNA binding and subsequent transcriptional activities. Furthermore, Nrf2/HO-1 signaling was also activated by CH. So, it means the anti-inflammatory mechanism of action of CH could be found in its ability to block NFκB through NFκB-IκB complex inhibition and the inhibition of NO synthase which together might have triggered the upregulation of Nrf2/HO-1 pathway for the antioxidant and anti-inflammatory effect [137].

Cisplatin-induced nephrotoxicity causes oxidative stress-mediated renal DNA adducts formation in rodents while Nrf2-dependent genes

(HO-1, NQO-1 and GLCL) as shown in Fig. 5 responded to exhibit cytoprotection against these electrophilic attack and oxidative stress. In this same study, wild-type and Nrf2-null mice plasma and kidneys were harvested after cisplatin administration in order to estimate the extent of renal injury and inflammation. Nephrotoxicity was more pronounced in Nrf2-null mice when compared with wild types after cisplatin treatment and this was attributed to the increased neutrophil infiltration accompanied by p65 NFκB binding with upregulated inflammatory mediator mRNA levels. Since cisplatin increased both the mRNA and protein levels of NQO-1, HO-1 and GCLC with other transporters like Mrp2 and Mrp4 in wild-type and not in Nrf2-null mice, we could infer that these genes are Nrf2-dependent and their functions in diabetic nephropathy could give insight on how they could be exploited for better therapeutic functions. Furthermore, when Nrf2 activator CDDO-Imidazole increased Nrf2 signaling in wild-type mice, cisplatin-induced toxicity was suppressed and the mice were protected against nephrotoxicity. Therefore, while Nrf2 activation might present novel therapy for kidney injury prevention, coordination of detoxification and drug transportation coupled with the inhibition of inflammation during cisplatin-induced nephrotoxicity might present defense mechanisms to eliminate nephrotoxicity promoters and therefore, induce proximal tubular remedy [138].

Antidiabetic nephropathy potential of 4-O-methylhonokiol (MH) (a biologically active ingredient from *Magnolia* stem bark) was investigated in order to elucidate if the treatment with MH could ameliorate DN and also explain whether its AMPK-related antioxidant and antiinflammatory capacities could explain the reasons behind this potential in T2D murine STZ-induced DN model, it was found that besides the obvious proteinuria in DN mice, renal lipid accumulation and lipotoxic-induced oxidative stress with inflammation-mediated fibrosis were also observed in DN mice when compared to the normal group. In addition to this, MH was able to prevent these changes in a three months treatment and three months post-MH withdrawal and this renal therapeutic effect was mechanistically attributed to their lipid metabolic improvement coupled with oxidative stress attenuation along with an increase in AMPK/PGC1α/CPT1β- mediated fatty acid oxidation and Nrf2/SOD2-mediated anti-oxidative stress. Although, these results show the antidiabetic nephropathy potential of MH, it also exposes that AMPK (Fig. 5), SOD2, PGC1α, and CPT1β may be Nrf2-dependent genes and their function in DN condition could be detected by checking their status in RNA-mediated Nrf2 silenced gene in HG-induced kidney cells or Nrf2-knockdown DN mice model [139]. Bach1 had been established as a competitive inhibitor of Nrf2 for the promoter region of Nrf2 target DNA and this causes the regulation of ARE-dependent gene expression. Bach1 (BTB and CNC homology 1) transcription factor is a member of Cap 'n' Collar basic region leucine zipper containing the BTB/POZ protein binding region with the C-terminal bZIP DNA binding domain that instigates the heterodimerization of Bach1 with sMaf proteins (MafF, MafG, and MafK) [140]. Bach1-Maf complex heterodimers, through the binding to the MAREs (Maf recognizing elements) at the promoter region, blocks the transcriptions of several oxidative stress-induced genes (e.g HO-1 and NQO-1) [141,142]. Four of the six CP (Cysteine-proline) motifs of Bach1 are located in the heme binding region close to the C-terminus. The interaction of heme with two of these CP motifs inactivates Bach1 thereby, causing the Bach1 nuclear exclusion and the promotion of Heme-oxidized IRP2 (Iron Regulatory Protein 2) ubiquitin Ligase-1 (HOIL-1)-stimulated ubiquitination and degradation. Bach1 nuclear export is also facilitated by Bach1 tyrosine 486 phosphorylation. This heme-induced Bach1 nuclear export is driven by chromosomal region maintenance 1 (Crm1) [143–145]. As earlier discussed, Nrf2 is bound to its cytoplasmic Keap1 repressor under basal/physiological condition while during oxidative stress or in the presence of Nrf2 activators/Keap1 inhibitors, Nrf2 dissociates from Keap1, translocates into the nucleus where it heterodimerically binds MAREs with sMaf, displacing Bach1 from MAREs thereby, activating Nrf2 downstream oxidative stress-response genes [146,147]. After the displacement of Bach1 from MAREs, Batch1 is exported out of the nucleus. Sirt6-mediated Nrf2 nuclear import and Bach1-MARE dissociation have been suggested to occur in hepatocytes. Another MARE site had been identified near the transcription start site of Bach 1 transcript variant 2. So, Bach1 functions as a potent Nrf2 inhibitor in the basal state whereas, Nrf2 restores Bach1 levels after oxidative stress-induced Bach1 nuclear export and degradation. With this modulatory mechanism, Bach1 mediates the regulation of Nrf2-orchestrated downstream transcripts [148–150]. Other genes targeted by Bach1 include NQO-1, GCLC, GCLM (glutamate-cysteine ligase modifier) and SLC7A11 (solute carrier family 7 members 11). All these genes have their roles in redox regulation including HO-1 which, besides the well-known oxidative stress regulation, is also responsible for cellular iron homeostasis in higher eukaryotes. When Heme level is low, HO-1 expression is being suppressed by Bach1 while higher heme levels blocked Bach1-DNA binding and promote Bach1 nuclear export and degradation which, therefore, induced HO-1 expression that subsequently degrades heme while generating antioxidant molecules including ferrous iron CO and biliverdin [151]. In addition, it has been shown that Bach1 overexpression promotes ROS production from mitochondria of endothelial cells and the ischemic limbs of mice, leading to apoptosis progression and angiogenetic decline. Therefore, it means that Bach1/HO-1 signaling feedback loop maintains heme homeostasis during oxidative stress.

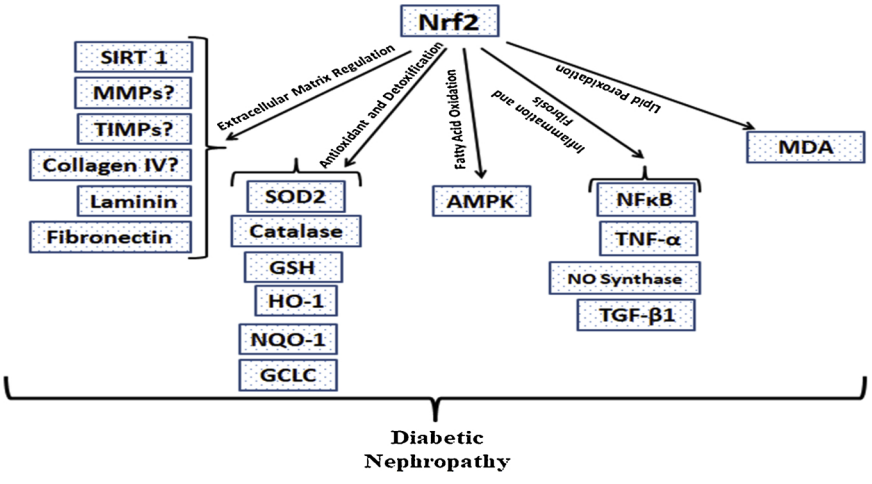

Fig. 5. Some Nrf2-related proteins and genes associated with Diabetic Nephropathy. With respect to this literature, the summary of key protein and genes related to Nrf2 transactivation associated with DN are presented above. Nrf2 transactivation induces the inhibition of fibronectin which is an extracellular matrix protein, therefore, other extracellular matrix protein turnover regulatory components (Metalloproteinases (MMPS), Tissue Inhibitor of Metalloproteinases (TIMPs) and Collagen IV and laminin) should be investigated. Upregulation of Nrf2 leads to the transcription of antioxidant and detoxifying genes (SOD2, catalase, GSH, HO-1, NQO-1, and GCLC). Nrf2 transactivation is linked with AMPK-activated β-oxidation regulation, inflammation, and fibrosis inhibition through the blocking of NFκB, TNFα, NO synthase, and TGF-β1 while lipid peroxidation biomarker malondialdehyde (MDA) is also being reduced by Nrf2 activation. These Nrf2 transactivation-orchestrated effects had being proved experimentally to ameliorate diabetic nephropathy either in cell lines, animal model or both.

5. Therapeutic strategies targeting Keap1/Nrf2/ARE signaling components

5.1. Nrf2 nuclear translocation and diabetic nephropathy

The first study that established the entrance of Nrf2 chemopreventive agents into the nucleus to exert their inhibitory prowess against Nrf2 ubiquitination in NBT-II cells, RT-4 cells and MEFs cells were done by Yun li and others [22]. In this research, CPDT (5, 6 dihydrocyclopentane-1, 2-dithiole-3-thione) and SF (sulphoraphane) Nrf2 prototypical chemical activators significantly activated Nrf2 protein level with elevated Nrf2 transactivation activity, but the modulation of Nrf2 gene transcription and keap1 dependent Nrf2 ubiquitination was not observed. They further detected that CPDT and SF inhibited keap-1 dependent Nrf2 ubiquitination but Nrf2 was not degraded through a keap1-independent pathway. Neither of these compounds dissociated Nrf2-Keap1 complex or disrupted ubiquitin ligase complex, but they both inhibited Nrf2 phosphorylation which is a probability for the sustained association of Nrf2 with Keap1. So, in this research, they demonstrated that the machinery orchestrating Nrf2 ubiquitination and proteasomal degradation exist in the nucleus, rather than the well-known elucidation of this same mechanism that emphasized its occurrence in the cytoplasm. One of the most abundant polyphenols chlorogenic acid (CGA) was reported to express its antioxidative potentials by promoting nuclear translocation of Nrf2, transcription of HO-1 and Nqo-1 in HBZY-1 cells and diabetic nephropathy mice model. In this investigation, CGA pretreatment of HG-induced HBZY-1 rats mesangial cell lines inhibited NFκB nuclear translocation, upregulated Nrf2 and its downstream HO-1. Meanwhile, when Nrf2 and HO-1 were downregulated by siRNA-mediated Nrf2 silencing and ZnPPIX respectively, nuclear translocation of NFκB increased along with the provocation of pro-inflammatory cytokines while PDTC (NFκB inhibitor) upregulated Nrf2 nuclear translocation [153]. In another research group, sodium butyrate (NaB) ameliorative effect against DN was studied to be perpetrated through the nuclear translocation of Nrf2 when STZ-induced diabetic C57BL/6 Nrf2 knockout WT mice were treated with NaB for 20 weeks. NaB downregulated histone deacetylase (HDAC) together with the translocation of Nrf2 which leads to its activation followed by the transcription of its downstream genes and therefore, the amelioration of DN. Therefore, in this research, it was concluded that the amelioration of DN could have been through the downregulation of HDAC that orchestrated the upregulation of Nrf2/ARE signaling [154]. A keto-carotenoid xanthophyll astaxanthine ameliorated diabetic nephropathy by reducing the protein expression of two diabetic nephropathy biomarkers (Fibronectin and Collagen IV) and lipid peroxidation biomarker malondialdehyde in the kidneys of STZ-induced diabetic nephropathy mice and also promoted nuclear translocation of Nrf2 followed by the increase in the downstream HO-1 and SOD1 activities. It was therefore suggested that the well-known antioxidant and renoprotective activities of astaxanthin might be controlled by the activation of Nrf2/ARE signaling [155]. Furthermore, another group of researchers investigated the renoprotective effect of astaxanthin using HG-induced glomerular mesangial cells in vitro model and STZ-induced diabetic mice as in vivo model. It was found that astaxanthin alleviated ICAM-1, fibronectin, TGF-β1 as well as ROS in these two DN models and to achieve these renoprotective effects, it was found that Nrf2 translocation and transactivation were also promoted which therefore led to the increased in some Nrf2 downstream transcripts including HO-1, NQO-1, and SOD1 just as presented in Fig. 5 [156]. Therefore, we could say that Nrf2 nuclear translocation could be at least, a part of the mechanism that might contribute to the ability exhibited by some small molecule activator of Keap1/Nrf2/ARE signaling in the amelioration of diabetic nephropathy and oxidative stress-related pathologies.

5.2. Nrf2 phosphorylation and diabetic nephropathy

It has been shown that there is an auto-regulatory feedback mechanism that oversees the cellular availability of INrf2 (Cytoplasmic Inhibitor of Nrf2 otherwise called Keap1) and Nrf2 [152]. During the physiological state, antioxidants and oxidants inhibit cytosolic INrf2/Keap1 leading to the increase in Nrf2 level [157–159]. This Nrf2 upregulation prompts its nuclear translocation where they bind ARE to activate a battery of chemopreventive genes. After this, Nrf2 undergoes nuclear export where it binds with cytoplasmic INrf2 after which it is being channeled for ubiquitine proteasomal degradation. This is referred to as the INrf2-dependent Nrf2 transcriptional modulation. Under the basal state, cytoplasmic Keap1/Cul3-RBX1 complex constantly degrades Nrf2. This same complex is also present in the nucleus under basal condition degrading Nrf2, although, INrf2-induced ubiquitination and degradation occur primarily in the cytosol [159,160]. Along with INrf2/Cul3-RBX1 complex, another nuclear negative regulator of Nrf2 which involves the Src subfamily A member (Fyn) (others are Yes and Fgr) phosphorylates Nrf2 at Y568 and leads to the Nrf2 nuclear export (As shown in Fig. 6) while Batch1 that competes with Nrf2 for ARE promoter region has been found to suppress ARE-

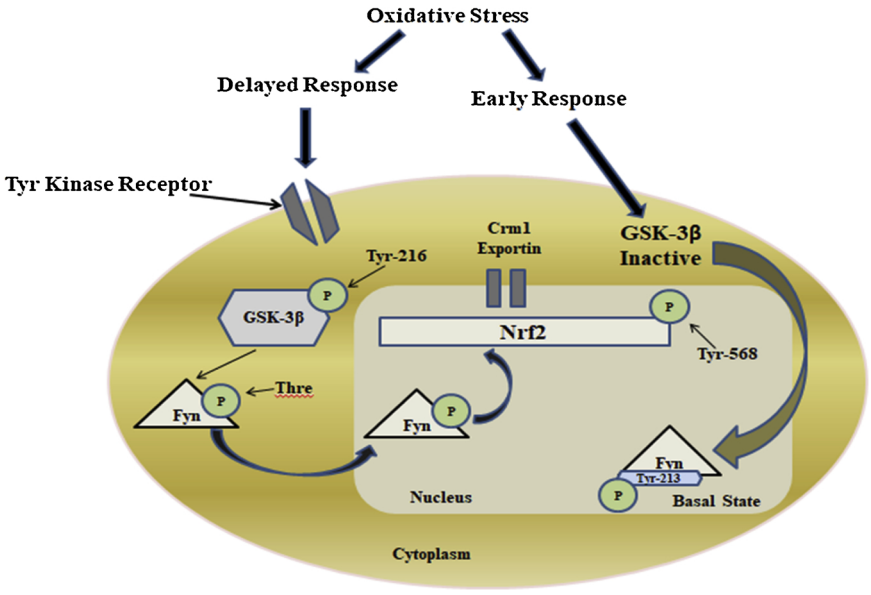

Fig. 6. Schematic diagram showing the mechanism of p-GSK-3β-mediated Nrf2 nuclear export. Oxidative stress induces the Tyr kinase receptor conformational change that activates GSK-3β phosphorylation at Ser216. p-GSK-3β then phosphorylates Fyn at threonine residue in a reaction that leads to nuclear translocation of Fyn where Fyn phosphorylates Nrf2 at Tyr residues after which Crm1/exportin exports Nrf2 into the cytoplasm for proteasomal degradation.

mediated gene expression [160]. It was later reported that cytosolic β-TrCP degrades GSK3β phosphorylated Nrf2. What actually occur is that, after gene induction, Chromosomal maintenance-1/Crm1 (Otherwise called exportin) facilitates the nuclear export followed by cytosolic degradation of Nrf2 after the phosphorylation of Fyn (SRC tyrosine kinase family) by glycogen synthase kinase 3β (GSK-3β) which makes Fyn undergoes nuclear translocation where it phosphorylates Nrf2 at Tyr568 residue as illustrated in Fig. 6 below [158–160]. Alternatively, GSK-3β could directly, at Ser335 and Ser338 phosphorylate Nrf2 at its Neh6 domain (Fig. 1), leading to its nuclear translocation recognized by β–transducing repeat which ultimately causes nuclear ubiquitination and Nrf2 [161]. Furthermore, in order to prevent the induction of genes depending on Nrf2, BTB and Bach1 transcription factor competitively bind to Nrf2 DNA binding sites, therefore, inhibiting Nrf2-orchestrated gene expression. These are the mechanisms explaining Nrf2 turnover system.

Nfe2l2 [162] consists of Neh1, Neh2, Neh3, Neh4, Neh5, Neh6, and Neh7 domains and each of these domains has specific functions. CNC-bZIP-containing Neh1 region coordinates the heterodimerizing interaction with the coactivator sMaf proteins and also promote the binding of Nrf2 to the DNA promoter region of Nrf2 target gene [158,162]; Neh2 (Fig. 1) helps in the negative regulation of Nrf2 through its binding with seven lysine residues (which serves as the target for ubiquitinylation and proteasomal degradation) and the upstream Keap1 repressor using its highly conserved DLG and ETGE (Fig. 2) motifs in a conformation that allows one molecule of Keap1 bind each of this region and therefore, trigger Nrf2 ubiquitination through the formation of the complex Keap1/Cul3 E3 ubiquitine ligase [163,164]. This occurs at the physiological state. C-terminal Neh3, Neh4, and Neh5 connive to perpetrate transcription and activation processes of Nrf2 transcripts [165,166] while Neh7 exploits retinoid X receptor to repress Nrf2 transcriptional proceedings [167]. Neh6 serine-rich domain is of great interest in Nrf2 phosphorylation because of its two conserved peptide motifs DSGIS and DSAPGS involvement in the negative regulation of Nrf2 stability in a reaction independent of Keap1. These two motifs are recognized by β- transducing repeat-containing protein (b-TrCP) [168,169]. After glycogen synthase kinase-3β phosphorylates DSGIS motif at the Neh6 domain of Nrf2 (Fig. 1), β-TrCP (The adaptor molecule for SCF E3 ubiquitine ligase complex) binds Neh6 to engage Skp1-Cul1-F-box protein (SCF) ubiquitin ligase complex thereby, promoting the negative regulation of Nrf2 through the proteasomal degradation [169–171]. When SNP-induced GSK-3β activation was executed to investigate if it could mitigate kidney injury in renal cells (SV-40 immortalized murine kidney MCT cells) and diabetic mice, it was found that in proximal tubular cells, SNP lowered high glucose-mediated laminin accumulation through the activation of GSK-3β. Three weeks of administration of SNP in mice with STZ-induced type 1 DM ameliorated kidney hypertrophy, ECM accumulation, and albuminuria without altering blood glucose levels. Further investigations established that diabetes inactivated GSK-3β through Src, Pyk2 and ERK activations while these reactions were abrogated by SNP. Although the short duration of this study could be a limitation as long term examination could suggest if GSK-3β regulation is time dependent but, it could still be concluded that SNP-induced GSK-3β activation reversed diabetic kidney conditions in renal cells and STZ-induced type 1 DN mice [172].

In an investigation to show the mechanism underlying the regulation of GSK3-mediated Nrf2 nuclear exclusion and degradation which is crucial to the switching off of Nrf2 antioxidant stress response after injury in the glomerular disease model of podocytes, doxorubicin elevated cell death and actin cytoskeletal disorganization and this was connected with GSK-3β overactivation and minimal Nrf2 activation. Small molecule SB216763-induced GSK3 selective inhibitor caused Nrf2-dependent antioxidant response protective effect which was marked by Nrf2 nuclear accumulation with Nrf2 and HO-1 upregulations. Deleted expression of GSK3β kinase-dead mutant in cultured podocytes brought back doxorubicin-mediated Nrf2 activation and therefore, prevented injury in podocytes. Contrary to this, a constitutive GSK-3β active mutant inhibited doxorubicin-propagated Nrf2 response and exacerbated injuries in podocytes and this was abrogated by SB216763 treatment. Furthermore, significant attenuation of albuminuria and reduced histological signs of podocyte injury including podocytopenia, podocyte marker loss, and podocyte de novo desmin expression were reported in murine models of doxorubicin nephropathy or nephrotoxic serum nephritis when GSK-3β was genetically targeted by doxycycline-induced podocyte-specific knockout or pharmacologic targeting through SB216763. So, this therapeutic effect was likely attributed to the enhanced Nrf2 signaling activation in glomerular podocytes because trigonelline (a selective Nrf2 antagonist) inhibited proteinuria-reducing and podocyte protective effects. It means the targeting of GSK-3β inhibition/Knocking out could fortify antioxidant potential of Nrf2 signaling after doxorubicin or NTS insults, reduced podocytopathy and glomerular injury with proteinuria attenuation. In summary, this research suggests that Nrf2 signaling and antioxidant response-mediated GSK-3β regulation might be an effective therapeutic measure for protecting podocytes and treating proteinuria in glomerulopathies (These are conditions associated with diabetic nephropathy) [172,173]. Therefore, Nrf2 phosphorylation at some specific amino acid residues could be exploited as a credible mechanism for the regulation of Keap1/Nrf2/ARE signaling. One of the posttranslational modifications of Nrf2 that could help its stability, nuclear translocation or transactivation through its association with other components of its signaling pathway involves its phosphorylation at certain serine, threonine or tyrosine residues [173]. Example of these extracellular signal-modulated kinases that could activate Nrf2 through GSK-3β inhibition includes ERK, p38MAPK, PI3K and PKC [174]. Nrf2 phosphorylation had been proposed with some uncertainties surrounding the proteins responsible for this action after establishing its housekeeping transcription of some basal genes (most especially GST). It was speculated that MAP kinases might be the key orchestrator of this action [174,175]. When alanine was substituted for serine/threonine residues that serve as major targets for MAPK-orchestrated phosphorylation, a decrease in Nrf2 transcriptional activation was reported, which was attributed probably to the reduction in nuclear Nrf2 translocation. Furthermore, it was noticed that Nrf2 phosphorylation does not affect keap1 in vivo, which implies Nrf2 stability remains intact. Therefore, besides the fact that direct phosphorylation of Nrf2 by MAPK is not the sole perpetrator of Nrf2 regulation, but indirect translational modulation of Nrf2 protein synthesis might contribute its quota [176], we could also suggest that MAP kinase phosphorylation of Nrf2 is partially/strictly responsible for nuclear translocation of Nrf2 for the transcription of its downstream genes.

In a study to investigate the cross-talking between Nrf2 and AMPK using LPS as inflammation inducer and BBR as AMPK inhibitor, it was established that activation of Nrf2 by phosphorylation was AMPK-dependent. As this research might underscore the link between bioenergetics and detoxification/cytoprotection, it also illuminates one of the key proteins that might be responsible for Nrf2 activation/modulation [177]. It was also discovered that in cells and mouse respectively, AMPK, at Ser558 and Ser550 directly phosphorylate Nrf2 which promotes Nrf2 nuclear accumulation necessary for ARE-directed gene expression. In addition to this, AMPK inhibits GSK3 thereby blocking the nuclear export of Nrf2 into the cytoplasm. Nrf2 phosphorylation at Ser40 could also dissociate Keap1-Nrf2 PPI thereby making Nrf2 available for nuclear translocation and subsequent downstream transcription detoxifying genes [177–179].

The involvement of protein kinase C (PKC) in the phosphorylation of Nrf2 which provokes nuclear translocation has been reported in response to oxidative stress. A synthetic peptide that mimics one of the binding sites of PKC was reported to competitively inhibit PKC, therefore, blocking the phosphorylation of a purified rat Nrf2 by PKC catalytic subunit. Furthermore, it was also noted that the mutation of Ser40 of Nrf2 to alanine could not be phosphorylated by PKC but this

mutation does not affect the in vitro binding of Nrf2/Maf to ARE. In this same research, it was reported that when Keap1 was overexpressed, Nrf2 activation was partially inhibited. Furthermore, the in vitro (HepG2 Cells) keap1 co-immunoprecipitated with Nrf2 while the phosphorylation of wild-type Nrf2 by PKC promoted its dissociation from Keap1 and Nrf2Ser40Alanine mutant remained associated [178,180,181]. As these results might establish the involvement of PKC in the phosphorylation of Nrf2 at Ser40 and its critical relevance to the signaling reactions that lead to the downstream transcription of antioxidant genes, it also stressed that some other reactions besides the PKC phosphorylation at Ser40 might contribute to the activation of Nrf2 and that the phosphorylation of Nrf2 at Ser40 could also promote its dissociation from its Keap1 repressor thereby provoking the transcription of antioxidant response element-orchestrated gene expression.

In order to investigate the possible crosstalking between PI3K/Akt and Keap1/Nrf2 signaling pathways in human cultured retinal pigment epithelial cells, different concentration of PI3K inhibitors were used to treat cultured ARPE-19 cells followed by their exposure to sulforaphane (a potent Nrf2 activator). In this research, a dose-dependent cellular and mitochondrial GSH reduction and downregulation of GCL modulatory unit were reported in cultured RPE cells and while basal and induced Nrf2 activities were inhibited by wortmannin and LY294002, there was overexpression of Akt that consequently caused Nrf2 activation. Furthermore, Nrf2-siRNA blocked Akt effect while LY294002 inhibited sulforaphane-induced Nrf2 nuclear translocation. Therefore, from these data, it is reasonable to conclude that PI3K/Akt signaling plays a significant role in the regulation of Nrf2/ARE signaling and that Akt and PI3K might be responsible for the activation by phosphorylation of Nrf2 responsible for the nuclear translocation that orchestrates the downstream transcription of antioxidant, detoxification and metabolizing gene. Other kinases that could phosphorylate Nrf2 include cyclin-dependent kinase (CDK) at Thr439, Jun-N terminal Kinase (JNK), p38, extracellular signal-regulated kinase (ERK) and double-stranded RNA activated protein kinase R (PKR) [182–184]. It then implies that different kinases could regulate keap1/Nrf2/ARE signaling through the phosphorylation of Nrf2 to keep the cellular homeostatic sanctity intact.

Therefore, as illustrated in Fig. 7, the direct or indirect phosphorylation of Nrf2 by different kinases could be tapped and amplified intracellularly to perpetrate some protective transcriptional responses that ultimately see to the regulation of the cellular homeostatic condition. While the off-target effects of these kinases might present some bottlenecks, nutraceuticals/drugs/pure compounds that could target these kinases might represent some therapeutic molecular targets towards the treatment/management of diabetic nephropathy.

6. Inhibition of Keap1-Nrf2 protein protein interaction (PPI)

The interruption of the association between keap1 and Nrf2, which consequentially initiate its nuclear translocation and accumulation, is one of the mechanisms exploited by natural and synthetic agents to upregulate ARE-regulated cytoprotective oxidative stress response enzymes for the development of therapeutic measures against various diseases [21]. One perceptive mechanism that uncovers the brain behind keap1 modification and dissociation from Nrf2 could be the inhibition of Nrf2 polyubiquitination or the dissociation of Cul3 from keap1 which involves the thiol modification of one or two of its cysteine residues. The modification of thiol residues in the IVR of keap1 (Fig. 2) has been observed to initiate the misalignment of lysine residues within Nrf2 that causes the inhibition of polyubiquitination [113,185]. These mechanisms exhibit the obligation to increase Nrf2 activation, nuclear translocation, and transcription of cytoprotective genes [186]. It was reported that Nrf2 activators stabilize Nrf2 by reacting with the cysteine residues of keap1 [187,188], and other studies emphasized that chemical modification of keap1 is not strong enough to perpetrate the dissociation of these two proteins [189,190]. The inhibition of ubiquitination and proteasomal degradation of Nrf2 is another mechanism exploited by chemical activators of Nrf2 to increase the degree of cytoprotection. Within the half-life of approximately 10 − 20 min, keap1 facilitates thedegradation and turnover of Nrf2. The hinge and latch model that explains the inhibition of Nrf2 polyubiquitination as a means of dissociating Nrf2 from its keap1 repressor also stressed the involvement of the low-affinity 29DLG31 (latch) and high-affinity 79ETGE82 (hinge) binding sites of Nrf2 in its degradation. Nrf2, through these 2 binding motifs in the Neh2 domain binds DGR site of keap1. This binding allows the free movement of Nrf2 despite the binding to the high-affinity site while the low-affinity site retards Nrf2 movement and conforms its lysineresidues within the Neh2 domain for ubiquitination [190,191]. The binding at both sites perfectly prepares the platform for poly-ubiquitination through the complex Cul3-E3-ligase which ultimately ensure low Nrf2 basal levels in cells through the 26S proteasome. Electrophiles or oxidative stress impose the modification of some cysteine residues in Keap1 which cause conformational alteration that forces the release of Nrf2 from the low binding latch site, thereby inhibiting ubiquitin transfer (One of the key reactions that prepare Nrf2 for degradation). Due to the blockage of Nrf2 degradation channel, keap1 is saturated with Nrf2 that are not to be degraded and newly synthesized free Nrf2 also accumulates in the cytosol, translocate into the nucleus, thereby increasing their propensity of binding to ARE in order to activate the transcription of genes that harbor the prowess to deliver the cells from oxidative challenges. One other controversy surrounding the release of Nrf2 from Keap1 explained that while Nrf2 ubiquitination ceases, there is a strong

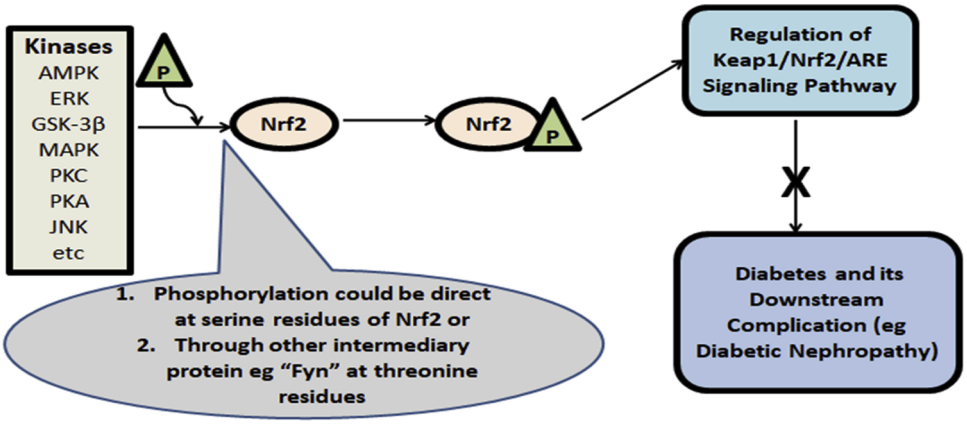

Fig. 7. Kinases that might be responsible for the activation of Nrf2 through the phosphorylation at specific amino acid residues which drives their nuclear translocation to initiate and propagate their binding to the ARE component of their target genes.

probability that keap1 ubiquitination increases under the spell of oxidative stress; however, this is only encountered with certain Nrf2 inducers [192]. Another point to consider erupted from the fact that Nrf2 is known to be a nuclear protein which left the inference that its nuclear localization comes first. Therefore, Nrf2 transcriptional activity is upstream to the degradation of Nrf2 through keap1 [191–194]. So, as researchers might pick up interest unraveling any of these conflicting hypotheses, one thing is crystal clear; Keap1 does not only set the stage for Nrf2 ubiquitin-proteasome degradation but, its downregulation could upregulate cytoplasmic Nrf2 and therefore, promote nuclear translocation and the transcription of its downstream genes.

Another considerable perception is the association of Keap1 with nuclear translocation and activation of Nrf2 and its subsequent target genes expression which involves the cysteine residues at position 151, 257, 273 and 288 due to their immense contribution to the conformational changes of keap1 which perpetrates the translocation process. These cysteine residues within the BTB/Kelch-repeat domain function as sensors for oxidative stress or inducers within the cellular environ. The downstream effect of inducing these cysteines is the conformational changes of keap1 which consequently makes Nrf2 available in the cytoplasm thereby, activating nuclear translocation [114,191,192]. Keap1 is critically sensitive to cellular stress and it has been reported that its high redox sensitivity is dictated by these cysteine residues [192–195] which could be subjected to electrophilic covalent modification. While Cys273 and Cys288 residues are responsible for Nrf2 basal and stress control, C151 is specific for stress [186–197]. The argument surrounding the mechanism of sulforaphane docking keap1 has resulted in the detection of C151 as one of the most potent residues. Cysteine, serine, tyrosine, asparagines, histidine and arginine residues at the keap1 ketch domain have been experimentally demonstrated to contribute to the interaction between keap1 and Nrf2 through the formation of bonds like hydrogen bonds and electrostatic interaction that consequently contribute to the binding affinity of Nrf2 peptide or small molecule ligands to this site.

The BTB-kelch family structural studies reveal the structural pocket and alternative peptide-binding conformations and interfaces and therefore illuminate how small molecules that could selectively interfere with keap1-Nrf2 complex through the inhibition of keap1 protein could be another therapeutic target [198–201]. So, keap1 is said to possess the intriguing potential to accommodate inducers of different physical characteristics and activities which led to the “cysteine code” that gives the basis for the regulation of Keap1/Nrf2 stress-sensing response. It is this response that is being targeted by small-molecule activator that expresses the potentials to activate Keap1/Nrf2 signaling in the course of providing therapeutic remedies for various diseases [202].

The eight keap1 cysteine residues (C38, C151, C368, C489, C77, C226, C319, and C434) that are readily modified by sulforaphane had been investigated to be recognized by other electrophilic inducers [202,203]. Other cysteine residues reported to have interacted with sulforaphane include C406, C23, C241, C391, C406 and C622. Isoliquiritigenin, xanthohumol, and 10-shogaol have also been investigated to induce ARE through their preferential modification of Keap1 at C151, C241, C273, C288, C319, C434 and C613 [202]. The irreversibility of the interaction between small-molecule inducers and Keap1 cysteine residues was also investigated to confirm the formation of stable adducts that could give better affinity and definitely, better therapeutic efficacy. It was reported that sulforaphane forms a reversible adduct with the sulfhydryl group of some keap1 cysteine residues which infers that those cysteine residues might not be an important targets for therapeutic measures [188]. However, binding of this same isothiocyanate sulforaphane to C38, C226, and C368 of Keap1 was detected to be less irreversible, meaning they could form stabilized adducts with sulforaphane which also infer that these sites might represent some therapeutic targets towards the search for novel drugs for the amelioration of several diseases.

There is a growing interest in the non-electrophilic regulators of Nrf2 than electrophilic activators and the former had been seen as a more crucial therapeutic target than the latter. These non-electrophilic activators of Nrf2 molecules activate Nrf2 in cell culture but are yet to be proven for the same activities in vivo [204]. Thermodynamically, it was analyzed that Keap1-DLGex binding is mediated by enthalpy and entropy while Keap1-ETGE binding is strictly enthalpy driven. This infers that Keap1-DLGex allows some disorderliness that ensures some kinetic destabilization which enforces fast-association and fast dissociation model while Keap1- ETGE binding presents a stable conformation. This exceptional attitude of DLGex motif does not only explains the cause of keap1/Nrf2 signaling to electrophile and oxidants but also illuminate the reasons why DLGex motif should be preferred to ETGE motif in the course of finding small molecules inhibitors of Keap1. In order to understand the thermodynamics behind the differential binding affinity of the ETGE and DLG motifs, systemic point mutation and Isothermal calorimetry (ITC) titration experiments analysis were carried out and the result revealed that a trend in the acidic residue was being exploited as Nrf2 destructive signal. While calorimetric analysis estimated the binding constant of 1×10^6 M^{-1} for DLG motif when interacting with Keap1-DC, ETGE motif has a higher binding affinity of 2×10^8 M^{-1} [205,206]. Furthermore, it was ascertained that the DLG motif is responsible for Nrf2 ubiquitination and stability. A correlation between the binding affinities of DLG mutants with their abilities to direct Keap1-dependent ubiquitination and Nrf2 stability was executed in 293 T cells to find out the functions of DLG motif in regulating Keap1-mediated Nrf2 ubiquitination. The evaluation of Nrf2 ubiquitination was carried out in 293 T cells and it was found that at the presence of Keap1, wild-type Nrf2 was highly ubiquitinated while all three DLG mutants (Gln26Ala, Asp27Ala, and Asp29Ala) expressed inhibited Nrf2 ubiquitination. Furthermore, the mutation of Asp-27 or Asp-29 (acidic residues) triggered repressed Nrf2 ubiquitination while binding calorimetric assay shows that Gln26Ala had a moderate effect on Keap1-DC association because its inhibitory potential to Nrf2 ubiquitination was found to be insignificant when compared to that of the acidic residues [206].

The crystal complexes that compares Keap1-DC domain and Nrf2DLG-Keap1 were found to be similar because the Keap1-DCdomain-DLG peptide with Keap1-DC-domain- ETGE peptide was visualized, binding in Nrf2ETGE-KeDLG peptide and Keap1-DC-domain- ETGE peptide motifs interact with the same basic surface of either Keap1-DC domain of the Keap1 homodimer. Furthermore, the binding interface between the weaker binding DLG motif and Keap1 was found that ETGE and DLG peptides in Keap1-DC are significantly similar with respect to substrate binding interface and Keap1-bound Neh2 motif hairpin backbone however, significant differences were reported in the recognition signal sequences between Keap1-DCETGE complex and Keap1-DC-DLG complex due to the involvement of differential negative electrostatic potentials in the recruitment of ETGE and DLG motifs [206]. Altogether, these show that preference for DLG motif while searching for small molecular inhibitors of Keap1 might present better therapeutic efficacy for stress-related diseases.

The pursuit of small molecule inhibitors of Keap1-Nrf2 PPI has been seen as a new therapeutic measure strategized to put oxidative-stress related diseases such as diabetes and its complications (including diabetic nephropathy) at hold. Series of novel hydronaphthoquinones Keap1-Nrf2 PPI inhibitors were screened and S01 and S05 were seen as the most potent compounds which release Nrf2 in H9c2 cells and LPS-inflammatory mouse models and also translocated Nrf2 into the nucleus in a dose-dependent pattern which as expected, significantly upregulated HO-1 and NQO-1, while ROS production dramatically reduced. These new compounds were predicted to form additional hydrogen bonding with S363 residue thereby, increasing their inhibitory grip. Furthermore, their mode of action and the protective role was confirmed using siNrf2 transfection method [207]. A novel Keap1-Nrf2 PPI inhibitor (JZ01) which was screened from compound library by fluorescent polarization assay, surface Plasmon resonance, molecular

docking and dynamic simulation could initiate Nrf2 nuclear translocation which consequently led to the increase in mRNA levels of Nrf2 downstream genes (HO-1 and NQO-1) and reduction in LPS-induced ROS production in cardiomyocytes [208].

Taking together, we could infer as shown in Fig. 8, that small molecule compounds that could downregulate Keap1 protein and mRNA expression might inhibit Keap1-Nrf2 PPI which could increase the availability of Nrf2 for nuclear translocation to drive the transcription of genes responsible for cytoprotection.

7. Clinical relevance of Keap1/Nrf2/ARE signaling to diabetic kidney disease

Even with the synergistic control of blood glucose, blood pressure, lipid-lowering and the inhibition of RAS in the interest of treating/managing diabetic conditions in patients, the progression of diabetic nephropathy remains unpreventable. This may implies that the complex crosstalk between Nrf2 signaling and other signaling pathways could be clinically related to some complex multifactorial molecular interaction-oriented diseases (e.g. diabetic complication) in human [209]. One of the Nrf2 activators that made its way into clinical practice in 2013 is BG-12 with a brand name Tecfidera (approved by FDA) for the treatment of multiple sclerosis. Its therapeutic mechanism of action involves Nrf2 transactivational process that consequently leads to the expression of its downstream genes responsible for cytoprotection, anti-inflammation and, antioxidation. Since it has been reported that diabetic patients express high phenotype for sustained ROS production as well as a diminished expression of antioxidant enzymes, therefore such mechanism that harnesses beneficial participation of Nrf2 signaling as a measure of therapeutic response thus showcases Nrf2 beyond its clinical significance as it further bolsters the critical evidence that Nrf2 signaling pathway could be a promising pathway that could expose some therapeutic options for oxidative stress-orchestrated diseases including T2DM and its downstream end stage complications [210,211]. However, it must be noted that Nrf2 signaling pathway components are yet to be fully exploited as biomarkers for T2DM and its complications but, credible evidence suggests this pathway could be harnessed in pre-diabetic and diabetic patients [212].

Small molecules that activate Nrf2 could directly interact with proteins and genes associated with renal fibrosis and functions. Nrf2 activator such as Bardoxolone methyl in a double-blind, randomized and placebo-controlled trial significantly improved estimated glomerular filtration rate in type 2 diabetic patients and impaired renal function (eGFR $20-45$ ml/min/1.73m^2). Hemodynamic effect of this method could be established because these changes were noticed within a month; however, after stopping the administration of this drug, the situation was reversed [213]. The success of this clinical trial represents a critical advancement over standard therapies because it impeded decline in renal function by < 1 ml/min/1.73m^2/year at best [214] and sustained improvements with this drug between $5-10$ ml/min/1.73m^2 [215]. The maintenance of serum creatinine-based estimated glomerular filtration rate (scb-eGFR) as an indicator for kidney function is one of the crucial biomarkers that must be considered when developing drugs for kidney diseases. Out of the eGFR-based classification for Chronic Kidney Disease (CKD) patients which include G1, G2, G3, G4 and G5, G5 may not escape dialysis and kidney transplantation. Therefore, in clinical studies, appropriate end-point is not only fundamental, but critical. The time required to reach end-stage is relatively long, therefore, its clinical usage cannot be practiced. Although, doubling of serum creatinine (53 %–57 % eGFR reduction) could also be used as an alternative end-point, however, it requires long-term studies of many patients and could contribute to the complications experienced in kidney disease drug discovery. Recently, 30 % or 40 % eGFR reduction from baseline following 2–3 years had been discussed in the US, European Union (EU) and Japan [216]. Worldwide, Japan had been ranked as number one in establishing guidelines using this endpoint which can enhance research in the area of drug development [217].

The reason behind the suggestion that the anticancer (clinically investigated) and antitumour CDDO-Me could be beneficial for chronic kidney disease (CKD) was because of its ability to notably improve eGFR [218]. It was later investigated that this synthetic triterpenoid-based natural product CDDO-methyl ester could stimulate Nrf2 activator and its transactivational processes through the inhibition of Keap1 ubiquitination activity [219]. In a phase-1 clinical trial where CDDO-methyl ester was used for the treatment of cancer and hematological malignancy [220], 26 % increase in eGFR was observed in patients administered with this compound. Therefore, there is high probability that CDDO-methyl ester could become one of the promising new drugs for renal diseases (including diabetic nephropathy). Furthermore, in order to investigate the positive role of CDDO-methyl ester on renal function in 227 patients with type 2 diabetes mellitus (T2DM), G3 and G4 stages of CKD, a BEAM study (also called phase-2 clinical trial) was carried out [218]. The result of this study showed that CDDO-methyl ester-treated patients had increased eGFR within the first 4 weeks of administration accrued by a durable improvement for at least 52 weeks. Most importantly, because the improvement of eGFR was maintained for 4 weeks after stopping CDDO-methyl ester's administration, it is now thought that CDDO-methyl ester could improve eGFR status of patients with kidney disease and this was attributed to their capacity to restore endothelial function and/or vasodilation, followed

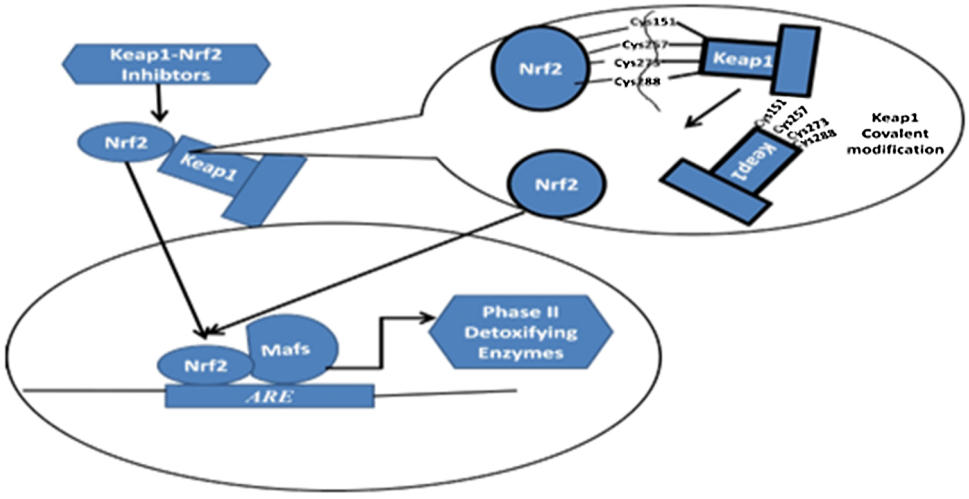

Fig. 8. Illustrates the how the cysteine residues of Keap1 are been disrupted to release Nrf2 for its subsequent transactivational processes.

Table 1
Antidiabetic and Keap1/Nrf2/ARE Activating Potential of Some Compounds.

S/No	Name	Target	Effects	References
1	CPDT (5, 6-dihydrocyclopentane-1, 2-dithiole-3-thione)	Modifies the cysteine residues of Keap1	Downregulation of Keap1, Upregulation of Nrf2 and Nrf2 translocation	[22]
2	Sulforaphane		Downregulation of Keap1 and upregulation of Nrf2, Nrf2 activation and translocation through Akt upregulation	[22]
3	Curcumin		Activates Nrf2 and HO-1 in NRK-52E and LLCPK1 cells	[227]
4	Quercetin		Nrf2 activation and NQO-1 increased expression in HepG2 Cells	[228]
5	Xanthohumol		Xanthohumol induces NQO-1 through the alkylation of Keap1 at some of its cysteine residues to promote detoxification in Hepa1c1c7 cells	[229]
6	CGA (Chlorogenic Acid)	Nrf2	Nuclear translocation of Nrf2, Nrf2 transcription and ARE-orchestrated gene expression that led to the amelioration of diabetic nephropathy in DN model and HBY-1 cells.	[153]
7	Sodium Butyrate (NaB)	Nrf2	Nrf2 nuclear translocation through HDAC repression that led to the amelioration of diabetic nephropathy in STZ-induced C57BL/6 Nrf2 KO WT mice	[154]
8	BBR (Berberine)	Nrf2	Nrf2 activation through AMPK upregulation	[177]
9	tBHQ	Nrf2	Nrf2 nuclear translocations and phosphorylation	[120]
10	C66	Nrf2	Nrf2 Upregulation through the miR-200a upregulation that saw to the amelioration of DN in STZ-induced Nrf2 KO and WT mice model	[230]
11	SP600125	Keap1	Keap1 downregulation and Nrf2 Upregulation and nuclear translocation. DN was arrested through Nrf2 translocation in WT HG induced SiRNA-JNK mesangial cells.	[231]
12	Radiation	Nrf2	Nrf2 activation through Akt upregulation and resultant treatment of DN in the kidney of STZ-induced C57BL/6L diabetes Type 1 mice	[232]
13	Isoliquiritigenin Xanthohumol 10-Shangaol	Cysteine residues of Keap1	ARE-directed gene expression through Keap1 downregulation	[202]
14	Hydronaphthoquinones (S01 and S05)	Keap1-Nrf2 PPI (Also forms H-bond with S363 of Keap1)	Nrf2 nuclear translocation, HO-1 and NQO-1 upregulation and significant ROS depletion	[207]
15	Astaxanthine	Nrf2	Nrf2 translocation and transactivation that led to the increase in HO-1, NQO-1 and SOD1with reduction in ICAM-1, Fibronectin, Collagen IV and MDA HG-induced GMCs and STZ-induced diabetic mice models	[233,234]
16	ZJ01	Keap1-Nrf2 PPI	Nrf2 nuclear translocation and ARE mRNA genes upregulation	[235]

by the preservation of the filtration bed area in the glomerulus [221,222]. This same trial found that CDDO-methyl ester increased albuminuria associated with cardiovascular risks even though Nrf2 activation-mediated kidney disease prevention was noticed [223]. In order to validate/confirm this result, when Cynomolgus monkeys were also administered with CDDO-methyl ester, they expressed marked increase in urine albumin-creatinine ratio, with decreased megalin expression- a critical regulator of urinary-albumin in the proximal convulated tubules- while blood urea nitrogen and creatinine were reduced without histological abnormalities in the kidneys [224]. Due to this trend of positive results gotten from the BEAM study, a phase-3 (also referred to as BEACON study) randomized clinical trial involving 2,185 stage 4 CKD and T2DM patients was conducted. Although, eGFR increment was consistent with those obtained in the BEAM study, it must be noted that BEACON study was terminated in 2012 due to high occurrence of cardiovascular issues [225]. These cardiovascular complications include hypertension (Placebo group), increase urinary albumin-to-creatinine ratio and B-type natriuretic peptide (BNP) and significant reduction in serum albumin and hemoglobin. The severity experienced in kidney disease may be associated to the incidence of cardiovascular issues in CDDO-methyl ester-treated patients because CKD in the BEACON study was more progressive than that in the BEAM study [226]. Therefore, besides the fact that the clinical mechanism of action of these small molecules in humans might also depend on the activation of Keap1/Nrf2/ARE signaling, it is noteworthy that the link between GFR and some occurrence of DN pathophysiology which include mesangial expansion stimulated by TGF-β1 and CTGF may implies that the antidiabetic mechanism of action of these molecules might be hidden in the modulation of signaling pathways associated with these two proteins such as TGF-β1/Smad3 signaling pathway. Furthermore, the mechanism through which these small molecules reduce GFR might be harbored in their capacity to activate Keap1/Nrf2/ARE signaling pathway (Table 1).

It is noteworthy that other Laboratories including ours (Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, People's Republic of China) found that some compounds, through various mechanisms have been experimentally (*in vitro* and *in vivo*) proven to express antioxidant and antidiabetic nephropathy therapeutic efficacy. While some were found to activate Nrf2 transcription factor (sarsasapogenin [236], sikokianin A [237], inotodiol [238], hesperetin [239], and mangiferin [240]), quercetin through phosphorylation mechanism activated Nrf2/ARE signaling pathway [182].

8. Conclusion

Hyperglycemia, oxidative stress, and hypertension are all associated with diabetes and its end-stage kidney disease (Fig. 3) while oxidative stress is central to its initiation and progression. In this review, we deduced that agents that activate Keap1/Nrf2/ARE signaling by fostering Nrf2 nuclear translocation, Nrf2 ser40 phosphorylation and dissociation of Nrf2 from its Keap1 repressor could abate oxidative stress-related disorders. Although Nrf2 transactivational processes are been linked with other complications like cancer and inflammation; however, pure compounds/drugs/Nrf2 activators that induce the modulation of extracellular matrix (ECM) proteins specifically expressed in DN pathology might present critical therapeutic measure. These extracellular matrix proteins include collagen IV, laminin and fibronectin while metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) are responsible for their turnover in the glomerulus. It has been reported that antioxidant agents could downregulate TGF-β1 and fibronectin which are linked to tubulointerstitial fibrosis and ECM accumulation in DN therefore, Nrf2 activators that could upregulate MMPs, downregulate TIMPs and inhibit ECM protein (collagen IV, laminin and fibronectin) accumulation as described in Fig. 4 might possess the mechanism to modulate ECM turnover thereby blocking the progression of glomerulosclerotic plague and ECM accumulation which are both perpetrators of diabetic nephropathy.

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Ethical approval

This article is a review manuscript and does not involve human and animal experimental studies performed by any of the authors.

Declaration of Competing Interest

All authors declare no conflict of interest.

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