Twenty years after ACEIs and ARBs: Emerging Treatment Strategies
for Diabetic Nephropathy

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Diabetic nephropathy (DN) is a serious complication of both type 1 and type 2 diabetes mellitus. The disease is now the most common cause of end-stage kidney disease (ESKD) in developed countries, and both the incidence and prevalence of diabetes mellitus is increasing worldwide. Current treatments are directed at controlling hyperglycemia and hypertension as well as blockade of the renin angiotensin system (RAS) with angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). Despite these therapies, DN progresses to ESKD in many patients. As a result, much interest is focused on developing new therapies. It has been over 2 decades since ACEIs were shown to have beneficial effects in DN independent of their blood pressure (BP) lowering actions. Since that time, our understanding of disease mechanisms in DN has evolved. In this review, we summarize major cell signaling pathways implicated in the pathogenesis of diabetic kidney disease as well as emerging treatment strategies. The goal is to identify promising targets that might be translated into therapies for the treatment of patients with diabetic kidney disease.

Key words: diabetic nephropathy, diabetes mellitus, cell signaling, oxidative stress
Introduction: Diabetic nephropathy (DN) is the leading cause of end-stage kidney disease in developed countries (123). Current clinical management is directed at strict control of blood glucose and blood pressure (BP) as well as inhibition of the renin angiotensin system (RAS) using angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). While several smaller human studies had suggested beneficial effects of ACEIs in DN, the beneficial actions of these agents were convincingly demonstrated in a large, randomized, controlled trial in 1993 (83). In this study, captopril attenuated the decline in renal function in type 1 diabetic patients with heavy proteinuria more effectively than BP control alone (83). Since this landmark study, data from several large human studies have consistently demonstrated renoprotective benefits of ACEIs and ARBs in diabetic patients with kidney disease (13, 84, 107). As a result, ACEIs and ARBs are now a mainstay of management for DN. Despite their clinical effectiveness, diabetic kidney disease continues to progress ultimately causing end-stage kidney disease (ESKD) in ~20% of patients (92, 97). Thus, new treatment strategies are needed. In this review, we examine major cell-signaling pathways implicated in the pathogenesis of DN beginning with G protein coupled receptors (GPCRs) and proceeding in rough chronological order to more recently identified signaling pathways that might be exploited for the treatment of diabetic kidney disease. Where applicable, we will discuss recently completed or ongoing clinic trials; however, the primary focus will be data arising from in vitro and in vivo experiments that may inform future clinical studies.

Targeting G protein coupled receptor (GPCR) signaling pathways in DN: GPCRs are the target of ~30-40% of all modern medicinal drugs (140). In this section, we discuss promising strategies to maximize RAS blockade as well as other GPCR systems that might be targeted for the treatment of diabetic kidney disease.

Maximizing RAS blockade: While targeting the RAS was a major advance in the treatment of DN (13, 83, 84, 107), initial attempts to maximize RAS blockade produced disappointing results (42, 170). For example, combined ACEI and ARB therapy was associated with an increased risk of the composite primary end-point of death from cardiovascular causes in the ONTARGET clinical trial (170). In a secondary analysis of renal outcomes, dialysis or doubling of the serum creatinine was similar in the monotherapy ACEI or ARB group but increased with combination treatment (94). Other investigators have targeted additional components of the RAS. For example, the direct renin inhibitor aliskiren showed promise as an adjunct to ACEI and ARBs in the treatment of DN (108, 112). Unfortunately, when the effect of aliskiren in combination with RAS blockade was tested in a large cohort of patients with type 2 diabetes and CKD, investigators observed no difference in the composite end-point of death from cardiovascular or
renal events (106). In this trial, aliskerin reduced albuminuria but increased the rate of decline in renal function and was associated with an increased risk of hypotension and hyperkalemia (106). An alternative approach to enhance RAS blockade is inhibition of the mineralocorticoid receptor (MR), a nuclear receptor that is not a member of the GPCR family. In vitro, aldosterone promotes apoptosis in podocytes (81) and induces expression of the inflammatory chemokine CCL2 (C-C motif ligand 2, formerly termed monocyte chemoattractant protein 1) in an NFκB dependent manner in both mesangial and proximal tubule cells (54). Furthermore, administration of MR antagonists to rodents with either type 1 or type 2 diabetes decreases albuminuria, and inhibits fibrosis (51, 54). In small clinical trials, short-term treatment with spironolactone decreased 24-hour urinary albumin excretion in type 1 and type 2 diabetic patients with persistent albuminuria despite RAS blockade (68, 127, 128). Similarly, combined treatment with an ACEI and the selective aldosterone inhibitor eplerenone reduced albuminuria to a greater extent than the ACEI alone without causing a significant increase in hyperkalemia (37). Additional MR antagonists have been developed (Table 1) and phase 2 clinical trials are currently evaluating these agents in patients with type 2 diabetes and albuminuria (Table 2). Ultimately, MR blockade may prove to be an effective treatment strategy for DN; however, larger trials with longer follow up are required to assess its effect on progression to ESRD and/or mortality.

A second promising approach to enhance RAS blockade is activating the vitamin D receptor (VDR), another nuclear receptor system. In addition to its well-known role in calcium homeostasis, the VDR ligand, 1,25-dihydroxyvitamin D3 has also been shown to negatively regulate renin gene expression, in vitro (169) and in vivo (86). VDR knockout mice treated with the β-cell toxin streptozotocin (STZ) develop more severe kidney damage than wild type mice (174), suggesting a protective role for VDR in diabetic nephropathy. Furthermore, treatment of diabetic mice with either paricalcitol or doxercalciferol abrogates renal damage by preserving glomerular basement membrane width as well as blunting inflammation and fibrosis (30, 173, 175). In both cases, a synergistic effect with ARBs was observed. The mechanisms mediating the beneficial effects of VDR activation in diabetes are not known with certainty; however, it may be due to anti-inflammatory effects (29, 121) and/or by inhibiting the compensatory increase in renin expression that occurs in the setting of ARB therapy (175). VDR activation has also shown promise in clinic trials. In the VITAL (selective VITamin D receptor activator for Albuminuria Lowering) study, paricalcitol (2 ug per day) decreased urine albumin excretion by 18% compared to placebo in patients with type 2 diabetes (26). As a result of these promising results, VDR agonists are currently being tested in additional clinical trials (Table 2).
**Endothelin receptors:** The endothelin receptors, ET\(_A\)R (ET\(_A\) receptor) and ET\(_B\)R, are G-protein coupled receptors that mediate the biological effects of endothelin peptides. The ET\(_A\)R and ET\(_B\)R are expressed throughout the kidney (12) and endothelin peptides are key regulators of blood pressure and sodium homeostasis (72). Both the ET\(_A\)R and ET\(_B\)R cause vasoconstriction; but the ET\(_B\)R is also a vasodilator due to ET\(_B\)R-induced generation of the vasodilator prostaglandins and nitric oxide (12). In the kidney, endothelins exert a net natriuretic effect that is largely mediated by the ET\(_B\)R, although the hemodynamic and natriuretic actions of endothelins and their receptors in the kidney is complex (72). In addition to effects on vascular tone and natriuresis, endothelin receptors promote kidney injury. Serum levels of endothelin A (ET-1) are increased in both type 1 and type 2 diabetes (52) and over-expression of the human ET-1 in mice results in glomerulosclerosis and tubulointerstitial fibrosis (59). Infusion of ET-1 into rats produces a dose dependent increase in glomerular permeability to albumin and increases glomerular expression of the inflammatory chemokine CCL2. These effects are blocked by a selective ET\(_A\)R antagonist (120). Furthermore, deletion of both ET\(_A\)R and ET\(_B\)R from podocytes protects against glomerulosclerosis (82), while diabetic ET\(_B\) receptor deficient rats exhibit higher levels of albuminuria and enhanced glomerular injury compared to wild type controls (115). Taken together, these data suggest that ET\(_A\)R receptor activation promotes podocyte damage and albuminuria.

This differential role of the ET\(_A\)R and ET\(_B\)R in the kidney is reflected in the effects of receptor antagonists. For example, treatment with the non-specific ET\(_A\)R/ET\(_B\)R antagonist bosentan did not improve renal pathology or albumin excretion in hypertensive diabetic rats (69). In contrast, selective ET\(_A\)R blockade with avosentan, alone or in combination with lisinopril, improved albuminuria and glomerulosclerosis in uninephrectomized, diabetic rats (43). ET\(_A\)R antagonists have also shown promise in clinical trials. Avasentan significantly reduced albuminuria in patients with either type 1 or type 2 diabetes and macroalbuminuria on standard RAS blockade (162). Similarly, treatment with the highly-selective ET\(_A\) antagonist atrasentan significantly reduced the urinary albumin to creatinine ratio in patients with diabetic nephropathy already receiving stable doses of RAS inhibitors (28, 71). The main impediment to using ET\(_A\) antagonists for the treatment of DN is fluid retention, which results in edema and may aggravate heart failure (28, 71, 93, 162). While promising, it remains to be seen whether these off target effects can be overcome more selective ET\(_A\)R antagonists.

**Chemokine receptors:** Another promising target for the treatment of diabetes includes the family of chemokine receptors, which are predominantly expressed on T-cells and monocytes (163). These GPCRs play a critical role in chronic inflammatory diseases (163). Accumulating evidence suggests that chronic inflammation also plays an important role in metabolic diseases.
linked to obesity including insulin resistance and diabetes mellitus (163). Adipose tissue produces both CCL2 (C-C motif ligand 2) and CCL5 (46, 143, 165), which bind to the chemokine receptors CCR2 and CCR5, respectively (163). Both ligands are up-regulated in obese rodents (46, 143, 165). In obese mice, enhanced chemokine levels are associated with an increased number of inflammatory cells in adipose tissue (161, 165), which ostensibly causes insulin resistance by indirect mechanisms. In addition, the CCL2-CCR axis may have a direct role in insulin resistance because treatment with CCL2 reduces insulin stimulated glucose uptake in cultured adipocytes (125). In support of an important role for CCR2 signaling in diabetes, knockout of CCR2 improves insulin resistance and fasting blood glucose levels in obese mice (161). Based on these results, small molecule CCR2 antagonists have been developed and are being tested in preclinical animal studies (67, 126, 135). Moreover, several pharmacologic agents have entered phase 2 clinical trials including studies of patients with overt diabetic nephropathy (see Tables 1 and 2) (55, 163). Preliminary results of these clinical studies suggest that CCR2 antagonism reduces both fasting plasma glucose and hemoglobin A1c levels (55). It remains to be determined, however, whether or not these pharmacologic agents will have beneficial effects on diabetic kidney disease independent of effects on blood glucose levels.

Adenosine receptors: Activation of adenosine receptor subtypes has both hemodynamic and anti-inflammatory actions in the kidney (79, 144). For example, glomerular hyperfiltration in diabetic kidney disease can be inhibited by the adenosine reuptake inhibitor dipyridamole (145). More recent studies using small molecule agonists and antagonists found that the adenosine A2A receptor mediated arteriolar vasodilation in diabetes-induced glomerular hyperfiltration (114). Other studies have investigated the anti-inflammatory effects of A2A receptor signaling in animal models of diabetic kidney disease. Awad et al (4) found that a selective A2A agonist reduced albuminuria, mesangial expansion and glomerular basement membrane width in diabetic rats. Moreover, knockout of the A2A receptor aggravated diabetic kidney disease in this same rodent model (4). Similarly, A2A receptor activation reduced proteinuria and promoted an anti-inflammatory phenotype characterized by reduced pro-inflammatory TNFα levels and enhanced anti-inflammatory IL10 levels as well as decreased macrophage infiltration and glomerular injury (113). Taken together, these data suggest that the A2A receptor may be a novel future therapeutic target for the treatment of DN.

Regulation of inflammation and fibrosis by transforming growth factor-β (TGF-β): The secreted protein TGF-β is a master regulator of inflammation and fibrosis. Activation of TGF-β in the kidney initiates a profibrotic program characterized by extracellular matrix production,
cellular hypertrophy, basement membrane thickening and apoptosis. These changes eventually lead to glomerulosclerosis and tubulointerstitial fibrosis (89). In CKD, the link between TGF-β and fibrosis has been well established and a full discussion of the topic is beyond the scope of this review. We will, therefore, focus on the therapeutic potential of TGF-β inhibition. In order to assess the role of TGF-β in early diabetes, Sharma et al (131, 133) treated mice with STZ and then measured the expression of TGF-β. Within 3 days of STZ administration, the expression of TGF-β increased in the kidney cortex. Treating the mice with an anti-TGF-β antibody blocked the increase and also inhibited glomerular hypertrophy and up-regulation of collagen α1, suggesting that TGF-β induction by hyperglycemia in vivo is an early event and highlighting the potential of a neutralizing anti-TGF-β antibody. In a long-term experiment, this same anti-TGF-β antibody was administered to db/db mice (a genetic model of type 2 diabetes) starting at the time when the mice become hyperglycemic. Again, anti-TGF-β antibody blocked the increase in collagen-α1 and fibronectin in the kidney cortex (179). However, it had no effect on albuminuria (179). Given that DN is often well established before renoprotective therapies are initiated, strategies to ameliorate established DN are required. In this regard, treatment with an anti-TGF-β antibody reduced glomerular basement membrane thickness and mesangial expansion in db/db mice with established diabetic kidney disease (17). In another study, diabetic rats were treated with either a murine or human anti TGF-β antibody in combination with lisinopril (8). In the absence of additional treatment, both murine and human antibodies decreased blood pressure, albuminuria and fibrosis. The human anti-TGF-β antibody was also able to abrogate glomerulosclerosis and had a synergistic effect with lisinopril (8). As a result, a phase 2 clinical trial is studying an anti-TGF-β antibody in patients with DN (Table 2) (1). The TGF-β pathway has also been targeted with pirfenidone, a synthetic compound that inhibits TGF-β promoter activity in vitro (117). Treatment of mouse mesangial cells with pirfenidone inhibited both TGF-β signaling and ROSs production in a dose dependent manner. Additionally, pirfenidone decreased mesangial expansion and the expression of collagen IV and fibronectin in db/db mice (117). A small randomized control trial to determine the effect of pirfenidone in diabetic nephropathy patients already on RAS blockade demonstrated a significant improvement in estimated glomerular filtration rate compared to placebo but there was a high drop-out rate in the pirfenidone group, which complicated interpretation of the study results (130).

Despite these promising findings, the use of TGF-β inhibitors for the treatment of fibrotic disease processes is tempered by the dual biological roles of TGF-β as both a profibrotic and anti-inflammatory mediator (167). For example, the use of a TGF-β neutralizing antibody in patients with systemic sclerosis was complicated by serious adverse effects including several deaths, with no evidence of a beneficial treatment effect (31). As a result, investigators have
explored other approaches. One promising strategy is amplifying bone morphogenic protein (BMP) 7 signaling (167). BMP7 activates members of the TGF-β receptor superfamily and inhibits both inflammation and fibrosis (167). BMP7 is highly expressed in the kidney and overexpression of BMP7 in podocytes and proximal renal tubular cells (RTCs) reduces albuminuria, prevents podocyte loss and decreases tubulointerstitial fibrosis (154). Similarly, pharmacologic doses of BMP7 reduce albuminuria and glomerulosclerosis in diabetic rats (153). Unfortunately, the bioavailability of BMP7 in the kidney is quite low (167). A potential strategy to circumvent this problem is targeting the predominant BMP7 receptor in proximal RTCs, activin-like kinase 3 or Alk3 (141). Consistent with an important role for Alk3 in regulating fibrosis, knockout of Alk3 in proximal RTCs enhanced TGF-β signaling and increased kidney fibrosis (141). Other treatment strategies include targeting endogenous inhibitors TGF-β signaling such as gremlin and uterine sensitization gene-1 (167). Thus, while much work remains to be accomplished, significant advancement has been made in exploiting the TGF-β superfamily to treat fibrotic disease process including diabetic kidney disease.

**Reducing oxidative stress in diabetes:** A large body of evidence links oxidative stress to the development of diabetic kidney disease (14, 139). The increase in reactive oxygen species is the result of both increased production as well as reduced and/or inadequate antioxidant function (139). Sources of ROSs include enzymatic activity including xanthine oxidase, cytochrome P450 enzymes, cyclooxygenases and uncoupled nitric oxide synthase (NOS) as well as NADPH oxidases and mitochondrial superoxide production (139, 166). A large component of the increase in ROSs generation in diabetes appears to be caused by hyperglycemia (139). Although not universally accepted (34), a prevailing theory suggests that hyperglycemia causes enhanced mitochondrial ROSs generation (15). In this model, metabolism of glucose generates the electron donors NADH and FADH2, which donate their electrons to the mitochondrial electron transport chain leading to the production of ATP and water (15). Under hyperglycemic conditions, metabolism of glucose leads to excessive electron transport, eventually overloading the pathway and resulting in donation of electrons to molecular oxygen, generating superoxide (15). Another major source of ROSs is NADPH oxidase (NOX) protein complexes (14, 139). Diabetes promotes increased expression of NOX proteins, resulting in enhanced superoxide generation. In diabetic animals, this pathway has been shown to be a major source of ROSs (7, 15, 48). In support of an important role for NOX proteins in the pathogenesis of DN, NOX inhibitors reduce renal injury in animal models of diabetic kidney disease (142).

Counterbalancing excessive ROSs generation is the antioxidant system, which includes superoxide dismutase, catalase, and the glutathione system (glucose-6 phosphate
dehydrogenase, glutathione reductase and glutathione peroxidase) (139). Numerous abnormalities of the antioxidant system have been described in animal models including increased, decreased and unchanged antioxidant levels (139). Overall, however, there does appear to be an inadequate antioxidant response either as a result of altered levels and/or abnormal function (139). One consequence of enhanced ROSs generation is excessive conversion of NAD to NADH (see above) resulting in depletion of cellular NAD. This cofactor is required to convert glyceraldehyde 3-phosphate to pyruvate during glycolysis resulting in blockade of the glycolytic pathway and accumulation of pyruvate precursor compounds (15, 139). The metabolic intermediates can then be metabolized to advanced glycation end products (AGE), sorbitol, glucose-6-phosphate and diacyl glycerol, an activator of protein kinase C (15, 139). These compounds have numerous adverse effects including enhancing expression of TGFβ, vascular endothelial growth factor (VEGF) and NADPH oxidases, activating NFκB and decreasing expression of endothelial NOS (eNOS) (15). Exacerbating the decrease in eNOS expression is depletion of cofactors required for eNOS enzymatic activity as a result of oxidation by ROSs (101). This leads to uncoupled eNOS activity, which results in generation of superoxide instead of NO and, in turn, enhanced ROSs generation (101).

The consequence of enhanced ROSs generation is to oxidize adjacent molecules including protein, lipids, nucleic acids and carbohydrates (139). As a result, multiple cellular processes are disrupted including organelle function, gene regulation and cellular signaling, which ultimately may affect cell survival (139). A variety of genetic and pharmacologic strategies have been used to “boost” the antioxidant system in diabetes. These strategies have shown promise in animal models of diabetic kidney disease. For example, treatment with the antioxidants vitamin E or probucol reduced ROSs generation in STZ-treated diabetic rats (74). Another antioxidant α-lipoic acid inhibits podocyte loss, decreases albuminuria and attenuates glomerulopathy in animal models of diabetes (137, 164). Alternatively, overexpression of an ubiquitously expressed superoxide dismutase transgene ameliorated kidney injury in diabetic mice (22, 32). Other groups have tried to target specific sources of ROSs generation. This strategy has been used to reduce uncoupled eNOS activity in diabetes by supplementing cofactors oxidized in the diabetic environment (19). These supplements reduced albuminuria and glomerular basement membrane thickness in a mouse model of type 2 diabetes (19). Unfortunately, the beneficial effects of antioxidant supplementation in animal models have not translated to beneficial effects in diabetic humans. For example, diabetic patients were treated with 400 IU vitamin E per day in the MICRO-HOPE clinical trial (microalbuminuria cardiovascular renal outcomes arm of HOPE) (90). In this study, vitamin E had no effect on either the development or progression of diabetic kidney disease (90). In contrast, treatment with the lipid-lowering and antioxidant probucol demonstrated a trend toward improved renal
function, reduced proteinuria and less ESKD in diabetic patients over a 3 year time period (36).

This trial was, however a small, open label study and it is difficult to discern if the effects were
directly related to probucol’s antioxidant properties. Results of additional clinical trials (Table 2)
may provide more definitive information on the efficacy of probucol in DN.

Another promising strategy to enhance the antioxidant system is targeting the
transcription factor Nrf2 [nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 or
Nrf2], a key regulator of antioxidant protein expression (44). Nrf2 interacts with Keap 1 (Kelch
like-ECH-associated protein 1), which promotes ubiquitination and proteosomal degradation of
Nrf2 (44). In diabetic rats, proteosomal inhibition up-regulates expression of both Nrf2 and
antioxidant genes (91). Thus, Nrf2 and the negative regulator Keap1 are attractive targets for
modulating antioxidant expression in diabetes. In support of an important role for Nrf2 in DN,
Nrf2 deletion exacerbates diabetic kidney disease in mice (66). Enthusiasm for this pathway in
treating human disease stimulated a number of clinical trials examining Nrf2 agonists and Nrf2
inducers in diverse disease processes (reviewed in reference 44). In this regard, bardoxolone
(Table 1) had shown great promise in an early phase 2 clinical trial in patients with moderate to
severe diabetic kidney disease (110, 111). Unfortunately, the phase 3 study was terminated
early due to a higher rate of cardiovascular events (27). In the phase 3 study, treatment with
bardoxolone improved glomerular filtration rate but increased albuminuria (27). Similarly,
bardoxolone treated monkeys also demonstrated enhanced proteinuria, likely as a result of
decreased megalin mediated protein uptake by the proximal tubule (119). It has been
suggested compounds such as bardoxolone may have a narrow therapeutic window and that
higher dosages of the drug may stimulate expression of pro-inflammatory genes (53). It is,
therefore, possible that titrating the dosage to maintain renal function, as opposed to improving
renal function, may have a more favorable side effect profile (53). There is, however, a general
concern that activation of the Nrf2 pathway may cause cancer cells to acquire a growth
advantage (157). Indeed, Keap1 expression is down-regulated in some cancers (157, 171).
Thus, targeting the Nrf2/Keap1 system holds great promise but further studies in animal models
are needed to optimize this therapeutic approach.

Lastly, while there is strong evidence that ROSs contribute to diabetic kidney disease, it
is important to understand that ROSs also play a key role in normal physiology. The system is
designed to maintain ROSs generation at some optimal level. For example, ROSs play a role in
cellular proliferation and overexpression of antioxidant proteins inhibits cell growth and
increases apoptosis (139). Another caveat is that blood glucose levels are often markedly and
chronically elevated in the animal models used to study diabetic kidney disease compared to
diabetic patients, who are usually receiving treatment for hyperglycemia. If ROSs generation is
largely driven by the severity of hyperglycemia, then the beneficial effects of antioxidant therapy
in animal models may overestimate the beneficial effects of antioxidants in humans with diabetes, at least over the short-term. While we acknowledge that the time-averaged effect over decades of the disease could be similar, it may be difficult to design clinical trials to study the effects of antioxidant therapies in diabetic humans if detectable effects of the treatment may take decades to discern.

**Dysregulated VEGF (vascular endothelial growth factor) expression in diabetes:** VEGF was discovered in 1983 and was initially named vasopermeability factor due to its ability to increase permeability of tumor-associated blood vessels (96). VEGF is now recognized as the critical angiogenic factor regulating endothelial cell migration, proliferation, differentiation and cell survival by inhibiting apoptosis (96, 104). The VEGF family is composed of secreted, dimeric glycoproteins including VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PGF) (21, 96, 129). The VEGF-A (hereafter referred to as VEGF) is recognized as the prototypical family member as the key regulator of physiological and pathological angiogenesis. Within the kidney, VEGF is expressed predominantly by podocytes but can also be detected in distal tubules, collecting ducts and, to a lesser extent, in the proximal tubules (21, 96, 129). Alternative splicing of VEGF results in multiple isoforms (96), which act by binding to receptor tyrosine kinases (RTKs) on target cells. The VEGF receptor 2 (VEGFR2, also termed Flk1 or KDR for kinase-insert domain receptor) is the RTK responsible for the known biological actions of VEGFR2 on endothelial cells (96, 104). A second receptor (VEGFR1 or Flt1) is also found on endothelium and seems to play a role in regulating endothelial cell VEGFR2 activity (104). VEGFR1 is, however, functional on other cell types. For example, VEGFR1 promotes hematopoietic stem cell recruitment and monocyte/macrophage migration (96, 104). In the kidney, VEGFR2 expression has been reported on glomerular endothelial cells, peritubular endothelial cells, medullary interstitial cells, mesangial cells and glomerular podocytes (10, 21, 96, 147). Expression of VEGFR2 on podocytes is, however, controversial with several groups unable to detect VEGFR2 mRNA or protein in cultured podocytes (18, 41) or in vivo (136) although all these studies were able to detect podocyte expression of VEGFR1 (18, 41, 136). Indeed, the biological effects of VEGF on podocytes may be mediated by activating VEGFR1 including anti-apoptotic actions (41) and stimulating synthesis of glomerular basement membrane components (18). Given the proximity of podocytes to glomerular endothelial cells and mesangial cells, both autocrine and paracrine signaling have been proposed to contribute to VEGF actions within the glomerulus (96). Although recent studies have questioned the presence of autocrine VEGF signaling under normal physiological conditions (136), the presence of both paracrine and autocrine signaling is supported by both cell culture experiments (18, 41) and in vivo studies (147), and might be mediated by activating VEGFR1.
In diabetes, renal VEGF expression is markedly upregulated with prominent VEGF expression in glomerular podocytes (21, 96, 129). This enhanced expression is in part related to activation of mammalian target of rapamycin (mTOR) target genes as well as blockade of glycolytic pathways [see oxidative stress above and (15)]. Within the diabetic kidney, the effects of VEGF are complex. In endothelial cells, VEGF induces eNOS expression and NO release, which under normal conditions, promotes vasorelaxation, inhibits VEGF induced capillary growth, blocks endothelial cell activation by inflammatory cytokines such as tumor necrosis factor (TNF) and inhibits release of pro-thrombotic von Willebrand factor from Weibel-Palade bodies (101). In the diabetic milieu, however, VEGF stimulates “uncoupled” NO generation and, in turn, NO production is decreased and generation of ROSs is enhanced (101). In this model, enhanced VEGF generation in diabetes might have adverse effects on endothelial cells by stimulating apoptosis-promoting ROSs generation, endothelial cell activation and intravascular thrombosis. In podocytes, the pro-apoptotic effect of ROSs may be mitigated by prosurvival, autocrine VEGF signaling. In this scenario, inhibition of paracrine VEGF signaling in endothelial cells would be predicted to have beneficial effects in diabetic kidney. In contrast, inhibition of autocrine VEGF signaling in podocytes would be predicted to enhance podocyte loss. Thus, global inhibition of VEGF in diabetes might have both adverse and beneficial effects on the disease process.

The role of VEGF inhibition in diabetic kidney disease has been studied using rodent models of diabetes. In this regard, VEGF inhibition attenuates albuminuria and some histological features of diabetic kidney disease in type 1 and type 2 diabetes (25, 39, 76). For example, a VEGF antibody ameliorates diabetic kidney disease in rodent models of type 2 diabetes (25, 39). Moreover, treatment with a soluble splice variant of VEGFR1 (sFlt1) reduced albuminuria, mesangial expansion and glomerular basement membrane (GBM), thickening in diabetic mice (76). In support of a pathogenetic role for VEGF in diabetic nephropathy, overexpression of VEGF in podocytes causes proteinuria, GBM thickening, and mesangial expansion (146, 147). Enthusiasm for these promising findings is, however, tempered by the observation that the absence of podocyte VEGF enhances glomerular injury in diabetic mice (138), suggesting some basal level of VEGF expression is required for glomerular maintenance.

The effects of VEGF are also regulated by additional angiogenic mediators. Angiopoietin-1 (Angpt1) is produced by specialized pericytes such as podocytes and mesangial cells (65). It binds to the Tie-2 receptor on endothelial cells and maintains endothelial cell quiescence and constrains angiogenesis and fibrosis following vessel injury (65). A second Angpt family member, Angpt2, is produced by endothelial cells and is the natural antagonist of Angpt1 (24). In diabetes, expression of Angpt1 and Angpt2 is altered resulting in an elevation in
the Agpt2/Angpt1 ratio (65). In support of an important role for the Angpts in DN, deletion of
glomerular Angpt1 enhances albuminuria and the severity of glomerular injury in diabetic kidney
disease. Moreover, enhancing glomerular expression of Angpt1 ameliorates glomerular
damage in diabetic mice (33, 80). Thus, angiogenic signaling is a complex process with
multiple, mutually antagonistic mediators.

While the Tie2 receptor system is also a potential target for developing therapeutic
agents, a large number of VEGF inhibitors have been developed and are used extensively for
the treatment of solid tumors including renal cell carcinoma, hepatocellular carcinoma,
gastrointestinal stromal tumors and soft tissue sarcomas (40). These drugs include blocking
antibodies and small molecule inhibitors (Table 1) (40). While the clinical use of these agents
has been associated with a low risk of adverse events (40), 1-2% of patients develop proteinuria
(178) and a small percentage develop thrombotic microangiopathy (38). A pathogenic role for
VEGF in the latter is suggested by the observation that animals lacking glomerular VEGF
develop a thrombotic glomerular disease (38). Given adverse effects of current VEGF inhibitors
as well as complexity of VEGF signaling within the glomerulus, a more complete understanding
of VEGF biology in diabetes will be essential for designing effective treatment strategies that
target angiogenic signaling in DN.

Activation of Janus kinases (JAKs) and STATs (signal transducers and activators of
transcription) in DN: JAKs are a group of tyrosine kinases that mediate cellular response to
cytokines and chemokines through interactions with STAT transcription regulators (47). The
JAK/STAT pathway is involved in several developmental processes, including hematopoiesis,
immune system development and cell growth (47). In canonical JAK/STAT signaling, the
pathway is activated by extracellular ligand binding to its receptor. This receptor-ligand
interaction results in JAK phosphorylation. Phosphorylated JAKs then phosphorylate and
activate cytoplasmic STAT proteins. Activated STATs translocate to the nucleus, where they
bind to target genes (57). One group of STAT target genes, the suppressors of cytokine
signaling (SOCS) family of proteins, inhibit JAK/STAT phosphorylation, thus creating a negative
feedback loop (57). Altered JAK/STAT signaling has been implicated in several human
diseases. For example, activating mutations in JAK2 were identified in myeloproliferative
disorders (6, 75). In DN, a role for the JAK/STAT pathway in disease pathogenesis was
described over a decade ago in a series of studies by Marrero and colleagues (95). These
findings have more recently been supported by microarray analysis of RNA isolated from human
kidney biopsies, which demonstrated significant up-regulation of multiple JAK/STAT gene family
members in both the glomerular and tubulointerstitial compartments (9). In this study, there was
a significant inverse correlation between expression of JAK/STAT genes and glomerular
filtration rate (9). The mechanisms promoting JAK/STAT activation in DN have not been fully elucidated; however, work by several groups found that angiotensin II (ANGII) and high glucose are upstream activators of JAK2, STATs 1, 3 and 5 as well as SOCS 1 and 3 in vitro (2, 95, 156). In vivo, ANGII infusion increases phosphorylation of JAK2 and STAT1 (58). Furthermore, treating diabetic rats with candesartan or the JAK2 inhibitor, AG490, decreases albuminuria (5). Taken together, these data implicate JAK/STAT family members in the pathogenesis of DN and suggest that inhibition of JAK/STAT might be beneficial. Currently, there are two JAK inhibitors on the market, tofacitinib, which preferentially inhibits JAKs 1 and 3 over JAK 2, and ruxolitinib, which blocks JAK2 (Table 1) (122, 148). A major hurdle to using JAK inhibition in patients with DN is the risk of myelosuppression (148). JAK2 in particular mediates signaling by several important hematopoetic cytokines including erythropoietin and GM-CS (105). If these agents are proven effective for the treatment of human diabetic kidney disease (Table 2), there is likely to be a significant risk-benefit ratio to consider and, in turn, this class of medications may only be considered for the patients with the greatest risk for disease progression.

Enhanced calcineurin (CN)/NFAT (nuclear factor of activated T cells) signaling in DN: CN is a calcium-activated phosphatase that plays a key role in diverse disease processes. An important CN substrate is the family of NFAT transcription factors (60). NFAT family members were originally discovered in cells of the lymphoid lineage, but abundant evidence indicates that NFAT isoforms are expressed in non-immune cells with some family members expressed ubiquitously (61). In quiescent cells, NFAT isoforms are phosphorylated and located in the cytoplasm (61). CN dephosphorylates NFAT, which permits translocation to the nucleus and stimulation of gene transcription.

A growing literature suggests that CN is activated in kidneys of diabetic rodents (50, 150). This increase in CN activation may contribute to the apoptosis of cell types that play an important role in the pathogenesis of DN including renal tubular cells (20) and podocytes (85, 150). CN directly promotes apoptosis through dephosphorylation of the pro-apoptotic protein BAD (155) and Drp1 (134). CN may also indirectly cause apoptosis by stimulating gene transcription. In this regard, a cell permeable peptide inhibitor termed VIVIT that specifically blocks CN-dependent NFAT activation attenuates apoptosis of cultured podocytes (85, 150). Indeed, expression of a constitutively active NFAT isoform specifically in podocytes promotes proteinuria, glomerulosclerosis and a decrease in podocyte numbers (159). Important gene targets of CN include (transient receptor potential cation channel C6), COX2 (cyclooxygenase 2) and RCAN1 (regulator of CN 1) (77, 152). These CN gene targets are upregulated in animal models of diabetic kidney disease and in patients with diabetes (73, 109). In support of a pathogenic role for CN in DN, inhibition CN ameliorates animal models of
diabetic kidney disease (50, 116, 172). For example, CN is required for glomerular hypertrophy and extracellular matrix accumulation in diabetic rats (50). In diabetic rodents, albuminuria, extracellular matrix accumulation and interstitial injury are attenuated by either pharmacologic CN inhibition (116) or the NFAT inhibitor VIVIT (172). The beneficial effects of VIVIT suggest that NFAT mediated gene transcription is, at least in part, responsible for the beneficial effects of CN inhibition in diabetic kidney disease.

Currently available pharmacological CN inhibitors include cyclosporine A and tacrolimus/FK506 (Table 1) (45). Both these drugs are immunosuppressive agents (45). As with JAK-STAT and mTOR inhibitors, the risk-benefit ratio will have to be carefully considered if CN inhibition is undertaken for treatment of DN. Moreover, both cyclosporine A and FK506 have adverse effects such as nephrotoxicity promoting the development of post-transplant diabetes. The development, however, of pharmacological agents that selectivity inhibit CN-NFAT signaling (3) without affecting dephosphorylation of other CN substrates may provide a therapeutic strategy for inhibiting the CN-NFAT signaling pathway while avoiding the adverse side-effects of pharmacologic CN inhibitors (168). In this regard, the cell permeable peptide NFAT inhibitor VIVIT prevented rejection of pancreatic islets in vivo without the adverse effects on glucose metabolism (102). Further research is needed but, if these initial findings are confirmed, then the results might provide the impetus for the development of highly specific pharmacological agents that block CN-dependent NFAT signaling without some of the adverse side effects of current pharmacological CN inhibitors. Indeed, a recent study identified dipyridamole as a small molecule inhibitor of CN-dependent NFAT activation that does not affect CN phosphatase activity (99).

Regulation of cell metabolism by mTOR/AMPK (5′-AMP activated protein kinase): The serine/threonine kinase mTOR regulates cell growth and metabolism. mTOR forms two distinct signaling complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (78). mTORC1 integrates input from both intracellular and extracellular signals to regulate several processes including lipid metabolism, protein synthesis and autophagy (35). Upstream regulators of mTORC1 include oxygen, growth factors, and amino acids (35, 78). Emerging evidence supports the role of mTORC1 in renal development and kidney disease. Embryonic deletion of the mTORC1 component Raptor in mouse podocytes causes robust albuminuria and glomerulosclerosis (49). This phenotype is present, though less severe when Raptor is deleted in adult mice (49). Moreover, genetic activation of mTORC1 by deletion of an upstream inhibitor in podocytes recapitulates histologic features of DN including mesangial expansion and glomerulosclerosis (62), and genetic inhibition of mTORC1 by deletion of one copy of Raptor confers resistance to albuminuria and mesangial expansion in a mouse model of type 2
In support of an important role for enhanced mTORC1 activity in diabetic kidney disease, phosphorylation of a downstream target of mTORC1 (p70S6 kinase) is enhanced in glomeruli from diabetic mice and in biopsy specimens from patients with DN (49, 98). Taken together, these findings support an important role for mTORC1 in diabetic kidney disease and suggest that a balance of mTORC1 signaling is required for maintenance of glomerular integrity. Targeting this pathway using pharmacological inhibitors may, therefore, have a narrow therapeutic window. Preclinical studies in rodents, however, suggest that this strategy is feasible because mTORC1 inhibition ameliorates mesangial expansion and interstitial fibrosis in streptozotocin (STZ) treated Sprague-Dawley rats (88). There are currently two mTOR inhibitors in clinical use, sirolimus (also known as rapamycin) and everolimus (Table 1). Unfortunately, these drugs are immunosuppressive agents (149), which may limit their use in all but high-risk patients. In addition, both drugs have been associated with development of proteinuria (100), consistent with the narrow therapeutic window required to obtain a beneficial therapeutic effect. While the mechanisms of this proteinuric effect are not known with certainty, mTORC1 target genes include vascular endothelial growth factor (VEGF) isoforms (49). As discussed above, VEGF plays a key role in maintenance of glomerular filtration barrier integrity. Alternatively, mTORC2 is required for activation of prosurvival Akt signaling (16, 118). While mTORC2 is classically thought to be insensitive to current mTOR inhibitors, recent studies suggest that both mTOR complexes can be inhibited by these agents (78, 124). Thus, one possible mechanism to reduce toxicity may be the development of more selective mTOR inhibitors, which preferentially target mTORC1 (149). mTORC2, however, also activates Rac 1 (64) and aberrant activation of Rho GTPases family members cause proteinuria (11, 151, 177). Thus, inhibition of mTORC2 signaling may also contribute to the beneficial effects of mTOR blockade in diabetic kidney disease. Additional studies will, therefore, be necessary to optimize this therapeutic approach.

Another therapeutic approach is to target upstream regulators of mTORC1 activity such as AMPK. In this regard, AMPK is a potent negative regulator of mTORC1 (56, 132) and strategies to augment AMPK activity could theoretically have therapeutic benefits in DN, perhaps without immunosuppressive effects. In this regard, the adipocyte secreted hormone adiponectin is a potent activator of AMPK activity (132). Adiponectin is a circulating plasma protein, and adiponectin serum levels negatively correlate with type 2 diabetes, coronary artery disease and obesity (132). In support of a role for adiponectin in DN, Sharma et al (132) found that plasma adiponectin levels were inversely correlated with albuminuria in obese patients. Moreover, knockout of adiponectin caused albuminuria and foot process effacement in mice, which was improved with adiponectin treatment in association with AMPK activation. These beneficial effects were not the result of improved glucose control because this knockout model
has normal glucose tolerance and insulin sensitivity. These data suggest that AMPK activation may be a useful therapeutic strategy for ameliorating the development of diabetes and DN. In support of this notion, treatment with the AMPK activator AICAR (5-aminoimidazole-4-carboxamide-1-b-D-ribonucleoside) (Table 1) improved albuminuria and kidney histology in two mouse models of diabetic kidney disease without altering blood glucose levels (34). Similarly, treatment with another AMPK activator metformin (Table 1) (56) reduced albuminuria and mesangial expansion in a rat model of type 2 diabetes (70). Unfortunately, this study did not control for the beneficial effects of reduced hyperglycemia observed in the metformin treated group. While these data are promising, additional basic research is needed. Moreover, given that metformin is already used clinically, examining this agent in humans with diabetic kidney disease may also be of benefit. Unfortunately, current guidelines limit the use of metformin in patients with chronic kidney disease (CKD) (63, 87), but carefully monitored clinical trials that control for effects of hyperglycemia in patients with DN and relatively well-preserved kidney function might be feasible.

Induction of Wnt/β-catenin signaling in DN: Wnt/β-catenin signaling was initially identified as a proto-oncogene in a mouse model of breast cancer (103). Since then activated Wnt signaling has been identified in a variety of cancers and diverse biological processes including embryonic patterning, bone biology and stem cell maintenance (103). Recent studies have implicated Wnt/β-catenin signaling in the pathogenesis of DN. In this regard, components of the Wnt/β-catenin signaling cascade are upregulated in proteinuric renal diseases including animal models of diabetic kidney disease and humans with DN (23). The mechanisms of Wnt/β-catenin gene induction are not known with certainty, but CN is activated in diabetic kidneys (see above) (50, 150) and expression of a constitutively active NFAT isoform in podocytes in vivo up-regulates Wnt genes (159). Moreover, Wnt/β-catenin upregulates multiple genes of the RAS, suggesting a potentially important role for this pathway as a major mediator of kidney damage in DN (175). Small molecule inhibitors Wnt/β-catenin signaling have been developed (Table 1) and treatment with these inhibitors has beneficial effects in animal models of glomerular disease (175). Thus, while the studies are still in their early stages, the Wnt/β-catenin signaling pathway looks promising as a potential target for the treatment of DN.

Summary and Conclusions: We have attempted to summarize a large body of literature on emerging treatment strategies for DN. For this review, we focused predominantly on signaling pathways that have been implicated relatively recently and selected pathways in which therapeutic agents and/or small molecule inhibitors were readily available (Table 1). Unfortunately, many of these inhibitors have significant risks associated with their use including
currently available inhibitors of JAK/STAT, mTOR, CN and VEGF signaling. As a result, using inhibitors of these pathways to treat diabetic patients will likely require identifying patients at high risk for disease progression. In contrast, other treatment strategies look more promising as a general approach to patients with DN. Combined therapies using aldosterone antagonists, endothelin inhibitors and chemokine receptor blockers with RAS blockade are an obvious approach that is currently being studied in clinical trials (Table 2). Another approach is the use of AMPK activators. Metformin activates AMPK and could be studied in clinical trials but it may be difficult to control for effects of treatment on hyperglycemia and insulin sensitivity. Moreover, the use of metformin is restricted to patients with relatively normal renal function (63, 87), although a number of investigators have suggested that the guidelines for using this agent in patients with CKD could be liberalized (63, 87). Additional AMPK activators are available that have beneficial effects in animal models of diabetic kidney disease without affecting blood glucose levels (34), but these agents would need to be approved for use in humans if clinical studies are considered. Vitamin D is another agent that has demonstrated beneficial effects in animal models of diabetic kidney disease (30, 158, 160, 173) and has been shown to lower albuminuria in macroalbuminuric patients with type 2 diabetes treated with RAS blockade (26). Vitamin D supplementation might, therefore, be a useful adjunction to current therapeutic approaches in DN that could also be rigorously tested in clinical trials (Table 2). Lastly, there may be strategies to reduce toxicities of other pharmacologic agents with a significant risk of adverse effects. For example, the development of mTOR inhibitors which are more selective for mTORC1 or CN inhibitors that specifically block CN-NFAT signaling without affecting other signaling cascades (see above). Modification of the dosing regimens used for bardoxolone-like drugs may also be of benefit (see above). In summary, promising new therapeutic approaches for the treatment of diabetic kidney disease are rapidly expanding. While more research is needed, a major challenge to the development of new therapies will be deciding which promising ideas to pursue and then translating these ideas into innovative health care strategies for treating patients with diabetic kidney disease.
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*Available drug products are dietary supplements or drugs approved for an indication other than DN. Investigational drug products are drugs used in animal studies or in early phase clinical trials. **5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside, †valine-isoleucine-valine-isoleucine-threonine
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*albumin creatinine ratio (ACR), †estimated glomerular filtration rate (eGFR), §angiotensin receptor blocker, additional information on these studies is available at: https://clinicaltrials.gov/