Recent insights into diabetic renal injury from the \( \text{db/db} \) mouse model of type 2 diabetic nephropathy

G. H. Tesch\(^{1,2} \) and A. K. H. Lim\(^{1,2} \)

Department of \(^{1}\)Nephrology and \(^{2}\)Monash University Department of Medicine, Monash Medical Centre, Clayton, Victoria, Australia

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The \( \text{db/db} \) mouse is the most widely used animal model of type 2 diabetic nephropathy. First described in 1966 (28), the \( \text{db/db} \) mouse is the most widely used animal model of type 2 diabetic nephropathy. Recent studies have utilized genetic backcrossing with transgenic mouse strains to create novel \( \text{db/db} \) strains that either lack or overexpress specific genes. These novel strains [ICAM-1\(^{-/-}\), CCL2\(^{-/-}\), MKK3\(^{-/-}\), osteopontin\(^{-/-}\), plasminogen activator inhibitor-1 (PAI-1)\(^{-/-}\), endothelial nitric oxide synthase\(^{-/-}\), SOD-Tg, rCAT-Tg] have provided valuable insights into the molecular mechanisms which promote diabetic renal injury. In addition, surgical removal of one kidney has been shown to accelerate injury in the remaining kidney of diabetic \( \text{db/db} \) mice. A number of novel therapeutic agents have also been tested in \( \text{db/db} \) mice, including inhibitors of inflammation (chemokine receptor antagonists, anti-CCL2 RNA aptamer, anti-c-fms antibody); oxidative stress (oxylkine, biliverdin); the renin-angiotensin-aldosterone system (aliskiren, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, eplerenone); advanced glycation end products (AGE; pyridoxamine, alagebrium, soluble AGE receptor); angiogenesis (NM-3, anti-CXCL12 RNA aptamer, soluble Flt-1); lipid accumulation (statins, farnesoid X receptor agonists, Ormacor); intracellular signaling pathways (PKC-\( \beta \) or JNK inhibitors); and fibrosis (transforming growth factor (TGF)-\( \beta \) antibody, TGF-\( \beta \) kinase inhibitor, soluble betaglycan, SMP-534, CTGF-antisense oligonucleotide, mutant PAI-1, pirfenidone), which have identified potential therapeutic targets for clinical translation. This review summarizes the advances in knowledge gained from studies in genetically modified \( \text{db/db} \) mice and treatment of \( \text{db/db} \) mice with novel therapeutic agents.

kx2 kid; gene knockout; uninephrectomy; therapy

C57BL/6J strain (27) and the FVB/NJ strain (12). In each of these strains, diabetes is more severe in males compared with females. Male C57BLKS/J \( \text{db/db} \) mice rapidly develop hyperglycemia (\( >16 \) mmol/l at 6–10 wk), albuminuria (10–12 wk), and declining renal function (15–18 wk) (13, 39), but their islet cells are sensitive to toxic effects of hyperglycemia, and these mice become dependent on insulin administration to control increasing blood glucose levels and allow survival. In comparison, the islets of the FVB/NJ \( \text{db/db} \) strain are more resistant to hyperglycemia-induced injury and their insulin production is better preserved in the presence of diabetes, which permits longer survival without intervention. FVB/NJ \( \text{db/db} \) mice develop hyperglycemia at about 4 wk of age, which is maintained at a similar level until at least 26 wk of age when mice develop albuminuria and significant renal histological lesions (11, 12). In contrast, the C57BL/6J \( \text{db/db} \) strain has a lower incidence of diabetes with \( \sim 50\% \) of males and 12% of females having significant hyperglycemia (\( >16 \) mmol/l) at 32 wk of age (8). C57BL/6J \( \text{db/db} \) males which develop diabetes have significant albuminuria, renal histological damage, and reduced renal function at 32 wk of age.

The rapid development of hyperglycemia and diabetic renal injury in C57BLKS/J \( \text{db/db} \) mice has resulted in this strain becoming the preferred \( \text{db/db} \) model for short-term interven-
tion studies. In contrast, the C57BL/6J db/db strain is more commonly used for studies examining the effects of single-gene deficiencies or gene overexpression on the development of diabetic renal injury, despite the slow rate of disease progression in this model. This is mainly due to the large range of genetic modifications available on the C57BL/6J background and the ease with which these modified strains can be back-crossed onto C57BL/6J db/db mice.

Acceleration of Nephropathy in db/db Mice by Uninephrectomy

Use of the db/db mouse as a research tool is sometimes limited by the rate of disease development and the severity of renal function impairment and kidney lesions. To accelerate diabetic renal injury in the db/db mouse, some researchers have developed a model in which one kidney is removed (uninephrectomized) in the early stages of diabetes, which puts a greater workload and stress on the remaining diabetic kidney.

The effects of uninephrectomy on glomerular injury were first reported in the C57BLKS/J db/db strain in 1980 (4). In this study, uninephrectomy was performed at 16 wk of age and db/db mice were analyzed at 6 mo of age. Compared with nonoperated db/db mice, the uninephrectomized db/db mice had a 70% increase in mesangial thickening based on semi-quantitative analysis of periodic acid-Schiff (PAS) staining. In subsequent studies, uninephrectomy of db/db mice has been performed at a much earlier age (6–10 wk) to obtain more robust disease and kidney lesions. To accelerate renal function impairment and kidney lesions, researchers have performed uninephrectomy at a much earlier age (6–10 wk) to obtain more robust disease and kidney lesions.

Recent characterization of the accelerated nephropathy in uninephrectomized db/db mice has resulted in growing use of this model for studies examining the effects of pharmacological inhibitors on the development of diabetic renal injury (26, 45, 59). One advantage of this model is that it allows pharmacological responses to be determined in db/db mice in a shorter time frame. However, when choosing this model, researchers need to consider the technical expertise required, the surgical mortality rate (~5%), and the limited amount of kidney tissue obtained.

Studies of Gene Deficiencies in db/db Mice

Deficiency of ICAM-1. ICAM-1 plays a major role in recruiting leukocytes from the circulation into the kidney. C57BL/6J db/db mice with genetic deficiency of ICAM-1 (ICAM-1−/− db/db) were found to have an 85% reduction in albuminuria at 32 wk of age compared with equally diabetic wild-type controls (ICAM-1+/− db/db mice) (10). This protection from renal injury was associated with reductions in glomerular leukocytes (63% ↓) and interstitial leukocytes (83% ↓) and marked attenuation of glomerular and tubular damage and kidney matrix accumulation (10). This was the first study to indicate a functional role for leukocytes in the development of diabetic renal injury in db/db mice.

Deficiency of MCP-1/Ccl2. Macrophages are the major leukocytes present in diabetic kidneys. Monocyte chemoattractant protein (MCP)-1, encoded by the gene Ccl2, is a major chemokine for monocyte/macrophages. C57BL/6J db/db mice deficient in Ccl2 have a reduced number of CD68+ macrophages in glomeruli (48% ↓) and the interstitium (78% ↓) at 32 wk of age compared with wild type db/db mice with equivalent diabetes (9). In addition, diabetic Cc12−/− db/db mice have reduced levels of albuminuria (62% ↓) and plasma creatinine (65% ↓), which are associated with protection from glomerular and tubular pathology and interstitial accumulation of myofibroblasts and extracellular matrix. These findings demonstrate a role for macrophage-mediated injury in the diabetic kidneys of db/db mice.

Deficiency of osteopontin. Osteopontin (OPN), also known as secreted phosphoprotein 1 (Spp1), is complex glycoprotein which can promote cell adhesion and migration and has some profibrotic properties. Its role in diabetic nephropathy has recently been examined in OPN−/− mice (129 × Black Swiss) which were crossed onto the C57BLKS/J db/+ strain to produce OPN−/− db/db mice (44). These OPN−/− db/db mice were found to have reduced albuminuria (at least 50% ↓) at 2–4 mo of age and decreased glomerular levels of collagen IV, fibronectin and TGF-β compared with equally diabetic OPN+/− db/db littermates. This study indicates that OPN promotes diabetic renal injury in db/db mice; however, the mechanisms responsible for this are still unclear and may involve effects on leukocyte accumulation and activation or damage to the glomerular filtration barrier induced by alterations to the glomerular structure.

Deficiency of MKK3. Elements of the diabetic milieu are known to induce proinflammatory and profibrotic responses in kidney cells via activation of the p38 MAPK signaling pathway. This pathway is activated in the kidneys of diabetic
patients and diabetic \(db/db\) mice (1). Because mice deficient in \(p38\) die in utero, the functional role of \(p38\) MAPK cannot be examined directly by genetic deletion in \(db/db\) mice. However, mice genetically deficient in MKK3 or MKK6, the upstream kinases of \(p38\) MAPK, are viable. C57BL/6J \(db/db\) mice deficient in mitogen activated kinase kinase-3 (MKK3\(^{-/-}\)/\(db/db\)) have reduced kidney activation of \(p38\) MAPK and develop less renal injury compared with equally diabetic wild-type controls (MKK3\(^{+/+}\)/\(db/db\)) (40). At 32 wk of age, diabetic MKK3\(^{-/-}\)/\(db/db\) mice have decreased levels of albuminuria (70% \(\downarrow\)) and are protected from the loss of renal function observed in diabetic \(db/db\) controls. In terms of kidney structure, diabetic MKK3\(^{-/-}\)/\(db/db\) mice have reductions in podocyte loss, tubular damage, and glomerular and interstitial accumulation of myofibroblasts and extracellular matrix. In addition, these mice have lower numbers of interstitial macrophages, which is associated with reduced kidney gene expression of \(Ccl2\). These findings support a role for MKK3\,-p38MAPK signaling in renal injury, inflammation, and fibrosis in the diabetic kidneys of \(db/db\) mice.

**Deficiency of plasminogen activator inhibitor-1.** Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor which is thought to function as a profibrotic mediator in diabetic nephropathy. PAI-1 inhibits tissue- and urokinase-type plasminogen activators. The effects of PAI-1 on diabetic renal fibrosis have been evaluated in PAI-1\(^{-/-}\) mice (B6/129) which were crossed onto the C57BLKS/J \(db/db\) strain to produce PAI-1\(^{-/-}\)/\(db/db\) mice (16). The kidneys of PAI-1\(^{-/-}\)/\(db/db\) mice showed 60% higher levels of urokinase activity compared with PAI-1\(^{+/+}\)/\(db/db\) littermate controls, indicating that higher PAI-1 levels in \(db/db\) mice suppressed urokinase activity. At 8 mo of age, PAI-1\(^{-/-}\)/\(db/db\) mice showed a trend toward reduced albuminuria; however, this was not statistically significant due to high variability. In addition, PAI-1 deficiency did not affect renal hypertrophy or blood urea nitrogen levels in \(db/db\) mice. Diabetic PAI-1\(^{-/-}\)/\(db/db\) mice also had a similar glomerular matrix fraction and total kidney collagen content to wild-type \(db/db\) controls. The investigators highlighted several concerning issues with the study. First, only females were studied because the PAI-1\(^{-/-}\)/\(db/db\) phenotype was associated with a greater risk of premature death and runtiness in males. PAI-1\(^{-/-}\)/\(db/db\) mice also had 15–25% lower blood glucose levels and a trend toward lower lipid levels than PAI-1\(^{+/+}\)/\(db/db\) mice, suggestive of other metabolic consequences of PAI-1 deficiency.

**Deficiency of endothelial nitric oxide synthase.** Endothelial dysfunction is implicated in the pathogenesis of diabetic nephropathy. Endothelial nitric oxide synthase (eNOS) modulates functions such as permeability, vascular tone, blood flow, and hyperfiltration. Mice with a genetic deficiency of eNOS are inherently hypertensive and develop slowly progressing renal damage (18). To develop an accelerated model of type 2 diabetic nephropathy, eNOS\(^{-/-}\) mice have been backcrossed onto the C57BLKS/J strain and then crossed with C57BLKS/J \(db/db\) mice to produce eNOS\(^{-/-}\)/\(db/db\) mice (63). The eNOS\(^{-/-}\)/\(db/db\) mouse has some phenotypic differences compared with eNOS\(^{+/+}\)/\(db/db\) mice. First, these mice develop moderate systolic hypertension, which is \(\sim 40\%\) higher than eNOS\(^{+/+}\)/\(db/db\) mice, but is not different to eNOS\(^{-/-}\)/\(db/db\) mice (41, 63). Second, they have lower fasting blood glucose levels (25% \(\downarrow\)) at 16 wk of age compared with eNOS\(^{+/+}\)/\(db/db\) mice, which is associated with fourfold higher insulin levels and increased islet hypertrophy (41). One study also found a twofold increase in plasma cholesterol level but no obvious difference in aortic atherosclerosis at 16 wk (41).

In terms of renal injury, eNOS\(^{-/-}\)/\(db/db\) mice have increased albuminuria at 16 (430% \(\uparrow\)) and 26 wk (570% \(\uparrow\)) and reduced renal function at 26 wk (inulin clearance 55% \(\downarrow\), plasma creatinine 48% \(\uparrow\)) compared with eNOS\(^{+/+}\)/\(db/db\) mice. These mice also showed increases in glomerular hypertrophy, glomerular matrix deposition, glomerular and tubular injury, and kidney macrophage accumulation, which was evident at both 16 and 26 wk (41, 63). Furthermore, characteristic features of human diabetic nephropathy, including arteriolar hyalinosis and nodular glomerulosclerosis, can be regularly seen in eNOS\(^{-/-}\)/\(db/db\), but are not normally detected in wild-type \(db/db\) mice; however, interstitial fibrosis remains relatively mild in \(db/db\) mice lacking eNOS. These findings indicate that eNOS\(^{-/-}\)/\(db/db\) mice represent an accelerated model of type 2 diabetic nephropathy which may prove useful for evaluating novel therapies.

**Effects of Gene Overexpression in \(db/db\) Mice**

Recent studies of gene overexpression in \(db/db\) mice have focused on increasing the production of antioxidant enzymes as a means of reducing the pathological effects of oxidative stress in diabetic kidneys. Hyperglycemia is known to induce reactive oxygen species (ROS) in kidney cells, which can directly damage DNA and protein, or function as signaling amplifiers to activate pathways of cellular stress.

**Overexpression of SOD-1.** C57BL/6J mice expressing the human Cu/Zn-SOD-1 transgene driven by the human SOD-1 promoter were crossed with C57BL/6J \(db/+\) mice to create the SOD-1\(^{+/+}\)/\(db/+\) genotype (SOD-Tg\(-db/+\)) (19). These transgenic mice have a greater than twofold increase in cortical SOD-1 levels compared with wild-type \(db/+\) mice, which is mainly localized to the glomerular endothelial and tubular epithelial cells. The SOD-Tg\(-db/+\) mice were completely protected from the threefold increase in albuminuria seen in wild-type \(db/+\) mice at 5 mo of age, despite their hyperfiltration (increased inulin clearance). Furthermore, SOD-Tg\(-db/+\) mice had reduced glomerular levels of mesangial matrix, TGF-\(\beta\), and nitrotyrosine compared with control \(db/+\) mice. The investigators also examined the effects of treatment with \(N^\omega\)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS on diabetic mice. Inulin clearance and urinary cGMP were suppressed by L-NAME in \(db/+\)-SOD-Tg but not in \(db/+\) mice, suggesting that the greater hyperfiltration in \(db/+\)-SOD-Tg mice was due to enhanced renal nitric oxide (NO) bioactivity. When cGMP responses to NO stimulation were examined ex vivo, glomeruli from \(db/+\) mice demonstrated greater quenching of NO compared with \(db/+\)-SOD-Tg mice. These findings demonstrate that superoxide mediates biochemical, functional, and structural changes in the diabetic kidneys of \(db/+\) mice, which appear, in part, to involve interactions with NO.

**Overexpression of catalase.** Apoptosis is seen in the proximal tubular cells of diabetic kidneys, which may contribute to the development of tubular atrophy during diabetic nephropathy. One potential cause of this apoptosis is the generation of ROS in tubular cells by hyperglycemia. A recent study has
examined whether overexpression of the antioxidant enzyme catalase (CAT) in proximal tubular cells is capable of preventing tubular apoptosis and renal injury in diabetic db/db mice (5). Transgenic C57BL/6J mice overexpressing rat catalase (rCAT) in their renal proximal tubular cells were crossed with the C57BL/6J db/+ strain to produce rCAT-Tg-db/db mice (5). In these mice, the expression of the rCAT-HA transgene is under the control of the kidney-specific androgen-regulated (KAP2) promoter, which is stimulated by circulating endogenous testosterone in males or exogenously administered testosterone in females. At 20 wk of age, there was a marked reduction in the ROS level (73%↓) of renal proximal tubular cells isolated from rCAT-Tg-db/db mice compared with those isolated from wild-type db/db mice. The db/db and rCAT-Tg-db/db mice developed similar obesity and hyperglycemia during the analysis period (8–20 wk). However, wild-type db/db mice showed mild hypertension (20–30 mmHg ↑ systolic blood pressure) after 12–14 wk of age compared with the rCAT-Tg-db/db or db/+ groups. Urine analysis showed that albuminuria was elevated 10–20-fold in db/db mice at weeks 18–20 compared with nondiabetic db/+ mice, but was reduced in the rCAT-Tg-db/db by 36–42% in males and by 67–83% in females. Examination of the diabetic kidneys at 20 wk found reduced tubular apoptosis and interstitial fibrosis in rCAT-Tg-db/db mice but no difference in glomerular hypertrophy compared with db/db mice. These findings indicate that intrarenal ROS plays a role in the development of albuminuria, tubular injury, and interstitial fibrosis in the diabetic kidneys of db/db mice.

Therapeutic Agents Used in db/db Mice

Inhibitors of inflammation. Studies in db/db mice deficient in ICAM-1 or MCP-1 have demonstrated a critical role for macrophage-mediated inflammation in the development of diabetic renal injury. In response to these findings, researchers have developed novel therapeutic strategies for targeting macrophages and have evaluated their effectiveness in db/db mice. Administration of BL5923, a CCR1 antagonist, to uninephrectomized C57BLKS/J db/db mice between 5 and 6 mo of age was found to reduce white blood cell counts and interstitial macrophage accumulation, as well as the development of tubular damage and interstitial fibrosis (46). However, treatment with BL5923 had no effect on albuminuria or glomerular pathology. In another study, uninephrectomized C57BLKS/J db/db mice receiving mNOX-E36–3PEG, an anti-CCL2 t- enantiomeric RNA aptamer, between 4 and 6 mo of age, were found to have normal white blood cell counts but showed a marked reduction in glomerular and interstitial macrophages. This RNA aptamer provided protection from declining GFR, glomerular and tubulointerstitial damage, and renal fibrosis (45). Macrophages have also been targeted in db/db mice using a neutralizing antibody to the macrophage CSF-1 receptor (c-fms). Treatment of C57BLKS/J db/db mice with a c-fms antibody (AFS98) from 12–18 wk of age had no effect on white blood cell counts but reduced both glomerular and interstitial macrophages (39). AFS98 also protected db/db mice from glomerular hyperfiltration, tubular injury, and mild interstitial fibrosis; however, established albuminuria and glomerular pathology were unaltered by this therapy.

Inhibitors of oxidative stress. Novel antioxidants have been tested in db/db mice to determine their potential for reducing oxidative stress and injury in diabetic kidneys. Oxykine is a melon extract rich in vegetal SOD which is protected from digestive degradation by a polymeric film of gliadin. Oral administration of oxykine to C57BLKS/J db/db mice between 6 and 18 wk of age has no effect on the development of diabetes but reduces urine levels of albumin and 8-hydroxydeoxyguanosine, and the mesangial matrix fraction (43). Biliverdin is a precursor of bilirubin, which is recognized as an endogenous antioxidant with a highly efficient radical-scavenging effect. C57BLKS/J db/db mice maintained on a diet supplemented with biliverdin from week 8 to week 10 or 20 were found to be protected from renal oxidative stress and albuminuria. Those mice treated for 12 wk also had reduced accumulation of mesangial matrix (22).

Inhibitors of the renin-angiotensin-aldosterone system. The use of an ACE inhibitor (ACEi) or an angiotensin II receptor blocker (ARB) to control hypertension is standard therapy for patients with diabetic nephropathy. The renoprotective effects of these therapies and other inhibitors of the renin-angiotensin-aldosterone system (RAAS) pathway have been examined in db/db mice. Treatment of C57BLKS/J db/db mice with the ARB losartan from 11–29 wk of age has been shown to partially reduce albuminuria and lipid peroxidation, but not mesangial matrix expansion (42). In another study, the effects of both an ACEi (benazepril) and an ARB (valsartan) were examined in normotensive C57BL/6 db/db mice to identify whether blocking these pathways had protective effects beyond inhibiting hypertension (64). Treatment of C57BL/6 db/db mice with benazepril and valsartan from 9 to 25 wk of age was found to reduce albuminuria, glomerular hypertrophy, and mesangial matrix accumulation. However, the same study found no effect when db/db mice were treated with an ACEi (enalapril) alone.

Administration of a direct renin inhibitor, aliskiren, to C57BLKS/J db/db mice between 6 and 12 wk of age was found to reduce circulating levels of angiotensin II but had no effect on blood pressure in these mice. Mice treated with aliskiren had reduced levels of albuminuria, renal oxidative stress, glomerular inflammation, mesangial matrix, and glomerular TGF-β1 and were protected from a loss of nephrin expression (20). The level of this protection was increased further when mice were treated with aliskiren in combination with valsartan, which reduced blood pressure.

Eplerenone, a specific antagonist of the mineralocorticoid receptor, has been used to examine whether aldosterone blockade can reduce diabetic renal injury. Administration of eplerenone to C57BLKS/J db/db mice between 8 and 25 wk of age had no effect on diabetes or systolic blood pressure, but decreased albuminuria, glomerular hypertrophy, and mesangial matrix accumulation (25). Eplerenone treatment also lowered kidney mRNA levels of OPN, CD68, and TGF-β1, suggesting that aldosterone promotes inflammation and fibrosis in diabetic kidneys.

Inhibitors of advanced glycation end products. Diabetes induces the formation of advanced glycation end products (AGEs), which can modify the function of proteins and stimulate pathologival cellular responses via AGE receptors. Increasing levels of AGEs, and their deposition in diabetic kidneys, correlate with the development of diabetic nephropa-
was able to decrease circulating levels of AGE-albumin in the administration of an anti-AGE-albumin monoclonal antibody A717. Mesangial matrix in female C57BL/6J db/db mice shown to reduce albuminuria, glomerular hypertrophy, and loss of nephrin protein in the renal cortex.

Several recent studies have tested the effectiveness of novel inhibitors of AGE formation for preventing the progression of diabetic renal injury in db/db mice. Cohen et al. (15) have examined a variety of AGE inhibitors in db/db mice. Their early studies showed that weekly administration of an anti-AGE-albumin monoclonal antibody A717 was able to decrease circulating levels of AGE-albumin in C57BLKS/J db/db mice, which reduced albuminuria, mesangial matrix and cortical gene expression of matrix components (15). Subsequent studies by this group have examined a number of small-molecule inhibitors of AGE formation as potential therapies, with the most recent being designated 23CPPA (14). Treatment of C57BLKS/J db/db mice with 23CPPA from 9 to 18 wk of age had no effect on diabetes but reduced kidney hypertrophy, urine excretion of albumin and collagen IV, and cortical levels of VEGF and VEGFR1. In addition, 23CPPA protected these db/db mice from declining renal function and loss of nephrin protein in the renal cortex.

Pyridoxamine, another inhibitor of AGE formation, has been shown to reduce albuminuria, glomerular hypertrophy, and mesangial matrix in female C57BL/6J db/db mice following treatment between 17 and 25 wk of age (64). Furthermore, this study also showed that the combined administration of an ACE inhibitor (enalapril) and pyridoxamine from 16–32 wk provided added protection against renal injury and mortality compared with the use of enalapril alone.

Alagebrum (ALT-711), an AGE cross-link breaker, has been used to treat female C57BLKS/J db/db mice, which develop diabetic nephropathy more slowly than their male littermates (48). In this study, 12 wk of alagebrum treatment commencing at either 3, 7, or 12 mo of age reduced serum AGE levels and the progression of albuminuria, glomerular hypertrophy, and mesangial matrix accumulation. In addition, kidney AGE deposition and glomerular basement width were diminished following treatment which began at 7 and 12 mo. These results suggest that alagebrum may prevent the progression of early and advanced diabetic nephropathy.

Diabetic kidneys have elevated expression of the receptor for AGE (RAGE) as well as an increased number of ligands for this receptor, including AGEs and S100/calgranulin. A soluble form of RAGE (sRAGE) is known to block AGEs and S100/calgranulin from binding to RAGE on cells and inducing pathological responses. Administration of sRAGE to C57BLKS/J db/db mice between 8 and 13 wk of age reduced kidney levels of RAGE, S100/calgranulin, VEGF, VCAM-1, and prevented glomerular macrophage infiltration (62). In comparison, longer term administration of sRAGE between 8 and 27 wk of age protected diabetic db/db mice from declining renal function and also decreased albuminuria, glomerular hypertrophy, mesangial matrix accumulation, glomerular basement membrane thickening, and expression of VEGF and TGF-β. These findings indicate that RAGE activation plays an important role in the development of diabetic nephropathy.

Inhibitors of angiogenesis. The progression of diabetic nephropathy is associated with an increase of angiogenic factors such as VEGF and angiopoietin (Ang)-2, which synergize to promote angiogenesis. Daily intraperitoneal injection of the antiangiogenic compound NM-3 to C57BLKS/J db/db mice between 8 and 16 wk of age lowered kidney the levels of VEGF-A and Ang-2 (29). This treatment reduced albuminuria, glomerular hyperfiltration, glomerular hypertrophy, mesangial matrix, the glomerular capillary area, glomerular macrophores, and nephrin mRNA expression. However, the impact of treatment on tubulointerstitial injury was not reported.

Chemokine (C-X-C motif) ligand 12 (CXCL12), also known as stromal cell-derived factor (SDF)-1, is known to induce angiogenesis, support vascular integrity similar to VEGF, and promote cell migration and survival. Administration of NOX-A12, an anti-CXCL12 L-enantiomeric RNA aptamer, to uninephrectomized C57BLKS/J db/db mice between 4 and 6 mo of age was found to reduce albuminuria, glomerulosclerosis, and podocyte loss, but had no effect on macrophage accumulation or cell proliferation within glomeruli (53).

Soluble Flt-1 (sFlt-1) is a variant form of the VEGF receptor 1, which lacks the transmembrane and intracellular domain. Binding of sFlt-1 to VEGF neutralizes its action on endogenous VEGF receptors. One study has used an adenoviral-associated virus as a vector to introduce sFlt-1 into 8-wk-old male C57BLKS/J db/db mice, which resulted in an attenuation of diabetes-induced elevation of phospho-Flt-1 after 8 wk of treatment (34). Low-dose gene therapy did not alter blood pressure, body weight, or hyperglycemia. However, albuminuria was attenuated by ~50% in sFlt-1-treated db/db mice, which was associated with protection from podocyte injury and depletion. In contrast, overexpression of sFlt-1 did not alter glomerular matrix accumulation and exacerbated tubulointerstitial injury, which included a loss of peritubular capillaries. This was associated with a reduction in tubular but not glomerular VEGF expression. These findings demonstrate the adverse effects of systemic blockade of VEGF and emphasize the difficulty in targeting the pathological effects of VEGF in diabetic kidneys.

Inhibitors of lipid accumulation. Statins are widely used cholesterol-lowering agents but have also been reported to have anti-inflammatory and antioxidative actions. Administration of pitavastatin to C57BLKS/J db/db mice between 12 and 14 wk of age was found to reduce albuminuria and kidney oxidative stress, which included a decrease in kidney NOX4 production (21). This report also demonstrated that long-term treatment from 12 to 24 wk decreased mesangial matrix and kidney mRNA levels of TGF-β1 and fibronectin. In a similar study, treatment of C57BLKS/J db/db mice with simvastatin from 8 to 24 wk of age reduced albuminuria and mesangial matrix accumulation in association with attenuation of kidney activity of the small GTPase protein RhoA (33). These findings suggest that statins protect against diabetic renal injury, in part, by inhibiting renal oxidative stress and RhoA activity.

The farnesoid X receptor (FXR) is a member of the nuclear hormone receptor superfamily, which is highly expressed in the kidney and liver and is known to regulate lipid metabolism by inhibiting the sterol regulatory element binding protein-1 (SREBP-1). Levels of SREBP-1 are increased in diabetic kidneys and correlate with the progression of renal injury. Treatment of 12-wk-old C57BLKS/J db/db mice with an FXR agonist (GW4064) for 1 wk had no impact on hyperglycemia but reduced plasma cholesterol and kidney mRNA expression of SREBP-1, Nox2, proinflammatory cytokines, and profibrotic molecules (31). In a follow-up study, C57BL/6 db/db mice were treated with another FXR agonist, cholic acid, between 12 and 24 wk of age, which reduced albuminuria and mesangial matrix accumulation. However, the control and treated C57BL/6 db/db mice were not overtly diabetic (31).
These findings indicate that FXR agonists may suppress the development of diabetic nephropathy due to systemic and renal effects on lipid metabolism and by decreasing oxidative stress, inflammation, and fibrosis in the kidney.

Investigators have also used Omacor, a solution containing n-3 polyunsaturated fatty acids, to suppress SREBP-1 activity in diabetic kidneys (7). Administration of Omacor to C57BLKS/J db/db mice between 8 and 10 wk of age did not alter diabetes but lowered kidney levels of SREBP-1 and reduced serum triglyceride, albuminuria, glomerular hyperfiltration, podocyte loss, and mesangial matrix. However, Omacor treatment also reduced food and water intake, suggesting that the observed protection involves regulation of metabolism as well as lipid signaling.

Inhibitors of intracellular signaling pathways. Diabetes regulates intracellular signaling responses, including those mediated by PKC-β and JNK. Both of these kinases are activated in diabetic kidneys, which has prompted studies to block their action in db/db mice.

Treatment of C57BLKS/J db/db mice with a PKC-β inhibitor (LY333531) from 9 to 25 wk of age reduced albuminuria, glomerular TGF-β production, and mesangial matrix accumulation (35). In comparison, when C57BLKS/J db/db mice were treated with a cell-permeable TAT-JNK inhibitor peptide from 8 to 18 wk of age, their insulin sensitivity improved and hyperglycemia declined compared with db/db controls (30). However, mice treated with the JNK inhibitor had increased albuminuria, reduced nephrin expression, and no effect on mesangial expansion. This finding indicates that therapies which reduce the severity of diabetes do not necessarily provide renal protection.

Inhibitors of fibrosis. TGF-β1 is known to be a major inducer of profibrotic responses in diabetic kidneys. An earlier study has shown that administration of a neutralizing monoclonal TGF-β1 antibody to C57BLKS/J db/db mice between 8 and 16 wk of age decreases plasma TGF-β1, mesangial matrix expansion, and kidney mRNA levels of collagen IV and fibronectin (65). In addition, this therapy prevented a loss of renal function but had no effect on the elevated albuminuria in db/db mice. More recently, investigators have used an inhibitor of TGF-β receptor kinase activity, GW788388, to treat C57BLKS/J db/db mice at 6 mo of age for 5 wk (49). This therapy reduced glomerular collagen staining and kidney mRNA levels of PAI-1 and collagen (I and III) but did not alter albuminuria. A study has also identified that TGF-β activity can be blocked in db/db mice using soluble betaglycan, which binds to TGF-β and prevents activation of its receptors (32). In this report, C57BLKS/J db/db mice were treated with soluble betaglycan from 8 to 16 wk of age and were found to be protected from albuminuria, declining renal function, and mesangial matrix expansion. Soluble betaglycan also reduced kidney mRNA levels of TGF-β isoforms, collagen (I and IV), and fibronectin. Using a different strategy, investigators have shown that administration of a naturally occurring inhibitor of TGF-β-induced SMAD signaling, N-acetyl-seryl-asparyl-lysyl-proline (Ac-SKD), to C57BLKS/J db/db mice between 10 and 18 wk of age reduces renal dysfunction, glomerular hypertrophy, mesangial matrix expansion, and overproduction of extracellular matrix proteins (56). However, Ac-SKD did not diminish albuminuria in these mice. In another approach, researchers administered a low-molecular-weight inhibitor of TGF-β1/p38 MAPK activity, SMP-534, to C57BLKS/J db/db mice between 9 and 25 wk of age (58). SMP-534 decreased mesangial matrix expansion and urine excretion of albumin and collagen IV. These findings indicate that TGF-β1 plays a critical role in diabetic glomerulosclerosis in db/db mice; however, its involvement in the development of diabetic albuminuria remains controversial.

Connective tissue growth factor (CTGF) is another profibrotic cytokine which promotes matrix accumulation in diabetic kidneys. Antisense oligonucleotides targeting CTGF have been shown as a therapy to C57BLKS/J db/db mice between 12 and 20 wk of age and were found to reduce CTGF levels in the renal cortex and urine (24). These mice were protected from increases in albuminuria, renal dysfunction, mesangial matrix accumulation, and kidney mRNA levels of collagen (I and IV) and PAI-1.

PAI-1 production is induced by TGF-β1 and CTGF and contributes to matrix accumulation in diabetic kidneys by reducing plasmin activity and matrix degradation. A human mutant form of PAI-1 (designated PAI-1R) has recently been shown to compete with PAI-1 for vitronectin binding. Administration of PAI-1R to uninephrectomized C57BLKS/J db/db mice between 20 and 22 wk of age increased kidney plasmin activity but reduced TGF-β1 protein, glomerular matrix, and mRNA and protein levels of collagen (I and IV) and fibronectin in the renal cortex (26).

In addition, PAI-1R therapy prevented an increase in albuminuria during the treatment period. This finding supports an important role for endogenous PAI-1 activity in the development of diabetic glomerulosclerosis in db/db mice.

Pirfenidone is a potent antifibrotic compound, whose mechanisms of action are still being defined. Treatment of C57BLKS/J db/db mice with pirfenidone from 17 to 21 wk of age reduced mesangial matrix accumulation and kidney cortex mRNA levels of collagen (I and IV) and fibronectin but did not decrease albuminuria (51). Subsequent analysis identified 21 proteins that are unique to pirfenidone-treated db/db kidneys and suggest that the antifibrotic effects of pirfenidone may include regulation of RNA processing.

Discussion

The growing incidence of type 2 diabetic nephropathy has driven a major effort to develop a greater understanding of the mechanisms underlying this disease and to explore novel intervention strategies as potential therapies. This has led to an increase in use of the db/db mouse model as a research tool. Recent advances in genetic manipulation and pharmacology have been applied to the db/db model to determine the importance of specific mechanisms in type 2 diabetic nephropathy and to identify therapeutic targets. In addition, the development of accelerated models of diabetic renal injury in db/db mice, using uninephrectomy or eNOS deficiency, provides increased flexibility when one is choosing a db/db model for exploring new mechanisms or therapies.

Studies in db/db mice have identified major mechanistic pathways of diabetic renal injury (Fig. 1), which can be suppressed by therapies which target key components of these pathways (see Table 2). These pathomechanisms also appear to be important in the development of rodent models of type 1 diabetic nephropathy. Lean mice which are genetically defi-
Fig. 1. Major mechanistic pathways of diabetic renal injury identified in db/db mice. The development of diabetes in db/db mice results in the formation of advanced glycation end products (AGEs), oxidative stress, and activation of the renin-angiotensin-aldosterone system (RAAS) within the kidney, which promotes progressive inflammation and fibrosis, leading to diabetic nephropathy and declining renal function. Intervention therapies described in Table 2 can inhibit each of these pathways in db/db mice.

cient in ICAM-1, MCP-1, or PAI-1, or genetically overexpress SOD, display protection against renal injury after induction of diabetes with streptozotocin, which is similar to that seen in obese db/db mice (17, 36, 60). In addition, therapeutic blockade of AGE formation (alagebrium), RAAS activation (losartan, eplerenone), or TGF-β-induced fibrosis (TGF-β antibody) can inhibit renal injury in the diabetic kidneys of db/db mice to a similar extent as those of streptozotocin-treated rodents with comparable diabetes (25, 52, 55, 61). This suggests that there are common mechanisms of renal injury which are critical to both type 1 and type 2 diabetic nephropathy. Furthermore, inhibition of diabetic renal injury by blocking AGE formation (pyridoxamine) or angiotensin II effects (losartan) demonstrates a role for inflammation, oxidative stress, and p38 MAPK signaling in the development of type 2 diabetic nephropathy, which have been supported by intervention studies with selective inhibitors (see Tables 1 and 2). In addition, the use of pharmacological inhibitors in db/db mice has identified additional molecular mechanisms that contribute to the progression of diabetic renal injury, including members of the RAAS pathway (renin, angiotensin II, aldosterone), the lipid signaling pathway (Fanesoid X receptor, SREBP-1), angiogenic factors (VEGF, CXCL12, angiopoietin-2), AGE, PKC-β signaling, and profibrotic molecules (TGF-β, CTGF, PAI-1).

Evidence provided from both genetic manipulation studies and therapeutic interventions in db/db mice have demonstrated a correlation between podocyte damage/loss and albuminuria levels (7, 14, 20, 29, 34, 39, 40), suggesting that podocyte injury is a critical factor in the early development of type 2 diabetic nephropathy. Furthermore, intervention studies in db/db mice with established albuminuria indicate that many strategies which effectively suppress mechanisms of renal injury are unable to reduce albumin excretion below the pretreatment level, or sometimes not at all (39, 49, 51, 56, 65). This emphasizes a difficulty in replacing podocytes which are lost from diabetic glomeruli. It also indicates that intervention therapies may provide significant renoprotection without reducing albuminuria, which suggests that albuminuria should not be the only end point used for intervention studies.

The role of hypertension in the development of diabetic renal injury in db/db mice has been controversial. Studies employing tail-cuff measurements have produced mixed results, identifying increased, decreased, or no change in blood pressure in db/db mice (5, 25, 35, 42). However, two reports using radiotelemetric probes have recently shown that there is a significant increase in mean arterial blood pressure (8–16%) at 14–16 wk of age in C57BLKS/J db/db mice compared with db/+ controls (54, 57). In contrast, radiotelemetry analysis has not identified any evidence of hypertension in C57BL/6 db/db mice.

Table 1. Summary of gene modifications in db/db mice with renal effects

<table>
<thead>
<tr>
<th>Modified Gene (and Strain)</th>
<th>Intended Target</th>
<th>Effect on Diabetic Nephropathy</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1 KO (db/db BL6)</td>
<td>Leukocyte recruitment</td>
<td>Reduces renal inflammation, albuminuria, glomerular and tubular injury, and kidney fibrosis.</td>
<td>10</td>
</tr>
<tr>
<td>MCP-1 KO (db/db BL6)</td>
<td>Macrophage recruitment and activation</td>
<td>Reduces renal inflammation, albuminuria, glomerular and tubular injury, and kidney fibrosis.</td>
<td>9</td>
</tr>
<tr>
<td>OPN KO (db/db BKS)</td>
<td>Cell motility and activation</td>
<td>Reduces albuminuria and glomerular fibrosis.</td>
<td>44</td>
</tr>
<tr>
<td>MKK3 KO (db/db BL6)</td>
<td>p38 MAPK signaling</td>
<td>Reduces podocyte loss, interstitial inflammation, albuminuria, histological damage and kidney fibrosis. Improves GFR.</td>
<td>40</td>
</tr>
<tr>
<td>PAI-1 KO (db/db BKS)</td>
<td>Matrix accumulation</td>
<td>Reduces hyperglycemia, cholesterol and glomerular matrix fraction.</td>
<td>16</td>
</tr>
<tr>
<td>eNOS KO (db/db BKS)</td>
<td>Endothelial function and vascular tone</td>
<td>Reduces hypertension. Exacerbates glomerular and tubular damage, albuminuria, inflammation and kidney fibrosis. Reduces GFR.</td>
<td>63</td>
</tr>
<tr>
<td>SOD-Tg (db/db BL6)</td>
<td>Oxidative stress</td>
<td>Reduces oxidative stress, albuminuria and glomerular matrix. Increases GFR.</td>
<td>19</td>
</tr>
<tr>
<td>rCAT-Tg (db/db BL6)</td>
<td>Oxidative stress in PTC</td>
<td>Reduces oxidative stress, hypertension, albuminuria, tubular injury and interstitial fibrosis.</td>
<td>5</td>
</tr>
</tbody>
</table>

KO, knockout; OPN, osteopontin; PAI-1, plasminogen activator inhibitor-1; eNOS, endothelial nitric oxide synthase; Tg, transgenic; GFR, glomerular filtration rate; PTC, proximal tubular cells.
mice (3). These findings indicate that it is feasible to evaluate the effects of therapies on hypertension in \( \text{db/db} \) strains which develop elevated blood pressure. However, the considerable expense of radiotelemetry suggests that it would only be sensible to use this analytic technique in \( \text{db/db} \) mice when the effects of a therapy or genetic deficiency which is known to have antihypertensive effects are being explored.

A limitation of recent studies of diabetic nephropathy in \( \text{db/db} \) mice is that they are predominantly focused on glomerular damage and albuminuria as end points and rarely report the development of tubulointerstitial injury. This is generally due to the mild nature of the interstitial fibrosis in this model. It is hoped that future development and characterization of accelerated \( \text{db/db} \) models will enable the study of interstitial fibrosis, which is featured in advanced diabetic nephropathy in patients. The importance of this lies in the knowledge that the interstitial fibrosis score is a strong predictor of end-stage renal failure in biopsied diabetic patients.

In conclusion, the recent application of genetic modifications, uninephrectomy, and pharmacological inhibitors to \( \text{db/db} \) mice has provided valuable knowledge about the pathomechanisms of diabetic nephropathy. Modifications of this model are continuing to evolve, which may provide a greater resemblance to human disease and be used to identify effective new therapies.

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### DISCLOSURES

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