European Journal of Pharmacology 908 (2021) 174378

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Diabetic proximal tubulopathy: Can we mimic the disease for *in vitro* screening of SGLT inhibitors?

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ARTICLE INFO

Keywords:
Diabetic kidney disease
Hyperglycemia
Proximal tubule
SGLT inhibitors
In vitro models

ABSTRACT

Diabetic kidney disease (DKD) is the foremost cause of renal failure. While the glomeruli are severely affected in the course of the disease, the main determinant for disease progression is the tubulointerstitial compartment. DKD does not develop in the absence of hyperglycemia. Since the proximal tubule is the major player in glucose reabsorption, it has been widely studied as a therapeutic target for the development of new therapies. Currently, there are several proximal tubule cell lines available, being the human kidney-2 (HK-2) and human kidney clone-8 (HKC-8) cell lines the ones widely used for studying mechanisms of DKD. Studies in these models have pushed forward the understanding on how DKD unravels, however, these cell culture models possess limitations that hamper research, including lack of transporters and dedifferentiation. The sodium-glucose cotransporters (SGLT) are identified as key players in glucose reabsorption and pharmacological inhibitors have shown to be beneficial for the long-term clinical outcome in DKD. However, their mechanism of action has, as of yet, not been fully elucidated. To comprehend the protective effects of SGLT inhibitors, it is essential to understand the complete functional, structural, and molecular features of the disease, which until now have been difficult to recapitulate. This review addresses the molecular events of diabetic proximal tubulopathy. In addition, we evaluate the protective role of SGLT inhibitors in cardiovascular and renal outcomes, and provide an overview of various *in vitro* models mimicking diabetic proximal tubulopathy used so far. Finally, new insights on advanced *in vitro* systems to surpass past limitations are postulated.

1. Introduction

Diabetic kidney disease (DKD) is the foremost cause of kidney failure worldwide (Alicic et al., 2017). The rising numbers in DKD parallel the increasing prevalence of diabetes mellitus (DM) worldwide. Globally, diabetic patients are expected to reach 642 million cases by the year of 2040, of which people with Type 2 DM account for 90 % of the cases (Zheng et al., 2018). DKD is characterized by albuminuria and accompanied by decreased glomerular filtration rate (GFR) and often causes an array of serious complications as a result of disturbances in hemodynamic and metabolic homeostasis (Thomas et al., 2015).

The kidney contributes to glucose homeostasis through different processes, including gluconeogenesis and glucose reabsorption. (Mather and Pollock, 2011). Glucose is filtered in the glomerulus, and under normal conditions, glucose is completely reabsorbed by the proximal tubule epithelial cells (PTECs) followed by diffusion into the peritubular capillaries. This transepithelial reabsorption process is accomplished by glucose transporters, of which the sodium-glucose co-transporter 1 (SGLT1) and SGLT2 are responsible for the uptake of glucose through a Na^+ gradient across the apical membrane, while the glucose transporter 1 (GLUT1) and GLUT2 facilitate the transport across the basolateral membrane into the systemic circulation (Fig. 1) (Ghezzi et al., 2018).

Owing to the fact that SGLT2 is responsible for the majority of glucose reabsorption, it has been widely studied as a therapeutic target to treat patients with Type 2 Diabetes Mellitus (T2DM). In case of hyperglycemia an excess of glucose is filtered by the glomerulus which leads to an increased expression of SGLTs mediating its reabsorption (Vallon and Sharma, 2010). With this in mind, a class of drugs was developed inhibiting SGLTs, also known as gliflozins, that decrease the risk of hyperglycemia by reducing the renal tubular reabsorption of

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https://doi.org/10.1016/j.ejphar.2021.174378
Received 10 May 2021; Received in revised form 15 July 2021; Accepted 21 July 2021
Available online 22 July 2021

glucose (Hsia et al., 2017). Table 1 summarizes the SGLT inhibitors currently prescribed to DKD patients, while providing an overview of the therapeutic benefits and the risks associated with this therapy.

DKD does not develop in the absence of hyperglycemia. Hyperglycemia induces activation of several pathways, including the polyol pathway and the protein kinase C pathway that contribute to oxidative stress (Dunlop, 2000; Noh and King, 2007). Hyperglycemia also causes tubulointerstitial fibrosis and inflammation, and accumulation of extracellular matrix proteins, such as collagen IV, fibronectin, and laminin (Reidy et al., 2014). Identification of the mediators implicated in the above mentioned processes will provide more information on the underlying molecular mechanisms of DKD, aiding in the development of new targeted therapies.

To investigate the mechanisms behind DKD both animal models and cell culture systems can be used. The use of animal models allows researchers to study the physiological aspects of the disease, however, these models fail to control biological changes of renal cells in real-time, while conveying expensive costs and being time consuming (King, 2012). *In vivo* studies also pose ethical issues, but most importantly, these models do not accurately correlate to human systems as they are either resistant to develop DKD or do not recapitulate all the different stages of the disease (Giralt-Lopez et al., 2020). For example, it is known that most diabetic rodent models can mimic key features of advanced human DKD in glomeruli, but they usually do not develop the characteristic widespread tubular atrophy and interstitial fibrosis. Even in models in which the renin-angiotensin-aldosterone system (RAAS) is strongly activated, the severity of tubular atrophy and interstitial fibrosis is still less than seen in human DKD (He et al., 2019).

To overcome this problem, 2D culture systems can provide some answers concerning the cellular effects of glucose. However, these models lack the complex 3D tissue architecture that is important for the correct understanding of the different disease states found *in vivo* (Caddeo et al., 2017). Recent advances in the stem cell field (Bai et al., 2019; Rogal et al., 2019; Tsakmaki et al., 2020) and microphysiological systems (Petrosyan et al., 2019; Wang et al., 2017) have shown promising clues on how to prevent DKD from progressing to kidney failure, however, as of yet a perfect *in vitro* model capable of replicating the disease does not exist.

With regard to treatment options, the pharmacological inhibition of SGLT2, the major player in glucose reabsorption, has shown renoprotective benefits (Kawanami et al., 2017). The SGLT2 inhibitors have been extensively studied both alone or in combination with other diabetic medication, however it is still yet to know whether these benefits are primarily due to SGLT2 inhibition or if off-target effects also play a role. To understand the protective effects of current therapies, such as SGLT2 inhibitors, as well as other pharmacological treatments currently

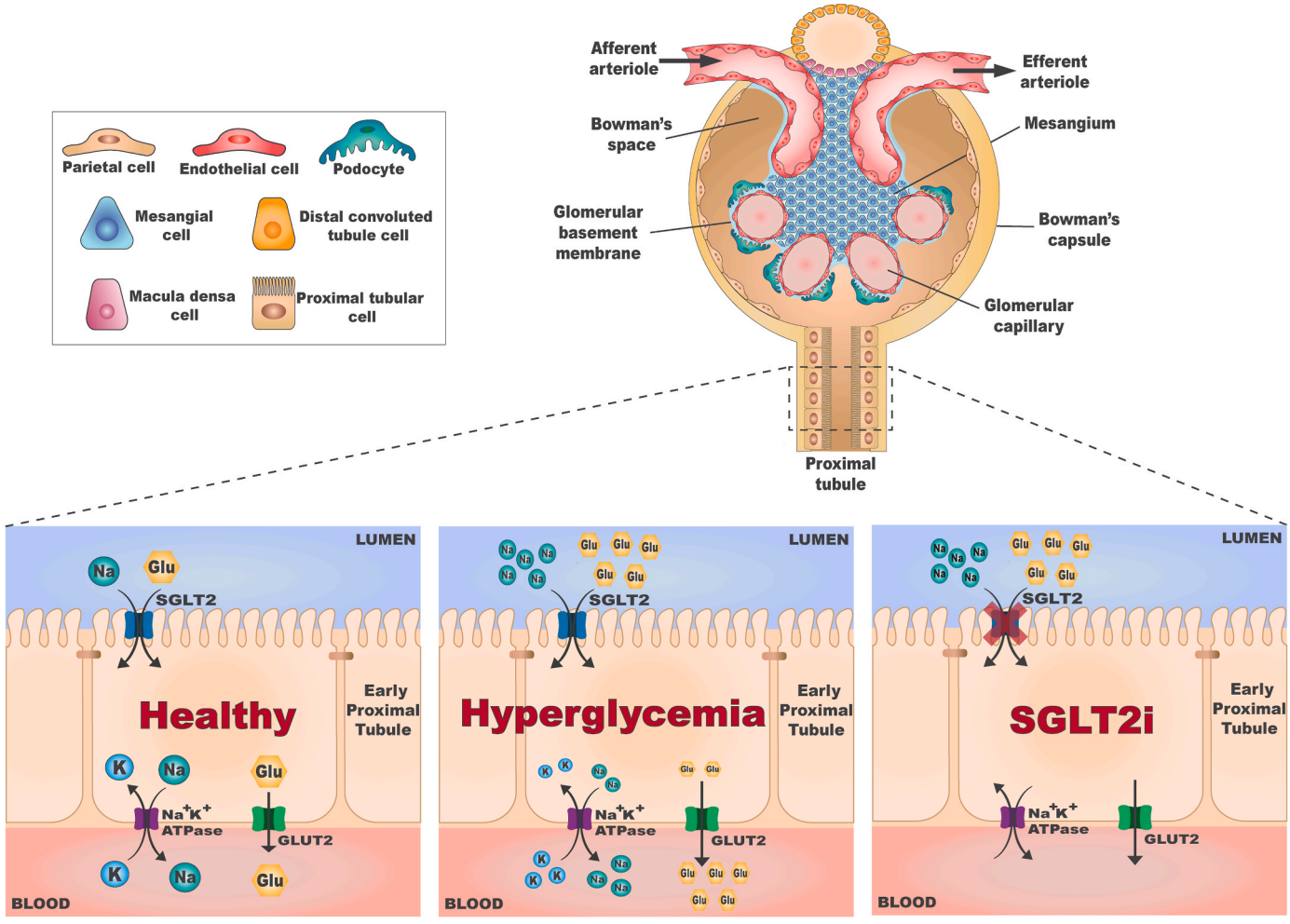

Fig. 1. Glucose handling in the kidney proximal tubule. The glomerulus filters glucose after which the proximal tubule is responsible for its reabsorption. In healthy conditions, SGLT2 reabsorbs roughly 90 % of glucose in co-transport with sodium. SGLT1 reabsorbs the residual 10 % in the late segments of the proximal tubule (not shown). In a hyperglycemic environment, elevated glucose filtration results in high glucose and sodium reabsorption. Increased tubular sodium reabsorption causes a decrease in sodium levels at the macula densa. This activates the tubuloglomerular feedback (TGF) mechanism, causing vasodilation of the afferent arteriole to elevate the glomerular pressure state. SGLT2 inhibitors reduce hyperfiltration via TGF, therefore minimizing the risks observed in DKD. Excessive glucose filtered, yet not reabsorbed, will cause glycosuria.

Table 1
Summary of the different SGLT inhibitors either commercially available or currently on clinical trials.

Drug	Mechanism of action	Human SGLT2 (IC_{50} nM)	Human SGLT1 (IC_{50} nM)	SGLT2 selectivity (fold)	Indication	Benefits	Side effects	References
Canagliflozin[a] (INVOKANA)	Selective SGLT2 inhibitor Mild intestinal and renal SGLT1 inhibition	4.4	684	155	T2DM	HbA1c reduction Reduced glomerular hyperfiltration via TGF	Hypoglycemia Dehydration	Liang et al. (2012)
Dapagliflozin[a] (FARXIGA)	Selective SGLT2 inhibitor	1.12	1391	1241	T2DM	Reduced albuminuria Reduction in plasma levels of uric acid	Urinary tract infections Ketoacidosis	Han et al. (2008)
Empagliflozin[a] (JARDIANCE)	Selective SGLT2 inhibitor	3.1	8300	2680	T2DM	Anti-inflammatory and anti-fibrotic properties		Grempler et al. (2012)
Tofogliflozin[b] (DEBERZA)	Selective SGLT2 inhibitor	2.9	8444	2912	T2DM	Reduction in natriuretic peptides		Ohtake et al. (2012)
Luseogliflozin[b] (LUSEFI)	Selective SGLT2 inhibitor	2.26	3990	1770	T2DM	Possible synergetic effect with anti-hypertensive drugs		Kakinuma et al. (2010)
Remogliflozin etabonate[c] (Remo™, Remozen™)	Selective SGLT2 inhibitor (prodrug)	12.4	4520	365	T2DM	Diuretic effect		Markham (2019)
Ertugliflozin[a] (STEGLATRO)	Selective SGLT2 inhibitor	0.9	1960	2178	T2DM			Mascitti et al. (2011)
Sotagliflozin[d] (ZYNQUISTA)	Dual SGLT1/2 inhibition	1.8	36	20	T1DM			Zambrowicz et al. (2012)

[a] FDA and EMA approved.
[b] Japan approved.
[c] India approved.
[d] EMA approved; T1/2DM Type 1/2 Diabetes Mellitus; Data retrieved from https://clinicaltrials.gov.

on market or possible future therapeutic candidates, a comprehensive study of the aetiopathogenesis of DKD is required.

In this review, we aim at providing readers an overview of what is known about the cellular and molecular pathophysiological mechanisms of diabetic proximal tubulopathy and the potential role of SGLT inhibitors. Using a literature search on the most relevant human proximal tubule *in vitro* models used to study the disease, we provide some recommendations for the requirements of a physiologically relevant *in vitro* model that allows studying DKD.

2. Pathophysiology of diabetic proximal tubulopathy

As mentioned before, the proximal tubule is a prime mover in the DKD pathogenic cascade. In diabetes, there is an excessive glucose flux which leads to an increased proximal tubular reabsorption of glucose via the sodium-glucose cotransporter 2 (SGLT2). This increased glucose reabsorption is dependent on the level of SGLT2 protein expression, as once a glucose transport maximum (T_m) is reached, there is a saturation of the transporter resulting in glycosuria (Poudel, 2013).

In case of hyperglycemia there is an increase in the formation of advanced glycation end products (AGEs) (Brownlee, 1995). Low molecular weight AGEs are filtered by the glomeruli and reabsorbed by the PTECs contributing to diabetic tubulointerstitial injury (Saito et al., 2005). The proximal tubule requires substantial amounts of energy to perform its role in waste removal and nutrient reabsorption. The active hyperreabsorption of sodium and glucose observed in the diabetic milieu is coupled with high levels of oxygen consumption, which may be accompanied by release of reactive oxygen species (ROS) by the mitochondrial electron transport chain (Forbes and Thorburn, 2018). Other major sources for ROS production include the accumulated AGEs (Yamagishi and Matsui, 2010), NADPH oxidase (NOX) and uncoupled nitric oxide synthase (NOS) (Sedeek et al., 2013).

Hyperglycemia has also been known to induce extracellular fluid volume depletion via glucose-induced osmotic diuresis. The volume depletion strongly stimulates the sympathetic nervous system that further promotes the activation of the RAAS, contributing to proteinuria and kidney disease progression (Thomas et al., 2015).

The understanding of how tubular (or tubulointerstitial) injury unravels will help researchers identify new targets for therapy along the way. A brief overview of the pathophysiological events of diabetic proximal tubulopathy is discussed below, whereas its underlying molecular events are shown in Fig. 2.

2.1. Oxidative stress and hypoxia

The balance between oxygen delivery and oxygen consumption (QO_2) is compromised in diabetic patients (Hansell et al., 2013) as the excessive reabsorption capability of the kidney involves a great demand for ATP production. However, the kidneys are not able to compensate for this inflation. The reduction in oxygen availability affects the cellular metabolism as it moves it towards a more glycolytic pathway that is less energy efficient, resulting in a lower ATP yield (Laustsen et al., 2014). During ATP generation, some electrons may escape from the respiratory chain and bind to oxygen to form ROS, such as superoxide radicals. ROS production occurs naturally via several cellular pathways; however, the elevated levels of ROS being generated in DKD surpass the local antioxidant capacity, causing an inflammatory cascade that ultimately leads to tubulointerstitial fibrosis (Forbes et al., 2008).

Besides oxidative stress, both tubular overload and increased QO_2 may cause intrarenal hypoxia (O'Neill et al., 2015). To counteract the effects of hypoxia, the hypoxia-inducible transcription factor (HIF) family promotes vasculogenesis via production of vascular endothelial growth factor (VEGF), causing an increase in oxygen delivery and alterations in cellular metabolism (Cerychova and Pavlinkova, 2018). HIFs are composed of two subunits: an α-subunit that is degraded in the presence of oxygen and a constitutive β-subunit. Due to a lack of oxygen, there is an accumulation of the α-subunit that, in turn, binds to the β-subunit, resulting in the transcription of hypoxia-responsive genes involved in cell regulatory mechanisms (*i.e.* angiogenesis, cell proliferation, etc.) (Catrina et al., 2004). The persistent hypoxia observed in

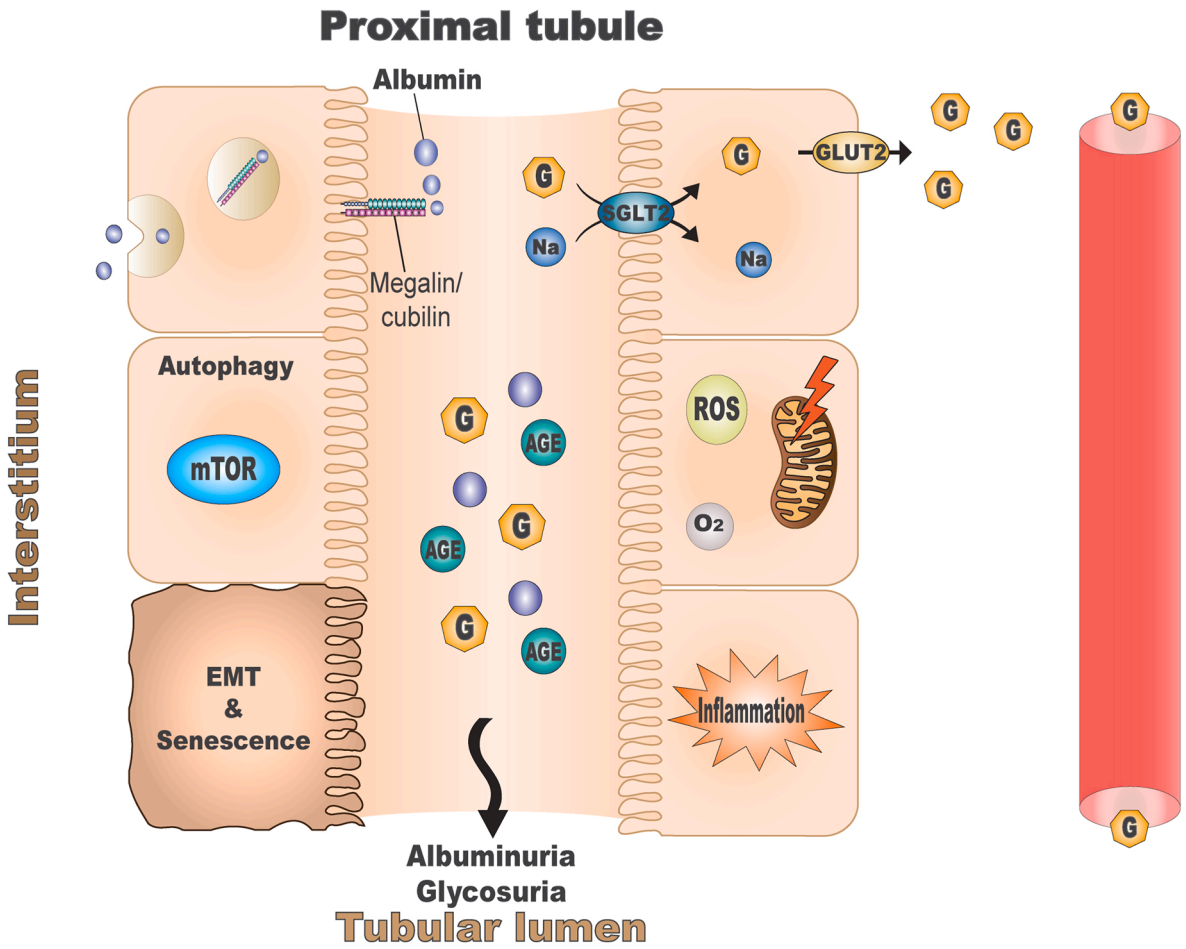

Fig. 2. Cellular and pathophysiological events in diabetic proximal tubulopathy. Damage to the glomerulus as a result of diabetes leads to impaired glomerular filtration barrier (GFB). Subsequently, excessive glucose (G), albumin, and advanced glycated end-products (AGEs) levels in the proximal tubule stimulate a process of inflammation, epithelial-mesenchymal transition (EMT) and senescence in the tubule cells. As a result of excessive tubular hyperreabsoprtion of glucose, elevated levels of reactive oxygen species (ROS) are produced together with an increase in oxygen (O2) consumption causing intrarenal hypoxia. Excessive albumin excretion due to damaged GFB cannot be fully reabsorbed by the apical megalin/cubilin complex, resulting in the secretion of pro-inflammatory cytokines by the proximal tubule epithelial cells (PTECs). The accumulation of glucose, albumin and AGEs contributes to secretion of pro-fibrotic mediators that in turn lead to fibrosis. Damaged PTECs lose their original phenotype, eventually start expressing α-SMA and transition into myofibroblasts. Autophagy is also disrupted by the accumulation of AGE, caused by the upregulation of mTOR in PTECs. Ultimately, these hyperglycemia-induced processes cause significant damage to the proximal tubule resulting in glycosuria and albuminuria, key markers of DKD.

DKD has been linked to low levels of HIF-1α expression, therefore inhibiting the activation of compensatory mechanisms to revert the state of hypoxia (Nordquist et al., 2015). The use of HIF stabilizers, such as prolyl hydroxylase inhibitors, have shown a renoprotective effect by altering diabetic renal metabolism in early stages of DKD (Hasegawa et al., 2020). For this reason, further investigation should be done throughout the different stages of the disease to really understand the renoprotective effect of these drugs.

2.2. Albuminuria

DKD is characterized by albuminuria due to a damaged glomerular filtration barrier and reduced tubular reabsorption (Dabla, 2010). Albuminuria is a predictor of DKD progression (Kim et al., 2013), therefore understanding its mechanism to prevent urinary protein leakage is of great interest. Filtered albumin is reabsorbed by PTECs via the apical megalin-cubilin complex, whereas the neonatal Fc receptor also plays a role in albumin transcytosis (Dickson et al., 2014). The increased tubular protein reabsorption is followed by an inflammatory cascade as PTECs start secreting an array of pro-inflammatory cytokines and chemokines, such as interleukins (IL)-8, IL-18, RANTES and monocyte chemotactic protein 1 (MCP1) (Vallon, 2011; Zoja et al., 1998). An increase in expression of adhesion molecules also occurs in the proximal tubule, including intercellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1). These adhesion molecules play a role in the initiation of renal inflammation (Navarro-Gonzalez et al., 2011). In addition, the NF-κB pathway has been associated as a regulator of the inflammatory pathomecanisms involved in protein tubular overload (Zoja et al., 2003). The proinflammatory response of PTECs also leads to the recruitment of macrophages and lymphocytes, sources of pro-fibrotic proteins such as transforming growth factor-beta (TGF-β), stimulating tubulointerstitial fibrosis progression (Eddy, 2001). It is still to be fully understood if albuminuria is either a cause or consequence of DKD progression as there is some debate on whether it is the albumin alone or the combination with other compounds, such as fatty acids, that actually lead to cytotoxic effects (Long et al., 2020).

With regard to treatments, patients diagnosed with albuminuria receive anti-hypertensive treatment, such as angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARBs), with the goal to block RAS, thus reducing hyperfiltration and consequently lowering urinary albumin levels (Basi et al., 2008).

2.3. Inflammation

The inflammatory response in the tubular compartment plays a role in disease progression (Matoba et al., 2019). The role of toll-like receptors (TLRs), mediators in the production of inflammatory cytokines and chemokines, have been linked to metabolic syndrome, such as hyperglycemia, with an emphasis for TLR2 and TLR4. Under diabetic conditions, cultured PTECs have shown to increase TLR4 expression via a PKC-dependent pathway, resulting in the upregulation of pro-inflammatory mediators (Dasu et al., 2010). Inflammasomes, together with TLRs, are key components of the innate immune system. In particular the NOD-like receptor protein 3 (NLRP3) inflammasome has been shown to play a role in DKD progression, a complex involved in mitochondrial disfunction (Han et al., 2018), albuminuria (Fang et al., 2013), and renal fibrosis (Song et al., 2018).

Another important mediator in the inflammatory cascade is the activation of the NF-κB pathway, activated upon different stimuli, including oxidative stress, hyperglycemia, AGEs, and albuminuria (Navarro-Gonzalez et al., 2011; Perez-Morales et al., 2019). Among cytokines, tumor necrosis factor (TNF)-α is a major driver of inflammation, therefore making it an ideal target for therapeutic intervention (Navarro and Mora-Fernandez, 2006). Studies have shown that pentoxifylline (PTF), inhibitor of TNF-α, has anti-inflammatory and anti-proteinuric effects (Garcia et al., 2015; Navarro et al., 2006). In addition, the combination of PTF and RAAS blockers led to a significant reduction in protein excretion (Harmankaya et al., 2003; Navarro et al., 2005). *In vitro*, hyperglycemia has also shown to increase the expression of tubular kallikrein 1 (KLK1) and bradykinin, mediators of the kallikrein-kinin system, resulting in tubular inflammation (Tang et al., 2010).

2.4. EMT and cellular senescence

Tubulointerstitial fibrosis is predominantly found in the diabetic kidney. PTECs' response to high glucose and albuminuria includes the secretion of TGF-β and extracellular matrix components (Diwakar et al., 2007; Stephan et al., 2004). TGF-β is the primary mediator of fibrogenesis as it activates the Smad pathway, specifically TGF-β1 activates Smad3 leading to fibrosis (Zhao et al., 2020). Epithelial-to-mesenchymal transition (EMT) is considered an initiating factor that triggers the development and progression of tubulointerstitial fibrosis. In DKD, EMT is mainly stimulated through the accumulation of AGEs (Zhao et al., 2014), hyperlipidemia (Toyama et al., 2014), and proteinuria (Wang et al., 1999). During this process, PTECs start losing their original phenotype as (1) cell polarity disappears, (2) cell-cell interaction is broken due to the disruption of tight junctions, (3) the basement membrane is slowly destroyed leading to the migration of these cells into the interstitium, and (4) PTECs start expressing α-smooth muscle actin (α-SMA), resulting in their transition into myofibroblasts (Loeffler and Wolf, 2015). The same stimuli that trigger EMT also promote the activation of several pathways, such as the JAK/STAT pathway (Liu et al., 2014), PKC pathway (Meier et al., 2007), MAPK pathway (Rane et al., 2010), Notch (Sirin and Susztak, 2012) and Wnt/β-Catenin (Mu et al., 2013) pathways, resulting in structural and functional changes in PTECs that aggravate the progression of renal fibrosis. Several microRNAs (miRNAs) have also been identified as promoters of TGF-β1/Smad3 activation, of which miR-21 (Chau et al., 2012) and miR-192 (Krupa et al., 2010) show the most aberrant effects on fibrogenesis progression, serving as potential targets in anti-fibrotic therapies. Hyperglycemia has also been linked to an increase in cellular senescence in PTECs, a process concerning the loss of regenerative capacity of cells in response to stress. This process is involved in ageing and metabolic diseases, such as diabetes (Palmer et al., 2015). The secretion of pro-fibrotic mediators during the course of DKD due to cellular senescence can lead to persistent fibrosis, which, when combined with other pathological processes, can lead to disease progression (Xiong and Zhou, 2019). For this reason, targeting senescent PTECs can provide new opportunities for the treatment of DKD.

2.5. Nutrient-sensing pathways

In DKD, the autophagy machinery is disrupted leading to abnormalities of several nutrient-sensing pathways, such as the mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), and sirtuins (SIRT) (Cetrullo et al., 2015). Type 2 diabetes is characterized by suppression of both AMPK and SIRT1, whereas mTOR is upregulated which causes autophagy flux to decrease in PTECs (Yang et al., 2018).

Autophagy upregulates lysosomal activity in PTECs upon AGE exposure leading to its degradation. However, the accumulation of AGEs observed in the diabetic kidney reduces lysosomal function due to the stagnation of autophagy (Liu et al., 2015). In addition, the transcription factor EB (TFEB), known mediator of the mTOR pathway, is downregulated upon AGEs overload leading to impaired autophagic activity in PTECs (Zhang et al., 2020). Similarly to rapamycin-mediated inhibition of mTOR, which can reverse the impaired autophagy, targeting TFEB can present a therapeutic strategy to activate autophagy.

3. SGLT inhibitors

The proximal tubule is accountable for the reabsorption of filtered glucose via SGLT1 and SGLT2, the latter being responsible for the majority of glucose reabsorption (Ghezzi et al., 2018). SGLTs are therefore perfect targets for anti-hyperglycemic therapies. With the goal to inhibit this hyperreabsorption of glucose, inhibitors of SGLT1 and SGLT2 have been developed (Table 1). At first, the O-glucoside phlorizin, a nonselective SGLT1/2 inhibitor, showed promising results as it normalized plasma glucose levels in diabetic rat models. These studies showed that phlorizin not only leads to glycosuria but also blocks intestinal glucose absorption, resulting in intestinal malabsorption complications (Ehrenkranz et al., 2005). This major drawback led to the development of a new class of glucose-lowering drugs, C-glucosides, that proved to be selective for SGLT2 with longer half-lifes compared to phlorizin (Isaji, 2011). The advantages of SGLT2 inhibition go beyond lowering glucose plasma levels, such as reduction in urinary albumin excretion (Cherney et al., 2017), blood pressure control (Mazidi et al., 2017), and diuretic effect (Ansary et al., 2019). The main disadvantage of this therapy is that it leads to glycosuria, therewith increasing the risk of urinary tract infections (Table 1) (Liu et al., 2017). Although very uncommon, SGLT2-induced glycosuria can cause euglycemic ketoacidosis due to reduced glucose availability and reduced insulin-to-glucagon ratio, enhancing lipolysis and lipid oxidation (Rosenstock and Ferrannini, 2015).

In terms of beneficial effects, SGLT2 inhibition also leads to improved cardiovascular outcomes. The protective effects of SGLT2 inhibition extends to different patient groups, whether diabetes is present or not. The DAPA-CKD trial (Heerspink et al., 2020) with dapagliflozin has shown that chronic kidney disease patients were associated with lower risk for cardiovascular death and heart failure, independently of the presence or absence of type 2 diabetes. In the EMPEROR-Reduced trial (Packer et al., 2020) with empagliflozin, the same therapeutic profile was confirmed. In addition, a sub-study of the latter trial has concluded that empagliflozin improved heart failure outcome regardless of CKD status (Santos-Gallego and Van Spall, 2021). Further studies in the clinical trials EMPA-TROPISM (Santos-Gallego et al., 2021) and EMBRACE-HF (Nassif et al., 2021), have shown evidence that empagliflozin may lead to reverse left ventricular remodelling, which could explain the cardiovascular benefits of empagliflozin seen in both diabetic and nondiabetic patients. Overall, these results demonstrate a broader therapeutic effect of SGLT2 inhibition, that not only proves to be beneficial in terms of cardiovascular outcomes but also to the therapeutic management of chronic kidney disease.

From a physiological point of view, SGLT2 inhibition causes an increase in the delivery of sodium to the macula densa, in turn, restoring the tubuloglomerular feedback and reducing hyperfiltration (Fioretto et al., 2016). By reducing hyperfiltration, the processes of oxidative stress, albuminuria, inflammation, EMT, cellular senescence, and eventually fibrosis are also minimized. On the molecular side, the protective effects of SGLT2 inhibition include increased antioxidant capacity via reduction of ROS production (Yaribeygi et al., 2018), anti-inflammatory and anti-fibrotic response due to a decreased expression of TLR2/4 and TGF-β, respectively (Heerspink et al., 2019). A possible role in RAAS activation is still under debate (Yaribeygi et al., 2019). In the heart, SGLT2 inhibition has been reported inhibit the Na^+/H^+ exchanger 1 (NHE1), a transporter involved in regulation of $[Na^+]$ and $[Ca2^+]$ intracellular levels, thus explaining a reduced risk in heart failure (Uthman et al., 2018). However, this hypothesis has been proven wrong in a new study (Chung et al., 2020) suggesting that the therapeutic effects of SGLT2 inhibitors are, as of yet, not fully understood.

Due to immense research done over the past years on SGLT2 inhibition, and having in mind that inhibition of SGLT2 leads to an upregulation of SGLT1 to limit glycosuria, new insights have been gained on the role of SGLT1 in the control of the tubuloglomerular feedback and glomerular filtration rate (Song et al., 2019; Zhang et al., 2019). In addition, a dual SGLT inhibition, sotagliflozin (Table 1), currently under phase 3 studies has shown similar advantages as, already available, SGLT2 inhibitors while providing delayed glucose absorption in the intestine (Cefalo et al., 2019). The SOLOIST-WHF trial (Bhatt et al., 2021) with sotagliflozin has concluded that this therapy is also associated with cardiorenal protective effects, including a decreased incidence in cardiovascular mortality and hospitalization for heart failure.

4. Can we replicate the disease *in vitro*?

To date, several *in vitro* models have been used to study the underlying mechanisms of proximal tubulopathy, both in 2D and in 3D. While 2D models have enabled great advances on the understanding of this pathology based on the cellular effects of high glucose, they fail to represent the *in vivo* state as single cell lines or primary cells are used, and therefore do not show the interplay between the proximal tubule

Table 2
Overview of the most prominent *in vitro* models capable of replicating features of diabetic proximal tubulopathy.

Model	Transporters/ Receptors	Pathways	Cons	References
HK-2	SGLT2 Na^+/K^+ ATPase AT1R, AT2R TGF-β receptor CTGF RAGE Megalin/cubilin complex HIF-1α LC3-I, LC3-II TLR2/4 β-galactosidase activity	AGE-RAGE EMT AMPK/ mTOR p38 MAPK HIF-1α-HRE TLR/NF-κB Polyol pathway PKC pathway	Absence of drug transporters (SLC22 family) Immortalization process	(Chen et al., 2018; Garcia-Pastor et al., 2019; Guo et al., 2016; Huang et al., 2019; Jiang et al., 2020; Kroening et al., 2009; Morigi et al., 2002; Sakai et al., 2019; Slattery et al., 2013; Valdes et al., 2020; Visavadiya et al., 2011; Zhu et al., 2019)
HKC8	SGLT2 Na^+/K^+ ATPase Megalin/cubilin complex LC3-II TGF-β CTGF AGER1 RAGE RANTES	AGE-RAGE EMT AMPK/ mTOR NF-κB/ RANTES PKC pathway	Absence of drug transporters (SLC22 family) Immortalization process	(Coffey et al., 2015; Diwakar et al., 2007; Feng et al., 2020; Kroening et al., 2009; Lee et al., 2017, 2019; Li et al., 2012; Racusen et al., 1997; Storch et al., 2017)
RPTEC/ TERT1	SGLT2 Na^+/K^+ ATPase Megalin/cubilin complex TGF-β SMAD3 HIF-1α	TGF-β/SMAD3 EMT AMPK/ mTOR NF-κB LPS/TLR4	Absence of AT1R	(Aschauer et al., 2013; Islam et al., 2019; Kim et al., 2019; Sallustio et al., 2019; Wieser et al., 2008; Wilmes et al., 2015; Zhou et al., 2018)
ciPTEC	Na^+/K^+ ATPase Megalin/Cubilin complex SLC22 family HIF-1α TLR4 NLRP3	LPS/TLR4 AMPK/ mTOR EMT	Absence of SGLT2	(Di Mise et al., 2018; Jansen et al., 2016; Mutsaers et al., 2015; Peters et al., 2015; Sun et al., 2020; Vriend et al., 2020; Wilmer et al., 2010)

AGER1, advanced glycation end product receptor-1; AMPK, AMP-activated kinase; AT1R, angiotensin II receptor type 1; ciPTEC, conditionally immortalized proximal tubular epithelial cell line; CTGF, connective tissue growth factor; EMT, epithelial-mesenchymal transition; HIF-1a, hypoxia-inducible factor 1-alpha; HK-2, human kidney 2; HKC8, human kidney clone-8; HRE, hypoxia response element; LC3, microtubule-associated proteins 1A/1B light chain 3; mTOR, mammalian target of rapamycin; NF-kB, nuclear factor-kB; NLRP3, NOD-like receptor protein 3; PKC, protein kinase C; RAGE, receptor for advanced glycation end products; RANTES, regulated on activation, normal T cell expressed and secreted; RPTEC/TERT1, renal proximal tubule epithelial cell/TERT1 immortalized; SGLT2, sodium/glucose cotransporter 2; SLC22, organic solute carrier 22 family; SMAD3, SMAD family member 3; TGF-b, transforming growth factor beta; TLR, toll-like receptor.

and the peritubular capillaries. The interaction between these two compartments is key to understand such a complex disorder, as peritubular capillary rarefaction is also correlated with DKD and decline in kidney function (Afsar et al., 2018). Different PTEC lines are presently available due to the importance of this nephron's segment for multiple studies. Table 2 summarizes the characteristics of relevant PTEC *in vitro* models, while providing an overview of DKD-related pathways studied over time in these models.

Adopting the most suitable model is vital as some models are not ideal for studying DKD. In addition, a combination rather than single exposures of diabetic mediators such as high glucose, TGF-β, AGE-albumin, hypoxia and angiotensin II will provide a better understanding of the disease (Slyne et al., 2015).

Based on what it was described earlier in this review regarding the pathophysiology of diabetic proximal tubulopathy, an ideal PTEC *in vitro* model should include 1) glucose transporters (SGLTs and GLUTs), 2) a functional megalin-cubilin protein reabsorption complex, 3) interaction between AGEs and its receptor RAGE, 4) angiotensin II receptor, 5) capability to undergo EMT and cellular senescence, and 6) susceptibility to hypoxia. The HK-2 cell line (Table 2) has been widely used as it expresses the glucose transporters, however, to investigate albumin reabsorption, specific culture conditions need to be met, as the endocytosis receptors, megalin and cubilin, appear not present (Slattery et al., 2013). When trying to recreate the hypoxic environment found *in vivo*, researchers found that the combination of high glucose and hypoxia (1 % O_2) led to an impaired HIF-1α stabilization as seen in the diabetic kidney (Valdes et al., 2020). This model, however, also lacks several relevant PTEC transporters involved in toxin removal (Mutsaers et al., 2011), and due to its oncogene-mediated immortalization, the cell line may be more resistant to PTEC toxicants than other more relevant cell lines. Although not as widely used, the HKC-8 cell line (Table 2) is another PTEC model possessing comparable biochemical properties as the HK-2 cell line, such as glucose transport, abrogated by phlorizin (Racusen et al., 1997). HKC-8 cells have also shown impairment of autophagy and mitochondrial dysfunction upon exposure to high glucose, both reverted by treatment with SGLT2 inhibitors and an activator of the AMPK pathway, respectively (Lee et al., 2017, 2019). A recent study by Khundmiri et al., in which several human and non-human cell lines' transcriptome were compared to native tissue, demonstrated a higher similarity of HK-2 cells to human kidney than HKC8 cells (Khundmiri et al., 2021). As this study was based on cells cultured in their standard media, it is still possible that upon exposure to different conditions, such as hyperglycemia, the transcriptomic profile of these cell lines differ. Although possessing key features of the native proximal tubule, the previously mentioned cell lines were immortalized using methods that can alter their characteristics over time. Immortalization using the human telomerase reverse transcriptase has since been employed as the resulting cell lines do not undergo senescence and still retain features of primary cells even at high passages (Wang et al., 2019). Examples of such cell lines include the RPTEC/TERT1 (Wieser et al., 2008) and the conditionally immortalized PTEC (ciPTEC) (Wilmer et al., 2010), both closely resembling the native proximal tubule. The latter does not express the glucose transporters (Table 2) important for studying DKD, however, similar to RPTEC/-TERT1, the remaining DKD pathways can still be extensively studied.

To highlight that for recreating the disease *in vitro*, researchers should be aware that media composition can influence the outcome of the study. Immortalized cell lines tend to have a higher metabolic rate when compared to primary cells, which reflects the high glucose concentration found in most commercially available cell culture media. Thus, lowering glucose levels in the media before exposure to DKD mediators is imperative to obtain clinically relevant results. The same issue arises from using fetal bovine/calf serum as it alters cell growth and functional behavior (Gstraunthaler, 2003).

Not only chemical methods, such as exposure to DKD mediators, but genetic methods can also be employed to study the pathophysiology of the disease. To this end, induced pluripotent stem cells (iPSCs) can be genetically modified via CRISPR-Cas9 technology to be more susceptible to developing the disease (Hurtado Del Pozo et al., 2018). With respect to 3D models, kidney organoids and iPSC-derived organoids have recently been highlighted for their potential use in diabetes research, however these models still resemble both genetically and structurally kidneys in fetal stages of development which hampers their use. Another disadvantage of organoids includes their heterogenous population that can detract from toxic effects, and due to their encapsulation in hydrogels, longer incubation times to DKD mediators may be required when compared to 2D culture systems (Tsakmaki et al., 2020).

Moving towards more advanced *in vitro* models where multiple cell types and the 3D architecture are included can definitely improve the knowledge acquired so far. Organ-on-chip (OoC) is the perfect example of such desirable model in which different cell types can be combined in a 3D microenvironment that most likely resembles the *in vivo* state (Wu et al., 2020). Different OoC models have been designed to study the reabsorption and secretion capacities of the proximal tubule (Homan et al., 2016; Jang et al., 2013; Jang and Suh, 2010; Lin et al., 2019; Vedula et al., 2017). Overall, these models successfully showed the possibility to recapitulate both function and structure of the proximal tubule *in vitro* while demonstrating epithelium-endothelium crosstalk via exposure to high glucose, however no efforts to mimic more complex molecular mechanisms involved in diabetic proximal tubulopathy were made. While OoC models are paving the way towards deeper understanding of such complex diseases, they face several challenges including standardization, technical problems (e.g., leak tight seals and robust connectors) as well as finding the appropriate culture medium to grow the different cell types (Rogal et al., 2017).

The development of *in vitro* models to study DKD has improved in recent years. A variety of *in vitro* models, each with its own advantages and disadvantages is currently available (Table 2). Whereas culture models, such as the HK-2 cell line, have provided valuable data on the effects of diabetic mediators, this model lacks important transporters that are required to understand the therapeutic effects of certain drugs. Empagliflozin, an SGLT2 inhibitor, is excreted into urine via the organic anion transporter 3 (OAT3) (Fu et al., 2018). The study of the glucosuric effect of Empagliflozin in the HK-2 cell line is not possible due to the lack of OAT transporters (Jenkinson et al., 2012), therefore limiting the use of this model for drug screening. Replacing this model with others better equipped with drug transporters, such as RPTEC/TERT1, can improve the quality and reliability of the results. Note that a full characterization of transporter expression in human-derived proximal tubule cell lines is of great need to understand the full potential of these *in vitro* models for the study of DKD (Khundmiri et al., 2021).

Not only replacing with better *in vitro* models, but also improving their functionality by increasing complexity with bioengineering approaches, such as co-cultures and pulsatile flow on-chip systems can lead to greater progress in research. With regard to co-culture models, the RPTEC/TERT1 cell line has been used to study the therapeutic effects of mesenchymal stromal cells as a treatment option for DKD (Islam et al., 2019), however with regard to vascularization and cell to cell interactions to study the different outcomes of the disease, such as inflammation, oxidative stress, and EMT, no co-culture systems have been applied so far. A kidney-on-chip model comprised of the proximal tubule (RPTEC/TERT1) and a vascular compartment has been designed and proved to show a functional tubular-vascular exchange, including glucose transport abolished by a SGLT2 inhibitor (Lin et al., 2019). This shows great promise for studying DKD, in which a model that closely resembles the *in vivo* situation can be tuned to respond to external stimuli, and possibly connected to microsensors that will allow a close look at the cellular changes in the environment (Kieninger et al., 2018). Given the growing list of candidate genes for DKD (Tziastoudi et al., 2020), engineered *in vitro* disease models may also help boost the knowledge on disease progression and treatment.

5. Conclusion and future perspectives

The impact of DKD in global health is a burden that weighs great responsibility on researchers into trying to find new approaches to prevent and treat the disease. Even though the complex clinical picture of DKD cannot be replicated by the use of a single nephron segment, attempts have been made to study the individual parts involved in the progression of the disease. Early changes in the diabetic kidney are mainly observed in the glomerulus and the proximal tubule, however, due to the important role of the latter in glucose reabsorption, more attention should be directed towards this nephron segment. Anti-diabetic drugs, such as the recently emerged SGLT2 inhibitors, have provided great benefits to diabetic patients, nevertheless the protective effects of these drugs are not fully understood due to very complex pathology of DKD.

To aid the understanding of how changes in the kidney proximal tubule lead to DKD progression, an *in vitro* model is crucial. High glucose exposure is key to mimic DKD; however, a cocktail of relevant disease-related mediators is crucial to properly understand its pathophysiology. A robust *in vitro model* not only has to show a consistent response to stimuli but it is also expected to respond to currently available treatments in a clinically relevant manner. The response to different exposure times should be further studied since acquisition of specific DKD markers may be transient in the early stages. Whereas studying glucose transport is important, further studies on the functionality of PTEC transporters should not be overlooked. The development of engineered *in vitro* disease models can also provide deeper insights on how the disease is unravelled. With regard to kidney organoids and iPSC-derived organoids, efforts to improve the maturity and relevance of these models is essential.

Overall, no reliable *in vitro* model to study DKD is currently available due to the complexity of the organ and its multiple structures and cell types, all playing a role in the development of diabetic complications. Choosing the 'right' model depends on the specific research question, however that model should consist of different cell types equipped with appropriate transporters and receptors to study specific mechanisms while being in a dynamically controlled microenvironment that closely resembles the *in vivo* state. All together, these features will provide a cheaper and more biologically alternative to animals to investigate the underlying molecular mechanisms of the disease and to identify potential drug candidates relieving DKD symptoms.

Author contribution

JF, KGFG, SMM and RM devised the main conceptual ideas, JF wrote the initial draft of the manuscript and prepared the figures. KGFG, TN, SMM and RM reviewed and edited the manuscript. KGFG, SMM and RM supervised the study.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This study has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant agreement no. 813839.

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