Prostaglandin E2 Signaling Through Kidney EP1 and EP4 Receptors; Implications in Diabetes and Hypertension

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Faculty of Graduate and Postdoctoral Studies
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Chronic kidney disease is defined as the appearance of kidney functional or structural injury. Cyclooxygenase and prostaglandin E2 have been implicated in the pathogenesis of diabetic nephropathy, the leading cause of chronic kidney disease. Beneficial in certain settings, inhibition of the cyclooxygenase pathway can however be detrimental in patients with compromised cardiac or renal function. Moreover, the quest for new therapies to treat diabetic nephropathy is hampered by the lack of appropriate rodent models. This doctoral thesis is a culmination of three studies, the first to determine the role of the prostaglandin E2 EP1 receptor in diabetic nephropathy, the second to elucidate the vascular prostaglandin E2 EP4 receptor's role in hypertension and lastly to establish and characterise a novel mouse model of diabetic nephropathy. The goal being to uncover new therapeutic avenues for the treatment of CKD, its causes and/or complications.

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Authorizations and author contributions

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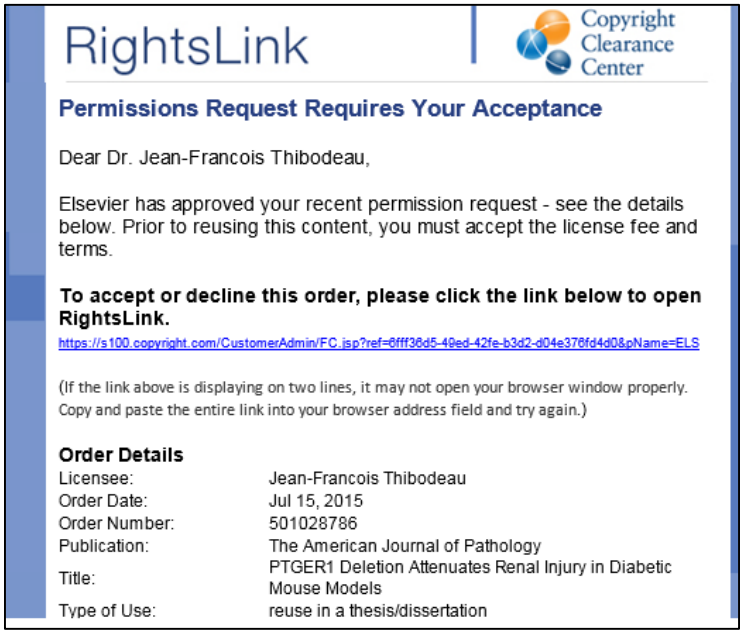
Authorization



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Jean-François Thibodeau	▪ Planned, performed and analyzed majority of experiments apart from those listed below ▪ Writing of manuscript
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Greg Cron	▪ MRI imaging and analysis for renal blood flow
Anthony Carter	▪ Surgical procedures for MRI imaging for renal blood flow
Alex Gutsol	▪ Kidney sections IHC staining
Ying He	▪ Mesenteric wire-myography experiments
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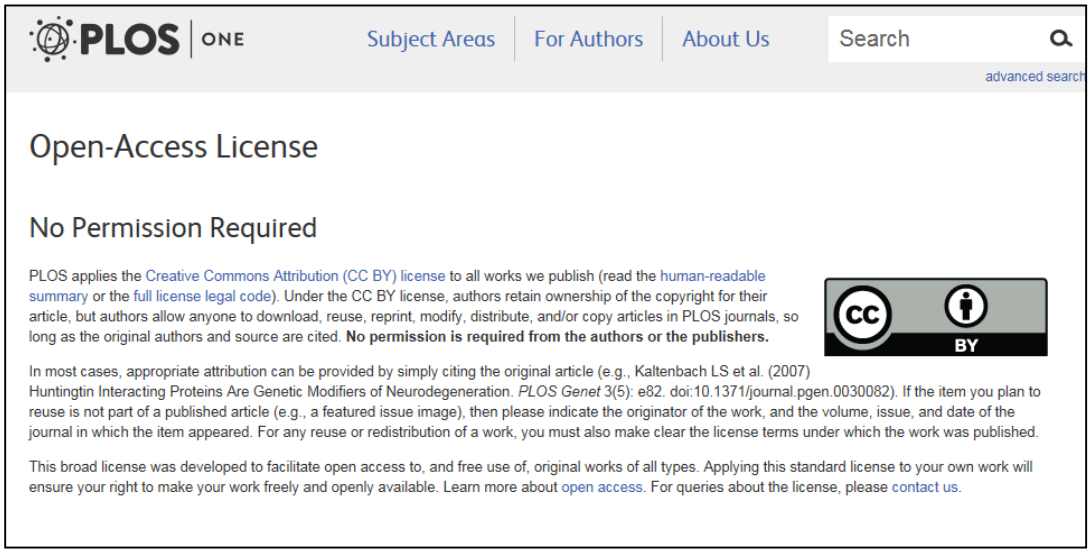

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Jean-François Thibodeau	▪ Mouse breeding, intercrossing and genotyping ▪ Planned, performed and analyzed majority of experiments apart from those listed below ▪ Writing of manuscript
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Dylan Burger	▪ Model characterization ▪ Analysis of physiological parameters
Naomi C Read	▪ Performed western blotting experiments
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Christopher RJ Kennedy	▪ Principal investigator, intellectual support, manuscript revision

List of journal articles (2009-2015)

1. R. Nasrallah, R. Hassouneh, J. Zimpelmann, A.J. Karam, **J.-F. Thibodeau**, D. Burger, K.D. Burns, C.R.J. Kennedy, and R.L. Hébert, *Prostaglandin E_2 increases proximal tubule fluid reabsorption, and modulates cultured proximal tubule cell responses via EP1 and EP4 receptors*. Lab. Invest., 2015 Jun 29. doi: 10.1038/labinvest.2015.79. *(in press)*

2. **J.-F. Thibodeau**, C.E. Holterman, G. Cron, A. Carter, A. Gutsol, Y. He, C.R.J. Kennedy, *Vascular smooth muscle cell specific EP4 deletion in mice exacerbates angiotensin II induced renal injury*. *submitted manuscript: Kidney International (August 2015).*

3. C.E. Holterman, J.-F. Thibodeau, and C.R.J. Kennedy, *NADPH oxidase 5 and renal disease.* (Review) Curr. Opin. Nephrol. Hypertens., 2014. 24(1): p. 81-7.

4. **J.-F. Thibodeau**, C.E. Holterman, D. Burger, N.C. Read, T.L. Reudelhuber, and C.R.J. Kennedy, *A novel mouse model of advanced diabetic kidney disease.* PLoS One, 2014. 9(12): p. e113459.

5. D. Burger, **J.-F. Thibodeau**, C.E. Holterman, K.D. Burns, R.M. Touyz, and C.R.J. Kennedy, *Urinary podocyte microparticles identify prealbuminuric diabetic glomerular injury.* J. Am. Soc. Nephrol., 2014. 25(7): p. 1401-7.

6. **J.-F. Thibodeau**, R. Nasrallah, A. Carter, Y. He, R. Touyz, R.L. Hebert, and C.R.J Kennedy, *PTGER1 deletion attenuates renal injury in diabetic mouse models.* Am. J. Pathol., 2013. 183(6): p. 1789-802.

7. C.E. Holterman, **J.-F. Thibodeau**, C. Towaij, A. Gutsol, A.C. Montezano, R.J. Parks, M.E. Cooper, R.M. Touyz, and C.R.J. Kennedy, *Nephropathy and elevated BP in mice with podocyte-specific NADPH oxidase 5 expression.* J. Am. Soc. Nephrol., 2013. 25(4): p. 784-97.

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List of abstracts (2009-2015)

1. Cron GO, **Thibodeau JF**, Melkus G, Carter A, Cameron IA, Schieda N, Shabana W, Kennedy CRJ. *Patients with high blood pressure should avoid aspirin: reduced renal perfusion in hypertensive EP4 knockout mice.* Int. Soc. for Magn. Res. In Med., 23rd Annual meeting. Toronto, Ont. 30 May- 5 June, 2015.

2. **Thibodeau JF**, Holterman CE, Carter A, Cron G, Gutsol A, He Y, Kennedy CRJ. *Vascular-specific EP4 receptor deletion in mice predisposes to angiotensin II induced renal injury.* Can. Soc. of Neph. 47th Annual meeting, 2015.

3. Croteau E, **Thibodeau JF**, Ismail B, Hadizad T, Renaud JM, Beanlands Rob, Hébert RLH, DaSilva J, Kennedy CR, deKemp R. *Reduced [^{18}F]FPyKYNE-losartan uptake confirms impaired renal AT_1 receptors in type 1 diabetic (OVE26) mouse.* Soc. Nucl. Med. 2015. (Oral abstract presentation by E. Croteau)

4. **Thibodeau JF**, Holterman CE, Carter A, Cron G, Kennedy CRJ. *Vascular-specific EP4 receptor deletion increases angiotensin II-induced renal injury through decreased glomerular filtration rate, increased albuminuria and kidney fibrosis.* Am. Soc. Neph. Kidney Week 2014. (Oral abstract presentation by JF Thibodeau)

5. Ismail B, Arksey N, Hadizad T, Croteau E, **Thibodeau JF**, Kennedy CRJ, Hébert RLH, Beanlands RS, deKemp RA and Da Silva JN. *Preliminary evaluation of [^{18}F] FPyKYNE-Losartan as a novel PET tracer for imaging kidney AT1 receptors in rats.* Soc. Nucl. Med., 2014. (Poster presentation by B. Ismail)

6. **Thibodeau JF**, Burger D, Holterman CE, Burns K, Touyz R, Kennedy CRJ. *A novel model of advanced diabetic kidney disease in mice.* Am. Soc. Neph. Kidney Week, 2013. (Poster presentation by JF Thibodeau)

7. Burger D, **Thibodeau JF**, Holterman CE, Burns KD, Kennedy CRJ. *Podocyte microparticle formation is increased in diabetic kidney disease.* Can. Soc. of Neph. 46th Annual meeting, 2014. (Poster presentation by D. Burger)

8. Burger D, **Thibodeau JF**, Holterman CE, Burns KD, Kennedy CRJ. *Podocyte ectosome formation is increased in diabetic kidney disease.* International Society for Extracellular Vesicles. 2013. (Oral presentation by D. Burger)

9. **Thibodeau JF**, Carter A, Kennedy CRJ. *The Prostaglandin E2 EP1 receptor promotes glomerular and tubular dysfunction in diabetic mice.* Am. Soc. Neph. Kidney Week, 2012. (Poster presentation by JF Thibodeau)

10. **Thibodeau JF**, Carter A, Kennedy CRJ. *Prostaglandin E2 EP1 receptor and its role in the development of diabetic albuminuria.* Am. Soc. Neph. Kidney Week, 2011. (Poster presentation by JF Thibodeau)

11. **Thibodeau JF**, Kennedy CRJ. *EP1 deletion in mice; implications for diabetic nephropathy.* Am. Soc. Neph. Kidney Week, 2010. (Poster presentation by JF Thibodeau.)

List of awarded internal and external scholarships

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List of abbreviations (alphabetical)

ACCORD	Action to control cardiovascular risk in diabetes
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
ACEi	Angiotensin converting enzyme inhibitor
ACR	Albumin to creatinine ratio
AGE	Advanced glycation end-product
AMDCC	American models of diabetic complications consortium
AngII	Angiotensin II
APC	Adenoma prevention with celecoxib
ApoE	Apolipoprotein E
APPROVe	Adenomatous polyp prevention on vioxx trial
AQP2	Aquaporin-2
ARB	Angiotensin receptor blocker
AT1	Angiotensin-2 receptor type 1
AT2	Angiotensin-2 receptor type 2
CD	Collecting duct
CKD	Chronic kidney disease
COL4A1	Collagen type-4 alpha 1
COX-1	Cyclooxygenase isoform 1
COX-2	Cyclooxygenase isoform 2
CTGF	Connective tissue growth factor
DCE-MRI	Dynamic contrast enhanced magnetic resonance imaging
DN	Diabetic Nephropathy
EC	Endothelial cell
eNOS	Endothelial nitric oxide synthase
EP1	E-type prostaglandin receptor 1
EP2	E-type prostaglandin receptor 2
EP3	E-type prostaglandin receptor 3
EP4	E-type prostaglandin receptor 4
ESRD	End stage renal disease

FITC	Fluorescein isothiocyanate
FMA	Fluorescence microangiography
GAD	Gadolinium
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFR	Glomerular filtration rate
HD	Hypertensive-diabetic
HIF	Hypoxia-inducible factor
ICAM	Intercellular adhesion molecule
INVEST	International verapamil-trandolapril study
K_f	Ultrafiltration coefficient
K_{trans}	Volume transfer coefficient
MC	Mesangial cell
MCT	Mouse proximal tubule cell line
NADPH	Nicotinamide adenosine dinucleotide phosphate
NFAT	Nuclear factor activator of transcription
NF-κβ	Nuclear factor kappa beta
NIDDK	National institute of diabetes and digestive and kidney diseases
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
P_{GC}	Glomerular capillary pressure
PGE2	Prostaglandin E2
PGEM	Prostaglandin E2 metabolite
PGI2	Prostacyclin I2
PT	Proximal tubule
PTGER1	Gene encoding prostaglandin E2 type 1 protein
PTGER2	Gene encoding prostaglandin E2 type 2 protein
PTGER3	Gene encoding prostaglandin E2 type 3 protein
PTGER4	Gene encoding prostaglandin E2 type 4 protein
RAAS	Renin angiotensin aldosterone system
RBF	Renal blood flow
ROS	Reactive oxygen species
SGLT	Sodium glucose co-transporter
SMA	Smooth muscle actin
STZ	Streptozotocin

T1DM	Type-1 diabetes mellitus
T2DM	Type-2 diabetes mellitus
TARGET	Treatment approaches in renal cancer global evaluation trial
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TXA2	Thromboxane A2
UUO	Unilateral ureter obstruction
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VIGOR	Vioxx gastrointestinal outcomes research trial
WT-1	Wilm's tumor 1

Thesis summary

Chronic kidney disease is defined as a progressive loss in renal function due to either primary kidney disease or of a condition of a non-specific nature. The most common cause of stage 5 chronic kidney disease known as end-stage renal disease is diabetic nephropathy, however non-diabetic kidney disease such as hypertension is also a major cause. The cyclooxygenase enzyme and its metabolites known as prostanoids are major contributors to renal inflammatory, fluid/ electrolyte and hemodynamic homeostasis. The cyclooxygenase-2 isoform is highly inducible in pathological conditions, subsequently enhancing prostanoid production primarily prostaglandin E2, which signals four G-protein coupled receptors dubbed E-type prostaglandin 1-4. The sporadic cellular localization of these receptors throughout the kidney governs what, where and when prostaglandin E2's effects will be. Cyclooxygenase and prostaglandin E2 have been implicated in the pathogenesis of diabetic nephropathy as non-steroidal anti-inflammatory drugs and selective cyclooxygenase -2 inhibitors can slow disease progression. Beneficial in certain settings, inhibition of the cyclooxygenase pathway can however be detrimental and is contraindicated in patients receiving blood-pressure lowering therapy or who have compromised cardiac or renal function. Moreover, the quest for new therapies to treat diabetic nephropathy is hampered by the lack of appropriate rodent models, as most do not fully develop the full spectrum of diabetic nephropathy-induced renal injury seen in humans. This doctoral thesis is a culmination of three distinct studies, the first to determine the role of the prostaglandin E2 EP1 receptor in diabetic nephropathy, the second to elucidate the vascular prostaglandin E2

EP4 receptor's role in hypertension and lastly to establish and characterise a novel mouse model of diabetic nephropathy. Data generated in these studies have contributed substantially to the renal field's literary arsenal and may help in the quest to validate current targets or to uncover new therapeutic avenues for the treatment of CKD, its causes and/or complications.

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Chapter 1: General introduction

The kidney

Vital physiological processes including but not limited to the removal of metabolic waste and toxins, maintenance of proper electrolyte balance and the regulation of blood pressure are dependent on proper kidney function. Structurally, the kidney can be divided into cortical and medullary regions. The cortex is the outer most part of the kidney, and is where reside the majority of the kidney's filters, known as the glomeruli. A glomerulus is the filtering unit of the nephron, the functional unit of the kidneys, and is composed of a dense capillary network surrounded by a membrane known as Bowman's capsule. Blood sent to the kidneys to be filtered travels through the renal artery, reaching interlobular and finally arcuate arteries which diverge into afferent resistance arterioles, feeding the glomeruli where it enters the capillary bed being filtered progressively at the level of the glomerular filtration barrier. Composed of a fenestrated endothelial cell monolayer, a glomerular basement membrane and specialized terminally-differentiated epithelial cells known as podocytes the glomerular filtration barrier is where plasma filtration occurs. Blood exits the glomerulus via the efferent arteriole, peritubular capillary network, vasa recta, ultimately reaching the renal vein and returning to circulation. In physiological conditions, only small molecules such as ions, small molecular weight proteins and water traverse the glomerular filtration barrier becoming the primary filtrate, to either be reabsorbed along the tubular system or eventually excreted as urine, while blood cells and large proteins such as albumin are returned to circulation. However, under pathological conditions, well characterized abnormalities seen in various layers of the glomerular

filtration barrier consequently lead to kidney dysfunction and urinary protein loss (proteinuria) (*Figure 1*).

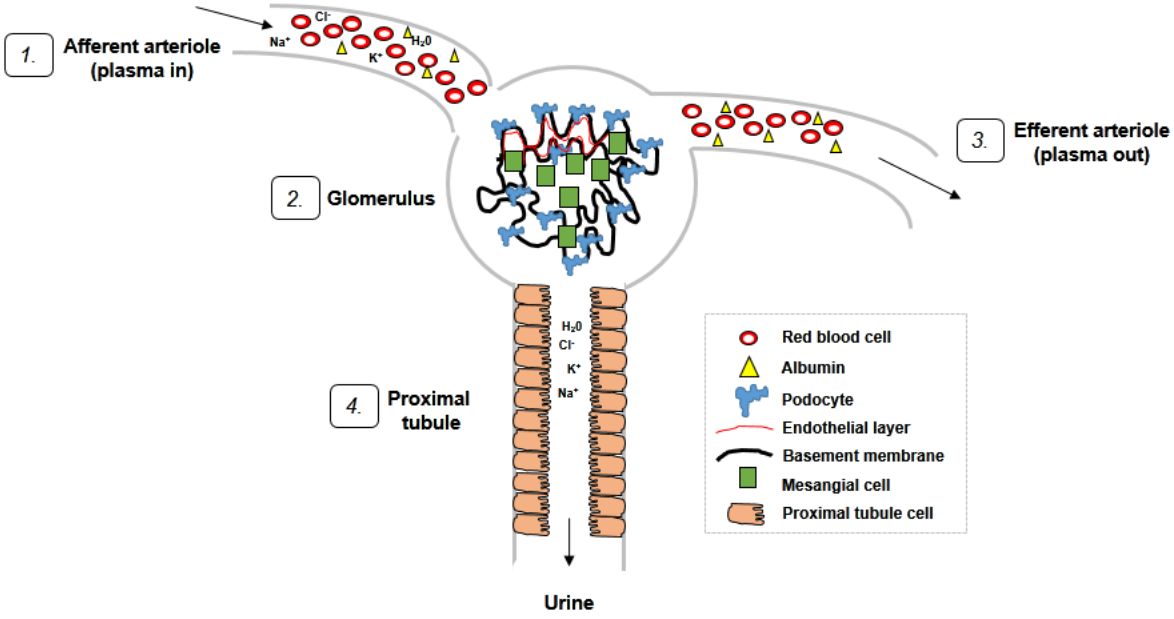

Figure 1: Glomerular filtration apparatus. Blood containing various blood cells, plasma proteins (notably albumin), electrolytes (Na^+, K^+, Cl^-, etc.) and water, enters the glomerulus via the afferent arteriole where it is sent through a network of capillaries, surrounded by podocytes and their foot processes. Podocytes restrict the passage of large blood constituents such as cells and albumin, which are returned to circulation by exiting the glomerulus through the efferent arteriole. Water and electrolytes as well as smaller molecular weight proteins traverse the glomerular filtration barrier into the proximal tubule, where the majority of solute reabsorption occur, to ultimately be excreted as urine.

The glomerular capillary network is surrounded by specialized epithelial cells commonly referred to as podocytes. Their roles include maintenance of the glomerular filtration barrier and the normal architecture of the glomerular capillary loops, remodelling of the collagen and laminin-based glomerular basement membrane, endocytosis of filtered proteins and counteracting changes in intraglomerular capillary pressure (P_{GC}) [1].

Primary and secondary foot processes emerging from the podocyte's main cell body are anchored to the glomerular basement membrane via proteins such as α3β1 integrins and dystroglycans [2] amongst others. Foot processes are highly organized structures containing bundled and cortical populations of actin. Adjacent foot processes from neighbouring podocytes interdigitate with each other to form a modified adherens junction known as the slit diaphragm. It's been established that the slit diaphragm is a crucial component of the filtration barrier since molecular or physical disruption of this junction leads to proteinuria. This is seen in congenital nephrotic syndrome of the Finnish type where mutations in the *NPHS1* gene encoding for nephrin, a critical member of slit diaphragm proteins, leads to mislocalization or absence of the slit diaphragm. This in turn translates itself into podocyte foot process effacement which impedes podocyte intercellular contacts and therefore the initial establishment or maintenance of proper slit diaphragm function leading to a compromised filtration barrier [1]. In addition, disruption of normal actin dynamics in podocytes impacts their ability to adhere to the glomerular basement membrane causing podocyte detachment, one of many possible podocytopathies. Thus, the kidney's primary function is the removal of waste and the fine-tuning of plasma fluids and electrolytes. The hallmark measurement of efficiency of renal function is known as the glomerular filtration rate (GFR), which in healthy individuals is maintained around 120 mL/min/1.73m^3 via several intrinsic mechanisms.

Following filtration at the glomerulus, filtrate travels towards the proximal tubule (PT), where the majority of sodium and glucose reabsorption occurs via sodium-glucose transporters 1 and 2 (SGLT1-2) and sodium-hydrogen exchanger 3 (NHE3). Along the nephron's tubule is where electrolytes are either actively or passively reabsorbed back

into circulation or secreted from circulation to be removed as urine. Various tubular segments are tasked with solute and water reabsorption/secretion, which ultimately dictates the extent of urine concentration and composition, depending on the body's needs. Following its passage through the loop of Henle and the distal tubule, the last fine tuning of urine occurs in the final segment of the nephron, known as collecting ducts. Each tubular segment comprises specialized and unique epithelial cells which express specific transporters which are tasked with reabsorbing various electrolytes or nutrients. Thus the kidney's maintenance of systemic fluid and electrolyte levels can be affected when injury to the glomerulus or tubular system occurs.

Chronic kidney disease

1.1.1 Definition and stages

The term chronic kidney disease (CKD) is defined as the presence of renal functional or structural injury. Regardless of the initial insult, CKD can be diagnosed when a patient's glomerular filtration rate (GFR) falls below 60 $mL.min^{-1}$ for a period spanning at least 3 months. Patients with CKD are faced with two major concerns, cardiovascular disease and the possibility of progressing to end-stage renal disease (ESRD), which is less common. ESRD is diagnosed when a patient's GFR dips to 15 $mL/min/1.73m^3$ which then requires renal replacement therapy (i.e. dialysis or renal transplant). The National Kidney Foundation's 2012 KDIGO guidelines have been established to guide clinical practices in treating CKD patients. Based on these guidelines, prognosis of CKD can be classified based on cause and category of GFR and albuminuria *(*

Table 1*)* [3]. Three classes exist for albuminuria classification, based on albumin to creatinine (ACR) values whereby values <30mg/g confer low risk, while ACR levels between 30 and 300 mg/g are classified as moderate. When ACR reaches >300 mg/g, this risk factor in itself, regardless of GFR is associated with high risk of developing CKD. However, when GFR is taken into account, this risk factor combined with ACR amplifies overall risk for CKD. For instance, low risk patients (<30mg/g ACR) with moderate decreases in GFR (50 mL/min/1.73cm^3) classifies them as having moderate risk (CKD category G3a). Thus these independent risk factors when combined are additive in increasing overall CKD risk.

Pathological or imaging abnormalities, persistent proteinuria, hematuria or GFR below 60 mL/min/1.73cm^3 on two separate occasions in euvolemic individuals are all indications of some form of kidney injury. Of interest, several large-scale observational studies reveal that even moderate reductions in GFR or the appearance of minute quantities of protein in the urine (micro-albuminuria) tend to increase all-cause and/or cardiovascular mortality rates in diabetic patients [4, 5]. However the majority of later stage CKD patients will not progress to stage 5, unless proteinuria, resistant hypertension or other kidney insults are present.

Table 1 : Prognosis of CKD by GFR and albuminuria category. *(adapted from KDIGO 2012, www.kdigo.org/clinical_practice_guidelines/ckd.php))*

				Albuminuria description and range		
				A1	A2	A3
				Normal to mild	Moderate	Severe
				<30 mg/g	30-300 mg/g	>300 mg/g
GFR categories (mL/min/ 1.73 m^2 Description and range	G1	Normal to high	>90	Low	Moderate	High
	G2	Mild decrease	60-89	Low	Moderate	High
	G3a	Mild to moderate decrease	45-59	Moderate	High	Very High
	G3b	Moderate to severe decrease	30-44	High	Very High	Very High
	G4	Severe decrease	15-29	Very High	Very High	Very High
	G5	Kidney failure	<15	Very High	Very High	Very High

1.1.2 Incidence and prevalence

The most common cause of stage 5 CKD known as ESRD, where glomerular filtration rate dips below 15 $mL.min^{-1}.1.73\ m^{2\ -1}$, is diabetic nephropathy (DN) [6, 7]. However non-diabetic causes such as hypertensive nephropathy is also a major contributor. DN and hypertension account for an estimated 45 and 26 % of newly diagnosed cases of ESRD, respectively. Additional risk factors implicated in CKD progression such as obesity, metabolic syndrome and insulin resistance have also been confirmed. Not only is CKD a health burden for developed countries, it is a major health concern in under-developed countries as costs associated with treating CKD, specifically ESRD, is exorbitant. In the United-States, the incidence of CKD had doubled between the late 90's and early 2000 but seems to have slowed in the last decade [7]. Also, incidence rates of all-cause ESRD or specifically due to diabetes are significantly higher in Blacks/ African Americans compared to Caucasians and Hispanics. While the diagnosis of new cases of ESRD seems to be stabilizing, the prevalence of ESRD continues to rise. There were an estimated 640,000 patients with ESRD in the US in 2012, an almost 4% year-to-year

increase. Of these, 75% required dialysis while 25 % underwent renal transplant [3]. As worldwide prevalence for diabetes is estimated to reach 366 million in the next decade or so, twice what was seen in the year 2000, CKD prevalence is expected to rise accordingly.

1.1.3 Causes

As mentioned above, the primary causes or risk factors associated with CKD are diabetes and hypertension. An estimated 20-40% of all diabetics will ultimately develop some form of renal complication (nephropathy), whereas some patients are resistant. In addition, a major proportion of the U.S.'s economic burden related to diabetes care is used to treat complications arising from kidney disease [8]. Recent data suggests that along with environmental factors, genetic vulnerability may explain the imparted vulnerability in some patients versus others as several genetic variants have been associated with DN. Inheriting risk alleles in susceptibility loci of certain genes such as *ACE* (angiotensin converting enzyme; renal function), *TNFalpha* (tumor necrosis factor alpha; inflammatory cytokine), *COL4AI* (Collagen type 4 alpha 1; extracellular matrix component), *eNOS* (endothelial nitric oxide synthase; endothelial function) and *APOE* (apolipoprotein E; lipid metabolism) is associated with DN [9]. Interestingly, if left untreated, the risk of progressing to overt nephropathy (>300 mg of albumin in urine/24 hrs.) is significantly higher in type-1 (80%) versus type-2 diabetics (20-40%) with concomitant microalbuminuria (30-300 mg of albumin in urine/24 hrs) [10].

DN pathogenesis implicates a myriad of maladaptive or overactive metabolic and/or hemodynamic pathways which ultimately lead to structural and functional renal injury[11]. Regarding renal hemodynamics in diabetes, it is well accepted that glomerular

hyperfiltration (aka glomerular hypertension) is a major contributor to DN pathogenesis, as evidenced, for example, by increased renal damage seen in diabetic-spontaneously hypertensive rats versus normotensive rats, which can be reversed using anti-hypertensive therapy [12, 13]. Research into the importance of hemodynamic control in the progression of DN and of the particularly fundamental role that the renin angiotensin aldosterone system (RAAS) plays in this regard has been well established by seminal studies by Barry M. Brenner. A hypothesis named on his behalf suggests that chronic injury to the kidney decreases the number of functional nephrons, which results in functional adaptation of remaining nephrons, increasing GFR via enhanced P_{GC} and blood flow, subsequently accelerating the progression of glomerular structural injury. The RAAS is a major player in this regard as hemodynamic alterations and glomerular injury can be reduced using agents which inhibit its activation or downstream signaling [14-16].

The classic RAAS pathway is an endocrine system tasked with maintaining BP through its main effectors, angiotensin II (AngII) which leads to vasoconstriction of blood vessels and aldosterone, which promotes sodium retention in the kidneys [17]. The RAAS is a very well characterized and critical regulator of renal and cardiovascular function. As shown in *Fig. 2*, the 'classic' RAAS-pathway commences by the production and release of angiotensinogen from the liver which is subsequently cleaved to angiotensin I via the renin enzyme, produced by cells of the kidney's juxtaglomerular apparatus. Angiotensin 1 is further converted to AngII by the activity of the angiotensin converting enzyme (ACE), localized in the lungs but also abundantly expressed in PT cell brush border. Our understanding of the classic RAAS pathway has been challenged by the discovery of additional enzymes and peptides, including but not limited to chymase, a major non-ACE

cascade involved in heart and vessel AngII formation [17, 18]. Also, a second ACE isoform, ACE2, expressed primarily in renal endothelium, epithelium and to a lesser degree in podocytes, has also been recognized, which has the capacity to metabolize AngII into a shorter peptide, Ang1-9, which can then be converted by ACE into angiotensin 1-7, for which vasodepressor [19, 20], apoptotic, anti-inflammatory and anti-fibrotic roles have been shown [21, 22]. The elucidation of the potential benefits of Ang1-7 in kidney health and disease is currently ongoing. Moreover, the idea that local or tissue RAAS systems function in several organs has also been put forth and confirmed. Most or all of the components of the RAAS have been localized in the kidneys, heart, brain, vasculature, digestive organs and adipose tissue [18]. Thus the complexity of this system has increased dramatically in recent years, and the locally expressed RAAS is now regarded as being distinct from its classic counterpart.

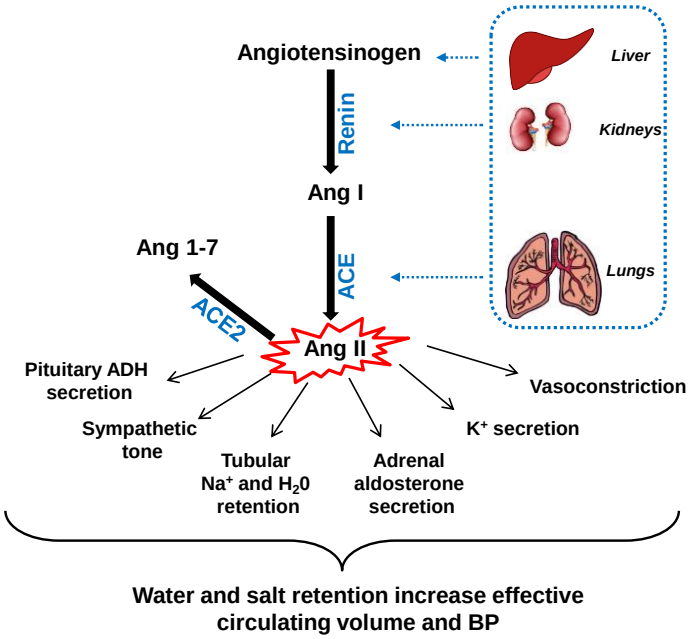

Figure 2: Classic RAAS pathway. ADH: Anti-diuretic hormone; AngI: Angiotensin I; AngII: Angiotensin II; Ang 1-7: Angiotensin 1-7; ACE: Angiotensin converting enzyme; ACE2: Angiotensin converting enzyme 2.

As previously mentioned, AngII is the main effector of the RAAS, and exerts its effects via activation of AngII receptors types 1 and 2 (AT1, AT2). These classic G-protein coupled receptors (GPCRs) are differentially expressed in the kidney and share a mere 30% homology at the amino acid level. The AT1 receptor is G_q coupled, which activates a myriad of well-known signaling pathways leading to effects such as enhancing phospholipase C (PLC)-mediated intracellular calcium levels and protein kinase C (PKC)-activity while inhibiting adenylate cyclase levels as well as participating in the phosphatidyl inositide kinase-mediated AKT and mammalian-target of rapamycin (mTOR) stimulation [23, 24]. AngII binding the AT1 also promotes the generation and release of reactive oxygen species (ROS) through activation of membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [25, 26]. On the other hand, AngII also signals via the AT2 receptor, however during maturation, renal expression of AT1 receptors becomes predominant. In fact, the majority of physiological and pathophysiological effects mediated by AngII occur through AT1 receptor stimulation, such as vasoconstriction, aldosterone release, tubular sodium reabsorption and the upregulation of pro-inflammatory, fibrotic and hypertrophic factors [18]. Characterisation of the AT2 receptor remains incomplete, yet has been linked to beneficial effects on BP through nitric oxide (NO) release [27] and to injurious pro-inflammatory cascades such as induction of NF-κβ [28]. In addition to its critical hemodynamic responsibility, the notion that local RAAS systems which operate independently from the systemic counterpart has been put forth. Observed in the heart, kidney, vasculature, skeletal muscle, pancreas, retina, adipose, neuronal and reproductive tissue, this local tissue RAAS generates AngII which acts in an autocrine and paracrine fashion [18]. In contrast

to systemic RAAS which its activation is required to maintain fluid and sodium levels when drastic drops in BP are sensed due to dehydration, salt depletion or hemorrage, the local RAAS is activated, more often than not, in pathological settings usually caused by organ injury. Hypoxia, dyslipidemia, hyperglycemia and ischemia are some pro-inflammatory conditions in which local RAAS activation occurs [29]. Thus diseases such DN and hypertension are clearly associated with the induction of the local, renal RAAS system, as its targeting remains the primary therapeutic strategy in CKD patients. As with the classic pathway, kidney RAAS activation involves the formation of ROS via NADPH oxidases, promotes tissue remodeling and production of extracellular matrix proteins which if untreated can promote end-organ damage. Thus the rationale behind intensive targeting of the RAAS pathway in CKD is based on decreasing renal injury through mitigation of BP increases but also on the inhibition of local deleterious pro-inflammatory and pro-fibrotic signaling cascades.

High blood glucose levels is the primary pathophysiological disturbance in diabetic nephropathy. Glucose may injure the kidney through either hemodynamic stress or metabolic actions. High-glucose in itself is injurious to various most if not all renal resident cell types, including podocytes, tubular and mesangial cells. Hyperglycemia activates several intracellular signaling cascades, transcription factors and triggers the production of injurious cytokines through metabolic and non-metabolic events *(Figure 3)*. These pathways ultimately affect the regulation of cell growth, survival, angiogenesis, extracellular matrix production which promotes aberrant glomerular filtration, permeability and tubular function. For instance, hyperglycemia promotes hypertrophy and MC extracellular matrix production [30, 31]. In podocytes, hyperglycemia increases

oxidative stress, promotes apoptosis [32], increases vascular-endothelial growth factor (VEGF) production and affects the architectural integrity of the cytoskeleton [10, 33]. Protein kinase C-mitogen activated protein kinases are also implicated in the formation of glomerular sclerotic lesions, as their inhibition confers renal protection in diabetic mice [34].

Altered renal hemodynamics is also a major player in the progression of DN-induced CKD. Briefly, renal autoregulation of renal blood flow (RBF) and GFR is a mechanism by which the kidney counteracts or handles wide variations in blood pressure to maintain renal function such as maintaining constant sodium reabsorption or fractional reabsorption. Two well described mechanisms are involved with autoregulation which occurs at the afferent arteriole, the first being the myogenic response, where changes in vascular pressure lead to rapid vasoconstriction of smooth muscle cells and the second is tubuloglomerular feedback (TGF). The TGF mechanisms operates by sensing changes in chloride delivery at the level of the macula densa cells in the juxtaglomerular apparatus. Elevated chloride levels are consistent with elevated GFR, thus activation of TGF decreases GFR via afferent arteriole constriction. Otherwise, low levels of chloride sensed at the MD will dampen TGF. Importantly, enhanced production of factors such as NO, VEGF, transforming growth factor beta 1 (TGFβ1), AngII and prostanoids in DN can affect the autoregulation of glomerular hemodynamics and have all been implicated in one way or other in DN pathogenesis [10].

Elevated circulating levels of glucose can also alter glomerular hemodynamic control, through dilation of pre-glomerular (afferent) arterioles thus increasing GFR, plasma flow and intraglomerular capillary pressure [14]. Loss of glomerular microcirculatory control

imposes a mechanical strain on the cells of the glomerular filtration barrier, which compromises its permselectivity to plasma proteins, leading to loss of albumin in the urine, a hallmark of kidney disease. The mechanism by which hyperglycemia affects afferent arteriolar tone is thought to implicate crosstalk with the local RAAS. Evidence to support this claim has been shown *in vitro* whereby culturing MC and tubular epithelial cells in hyperglycemia media upregulates renin and angiotensinogen, most likely participating in an overproduction of AngII and activation of the local kidney RAAS [17, 35]. In addition, increases in the aforementioned compounds and hemodynamic alterations also contribute to increased MC extracellular matrix production and podocyte-specific damage via local release of damaging cytokines and growth factors including TGFβ, CTGF, IL-6, MCP1 and VEGF [11, 36].

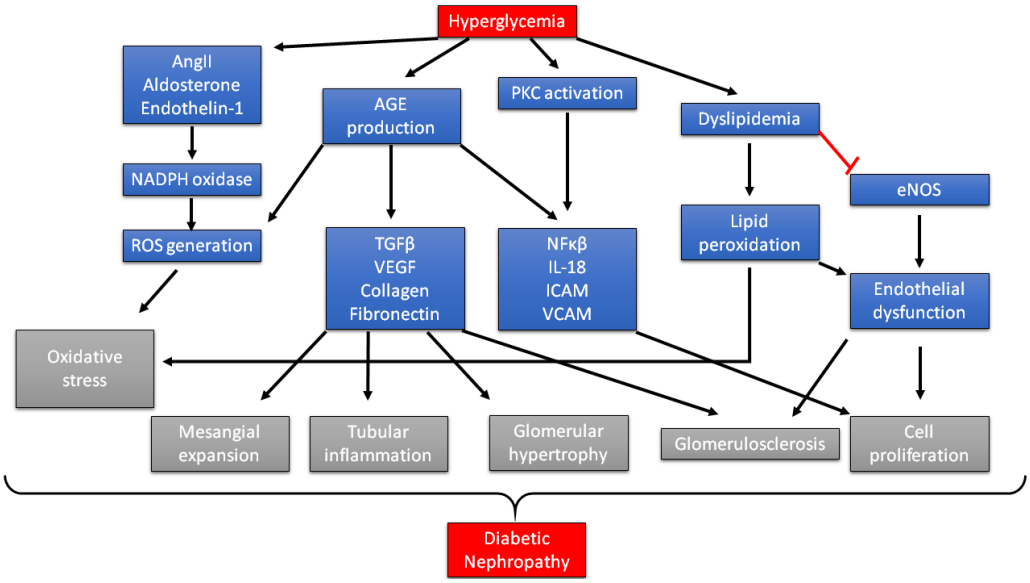

Figure 3: Hyperglycemia and pathophysiology of DN. AngII: Angiotensin II; NADPH: Nicotinamide adenine dinucleotide phosphate; ROS: Reactive oxygen species; AGE: Advanced glycation end-products; TGFb: Transforming growth factor beta; VEGF: Vascular endothelial growth factor; PKC: Protein kinase C; NFkB: Nuclear factor kappa-light-chain-enhancer of activated B cells; IL-18: Interleukin 18; ICAM: Intercellular adhesion molecule 1; VCAM: Vascular cell adhesion molecule 1; eNOS: Endothelial nitric oxide synthase.

The second major cause of ESRD is hypertension, which is a general term encompassing several contexts in which arterial blood pressure (BP) is elevated in a sustained manner. Regulation of BP is complicated, involving the cardiovascular and central nervous systems, kidneys and adrenal glands [37]. These aforementioned organs maintain cardiac output, fluid levels and peripheral vascular resistance, all major determinants of systemic BP. The majority (90-95%) of hypertension diagnoses are of idiopathic nature, in that no specific underlying medical cause can be determined. Known as the 'silent killer', hypertension in itself is associated with cardiovascular dysfunction including stroke, aneurysms and coronary artery disease [38, 39]. Hypertension is commonly associated with CKD progression, but is however often misdiagnosed as the cause, rather than a consequence of CKD. Kidneys are major players in systemic BP regulation through electrolyte and fluid balance. Of importance, the pressure natriuresis relationship is considered a primary mechanism by which the kidneys maintain BP through tubular sodium and water handling. This infinite gain mechanism stipulates that increased pressure in the renal artery will stimulate renal sodium and water excretion, reducing extracellular fluid volume and thus normalizing BP. Thus deficiency in this relationship participates in sustained BP elevations and the maintenance of hypertension. Furthermore, classic studies by Goldblatt in 1933 were seminal in establishing a role for the kidneys in long-term BP regulation. He and colleagues used a clamp to reduce blood flow causing ischemic damage in canine renal arteries leading to rapid and persistent hypertension. These experiments demonstrated that renal ischemic damage was sufficient to significantly elevate systemic BP [40]. Several elegant studies involving renal transplantation from genetically-hypertensive donor rats into healthy recipient rats have

confirmed that primary hypertension is kidney-dependent [41]. These experiments have successfully and repeatedly shown that kidneys obtained from either Milan, Dahl salt-sensitive or spontaneously hypertensive rats confer hypertension when transplanted into healthy donor rats, suggesting that 'BP travels with the kidney'. Hypertension affects 1 in 3 adults in the United-States, and is prevalent in 80% of patients diagnosed with CKD [39]. Of interest, hypertension incidence follows CKD disease progression as higher rates are reported in later stages of CKD. The etiology of hypertension is based on the notion that unrelenting elevations in BP initiates and/or exacerbates organ injury through overwhelmed vascular hemodynamics [42]. Specifically, increased BP enhances hemodynamic load to the vasculature, first encountered by the EC layer. Changes in EC signaling and gene expression ensue, which enhances the activity of various ion channels, integrins, tyrosine kinases and hormone production (including AngII) and growth factors [43, 44]. Hemodynamic overload can also lead to an overproduction of extracellular matrix, ROS generation and VSMC proliferation. This context promotes vessel permeability to leukocyte infiltration and thus local inflammation [45].

In a healthy kidney, autoregulatory processes such as the myogenic and tubuloglomerular feedback mechanisms (TGF) buffer the transfer of high arterial pressure to elevate P_{GC}. The role of autoregulation is to maintain GFR and RBF over a wide range of varying BP. The myogenic response is considered to be a rapid reaction by vascular smooth muscle cells to changes in BP while the TGF system is a slower more graded response. TGF relies on the anatomical positioning of glomerular microcirculation to the distal tubules MD forming the juxtaglomerular apparatus. This region allows for control of GFR via alteration (increase or decrease) of afferent arteriolar resistance in response to changes in

distal tubule chloride delivery to the MD. In conditions where GFR is elevated, distal tubule delivery of chloride is higher which is sensed by MD, triggering a signaling cascade resulting in the local release adenosine. Its subsequent binding to its adenosine-1 receptor expressed on VSMC's of the afferent arteriole results in vasoconstriction returning GFR to appropriate levels. The TGF system can also be suppressed in conditions of low GFR. It is well established that in DN, these autoregulatory processes are impaired [46, 47]. In DN, one mechanism which may explain classic early hyperfiltration is enhanced PT reabsorption which results in decreased distal delivery and suppressed TGF. Resistance of glomerular afferent arterioles is also diminished in this disease due to several vasoactive factors including VEGF, prostanoids, NO [11]. This promotes increased P_{GC} and hyperfiltration (aka glomerular hypertension). Thus elevated P_{GC} increases GFR which promotes glomerular filtration barrier injury and albumin leakage.

In hypertension, chronic elevations in BP and Pgc cause microvascular damage in the glomerulus and kidney through damage to either the endothelial cell layer or the vascular smooth muscle. Decreased blood flow impairs tissue oxygenation and nutrient delivery. Erroneously sensed by the kidneys as a state of dehydration, RAAS activation ensues which leads to aldosterone release from the cortex of the adrenal gland to signal via mineralcorticoid receptors expressed in the collecting duct, promoting the retention of salt and water, increasing blood volume and systemic vascular resistance. Although the RAAS is critical in maintaining systemic BP in certain conditions, it is clear that chronic activation of this pathway in CKD promotes the development of hypertension associated renal injury.

1.1.4 Rodent models of DN

The use of rodents for modeling DN disease progression has been refined over the years. Due to their short life span, tendency for mating and lower cost associated with housing, mice actually represent an estimated 65-70% of all animal species used in biomedical research. Rodent models designed to replicate disease progression are useful tools to test new therapeutic avenues or to elucidate molecular pathways involved in disease progression. Regarding DN, although several artificially induced, genetic or spontaneous mouse and rat models of DN are available, their utility is hampered by the fact that most of them fail to develop the majority of injurious processes which characterize the later stages of human DN-progression. Guidelines established by the American Models of Diabetic Complications Consortium (AMDCC) stipulate that an adequate murine model of DN should display most or all of the following characteristics: a 50% decline in GFR over a lifetime, a 10-fold increase in albuminuria compared to age and gender-matched healthy controls, a 50% increase in glomerular mesangial matrix, hyalinosis of the arterioles, a 25% thickening of the glomerular basement membrane and the presence tubulointerstitial fibrosis. Since no current murine model of DN meets all of these requirements, this area of research remains quite active.

Rodent models of T1DM, where mice are either born or rendered hypoinsulinemic, include intraperitoneal injections of alloxan or β-cell toxin streptozotocin (STZ) or through, β-cell specific overexpression or mutation of calmodulin and insulin respectively in OVE26 and Akita models [48, 49]. T2DM rodent models are typically obtained by rendering these animals obese using a high fat diet, or by disrupting the satiety hormone leptin via mutations within the leptin gene (ob/ob mice), or leptin

receptor (db/db mice, Zucker rats). While the above models display the majority of abnormalities associated with early DN, such as albuminuria, renal hypertrophy and glomerular scarring, they fail to develop late features human disease, including declining GFR and arteriolar hyalinosis [50]. The discrepancy found in the severity of renal injury observed in rodent models may explain why some treatments are effective in experimental (animal) models but fail to impart beneficial effects in human trials. Several factors must be taken into consideration when choosing to exploit a specific model vs. another. Of these, the susceptibility imparted by the strain of rodent chosen and whether or not the model develops hypertension are both major requirements. In mice for instance, it is well established that equally obese and hyperglycemic type-2 diabetic db/db mice develop substantially more renal injury when bred onto an Fvb/n background, compared to the resistant C57BL/6J mouse strain. The same holds true for models of T1DM, as demonstrated by enhanced glomerulosclerosis and albuminuria when OVE26 mice are bred onto the susceptible Fvb/n background [51]. Also, hypertension is not commonly observed in murine DN models, while it is a common development in advancing renal injury in human DN. Thus, it is hypothesized that the absence of hypertension in most murine models of DN may partly explain the apparent resistance to developing advanced DN-induced renal injury. Therefore, several recently developed models have employed various strategies to superimpose hypertension onto either available or newly generated DN models. For example, markers of renal structural and functional injury are exacerbated in T2DM db/db mice lacking eNOS expression ($eNOS^{-/-}$ db/db) compared to equally hyperglycemic $eNOS^{+/+}$db/db controls [52]. In addition, RAAS-activation through either exogenously or endogenously enhanced renin-dependent

AngII production has also provided a means to overlay hypertension onto a diabetic phenotype. One example, TTRhRen mice developed by Dr. Reudelhuber in Montreal, express a modified human pro-renin gene specifically expressed in the liver which leads to an overproduction of active renin and thus promotes AngII-dependent hypertension.

1.1.5 Current treatments

As the key causes of CKD are diabetes and hypertension, treatments for patients with CKD logically include drugs designed to tightly control glycemia and BP. Regarding the latter, uncontrolled BP can have serious consequences on the kidneys' ability to regulate electrolyte and fluid handling, which ultimately in itself promotes hypertension through volume expansion and increased vascular stress. As described above, the RAAS pathway is a major player in this regard. Thus, current first line BP lowering agents which include angiotensin-converting enzyme inhibitors (ACEi), angiotensin type 1 receptor blockers (ARB) and renin inhibitors are also effective in lowering albuminuria in hypertensive patients with or without diabetes with concomitant ACR values of at least 200 mg/g [39]. These treatments are often combined with additional drugs, such as diuretics, beta-blockers, calcium channel blockers and vasodilators to achieve a specific BP goal, usually below 130 mmHg systolic and 80 mmHg diastolic. Thus proper BP control translates to a reduction in renal injury in patients with DN. However these drugs can only slow disease progression and are thus deemed incompletely effective. In addition, these drugs may lead to hypotension if higher doses are used in cases where the desired anti-albuminuric effect is not achieved. Therefore the search remains for effective DN therapies which are BP-independent.

As diabetes is a disorder in which circulating glucose levels are elevated due to either an inability to produce insulin (type 1 diabetes mellitus; T1DM) or the incapacity of cells to utilize this insulin (type 2 diabetes mellitus; T2DM) to take up extracellular glucose. Thus, while patients with T1DM rely on some form of insulin replacement therapy, treatment of T2DM includes the use of biguanides, thiazolidinediones and sulfonylureas which respectively lower liver glucose production, enhance peripheral tissue glucose reuptake and stimulate pancreatic insulin secretion. These drugs are thus effective in restoring blood glucose levels to a normal range found between 80-110 mg/dL or 4.5-6.0 mM. However in susceptible diabetic patients diagnosed with some form of CKD, tight glycemic control is not recommended. Data extrapolated from patients with T2DM in the large Action to Control Cardiovascular Risk in Diabetes (ACCORD) study found a positive association between strict glycemic control and increased all-cause and cardiovascular associated deaths [53]. Moreover, a post-hoc analysis of the ACCORD trial found that intensive glycemic control elevated the risk of mortality in T2DM patients with concomitant mild to moderate CKD [54]. As the kidney has a major role in the metabolism of insulin and the clearance of drugs, characteristically decreased renal function in CKD may lead to sustained effects of anti-diabetic drugs and increase insulin half-life, promoting hypoglycemia in patients with mild to moderate CKD [55]. Great care and consideration should be taken when choosing a therapeutic approach to treat diabetes in patients with underlying renal dysfunction or predisposed to kidney injury.

Cyclooxygenase system

1.1.6 COX-1 and COX-2

Since its isolation from ram seminal fluid in 1988 [56], the COX enzyme also known as prostaglandin synthase G2/H2, has been of major therapeutic and pathophysiological interest. COX is the rate limiting enzyme responsible for the conversion of cell membrane arachidonic acid to produce prostaglandins and thromboxane. Further studies identified the existence of two COX isoforms, which led to the current nomenclature COX-1 and COX-2 [57]. COX-1 is generally regarded as ubiquitously and constitutively expressed and as having homeostatic effects on kidney blood flow, maintaining gastro-intestinal tract integrity and normal platelet function [58]. In the human kidney, COX-1 is highly expressed in mesangial cells, arteriolar endothelial cells and both cortical and medullary collecting ducts [59]. COX-2 is referred to as the inducible isoform, and while it is strongly expressed in the developing human kidney (macula densa, thick ascending limb), its expression in basal conditions is almost non-existent in adult kidneys [60]. COX-2 is highly upregulated in a number of inflammatory conditions triggered by growth factors, phorbol esters and bacterial lipopolysaccharides [58]. Both COX enzymes are responsible for the production of either prostaglandins (E_2, D_2 and $F_{2\alpha}$), prostacyclin (PGI_2) or thromboxane A_2 (TXA_2).This doctoral thesis will focus on the action of PGE2-mediated renal injury through specific receptors in experimental models of DN and hypertension.

1.1.7 PGE_2 and EP receptors

Along with its participation in a myriad of systemic physiological processes such as immune function, blood pressure regulation, gastrointestinal integrity (GI) and fertility,

PGE2 is the most abundant renal prostanoid, which targets practically the entire renal cell population [61]. In the kidney, PGE_2 has a multitude of effects relating to inflammation, volume homeostasis, electrolyte handling, vascular tone and glomerular hemodynamics [62]. PGE_2 signals through four well characterized GPCR's known as E-type prostaglandin receptors (EP1-4). EP receptors 1 and 3 are generally regarded as being vasoconstrictive while EP2 and EP4 are usually associated with vasorelaxation. The mouse EP1 receptor, encoded by the *PTGER1* gene, is a 405 amino acid protein which is coupled to the Gq alpha-subunit signal transduction pathway. PGE_2 binding to EP1 activates PLC, which cleaves phosphatidylinositol 4,5-bisphosphate into diacyl glycerol and inositol 1,4,5-triphosphate (IP_3) which then binds to IP_3 receptors expressed on the endoplasmic reticulum increasing intracellular calcium levels ($[Ca^{2+}]_i$). Cell culture studies suggests EP1-mediated increased $[Ca^{2+}]_i$ may not entirely depend on usual IP_3 turnover, rather it may require synergism with other families of calcium gating G-proteins [63]. While mRNA levels are highest in the collecting duct (CD), EP1 expression has also been described in the vasculature, glomerulus and proximal tubule cells [64, 65].

Signaling via the 366 a.a. EP3 receptor (*PTGER3* gene) activates the classic G_i signal transduction pathway, which hampers generation of cyclic adenosine monophosphate (cAMP) from ATP via inhibition of adenylate cyclase. Thus cAMP associated kinase activation is also decreased. Post-transcriptionally, EP3 can be alternatively spliced generating 3 or as much as 9 distinct but functionally similar EP3 isoforms in mouse and humans respectively, all of which bind PGE2 with similar affinities [66]. Consistent with

human tissue, expression of EP3 in the mouse kidney is restricted to tubular epithelial cells, thick ascending limb and cortical collecting ducts [67].

Distinct from EP receptors 1 and 3, the 362 and 513 a.a. EP2 and EP4 receptors are considered vasodilatory as they ultimately couple to signal transduction cascades which increase cAMP levels. EP2 and 4 activation via PGE_2 increases the activity of adenylate cyclase, stimulating the conversion of ATP to cAMP and subsequent cAMP-dependent kinase activation. This ultimately promotes vasorelaxation through downstream phosphorylation/inhibition of myosin light-chain kinase and thus dephosphorylation of myosin light chain found in muscle. EP2 mRNA transcripts are low to undetectable in mouse kidneys, while predominating in medullary regions in rats and humans. In contrast, EP4 mRNA expression is predominantly found in the glomerulus, collecting duct, vasa rectae and pre-glomerular arterioles in murine and human kidneys [65, 67].

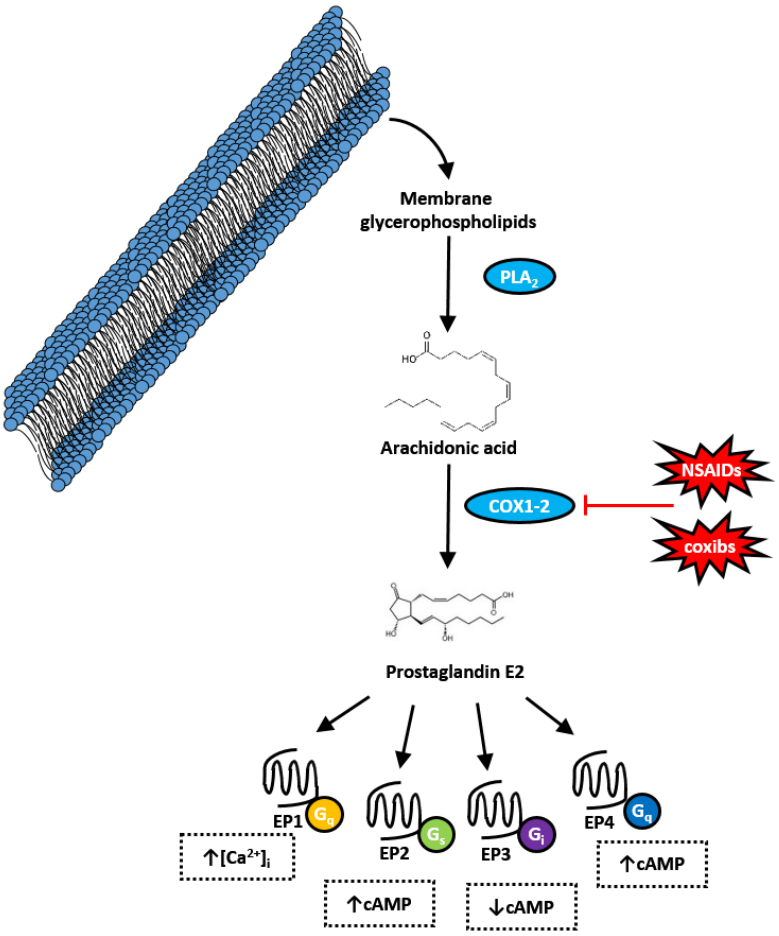

Figure 4: COX-derived PGE2 and EP receptors. Prostaglandin E2 (PGE2) is a product of cyclooxygenase (COX) mediated conversion of arachidonic acid, cleaved from membrane phospho-lipids by phospholipase A2 (PLA_2). PGE2 then acts upon four G-protein coupled EP receptors (EP1-4) which affect downstream second messenger formation i.e. cyclic adenosine monophosphate (cAMP) and intracellular calcium (Ca2+).

PGE_2 in health and disease

1.1.8 PGE_2 and renal function

PGE_2 is the most abundantly produced prostaglandin systemically and in the kidney. It is well established that in the kidney, PGE_2 promotes natriuresis and diuresis through activation of various EP receptors along the nephron. Along these lines EP1 activation via PGE_2 inhibits Na^+ and H_20 reabsorption in the mouse collecting duct [68, 69] while in the renal medulla, hypertonicity induces PGE_2 production and natriuresis via EP2

activation [70]. PGE_2 also participates in regulating potassium excretion through its ability to activate the RAAS system via EP4 receptor-mediated renin secretion by juxtaglomerular cells, consequently triggering aldosterone mediated urinary potassium excretion [71-74]. In a properly hydrated individual, PGE_2 plays a minimal role in Na^+ and H_20 handling, however in conditions of dehydration or decreased effective circulating volume due to hemorrhage, congestive heart failure, diuretics and salt-restriction, PGE_2 plays a major adaptive role in maintaining renal and thus systemic fluid balance [73, 75].

1.1.9 COX-inhibition

The mechanism by which acetylsalicylic acid, commonly referred to as aspirin, and other salicylates wield their pain-relieving and anti-inflammatory actions remained elusive for 74 years after its original synthesis by Felix Hoffman of Bayer laboratories in 1897. In 1971, John Vane described how this class of drugs blocked the formation of prostaglandins by inhibiting the COX enzyme [76], and was eventually awarded the Nobel prize in physiology and medicine in 1982 for his discovery. NSAIDs are regularly used worldwide to treat pain and inflammation. Through their ability to non-selectively block both COX enzyme isoforms, NSAIDS inhibit prostaglandin production and subsequent downstream pro-inflammatory signaling, including but not limited to tumor necrosis factor alpha (TNFα) [77]. However, due to the homeostatic nature of prostaglandins on GI health such as maintaining the integrity of the gastric mucosa, NSAID-use is associated with several gastrointestinal side effects. Also, adverse effects of NSAID use on renal function are well described, which include altered hemodynamics, while increased BP, edema and interstitial nephritis often occur with these drugs. The

discovery of COX-2 selective drugs (coxibs) in the 1990s was fueled by the need to protect the GI tract from undesirable side effects associated with loss of COX-1. However GI-friendly, coxib use has been linked to increased risk of developing cardiovascular injury, especially in susceptible individuals [78-81]. These undesirable side effects implicate impaired endothelial COX-2 derived production of prostacyclin (PGI2) while sparing COX-1 derived production of TxA2, respectively involved in inhibition and activation of platelet aggregation predisposing to stroke or atherosclerotic lesions [82]. Importantly, four major clinical trials (APC, VIGOR, APPROVe and TARGET) performed in the last 15 years were critical in the Food and Drug Administration's decision to remove rofecoxib (aka Vioxx) and valdecoxib (Bextra) from the market, due to increased risk of developing cardiovascular events and mortality, while celecoxib remained available albeit with new labelling requirements [83].

Renal hemodynamics are seemingly unaltered in healthy individuals treated with NSAIDS or coxibs [84]. However, it seems specific inhibition of COX-2 activity may predispose the kidney to injury or affect its function in certain situations over others. For example, COX-2 inhibition using celecoxib in hyperfiltering DN patients reduces GFR, whereas in normofiltering diabetic patients, celecoxib has the opposite effect, increasing GFR [85]. Moreover, cortical or medullary blood flow is unaffected by COX-1 inhibition, while COX-2 inhibition lowers medullary blood flow in mice, and increases BP in high-salt diet fed animals [86]. Furthermore, reductions in RBF induced by AngII infusions are exacerbated in mice pretreated with COX-2 inhibitor, but not COX-1[87]. Renal COX-2 expression and subsequent prostaglandin production is increased in isolated glomeruli and cultured MC derived from rats with streptozotocin (STZ)-induced

type-1 diabetes mellitus (T1DM) [88, 89]. Also, NS-398-mediated selective COX-2 inhibition blunts glomerular hyperfiltration in STZ-treated rats, which also correlates with decreased glomerular prostaglandin production [90]. Work by Harris and colleagues demonstrate that cortical COX-2 expression increases in the rat remnant kidney model (partial nephrectomy), and that proteinuria and glomerulosclerosis can be attenuated using another selective COX-2 inhibitor, SC-58326 [91, 92]. In addition, SC-58326 administration decreases markers of DN, including mesangial matrix expansion and proteinuria in diabetic salt-sensitive hypertensive rats [93]. Likewise, NS-398 reduces albuminuria, GFR and kidney fibronectin expression in the TIDM Akita mouse model [94]. In contrast to its beneficial effects in DN, NSAID use and/or COX-2 inhibition is contraindicated in patients diagnosed with hypertension as it may predispose them to dangerous drops in RBF and GFR or impair diuresis/natriuresis and increase otherwise elevated BP by 5-6 mmHg [95]. A recent study found increased adverse side effects in hypertensive patients with coronary artery disease who were also chronic NSAID users [96]. Also, risk of new onset hypertension increases significantly in elderly patients treated with the COX-2 selective rofecoxib, which doubles in cases where existing renal, liver or heart disease is present [97]. NSAIDs can also interact with impair the actions of anti-hypertensive medications including angiotensin-converting enzyme inhibitors and diuretics [98]. Thus COX-2 inhibition appears to be a notable target in mitigating glomerular hemodynamic injury such as hyperfiltration in DN; however adverse renal side effects associated with COX-2 inhibition in predisposed individuals (i.e. hypertension) may preclude their use in patients with CKD.

1.1.10 EP receptors: regulation of BP and renal hemodynamics

It is well established that COX-derived prostanoids are critical players in the regulation of renal blood flow and GFR, especially in certain pathophysiological conditions. When effective circulating volume is decreased as in congestive heart failure, nephrotic syndrome or cirrhosis, adequate renal function becomes highly reliant on prostanoids and downstream signaling, as evidenced by drastic drops in GFR when patients with the aforementioned afflictions are treated with not only non-selective COX inhibitors, but also coxibs. The precise mechanisms by which prostanoids maintain renal hemodynamics is currently unresolved, however several targets have been put forth. Catecholamines, AngII and vasopressin are all involved in vasoconstriction of peripheral and renal arteries in diseases which affect effective circulating volume. Also, low-dose infusion of PGE2 in the renal artery leads to vasodilation and can counteract the actions of vasoconstrictive agents such as AngII, catecholamines and vasopressin [65, 67, 99, 100]. PGE2 can also buffer the vasoconstrictive response of AngII on isolated rat pre-glomerular vessels, an effect associated with maintenance of GFR and RBF [101]. Of importance, recent studies in rats have identified the EP1 and EP4 receptors as being responsible for transient vasoconstriction and sustained vasodilation, respectively, in pre-glomerular afferent arterioles [102]. Alongside its role in maintaining GFR, EP4 receptor expression is also elevated in the medulla, where it colocalizes with COX-2 in human medullary blood vessels i.e. vasa recta and capillaries [103]. Accordingly, EP4-null mice challenged with a low-salt diet have reduced medullary blood flow due to enhanced constriction of medullary vasculature. Regarding BP regulation, in contrast to the EP2 receptor, which is likewise Gs-coupled, the EP4 receptor play's a minimal role in this regard. Previously

published data has clearly shown unchanged SBP in healthy, or low-salt fed, or diuretic-challenged EP4-/- mice [71, 73].

1.1.11 EP1 and EP4 targeting in renal disease

Based on their sporadic expression pattern in the kidney and on the context-dependent beneficial or detrimental nature of COX-inhibition, EP-specific contributions to disease progression remain a constantly evolving area of research. Our knowledge regarding the role of each individual EP receptors in various renal diseases has been based on studies employing many commercially available pharmacological compounds or by exploiting global or tissue-specific EP knockout mice. In experimental models of hypertension, EP1-antagonism appears to be beneficial in that it lowers the vasculature's response to acute and chronic AngII-stimulation. In spontaneously hypertensive rats, treatment with an EP1-selective antagonist (SC51322) reduces BP [104], while recently published data found that EP1 deletion decreased mortality rates due to fewer aortic aneurysms and attenuated end-organ damage in severly hypertensive high-salt treated, partially nephrectomized (5/6) and AngII infused mice [105]. These data suggest EP1-selective targeting may be beneficial in hypertension associated renal disease. Consistent with hypertension models, a detrimental role for the EP1 receptor in the pathogenesis of DN has been proposed, as pharmacological antagonism attenuates DN-induced fibrosis and albuminuria and overall renal injury in diabetic rats [106]. The existence of a fourth EP receptor, EP4, was discovered when Coleman and colleagues ruled out EP's 1-3 as being responsible for PGE2-induced relaxation of piglet saphenous veins [107]. Its role in normal renal physiology and various models of renal diseases has been studied by numerous research groups and involves the regulation of cell proliferation and migration,

vascular tonicity and renin secretion [71, 74]. Generally regarded as a beneficial receptor in the renal vasculature tasked with maintaining GFR and vascular tone, the EP4 receptor's role in disease appears to be context dependent. For instance renal injury in DN rats treated with a pharmacological EP4 agonist is worsened, while in acute (ischemia reperfusion) and chronic (subtotal nephrectomy) models of renal injury, EP4 receptors appear to be protective as antagonism is detrimental to glomerular and tubular integrity [108, 109]. Moreover, anti-inflammatory effects driven by local EP4 activation have been observed in the tubular epithelium, as EP4-/- mice show increased susceptibility to unilateral uretal obstruction (UUO)-induced tubulointerstitial fibrosis [110]. On the other hand, podocyte-specific EP4 overexpression worsens while deletion ameliorates subtotal nephrectomy-induced renal injury in mice, highlighting a detrimental role in this cell-type when glomerular capillary pressure or single nephron GFR is elevated [111]. Therefore EP1 and EP4 receptors clearly participate in a number of physiological and pathophysiological processes which makes them potential targets in the quest for new treatments to combat CKD progression.

The rationale behind this doctoral thesis is clearly justified as COX inhibition is a promising target to alleviate renal injury in various diseases, however the myriad of side-effects associated with NSAIDS and COX-2 inhibitors indicate that context-dependent receptor-specific targeting may offer additional protection, while mitigating unwanted side-effects.

Research questions and objectives

In the first study (chapter 2), the main research questions were the following: does the PGE2 EP1 receptor contribute to the development of diabetic kidney disease (aka DN) in

type-1 diabetic mice and through which cell-type(s) are these effects mediated. To answer these questions, our objectives were to subject mice lacking the EP1 receptor (EP1-/-) to T1DM using two distinct models, and to compare disease progression with age and gender-matched diabetic wild-type mice. We hypothesized, based on previous observations using pharmacological antagonists, that deletion of the EP1 would offer protection to the kidney in DN.

The second study focused on the impact brought on by EP4 receptor activation in the vasculature and on the ability of the kidney to withstand injury in response to AngII-dependent hypertension when this receptor is absent. Based on the fundamental role that PGE2 plays on regulating glomerular hemodynamics, we generated mice with vascular-specific deletion of the EP4 receptor ($EP4^{VSMC-/-}$) and subject them to the AngII-dependent model of hypertension. Secondary objectives included the assessment that this genotype would have on renal/ glomerular functional and structural injury in this hypertensive-context. In accordance with previously published work done by others, we hypothesized that the vasodilatory EP4 receptor would be critical in maintaining adequate glomerular and kidney hemodynamic function in a hypertensive, pro-vasoconstrictive environment.

The final study contained in this doctoral thesis was a collaborative effort in which we questioned whether we could develop a robust mouse model of DN by superimposing T1DM onto a genetically-hypertensive mouse background. We speculated that the lack of effective and available rodent models of DN is based on the fact that these animals seldom develop arterial hypertension or other signs of advanced human DN and thus by generating a type-1 diabetic/ hypertensive mouse, we may be able to increase the severity

and accelerate disease progression. We employed hypertensive mice which harbor a metabolically overactive form of human-renin, the rate limiting enzyme in the production of the pro-hypertensive AngII hormone. We rendered these genetically hypertensive mice diabetic (type-1) using two distinct models and compared DN pathogenesis.

Chapter 2: *PTGER1* deletion attenuates renal injury in diabetic mouse models

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PTGER1 Deletion Attenuates Renal Injury in Diabetic Mouse Models

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Description

Current therapies aimed at treating diabetic kidney disease (i.e. DN) can only slow disease progression. While tight control of blood pressure and glucose levels are central in mitigating diabetes-induced renal injury, additional drugs may be of benefit, targeting newly discovered or classic therapeutic pathways. NSAIDS and selective COX-2 inhibition have shown promise in alleviating some indices of DN-induced renal injury, such as the development of albuminuria and glomerular scarring. However their association with unwanted side effects, such as edema, increased BP and overall nephrotoxicity, preclude their use as effective anti-proteinuric DN drugs. COX generates prostaglandin E2 which activates downstream cell surface EP receptors. We speculate the beneficial impact of COX inhibition on proteinuria may occur via impaired activation of a specific downstream EP receptor. Accordingly, one study found that pharmacological antagonism of the EP1 receptor decreased DN-induced renal injury in rats. We therefore induced type-1 diabetes in either wild-type or EP1-/- mice and compared the progression of structural and functional renal injury.

Abstract

Cyclooxygenase (COX)-derived prostaglandin E2 (PGE2) synthesis and downstream E-Prostanoid (EP) receptor activation contributes to diabetic nephropathy (DN). Given that pharmacological EP1 receptor (EP1) antagonism is beneficial in diabetic rats, we hypothesized that the Gq-coupled EP1 promotes glomerular and/or tubular damage in DN. Here we rendered EP1 knockout (EP1-/-) mice diabetic using the streptozotocin (stz) and OVE26 models of type-1 diabetes. At 16 and 26 weeks respectively, albuminuria, mesangial matrix expansion and glomerular hypertrophy were each blunted in both EP1-/- stz and OVE26 cohorts as compared to wild type counterparts. Glomerular hyperfiltration was unaffected in the stz study, while OVE26EP1-/- mice hyperfiltered to a lesser degree. Although diabetes-associated podocyte depletion was unaffected by EP1 deletion, EP1 antagonism with ONO-8711 in conditionally-immortalized podocytes decreased angiotensin II (AngII) -mediated superoxide generation suggesting that EP1-associated injury of remaining podocytes *in vivo* could underlie filtration barrier dysfunction. Accordingly, EP1 deletion in OVE26 mice prevented nephrin mRNA expression downregulation while also reducing glomerular basement membrane thickening and foot process effacement. Moreover, EP1 deletion reduced diabetes-induced expression of cortical fibrotic markers fibronectin and α-actin while EP1 antagonism decreased fibronectin expression in cultured proximal tubule (PT) cells thereby suggesting that PGE2 acts directly on this nephron segment. Consistent with this finding, PT megalin expression was reduced by diabetes, but was preserved in EP1-/- mice. Finally, a role for EP1 receptor in the diabetic vasculature was suggested as the diabetes-associated increase in AngII-mediated vasoconstriction of isolated mesenteric arteries was blunted in OVE26EP1-/- mice. These data suggest that EP1 activation

contributes to DN progression at several locations including podocytes, proximal tubule and the vasculature. Some of the effects of the EP1 appear to facilitate the actions of AngII thereby suggesting that targeting of both the renin-angiotensin system and the EP1 receptor could be beneficial in DN.

Introduction

Accounting for nearly 40% of newly diagnosed cases of end-stage renal disease requiring dialysis or renal transplantation and occurring in almost one third of diabetes patients, diabetic nephropathy (DN) currently represents a major global healthcare burden [112]. Functional abnormalities commonly associated with DN include an early hyperfiltration phase followed by a declining glomerular filtration rate (GFR) and the onset of albuminuria, which can progress to overt proteinuria as disease worsens. Albuminuria is a hallmark clinical marker of DN and is an independent risk factor for the development of cardiovascular disease in diabetic and hypertensive patients [113]. Several factors contribute to DN including but not limited to activated protein kinase-C - mitogen activated protein kinase [34], increased pro-fibrotic transforming growth factor beta (TGFβ) [114, 115] and an abnormal/ overactive renin angiotensin aldosterone system (RAAS) [116-118]. The latter is currently the main therapeutic target in DN, as angiotensin II (AngII) type 1 receptor blockers and angiotensin converting enzyme inhibitors reduce albuminuria and preserve renal function in humans and rodents [119-122]. However, since these agents only slow DN progression, the search for novel or complementary therapies is warranted.

Lipid mediators are factors implicated in DN. Cyclooxygenases (COX) catalyzes the metabolism of arachidonic acid to unstable endoperoxide intermediates, which are then

isomerized into prostanoids via tissue-specific synthases. Prostaglandin E2 (PGE2) is the most abundant renal prostanoid synthesized [123] and exerts its effects through four distinct G-protein coupled E-type receptors (EP1-4) encoded by *PTGER1-4* genes [124-127]. PGE2 acts in an autocrine/ paracrine manner, promoting a variety of cell signaling and physiological responses, depending on prevailing local EP expression profile [62]. COX-derived prostanoids are critical for regulation of salt excretion and blood pressure, as high salt diet induces medullary COX-2 expression in mice [128]. Moreover, COX-2 inhibition impairs renal sodium excretion, in part, by blunting PGE2/ EP2 activation in the collecting duct [70, 129]. However, in addition to its homeostatic function, COX-2 has been implicated in several diseases, including DN [58].

Renal COX-2 expression and prostaglandin levels are elevated in diabetes. Prostaglandin production is increased in isolated glomeruli and cultured mesangial cells (MC) derived from rats with streptozotocin (stz)-induced type-1 diabetes mellitus (T1DM) [88, 89]. In a separate study, NS-398-mediated COX-2 inhibition blunted glomerular hyperfiltration in stz-rats, which correlated with decreased glomerular prostaglandin production [90]. The latter study supports the notion that glomerular hemodynamic alterations early in DN involves the actions of COX-2 derived prostaglandins [130]. As for progressive renal disease, work by Harris and colleagues demonstrated that cortical COX-2 expression increases in the rat remnant kidney model, and that proteinuria and glomerulosclerosis can be attenuated using SC-58326, a selective COX-2 inhibitor [91, 92]. In addition, SC-58326 administration decreased markers of DN, including mesangial matrix expansion and proteinuria in diabetic salt-sensitive hypertensive rats [93]. Likewise, NS-398 reduced albuminuria, GFR and kidney fibronectin expression in TIDM Akita mice [94].

Taken together, these data suggest that COX-2 derived PGE2 signaling through specific EP receptors promotes renal dysfunction in hypertension and/or diabetes.

Which of the four EP subtypes mediates the actions of PGE2 in these disease contexts remains incompletely resolved. Our lab showed that the actions of the podocyte EP4 receptor (EP4) are maladaptive since podocyte-specific EP4 deletion ameliorated, while overexpression of an EP4 mutant resistant to ligand-induced desensitization exacerbated albuminuria and glomerulosclerosis following 5/6 nephrectomy in mice. In addition, adenoviral-mediated EP4 overexpression in cultured mouse podocytes led to an adhesion defect when challenged with mechanical stretch, an *in vitro* surrogate of intraglomerular forces exacerbated by hyperfiltration in DN [131]. Conversely, inhibition of the Gq and Gi-coupled EP1 and EP3 receptors has proven to be beneficial in preventing end-organ damage in severely hypertensive mice [132] and AngII-mediated hypertension [133]. Interestingly, a role for EP1 in DN was suggested by Makino and colleagues who treated stz-induced TIDM rats with an orally-active EP1 antagonist which resulted in improved albuminuria and decreased fibrotic glomerular damage [106], an effect attributed to decreased mesangial fibronectin and TGFβ production. However possible non-glomerular effects of this compound cannot be fully discounted since renal EP1 expression has been described in the vasculature, cortical collecting duct and in proximal tubule cells [64, 65]. Given that pharmacological EP1 inhibition improves renal function and filtration barrier integrity in DN rats [106], we hypothesized that gene-targeted EP1 deletion would attenuate DN-induced glomerular and/or tubular damage in diabetic mice. To this end, T1DM was induced in wild type (WT) and EP1-null (EP1-/-) mice on an FVB/n background using either low-dose stz or genetic OVE26 models. Compared to their

diabetic WT counterparts, diabetic EP1-/-stz or OVE26EP1-/- mice were significantly less albuminuric and had decreased glomerular and tubular damage. Our data suggest that the PGE2 EP1 receptor promotes glomerular and/or tubular dysfunction in diabetic mice further implicating COX-derived PGE2 in mediating deleterious consequences in diseases characterized by compromised renal hemodynamics.

Materials and methods

Antibodies and chemical reagents

Polyclonal goat anti-megalin (P-20); polyclonal rabbit anti-connective tissue growth factor (CTGF, H-55), anti-Wilm's Tumor 1 (WT1, C-19) and monoclonal anti-alpha actin (1A4) purchased from Santa Cruz biotech (*Santa Cruz, Ca.*); polyclonal rabbit anti-fibronectin (F3648) was from Sigma (*St. Louis, MO*). Secondary fluorescent Alexafluor-488 donkey anti-rabbit antibody purchased from Molecular probes (*Burlington, Ont.*). The EP1 antagonist AH6809 was purchased from Cayman Chemical (*Ann Arbour, MI*) and ONO-8711 was supplied by Dr. Richard Hébert (University of Ottawa).

Animals

Global EP1-knockout mice (EP1-/-), generated and characterized by the Breyer group in 2007 [68] were used in this study following backcrossing for 10 generations onto the FVB/n background. Following guidelines established by the Diabetic Complications Consortium, T1DM was induced in WT and EP1-/- mice via the low-dose streptozotocin model[134]. Briefly, 8-10 week old male mice were subjected to 5-day intraperitoneal injections of stz (Sigma, 50mg kg^{-1} BW^{-1}) or 0.1 M Na-Citrate buffer pH 4.5 as vehicle. Mice were followed for 16 weeks post-stz until sacrifice. The transgenic OVE26 model of T1DM was also studied. Previously characterized [135] and commercially available OVE26 mice (Jackson Labs) on an FVB/n background were obtained at 4 weeks of age and intercrossed with EP1-/- mice yielding an OVE26EP1-/- genotype. These groups were studied up to 26 weeks of age. Experimental animals were housed and cared for in the Animal Care Facility at the University of Ottawa with free access to food and water. All surgeries were performed under anesthesia. Protocols were approved by the

University of Ottawa Animal Care Committee and conducted according to the guidelines of the Canadian Council on Animal Care.

Physiological data, plasma analysis and urinary PGE2

At sacrifice, blood was collected via heparinized syringes kept on ice and centrifuged at 9000 *x*g for 10 minutes at 4°C. Collected plasma was immediately frozen and kept at -80°C until subsequent analysis. For the stz study, plasma glucose levels were determined by glucometry while OVE26 study plasma samples were analyzed commercially (IDEXX labs, *Toronto, Ont.).* Urine was collected in metabolic cages and 24 hour volumes were recorded for each mouse. The urine was stored at -80^{o}C. The prostaglandin E2 urinary metabolite 13,14-dihydro-15-keto PGE2 (PGEM) was assayed by a competitive enzyme immunoassay (Cayman Chemical) according to manufacturer's instructions. Briefly, urine samples and the PGEM standard were derivatized overnight at 37^{o}C and assayed in triplicate using a 1:2 dilution of the original sample. Quantification is based on a colorimetric reaction catalyzed by acetylcholinesterase, following a 90 min incubation with Ellman's reagent. The plate was read at 420 nm, and PGEM was determined using the corresponding standard curve. All samples were expressed as picogram PGEM/24 hours.

Systolic blood pressure measurement and FITC-inulin clearance

Prior to sacrifice, systolic BP was measured via tail-cuff plethysmography (BP 2000, Visitech systems, *Apex, NC*) in a subset of mice from each group. Following a five-day training regimen (10 BP readings/ day), average daily systolic BP was calculated from five consecutive days of measurements (5 preliminary, 10 actual BP readings/ day). In parallel, FITC-labeled inulin clearance was used to estimate GFR. Briefly, 5% (w:v)

FITC-inulin (Sigma) dissolved in 0.9% (w:v) saline was dialyzed overnight and sterilized by filtration. Anesthetized mice received a bolus (3.74 μL/g BW) of FITC-inulin retroorbitally. Blood samples (≈20 μL) were collected from the saphenous vein into heparinized capillary tubes, and centrifuged for 10 minutes at 10000 RPM. Blood sampling was carried out at 3, 7, 10, 15, 35, 55 and 75 minutes post injection. Samples were buffered in 500 mM Hepes pH 7.4 and plasma fluorescence was measured (Ex. 488 nm/ Em.538 nm). A two-compartment clearance model was used to calculate GFR as previously described [136] using statistical analysis software (Graphpad Prism, San Diego, Ca.).

Albuminuria

At selected time points, non-diabetic and diabetic mice were subjected to 24 hour urine collection in metabolic cages for subsequent urinalysis. At the 8 week time point, mice were acclimatized to the metabolic cages for 4 hours on the morning of collection. Mice had free access to drinking water and chow. Following collection, samples were kept on ice, centrifuged at 3000 RPM / 10 minutes to pellet urinary sediment and aliquots stored at -80°C until analysis. Albuminuria was measured using the Mouse Albumin Elisa Kit (Bethyl labs, Montgomery, TX.) following manufacturer's protocol. Extrapolated albumin concentrations were normalized to 24 hour urine volume and creatinine concentrations as determined by the Creatinine companion kit (Exocell, Philadelphia, PA).

PAS scoring and immunostaining of kidney sections

At sacrifice, mice were anesthetized, perfused with phosphate buffered saline (PBS) and kidneys were excised, dissected and immediately fixed in 4% paraformaldehyde (PFA).

Paraffin-embedded kidney sections (3 μm) were obtained and stained with periodic-acid (PAS) Shiff reagent. All sectioning, paraffin embedding and PAS-staining were performed by the University of Ottawa's pathology department. PAS-stained kidney sections were viewed under light-microscopy at 400x magnification (Axioskop 2 Imager A1, Zeiss, Germany). Representative glomerular cross-sectional profiles for each group were analyzed in a blinded manner. Imaging software (Axiovision v4.8, Carl Zeiss, Germany) was used to calculate relative mesangial matrix/ glomerular area.

Fibronectin, megalin and α-actin immunohistochemistry was performed on paraffin-embedded sections mounted on glass slides. Sections were deparaffinized in mixed xylenes (Fisher), and rehydrated through a gradient of ethanols and distilled water. Sections were washed 3x in PBS, boiled for 20 minutes in 0.1 M Na-citrate buffer (pH 6.0) for antigen unmasking and endogenous peroxidase activity quenched by 0.3 % H_20_2 in methanol. Sections were blocked in 5% rabbit or goat serum (Vector labs.) for 1 hour and incubated with either anti-fibronectin (1:200), anti-megalin (1:100) or anti-α-actin (1:200) overnight at 4°C. Slides were then incubated with HRP or FITC-labelled rabbit or goat secondary antibodies respectively. Sections were then processed for Vector ABC/3,3'diaminobenzidine (DAB) staining according to manufacturer's instructions (Vector). DAB exposure times were identical for all samples. Slides were then dehydrated and covered with mounting media (Vector) and coverslips. Slides were visualized under light or fluorescence microscopy whereby representative cortical profiles from each group were obtained in a blinded manner. Positive signal area was calculated using the AlphaView software suite (Alpha Innotech).

Immunofluorescence (IF) detection of podocyte Wilm's tumor-1 (WT1) was used to estimate podocyte numbers. Frozen optimal-cutting-temperature (OCT) embedded kidneys were sectioned (8 um) and processed for WT1 staining. Following brief acetone fixation, slides were dried at room temperature, washed in PBS and blocked with 5% donkey serum (Jackson), followed by incubation with a primary anti-WT1 antibody diluted at 1:200 overnight. After several washes, a fluorescently conjugated secondary donkey anti-rabbit antibody (1:1000) was added for 1 hour at room temperature. Sections were covered with fluorescent mounting medium (Vector) and coverslips. Podocyte quantification and analysis was performed in a blinded manner by fluorescence microscopy (Zeiss).

RNA extraction and qPCR

Snap frozen kidney cortex was mechanically homogenized using the TP-103 Amalgamator COECapmixer (GC America, Inc.). Capsules and ceramic beads were dipped into liquid nitrogen prior to sample addition. Cells were homogenized using QIAshredder columns (Qiagen). RNA was extracted using the Qiagen RNEasy minikit as per manufacturer's instructions. Extracted RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) with 500 ng starting material per reaction. Quantitative PCR (qPCR) was performed using an ABI Prism 7000 Sequence Detection System with SYBR Advantage qPCR Premix (Clontech) according to manufacturer's instructions. Primers used: Nephrin sense (5'-CCC AAC ACT GGA AGA GGT GT-3'), antisense (5'-CTG GTC GTA GAT TCC CCT TG-3'); Megalin sense (5'-AGG CCA CCA GTT CAC TTG CT-3'), antisense (5'-AGG ACA CGC CCA TTC TCT TG-3').

Western blotting

At sacrifice, kidney cortex was immediately snap-frozen in liquid nitrogen and kept at -80^{o}C. Tissue was homogenized by rotor-stator in tissue lysis buffer (150 mM NaCl, 1% Triton X-100 and 50 mM Tris pH 8.0, 1% protease inhibitor cocktail (Sigma)), followed by brief sonication on ice. Samples were centrifuged at 13000 rpm for 10 minutes at 4^{o}C. Protein concentration was determined by Bradford method (Biorad reagent) and equal amounts were boiled for 5 minutes and resolved by 8% SDS-page for fibronectin and connective tissue growth factor (CTGF), and by commercially available 4-12% gradient gels (Biorad) for megalin. For fibronectin and CTGF, SDS-page gels were transferred to nitrocellulose membranes, which were blocked in 5% milk, probed with primary antibody overnight and HRP-conjugated secondary antibody (Jackson) for 1 hour at room temperature. For megalin, gels were transferred overnight (25V- 18 hours) at 4^{o}C and immunoblotting occurred as described above. Detection was effected by enhanced chemiluminescence (GEhealthcare) and densitometry performed using the Alpha Imager system (Alpha Innotech).

Previously characterized mouse proximal tubule cells (MCT), provided by Dr. E. Neilson (Vanderbilt University) [137], were grown to confluence, incubated with PGE2 (1uM) and/or ONO8711 (100 nM) and lysed in RIPA containing: 0.5mM PMSF, 1% protease inhibitor cocktail, 1mM sodium pyrophosphate, 10 mM sodium fluoride and 100 μM sodium orthovanadate, and briefly sonicated. Protein was quantified with Bradford reagent (Bio-Rad). Samples were denatured at 70°C for 15 minutes, separated by electrophoresis and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% milk for 90 minutes and incubated overnight with 1:5000 anti-fibronectin

followed by 1:2000 anti-rabbit (Promega) for 90 minutes. Super Signal West Pico Chemiluminescent reagents (Pierce) was applied and β-actin was detected as a loading control for densitometry.

Lucigenin assay for superoxide production

Conditionally immortalized human podocytes [138] were grown at 33 °C on type-I collagen (BD Biosciences) coated plastic culture dishes in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, 100U/ml penicillin/streptomycin, and 10U/ml recombinant-interferon-γ. Differentiation was induced by maintaining the cells at 37 °C in the above media without recombinant interferon for 10-14 days. Cells were maintained in RPMI-1640 medium supplemented with 2% FBS and 100 U/ml penicillin/streptomycin for 2 days prior to treatment with the EP1 antagonist AH6809 for 1 hour with subsequent stimulation with 500 nM AngII (EMD Millipore) for 2 hours to induce superoxide production. Cells were harvested in ice cold phosphate buffer (50mM KH2PO4, 1mM EGTA, 150 mM sucrose) pH 7.4 with protease inhibitors. 50 μL of cell lysate was added to 175 μL buffer and 1.25 μL 1mM lucigenin (ENZO Life Sciences). Baseline activity was measured. Cells were stimulated by addition of 25 μL of 1mM NADPH and active levels were measured. Baseline activity reported as relative light units (RLU) was subtracted and adjusted RLU was normalized to protein concentration.

Myography

Wire myography was used to assess microvascular contractility in response to AngII in experimental mice. Briefly, second order branches of mesenteric arteries were removed from anesthesized mice, placed in Krebs solution and cleaned of connective tissue. Arteries were mounted in a Multi Wire Myograph System (DMT). Maximal vessel

contractility was assessed upon addition of KCl 60 mM + norepinephrine 10 μM. Arteries were then washed and challenged with AngII (10 nM). The AngII-induced contraction response was calculated as a percentage of maximal constriction.

Electron microscopy

Ultrastructural analysis of the glomerular filtration barrier was assessed by transmission electron microscopy (TEM). Kidneys were immersion-fixed in cold 2.4% glutaraldehyde in PBS buffer, post-fixed in 2% buffered osmium tetroxide, dehydrated in graded ethanols and embedded in Spurr resin. Samples were sectioned at 70 nm, placed on copper for TEM, and stained with uranyl acetate and lead citrate. Samples were screened on a Hitachi H-7100 TEM. Representative profiles at 5000x and 20000x from 2-3 glomeruli were assessed in 3 mice/group. Glomerular basement membrane (glomerular basement membrane) measurements were taken in random capillary loops, while avoiding proximity to mesangial cells.

Statistical analysis

The values are presented as means ± SE. Statistical comparisons between two-groups was performed using the unpaired Student's *t*-test, while analysis of variance (ANOVA) was used for three or more groups, followed by a Newman-Keuls posttest. Statistical significance was achieved when $P < 0.05$.

Results

Table 1: STZ-study physiological parameters

	WT	WT stz	EP1-/-	EP1-/- stz
Bodyweight (g)	30.2 ± 0.5	25.8 ± 0.7 **	29.4 ± 0.7	24.5 ± 0.5 **
Kidneys/ BW (mg/g)	12.6 ± 0.2	20.5 ± 0.9 **	14.2 ± 0.2	21.1 ± 0.6 **
Plasma glucose (mM)	6.8 ± 0.5	30.6 ± 1.4 **	7.7 ± 0.2	31.5 ± 0.4 **

**= $p<0.01$ vs. healthy control

Table 2: OVE26-study physiological parameters

	WT	OVE26	EP1-/-	OVE26EP1-/-
Bodyweight (g)	27.1 ± 0.9	22.8 ± 1.1 *	26.6 ± 1.1	21.2 ± 0.9 *
Kidneys/ BW (mg/g)	14.5 ± 0.6	21.7 ± 0.6 †	13.5 ± 0.2	18.9 ± 1.5 †‡
Plasma glucose (mM)	9.5 ± 0.8	38.3 ± 3.1 †	10.6 ± 0.6	38.6 ± 2.0 †

* = $p<0.05$ vs. healthy control

† = $p<0.001$ vs. healthy control

‡ = $p<0.01$ vs. OVE26

EP1 deletion ameliorates albuminuria in two distinct models of T1DM

Pharmacological inhibition of the PGE2 EP1 receptor using an orally active antagonist decreases albuminuria in stz-rats [106]. Therefore we hypothesized that mice with global EP1 deletion would likewise be protected. Low-dose stz was used to induce T1DM. Twenty-four urine samples were collected and albuminuria determined at 8 and 16 weeks post-injection. As shown in Figs. 1a) and b), both WTstz and EP1-/-stz groups had similar albeit elevated urinary albumin levels compared to healthy controls at the 8-week time point. However, at 16 weeks, WTstz mice developed more severe albuminuria, while EP1-/-stz values did not increase to a similar degree, as measured by 24 hour urinary albumin excretion rates (WTstz, 1546 ± 282 vs. EP1-/-stz, 525 ± 110 μg/ 24 hrs., $p<0.001$). Both stz groups developed T1DM phenotypes characterized by slight weight loss, kidney hypertrophy and polyuria (Table 1). Plasma glucose levels were elevated similarly in WT and EP1-/- stz-animals consistent with equivalent diabetes induction in both groups.

Next, we extended our findings to the more robust OVE26 transgenic model of DN [48, 135]. EP1-/- mice were intercrossed onto the OVE26 diabetic transgenic line (OVE26EP1-/-) and followed until 26 weeks of age. OVE26EP1-/- mice were protected from albuminuria as early as 8 weeks of age (1c). OVE26 mice continued to be significantly more albuminuric than OVE26EP1-/- mice at 26 weeks of age (OVE26, 2762 ± 767 vs. OVE26EP1-/- 1022 ± 395 μg/24 hrs., $p<0.05$). At 26 weeks of age, kidney hypertrophy was exacerbated in OVE26 mice as compared to OVE26EP1-/- mice, while plasma biochemistry confirmed that the protective effect was independent of the diabetes-associated hyperglycemia, which reached similar degrees in both groups (Table

2). The observed reduction in overall urinary albumin excretion in the OVE26EP1-/- cohort was not a consequence of altered renal PGE2 production, as urinary levels of the PGE2 metabolite, PGEM were similarly increased in both diabetic groups (Fig.2). Deletion of the *PTGER1* gene did not alter mRNA expression of the other EP receptor subtypes (data not shown).

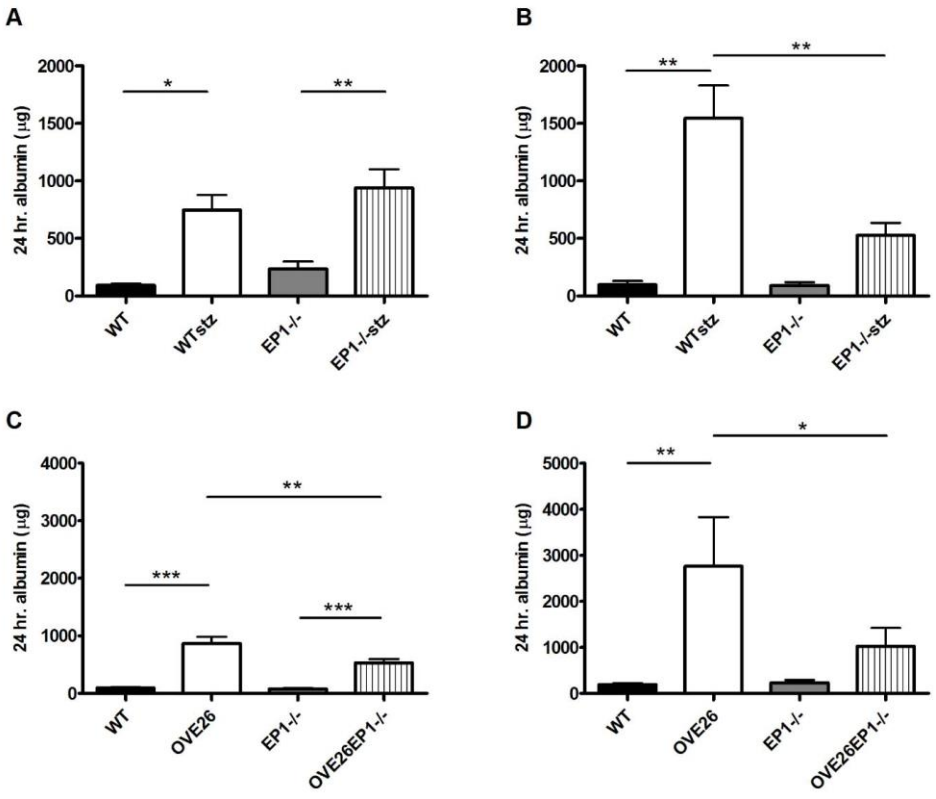

Figure 1: 24 hr. urinary albumin excretion in stz and OVE26 models of T1DM. Albumin concentration was measured by ELISA and normalized to 24 hr. urine volume. A) 8 weeks post-stz. B) 16 weeks post-stz. C) OVE26 study at 8 weeks of age. D) OVE26 study at 26 weeks of age. Data represented as means of duplicate samples ± SEM. (stz study: WT, n=8; WTstz n=9; EP1-/-, n=6; EP1-/-stz, n=11.; OVE26 study: 8 weeks: WT, n=5; OVE26, n=14; EP1-/-, n=8; OVE26EP1-/-, n=9; 26 weeks: WT, n=6; OVE26, n=6; EP1-/-, n=8; OVE26EP1-/-, n=7); *=p<0.05, **=p<0.01, ***=p<0.001.

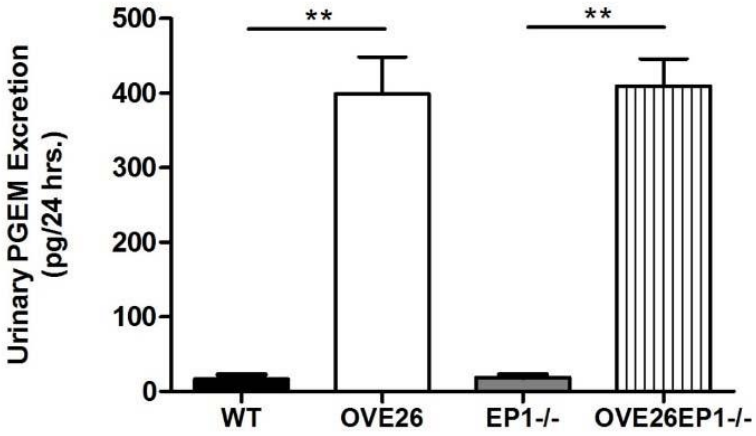

Figure 2: Urine PGE2 levels in OVE26 mice at 26 weeks. Samples were obtained by 24 hr. metabolic cage collection and subjected to PGEM EIA assay. Values were normalized to 24-hour urine volume. Statistical data represented as means ± SEM. (n=5 mice per group.) *=$p<0.05$, **=$p<0.01$.

Decreased albuminuria in EP1-/-stz mice occurs independently of GFR and BP

In order to determine whether the increase in albuminuria observed in the WTstz mice compared to EP1-/- mice after 4 months of diabetes was due to differences in glomerular hyperfiltration, we estimated GFR based on FITC-inulin clearance. As expected, hyperfiltration was evident in both WTstz and EP1-/-stz cohorts, with 2.5-fold increases in GFR (WT, 10.3 ± 2.0 vs. WTstz, 26.1 ± 3.0 and EP1-/-, 11.1 ± 2.5 vs. EP1-/-stz, 28.6 ± 3.8 $\mu L.min^{-1}.g\ BW^{-1}$, $p<0.05$) at 16 weeks post-stz (Fig. 3a**)**. Furthermore, although slightly elevated in all groups, no significant changes in tail-cuff estimated systolic BP were noted between any of the groups (Fig.3b), suggesting that the hyperfiltration due to altered stz-induced renal hemodynamics was unaffected by EP1 deletion in this model at this stage of disease progression.

OVE26EP1-/- mice hyperfilter to a lesser extent than OVE26 at 26 weeks, independently of BP

FITC-inulin clearance was also used to estimate GFR in OVE26 mice at 26 weeks of age. As shown in Fig.3c, a 2.5 fold increase in FITC-inulin clearance was observed in OVE26 mice, indicative of glomerular hyperfiltration, however diabetic EP1-/- mice exhibited milder hyperfiltration vs. OVE26 mice (WT, 10.2 ± 2.1; OVE26, 23.7 ± 1.4; EP1-/-, 10.9 ± 1.3 and OVE26EP1-/-, 15.9 ± 1.7 $\mu L.min^{-1}.g\ BW^{-1}$, $p<0.05$). Diabetes had no effect on systolic BP in each of the groups (Fig. 3d).

EP1 deletion reduces the extent of mesangial matrix expansion and glomerular hypertrophy in both stz and OVE26 models

Structural analysis of PAS-stained paraffin-embedded kidney sections was performed to determine whether the protective effect of genetic EP1 deletion on DN-induced albuminuria was associated with decreased glomerular damage. As shown in Fig. 4 and 5, both the stz and OVE26 models presented with increased mesangial matrix deposition which was attenuated in EP1-/- mice (stz study: WT, 22±1; WTstz, 36±6; EP1-/-, 22±1; EP1-/-stz, 26±4 and OVE26 study: WT, 22±4; OVE26, 35±8; EP1-/-, 23±6; OVE26EP1-/-, 26±8, % of glomerular area). Similarly, while glomerular hypertrophy was elevated in both diabetic models, it was lower in the EP1-/- groups. (stz study: WT, 6±1; WTstz, 10±3; EP1-/-, 8±2; EP1-/-stz, 8±3, and OVE26 study: WT, 7±1; OVE26, 11±3; EP1-/-, 7±2; OVE26EP1-/-, 8±3, glomerular area in mm^2). These data suggest that EP1 receptor ablation may delay the development of early diabetic glomerular structural damage.

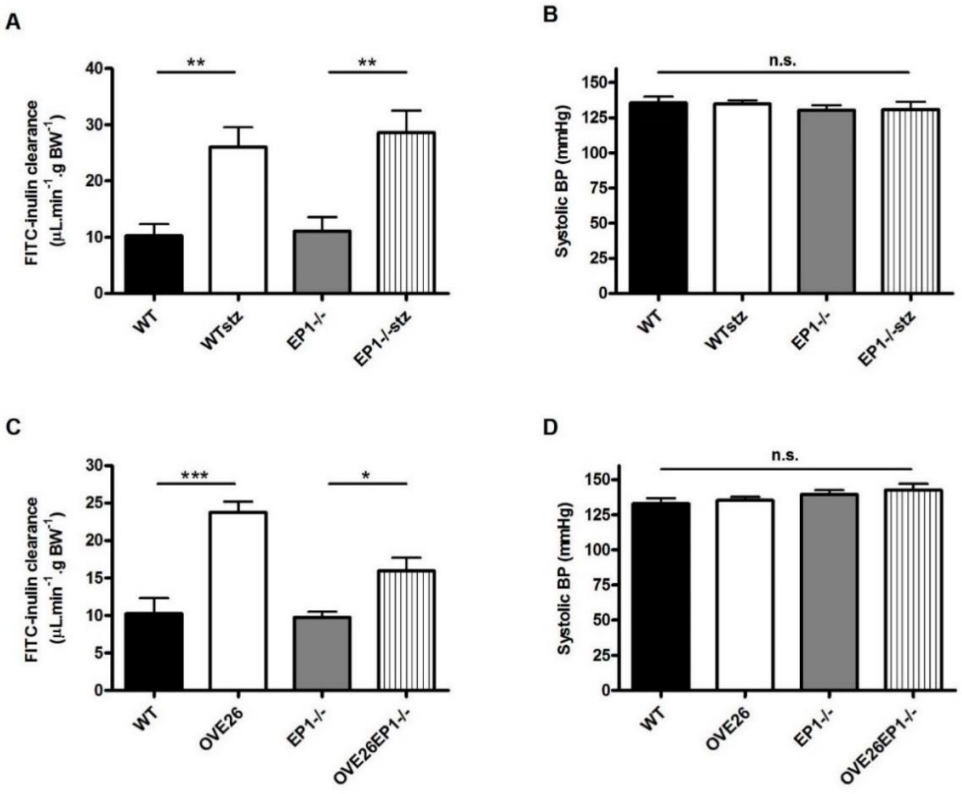

Figure 3: FITC-inulin clearance and systolic blood pressure measurement. (A,C) Prior to sacrifice, mice were subjected to FITC-inulin injections and plasma FITC fluorescence was measured at 488 nm. Data represented as mean ± SEM. (stz-study: n=5 mice/ group; OVE26 study: WT, n=5; OVE26, n=13; EP1-/-, n=9; OVE26EP1-/-, n=5). (B,D) Systolic blood pressure (BP) was measured by tail-cuff plethysmography. Data represented as mean ± SEM of 5 days of independent measurements, 10-readings/ day. (stz study: WT, n=8; WTstz, n=8; EP1-/-, n=9; EP1-/-stz, n=8; OVE26 study: WT, n=11; OVE26, n=8; EP1-/-, n=7; OVE26EP1-/-, n=6), *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

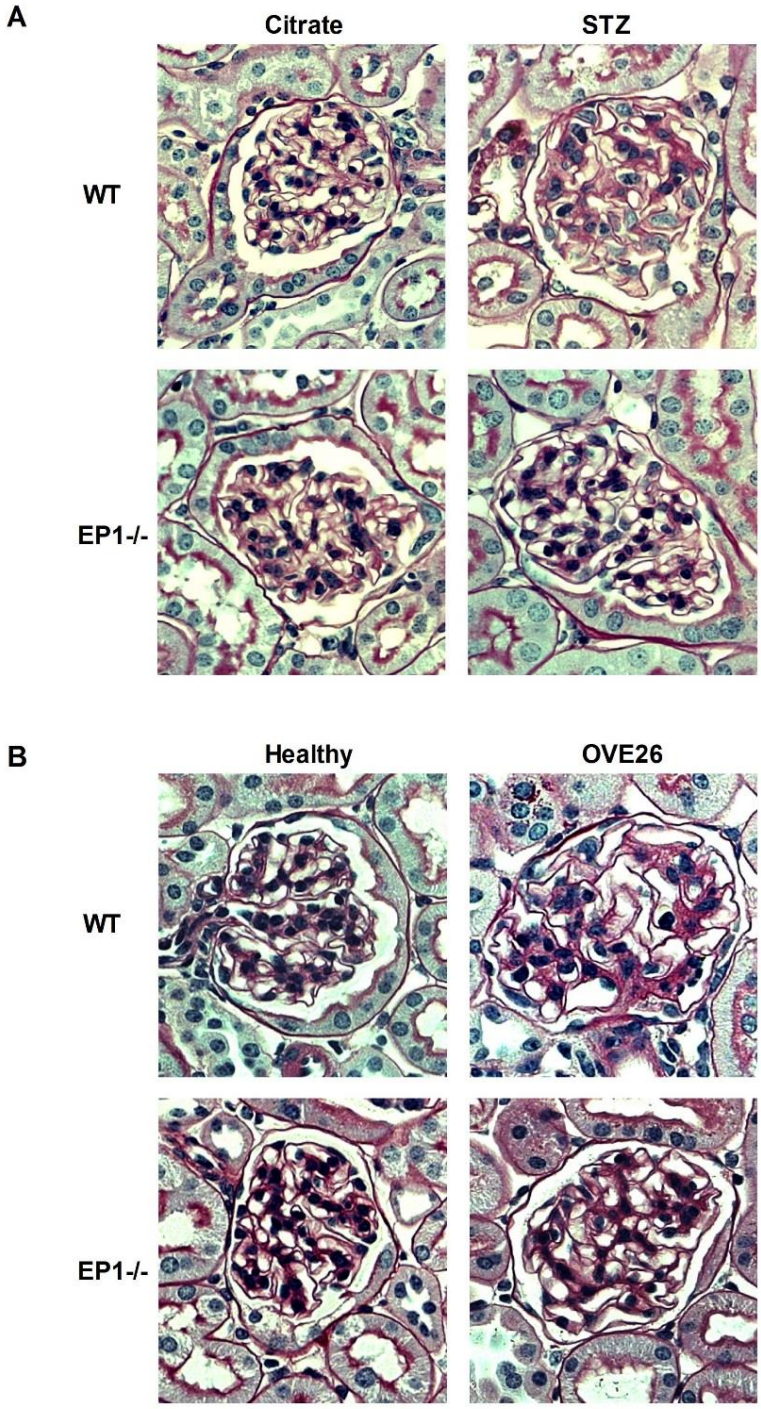

Figure 4: PAS staining in both studies. Representative images of paraformaldehyde-fixed paraffin-embedded kidney sections stained with PAS at 16 weeks for the stz study (A) and 26 weeks for the OVE26 study (B).

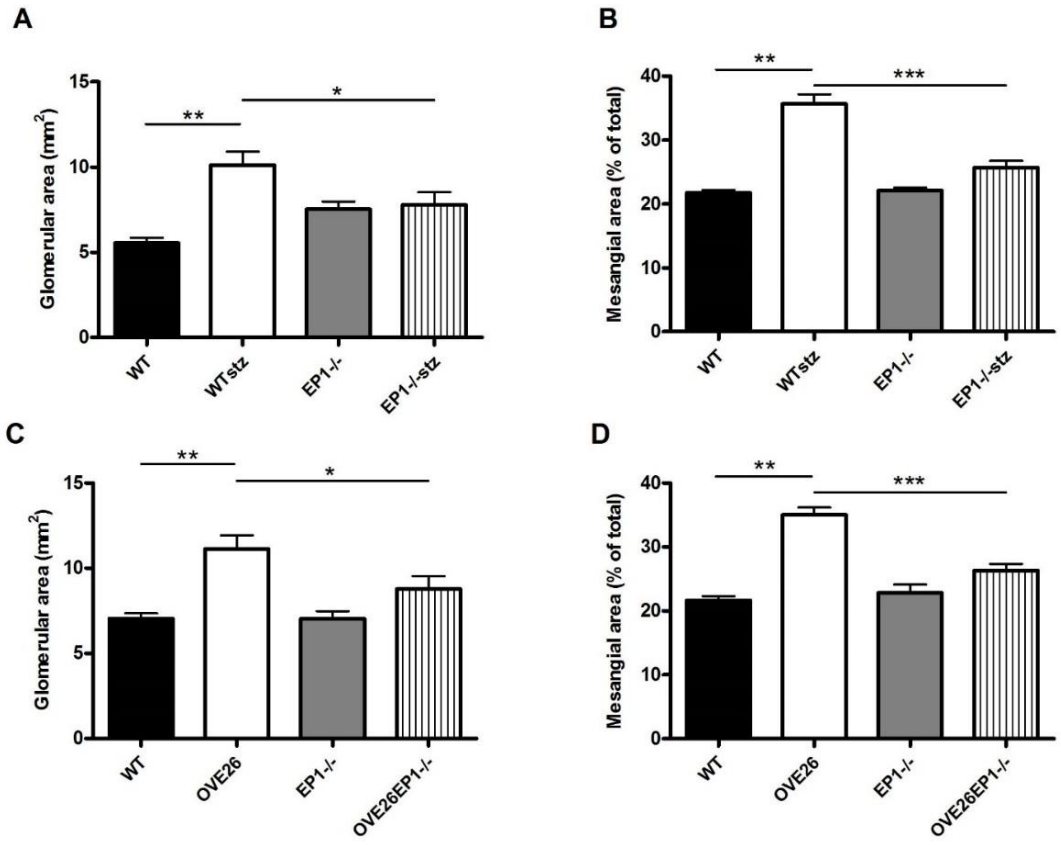

Figure 5: Glomerular mesangial expansion and hypertrophy measurements in stz and OVE26 models of T1DM. Glomerular surface area (A, C) and mesangial matrix scoring (B, D) in stz and OVE26 study at 16 and 26 weeks respectively. Statistical data represented as means ± SEM. (n=5-7 mice per group, 25 fields/mouse @ 200X magnification), *=p<0.05, **=p<0.01, ***=p<0.001.

WT1-positive cell counts are similar in diabetic WT and EP1-/- glomeruli

Podocyte depletion, caused by either apoptosis or detachment, is commonly observed in DN, [10, 32, 139]. To further evaluate the extent of glomerular filtration barrier damage, we counted podocyte numbers in both stz and OVE26 mouse cohorts. As depicted in Fig. 6, diabetic EP1-/- mice had a similar reduction in WT-1 positive nuclei compared to WT mice in both diabetic models (stz study: WT, 15.2 ± 0.6; WTstz, 11.5 ± 0.8; EP1-/-, 15.9 ± 0.8; EP1-/-stz, 11.8 ± 0.6; OVE26 study: WT, 17.1 ± 0.5, OVE26, 14.2 ± 0.3; EP1-/-, 18.2 ± 0.6; OVE26EP1-/-, 13.6 ± 0.4, WT1+ cells/ glomerulus, $p<0.05$). Since the severity of DN-induced podocyte depletion is similar in both cohorts, EP1 activation may impact podocyte structure/function in ways that do not affect cell numbers.

DN-induced glomerular basement membrane thickening and foot process derangement are reduced in OVE26EP1-/- mice

Transmission electron microscopy was used to assess glomerular filtration barrier integrity in a subset of mice from the OVE26 study. Increased albumin leakage and glomerular structural damage seen in these diabetic mice was associated with foot process effacement (Figure 7a) and augmented glomerular basement membrane width (Figure 7b), which were not apparent in the OVE26EP1-/- cohort (WT, 159±3; OVE26, 214±36; EP1-/-, 160±2; OVE26EP1-/-, 175±10 nm, $p<0.05$). These data further confirm a protective effect of EP1 receptor deletion on the podocyte and the glomerular filtration barrier in this DN model.

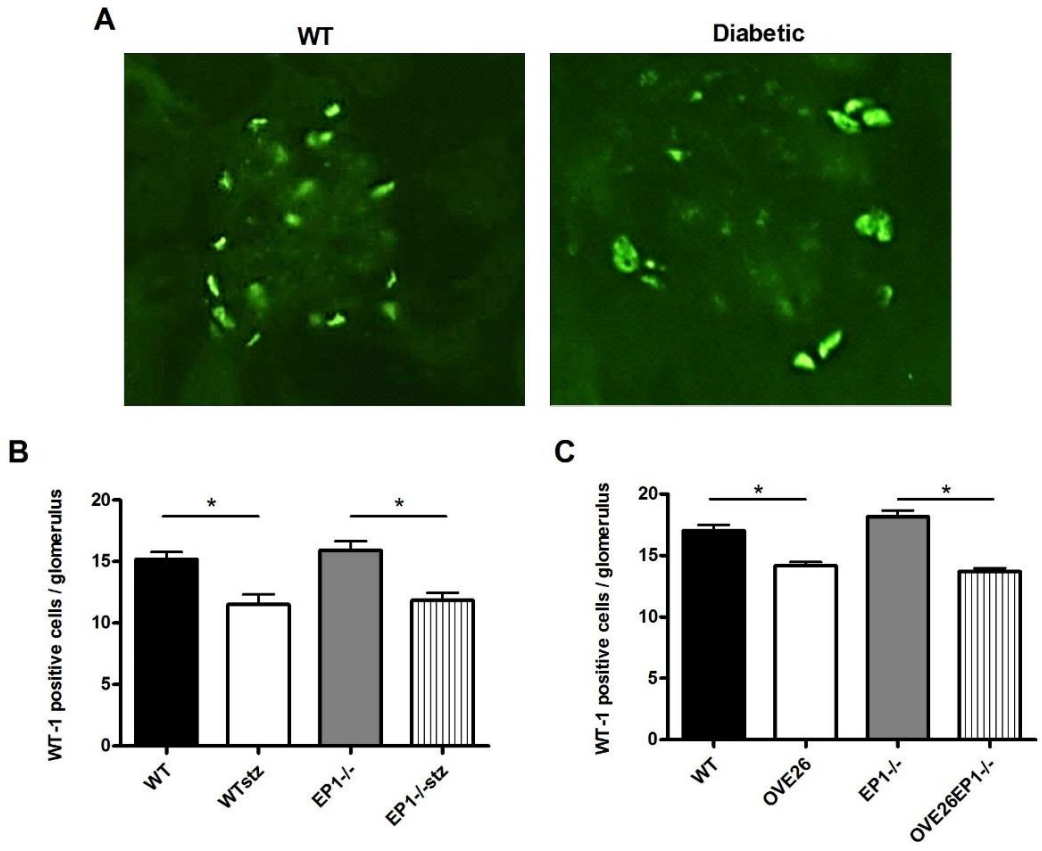

Figure 6: Glomerular podocyte estimation in stz and OVE26 models. Mice were sacrificed and kidneys processed for frozen sectioning and WT-1 antibody immunofluorescence microscopy. A) Representative glomeruli from healthy and diabetic mice; B) Graphical representation of stz study and C) OVE26 study WT-1 positive podocytes per glomeruli. A total of 15-20 glomeruli per mouse were assessed in each group. Statistical data represented as means ± SEM. (n=4-6 mice per group), *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

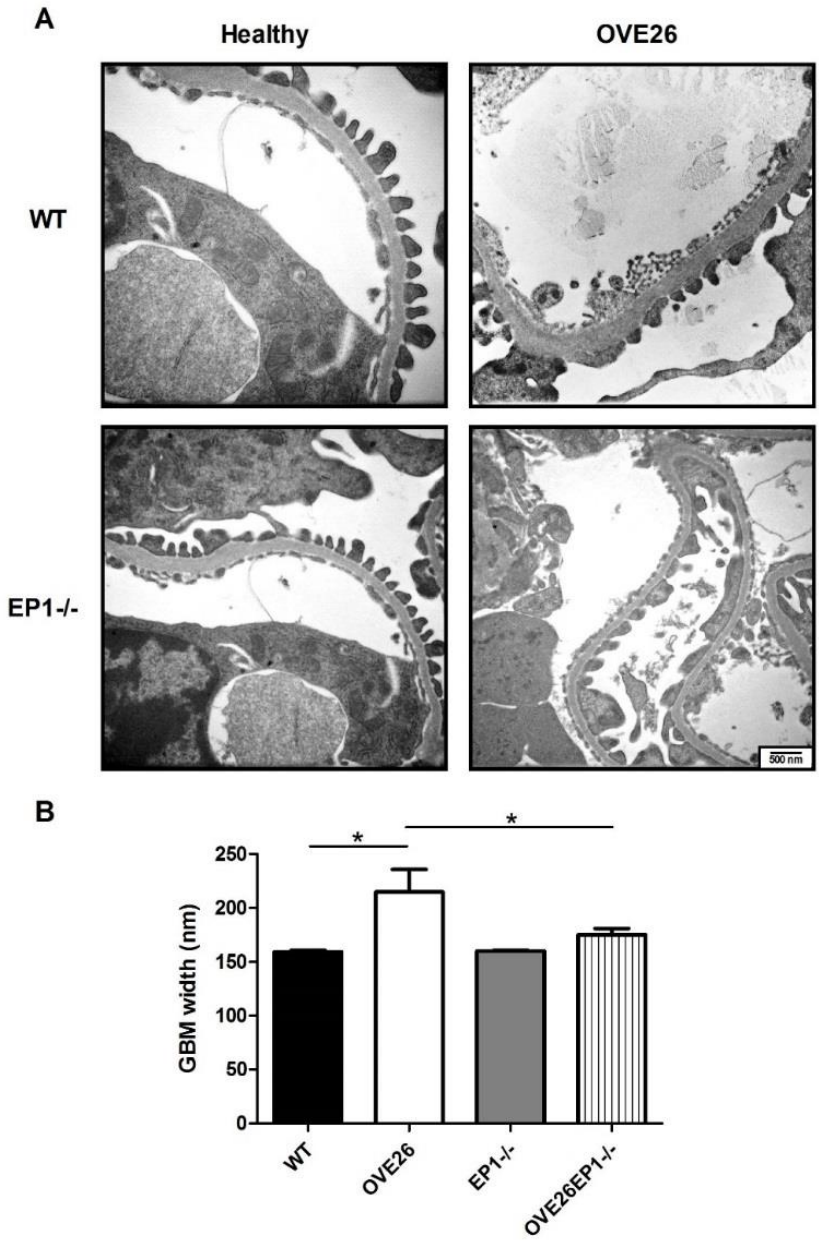

Figure 7: Transmission electron microscopy in the OVE26 study. Glutaraldehyde-fixed cortex samples were processed for TEM as described above. A) Representative micrographs from each group and B) glomerular basement membrane thickness measurements. A total of 20-30 representative micrographs from 2-3 glomeruli per mouse, n=3 mice per group. Statistical data represented as means ± SEM. *=$p<0.05$.

EP1-antagonism reduces DN-induced nephrin mRNA downregulation in diabetic mice and Ang-II induced podocyte-derived superoxide production in culture

Since deletion of the EP1 was associated with glomerular filtration barrier protection, we hypothesized that EP1 targeting may have a beneficial effect on the podocyte slit diaphragm in DN. To this end, we measured nephrin mRNA levels in the cortex of the OVE26 study mice. Renal nephrin mRNA levels were significantly reduced in OVE26 mice, but were preserved in OVE26EP1-/- mice (Figure 8a). Higher nephrin levels may be indicative of preserved function in remaining podocytes, consistent with improved filtration barrier integrity in diabetic EP1-/- mice.

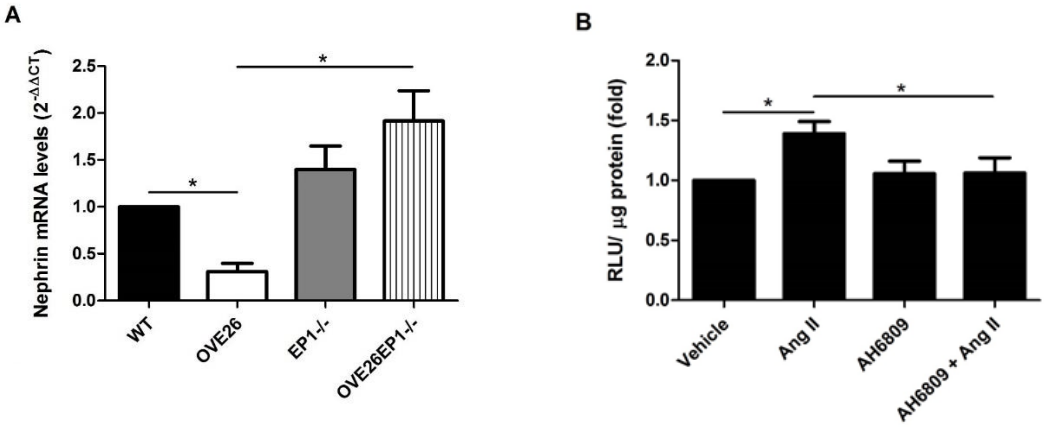

Figure 8: Nephrin qPCR in the renal cortex and ROS generation in human podocytes. A) Kidney cortex RNA was reverse transcribed and qPCR was performed using SYBR Green. Data are reported using the delta deltaCT method, and expressed as fold WT, normalized to GAPDH. Statistical analysis represented as means ± SEM from 4-6 mice per group assayed in triplicate. *=$p<0.05$. B) Cultured human podocytes were differentiated for 14 days, and subjected to 2 hr. AngII (500 nM) stimulations with or without pre-treatment with the EP1 receptor antagonist, AH6809. Data are represented as mean RLU/ µg protein ± SEM from 4 experiments. *=$p<0.05$.

Recent studies showed that AT1 receptor activation is partly dependent upon EP1 and/or EP3 receptor signaling [133]. While the specific interaction between these two receptor families remains incompletely described, they often act in concert to promote synergistic effects, including reactive oxygen species generation and/or increasing vascular dysfunction [140-142]. Since increased oxidative stress, due to higher levels of reactive oxygen species can result in podocyte damage and ultimately the development of albuminuria [143], we determined whether EP1-ablation would reduce AngII-mediated reactive oxygen species generation. As shown in Figure 8b, AngII induced superoxide production in conditionally-immortalized human podocytes, as assessed by lucigenin assay, showed that antagonism of the EP1 receptor with AH6809 abrogated the AngII-mediated superoxide production. These findings suggest that the EP1 and AT1 may act in concert to enhance damage-inducing podocyte superoxide production.

EP1-/- diabetic mice have reduced expression of renal fibrosis markers

In addition to filtration barrier damage, diabetes promotes tubular dysfunction leading to interstitial fibrosis. We therefore measured cortical fibronectin expression as an indication of kidney fibrotic damage. Immunoblotting of renal lysates revealed that fibronectin expression was upregulated in WTstz, but was unchanged in EP1-/-stz mice compared to healthy controls (Fig.9 WT, 809 ± 82 vs. WTstz, 2060 ± 212 and EP1-/-, 1335 ± 232 vs. EP1-/-stz, 1327 ± 75 a.u., $p<0.01$). In parallel, fibronectin expression as detected by immunohistochemistry revealed elevated staining in WTstz mice with a trend towards reduction noted in the EP1-/- stz mice as compared to healthy controls. However these data did not reach statistical significance. The above findings suggest the EP1 may be involved in profibrotic renal damage in DN.

In the OVE26 study, markers of DN-induced renal damage which were assessed included fibronectin, connective-tissue growth factor (CTGF) and α-actin. The OVE26 phenotype was associated with an increase in renal fibronectin **e**xpression; however immunoblotting revealed that EP1-/- mice had decreased overall fibronectin levels (WT, 2957 ± 421; OVE26, 6247 ± 444, EP1-/-, 1790 ± 212, OVE26EP1, 2895 ± 299, a.u., $p<0.01$). As the OVE26 model typically induces a more robust diabetic phenotype as compared to stz, we measured renal CTGF expression. CTGF protein expression was decreased in OVE26EP1-/- mice compared to OVE26 mice (WT, 1987 ± 150; OVE26, 3528 ± 313; EP1-/-, 2105 ± 271; OVE26EP1-/-, 2212 ± 313, a.u. $p<0.05$). Moreover, α-actin staining was assessed as an additional marker of interstitial fibrosis. As shown in Fig. 11, basal α-actin staining was observed in vascular structures in all mice, however the presence of α-actin positive cells was markedly elevated in the interstitium of OVE26 mice, an effect observed to a lesser extent in the EP1-/- cohort.

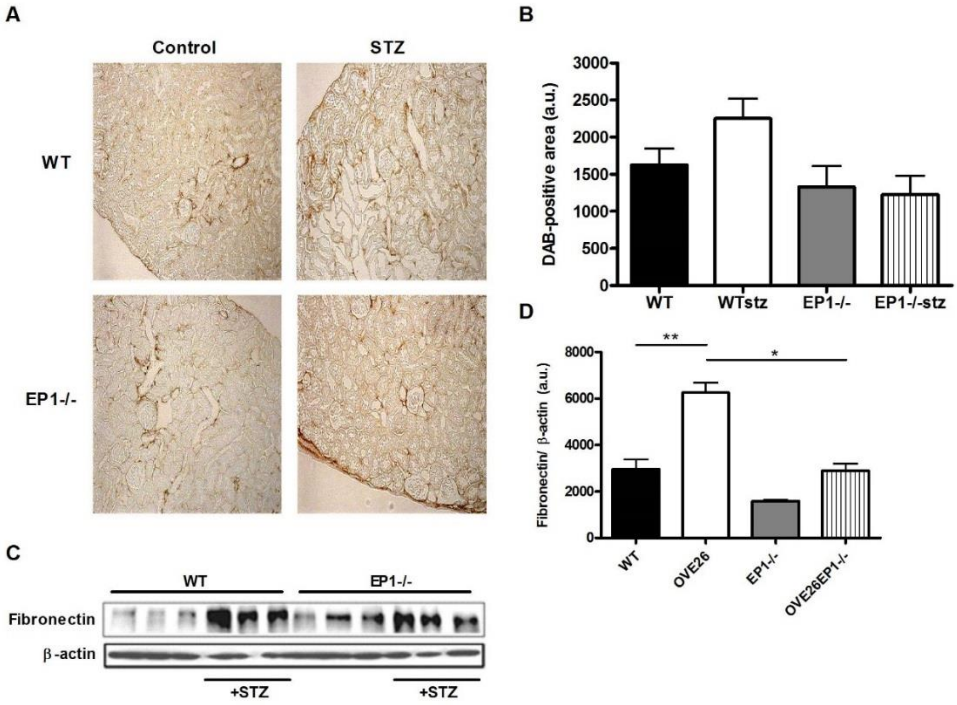

Figure 9: Renal fibronectin expression in STZ mice. Paraffin-embedded kidney sections were processed for immunohistochemistry (IHC) with anti-fibronectin antibody and visualized by light-microscopy. A) Representative image of fibronectin IHC in the cortex (200X mag.). B) Analysis of immunodetectable fibronectin expression. C) Representative fibronectin and β-actin western blots in mouse kidney cortex. Protein (10 μg) resolved by 8% SDS-Page Tris-HCl gel. D) Graphical representation of fibronectin western blot as determined by densitometric analysis. Statistical analysis represented as means ± SEM in 3-5 mice per group. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

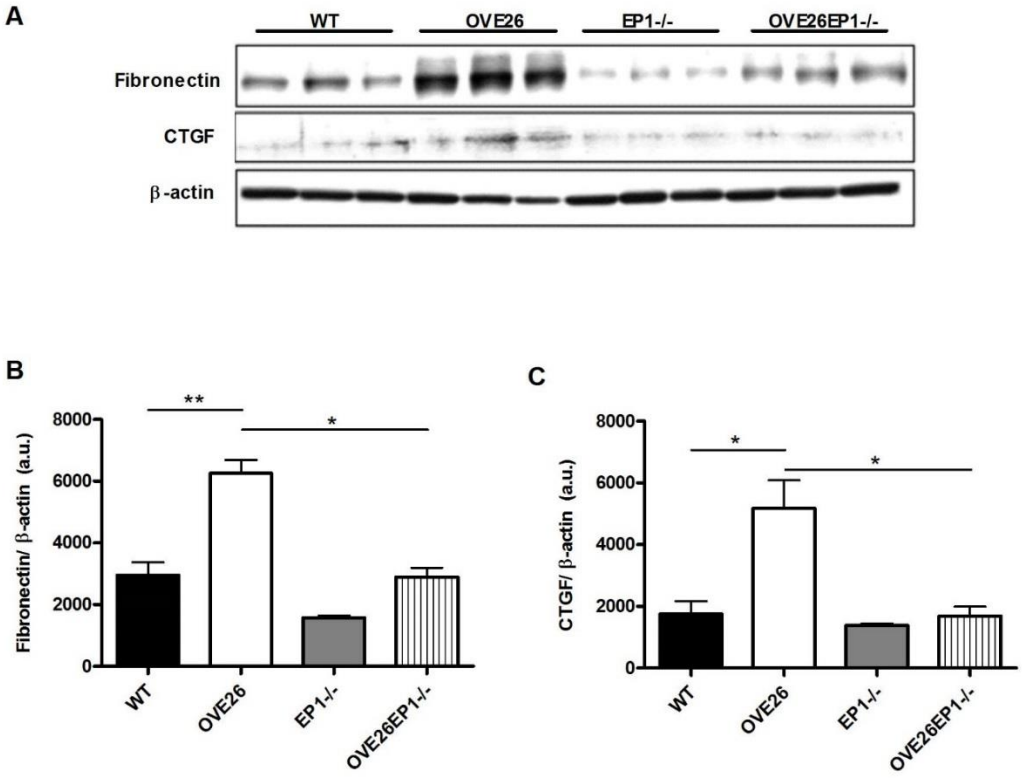

Figure 10: Renal fibronectin and CTGF Immunoblotting in OVE26 mice. A) Representative western immunoblot of fibronectin and CTGF protein expression in mouse kidney cortex. Protein (15 μg) resolved by 8% Tris-HCl SDS-Page. Graphical representation of fibronectin (B) and CTGF (C) western blot as determined by densitometric analysis. Statistical analysis represented as means ± SEM in 4-6 mice per group. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

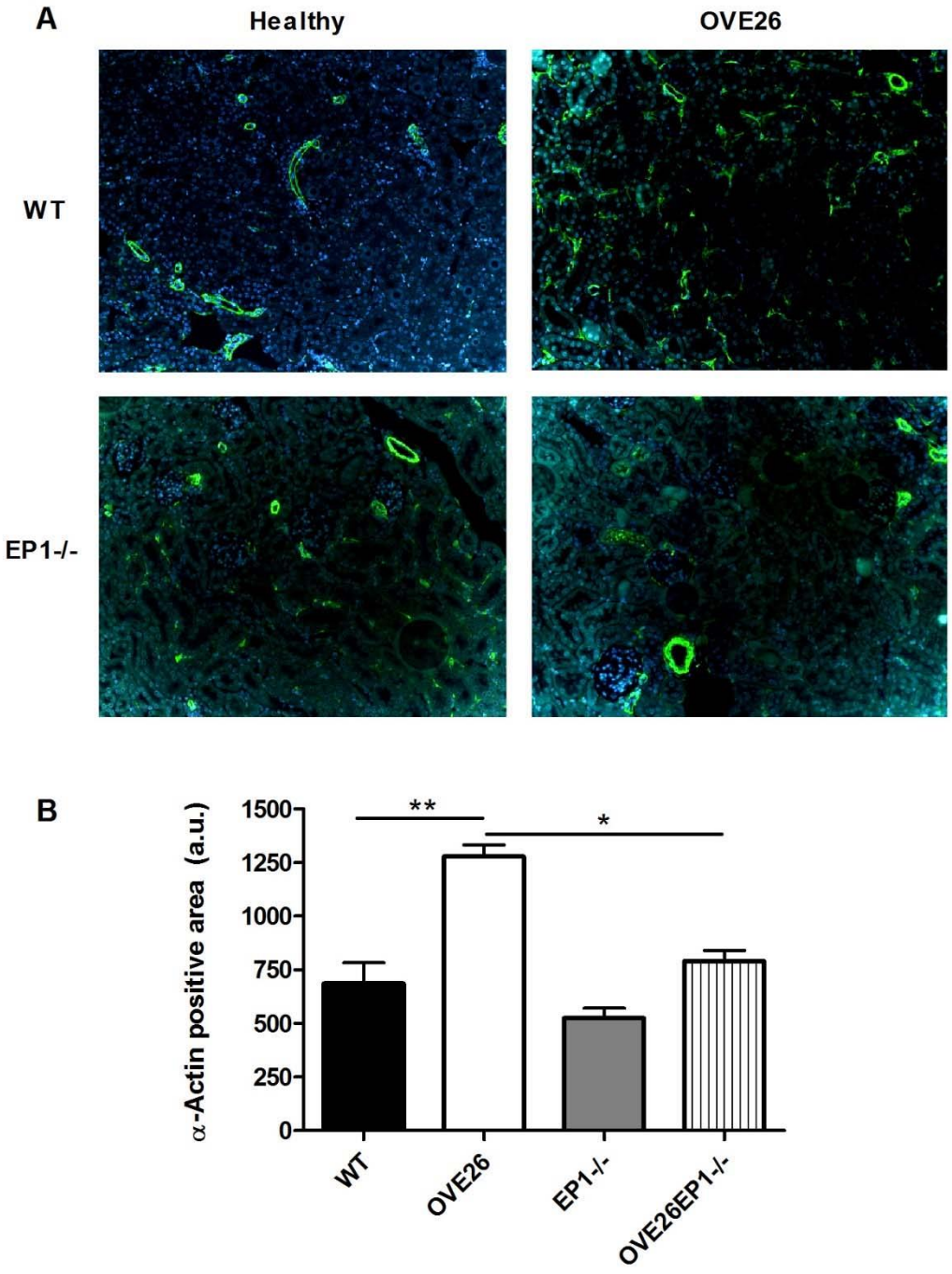

Figure 11: α-actin staining in OVE26 study. Paraffin-embedded kidney sections were processed for immunofluorescence staining with anti-α-actin antibody and visualized by fluorescence microscopy. A) Representative images of α-actin in the cortex (200X mag.). B) Analysis of immunodetectable α-actin expression. Statistical analysis represented as means ± SEM in 3-5 mice per group. *=p<0.05, **=p<0.01, ***=p<0.001.

EP1 receptor mediates PGE2-induced fibronectin expression in cultured PT cells

In order to further explore the protective effect of EP1 deletion on diabetes-induced cortical fibronectin upregulation, we tested whether PGE2 stimulates fibronectin expression in cultured mouse PT cells. To this end, MCT cells were stimulated with either PGE2 alone or in combination with the EP1 receptor antagonist ONO8711. PGE2 stimulated fibronectin expression by two-fold at 24 hours which was abrogated entirely by ONO8711 (Fig. 12). These data suggest that PGE2 acting via its EP1 receptor participates in the PT`s profibrotic response in the diabetic kidney.

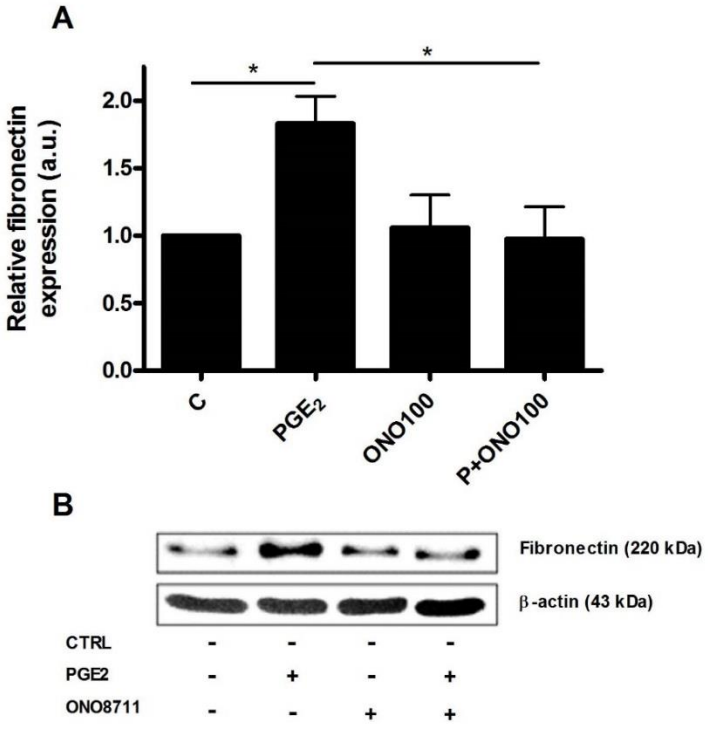

Figure 12: MCT cell fibronectin expression. MCT cells were grown to confluence and stimulated with either vehicle, PGE2 (1μM), ONO8711 (100 nM) or both for 24 hours and samples subjected to Western immunoblotting. Densitometric analysis (A) and graphical representation (B) of fibronectin and β-actin are shown. Statistical analysis represented as means ± SEM from 4-6 experiments. *=$p<0.05$.

EP1-/-stz and OVE26EP1-/- mice have preserved tubular megalin expression

Tubular damage in diabetes is often associated with increased glucose and sodium reabsorption, cellular hypertrophy and impaired albumin reabsorption [144, 145]. Since megalin participates in post-glomerular albumin processing along the PT, we tested whether the reduced albuminuria seen in diabetic EP1-/- mice was accompanied by attenuated tubular damage, as measured by megalin expression. Immunodetectable megalin protein was significantly decreased in OVE26 and stz mice, whereas diabetic EP1-/- mice were protected against megalin loss (stz study: WT, 1135 ± 89; WTstz, 791 ± 48; EP1-/-, 1062 ± 63; EP1-/-stz, 1091 ± 124; OVE26 study: WT, 993 ± 148; OVE26, 630 ± 101; EP1-/-, 940 ± 53; OVE26EP1-/-, 1094 ± 185, a.u.). Similar findings were observed by immunoblotting for megalin protein in stz-study cortex samples (Fig. 13e), while mRNA levels were decreased in both cohorts (Figure 13f).

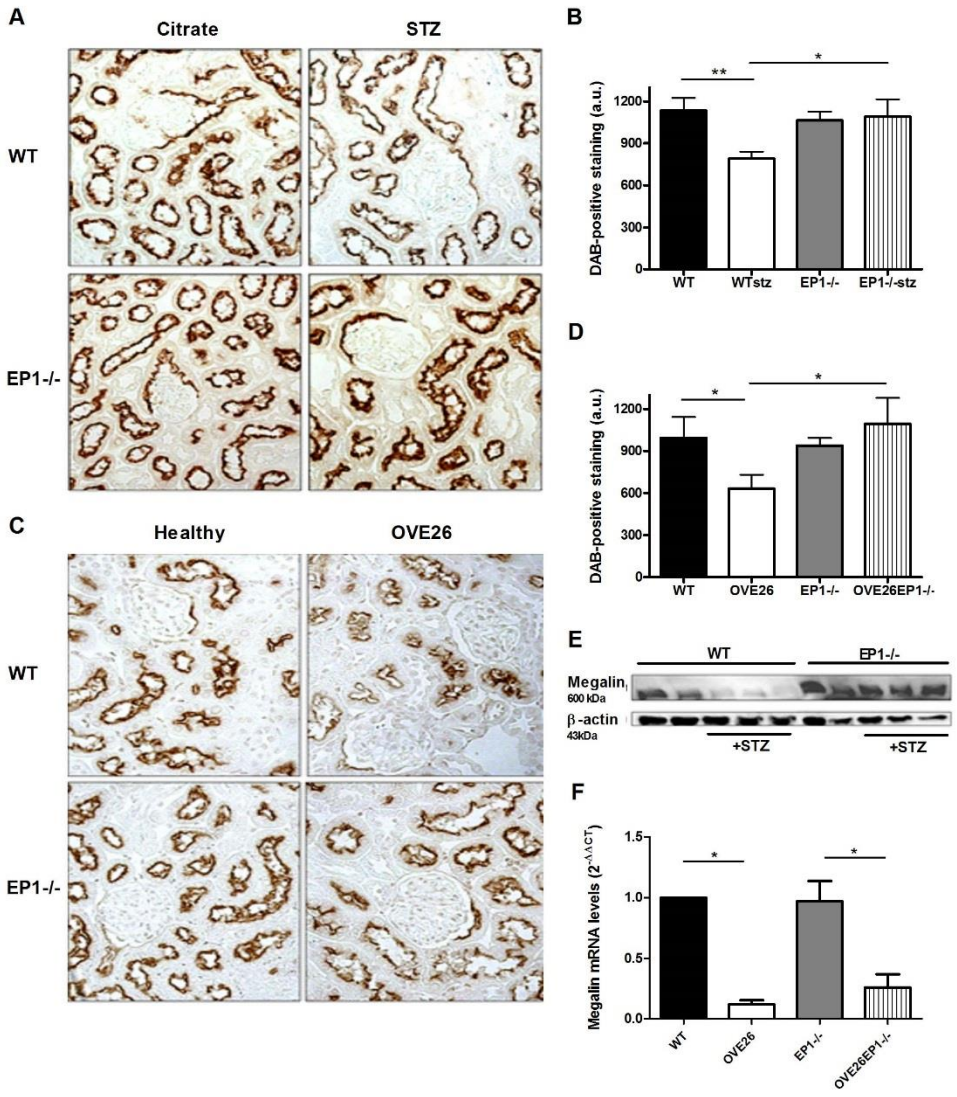

Figure 13: Kidney megalin expression in STZ and OVE26 models of T1DM. Representative images of immunodetectable megalin protein by IHC (A, C) Graphical representation of IHC scoring (B, D). E) Megalin immunoblotting. Kidney cortex protein (15 μg) was resolved in 4-12% gradient Tris-HCl gel. (n=4-6 per group) and probed with a megalin antibody. F) Megalin qPCR. Kidney cortex mRNA was isolated and megalin pPCR was performed in the OVE26 study using SYBR green on cDNA (n=5/ group). Statistical analysis represented as means ± SEM. *=p<0.05, **=p<0.01, ***=p<0.001.

OVE26EP1-/- mice are less sensitive to AngII – induced mesenteric artery vasoconstriction

In addition to expression in glomerular and tubular cells, the EP1 receptor is also found in vascular smooth muscle cells along with the angiotensin AT1 receptor where it likely contributes to vasoconstriction. To assess whether loss of EP1 receptor expression would affect AngII-mediated vasoconstriction, we isolated mesenteric arteries from a subset of the OVE26 study mice at 30 weeks of age/ diabetes and subjected them to wire myography. As represented in Fig.14, mesenteric arteries isolated from OVE26 diabetic mice exhibited a significantly enhanced AngII-induced vasoconstriction. However, both the maximal AngII-induced contraction and the rate of vasoconstriction were markedly reduced in vessels obtained from OVE26EP1-/- mice. Thus PGE2/EP1 signaling appears to enhance AT1 signaling in the diabetic vasculature. However whether this phenomenon occurs in renal arterioles will require further investigation.

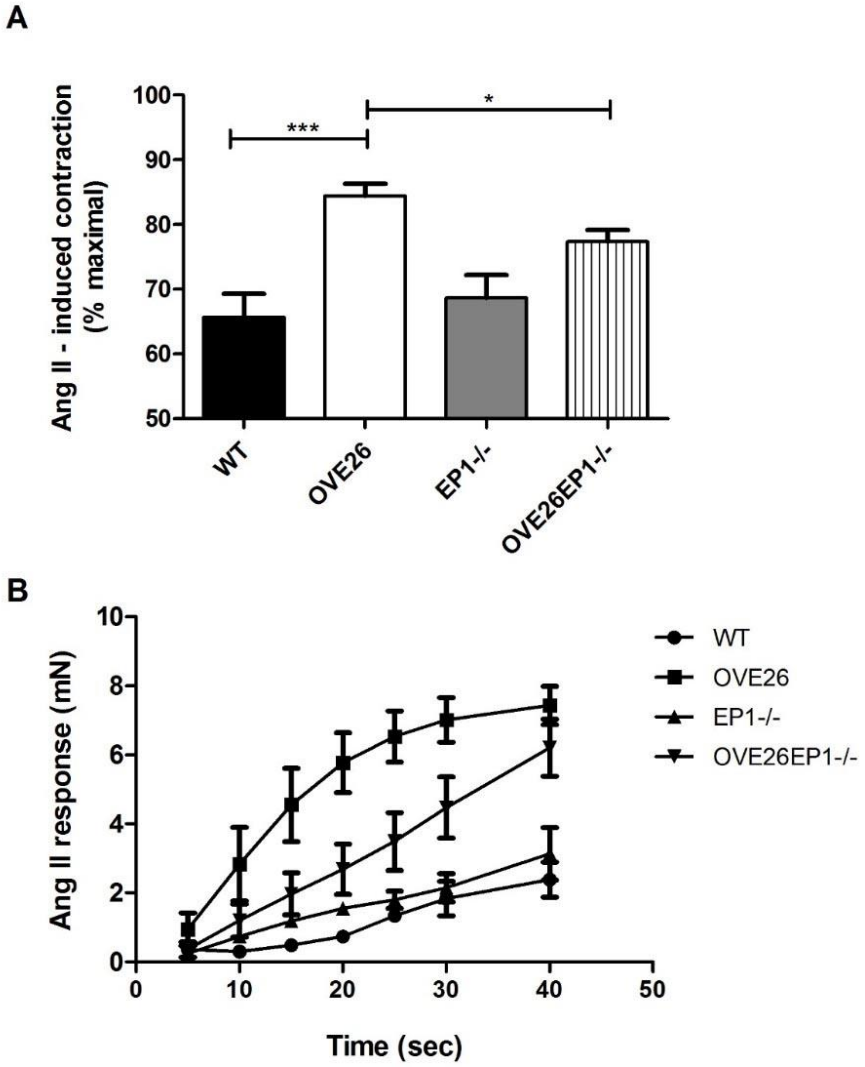

Figure 14: Myography on isolated mesenteric arteries from OVE26 study. Mesenteric arteries were removed from anesthesized mice, placed in Krebs solution and mounted in a wire Multi Myograph System. Maximal contraction was achieved by stimulation with KCl (60 mM) and norepinephrine (10 uM). Arteries were then washed and stimulated with AngII (10 nM). Data represented as A) percent of maximal contraction achieved by AngII stimulation and B) AngII response curves as a function of time. Statistical analysis represented as means ± SEM. WT, n=3; OVE26, n=6; EP1-/-, n=4; OVE26EP1-/-, n=6). *=p<0.05, **=p<0.01, ***=p<0.001.

Discussion

COX inhibitors (e.g., NSAIDs) which block the synthesis of prostaglandins, thromboxanes and prostacyclins (i.e., the prostanoids) - reduce DN-associated proteinuria [146, 147]. Although COX inhibition is anti-proteinuric, NSAIDs and the recently developed gastrointestinal-sparing COX-2 selective inhibitors (coxibs) can be nephrotoxic for renal disease patients [148, 149]. By blocking the synthesis of vasodilatory prostaglandins these drugs can elicit a precipitous decline in renal blood flow and GFR [126, 150-153]. Moreover, some of the clinical data with COX-2 inhibitors have failed to demonstrate beneficial anti-proteinuric effects [154]. Such discrepant outcomes are likely due to the fact that NSAIDs block the synthesis of an entire family of COX-derived prostanoids, which exert numerous biological actions through a host of cell surface receptors. More effective strategies might therefore focus downstream of COX blockade and differentiate between those prostanoids and their respective receptors that deliver protective effects from those that impair renal function.

Presently, we studied the role of the EP1 on the progression of DN in mice. Using two distinct models of T1DM, we observed a 60% reduction in urinary albumin excretion and decreased renal structural and ultrastructural damage in mice with global EP1 deletion, suggestive of partial yet significant preservation of glomerular filtration barrier integrity. Furthermore, our data show that the PGE2 EP1 receptor promotes renal and glomerular hypertrophy, mesangial matrix expansion and indications of tubulointerstitial fibrosis. The limited renal damage in EP1-/- cohorts was independent of the diabetic status of the mice, as all groups displayed similar hyperglycemia. Our results using a gene-targeted approach are consistent with previously published data that showed beneficial effects of

pharmacological antagonism of the EP1 with ONO8713 on albuminuria and mesangial cell dysfunction in stz-rats [106]. To our knowledge, our study is the first to identify EP1 receptor actions at the PT as immunodetectable levels of megalin were significantly preserved in diabetic EP1-/- mice, and that EP1 antagonism reduced PGE2-mediated fibronectin upregulation in a PT cell line. Furthermore, recent data implicates the EP1 in promoting end-organ damage in severely hypertensive mice, due to increased susceptibility to developing aortic aneurysms [132]. Abolishing EP1 expression in our diabetic mice had no effect on BP, as it remained unchanged with the onset of diabetes. However a striking disparity was noted regarding mouse survival, as OVE26EP1-/- mice fared better than age-matched diabetic controls (data not shown).

The *PTGER1* gene encodes a seven transmembrane receptor that utilizes the Gαq (Gq) signaling axis whereby PGE2 binding to the EP1 leads to the activation phospholipase Cβ, which catalyzes phosphoinositide hydrolysis, calcium mobilization and protein kinase C activation [126]. Renal EP1 expression has been described in mesangial cells (MC), podocytes, collecting duct cells, the vasculature and in proximal tubule cells [64, 106, 125]. Makino and colleagues attributed the beneficial effect seen by EP1 antagonism in with ONO8713 in DN rats to decreased MC fibronectin and TGFβ production at the transcriptional level [106]. Other studies showed that the hypertrophic response of cultured rat MCs to angiotensin II could be blocked by pharmacological EP1 antagonism [141]. Our findings are consistent with such observations as glomerular hypertrophy and mesangial matrix expansion were significantly blunted in diabetic EP1-/- mice. While the impact of altered MC homeostasis is important as these cells help support glomerular architecture, the extent of podocyte damage or loss was not investigated in those studies.

Podocytes maintain filtration barrier integrity by establishing the size and charge selective 40 nm-wide slit diaphragm [155]. Increased Gq signaling can be detrimental to podocyte health, as constitutive Gq signaling induces COX-2 via calcineurin/ NFAT activation and promotes podocyte apoptosis [156, 157]. A role for COX-2 in podocyte injury has also been proposed, whereby podocyte-specific overexpression of COX-2 in mice increases adriamycin-induced albuminuria and foot process effacement, an effect, which can be blocked using the COX-2 selective inhibitor, NS398 [158]. Since Gq activation predisposes podocytes to damage via COX-2 induction, the renoprotective effect of EP1 deletion may be due in part to decreased signaling of this receptor subtype in podocytes. In our study, we observed minimal podocytopenia in diabetic mice with no significant differences noted between WT and EP1-/- mice. Yet in culture, EP1-antagonism had an inhibitory effect on AngII-mediated podocyte superoxide generation. In addition, TEM revealed DN-induced ultrastructural damage to the filtration barrier's glomerular basement membrane and podocyte foot processes was significantly reduced in OVE26EP1-/- mice, suggesting a direct detrimental effect of PGE2/EP1 signaling on podocyte health and glomerular filtration barrier integrity. Thus, activation of the podocyte EP1 receptor may lead to morphological changes while providing additional Gq-signaling input, promoting a pro-oxidant context leading to further filtration barrier damage. Whether the reduced albuminuria seen in diabetic EP1-/- mice was primarily governed by a loss of podocyte EP1 activity, thereby directly preventing damage to this final layer of the filtration barrier will require further investigation.

While filtration barrier injury likely accounts for the majority of the urinary albumin content in DN, the PT may also play a role in the early stages [159, 160] . PT-mediated

albumin reabsorption occurs at the level of the brush border where the megalin-cubilin endocytic protein complex is abundantly expressed. In healthy individuals, minute amounts of albumin (600 mg/day) leak through the glomerulus and reach the PT, yet less than 30 mg are detected in the urine, which implies that the PT reabsorbs 95% of filtered albumin. In a small T1DM study cohort, microalbuminuria was associated with increased megalinuria and cubilinuria, possibly due to increased matrix-metalloprotease-induced shedding in the PT lumen [161]. Furthermore, diabetic rats have decreased PT-mediated albumin reabsorption, an effect that can be blocked by antagonizing RAAS activation [145, 162]. We measured cortical megalin expression in our TIDM mouse cohorts in order to assess the impact of EP1 deletion on PT integrity. Our results show decreased immunodetectable renal megalin for both diabetic models. EP1 deletion prevented megalin protein downregulation but not mRNA expression, thereby suggesting post-translational regulation of this gene product. It remains unclear whether activation of the EP1 on PT cells directly impacts megalin expression or instability, thereby decreasing albumin reabsorption or if increased glomerular albumin leakage coupled with toxic luminal albumin concentrations may result in megalin downregulation and associated PT dysfunction. Of interest, activation of the functionally similar, Gq-coupled AT1 receptor has been shown to downregulate PT-megalin expression [163]. In these studies, AngII infusion reduced both megalin expression and albumin endocytosis in proximal tubules of stz-rats [162], an effect which was prevented by angiotensin converting enzyme inhibition or AngII receptor blockers. In fact, a growing body of evidence suggests the involvement of the COX/PGE2/EP1 pathways in modulating the RAAS system. PGE2/ EP1 activation facilitates AngII-mediated oxidative stress and endothelial damage in the

cerebral vasculature [164] and is a major player in hypertensive renal damage [104, 132, 140, 165]. In agreement with these studies, our work shows that EP1 deletion reduced AngII-mediated contractility of isolated mesenteric arteries and attenuated AngII-stimulated oxidative stress in cultured podocytes. The precise molecular mechanism that accounts for such interactions awaits investigation. Moreover, while the present studies were conducted using mesenteric arteries, whether such receptor interactions occur in glomerular vessels remains unknown. However, it is not unreasonable to speculate that EP1/AT1-stimulated vasoconstriction of the efferent arteriole could contribute to intraglomerular capillary pressure elevations thereby contributing to filtration barrier damage in DN. Taken together, if EP1/AT1 dependency represents a general phenomenon, occurring wherever these two receptors are co-expressed, our data would suggest that inhibiting PGE2/ EP1 signaling may complement existing RAAS blockade treatments thereby conferring additional renoprotection in DN.

Summary

In conclusion, abolishing EP1 signaling is protective against the onset and progression of early DN in type-1 diabetic mice, as it reduces the extent of renal structural and functional damage. It remains unclear whether PGE2/EP1 signaling is detrimental to a specific resident renal cell type, since both glomerular and tubular compartments benefitted from abrogated EP1 activation. Further studies should be undertaken to fully elucidate the role of the EP1 receptor in other renal compartments including the vasculature in DN. Targeting the renal EP1 may represent a worthy therapeutic target in order to circumvent undesirable side effects associated with current COX modulating drugs.

Chapter 3: Vascular smooth muscle-specific EP4 deletion exacerbates angiotensin II-induced renal injury

VASCULAR SMOOTH MUSCLE-SPECIFIC EP4 RECEPTOR DELETION IN MICE EXACERBATES ANGIOTENSIN II-INDUCED RENAL INJURY

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Running Title: Vascular EP4 receptor is beneficial in hypertension

Key words: prostaglandin E2, EP4 receptor, hypertension, renal blood flow

Abstract: 237

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Description

The COX enzyme and its derived prostaglandins play major roles in maintaining systemic fluid and electrolyte balance, glomerular hemodynamics and vascular tonicity. The effects brought on by activation of this system are increasingly relevant in conditions of decreased circulating fluid volume or other states in which kidney function is challenged. In the current study, we hypothesized that negative renal side effects associated with COX-inhibition may due to loss of PGE2/EP4 signaling in the vasculature. To this end we generated mice with targeted *PTGER4* (EP4) gene deletion specifically in vascular smooth muscle cells and subjected these mice to the AngII-model of hypertension.

Abstract

Cyclooxygenase -inhibition by chronic non-steroidal anti-inflammatory drug use is contraindicated in hypertension as it may reduce glomerular filtration rate and diminish renal blood flow. Accordingly, loss of cyclooxygenase-derived prostaglandin E2 acting via E-Prostanoid 4 receptors which normally dilates the renal vasculature by counteracting pressor hormones such as angiotensin II could account for such non-steroidal anti-inflammatory drug-associated effects on renal function. We hypothesized that EP4 receptor deletion from vascular smooth muscle cells) would predispose to renal injury in a model of hypertension. We generated mice with inducible vascular smooth muscle cell-specific EP4 receptor deletion ($EP4^{VSMC-/-}$) under control of the tamoxifen-sensitive smooth-muscle actin promoter and subjected them to angiotensin II-induced hypertension by osmotic mini-pumps .EP4 deletion was verified by qPCR of aorta and renal vessels, as well as functionally by the loss of prostaglandin E2-mediated mesenteric artery relaxation by wire myography. After 4 weeks both angiotensin II-treated wild type ($EP4^{VSMC+/+}$) and $EP4^{VSMC-/-}$groups were similarly while albuminuria was exacerbated in AngII-treatment in $EP4^{VSMC-/-}$ mice but not in $EP4^{VSMC+/+}$ mice led to severe glomerular scarring and tubulointerstitial fibrosis. AngII significantly lowered glomerular filtration rate in $EP4^{VSMC-/-}$ mice, but not in $EP4^{VSMC+/+}$ mice. Lastly, AngII-treated $EP4^{VSMC-/-}$ mice showed evidence of capillary damage and reduced renal blood flow as measured by fluorescent bead microangiography and dynamic contrast-enhanced magnetic resonance imaging, respectively. Our data suggest that renovascular EP4 receptors buffer the actions of AngII upon renal hemodynamics and thereby protect against hypertension-associated structural damage.

Introduction

Renal side effects associated with non-steroidal anti-inflammatory drug (NSAID) use include acute renal failure, interstitial nephritis, papillary necrosis and hyperkalemia [166]. NSAIDs are therefore contraindicated in hypertensive patients as they can impair diuresis/natriuresis and increase otherwise elevated blood pressure (BP) by 5-6 mmHg [95]. Post-hoc analysis of the INVEST trial found increased adverse side effects in hypertensive patients with coronary artery disease who were also chronic NSAID users [96]. Not only is NSAID use associated with modest increases in blood pressure in healthy individuals, but can also inhibit the actions of anti-hypertensive medications including angiotensin-converting enzyme inhibitors and diuretics [98].

Prostaglandin E2 (PGE2) is the major product of COX-mediated processing of arachidonic acid, and exerts its potent actions via four E-type prostaglandin G-protein-coupled receptors 1-4 (EP1-4) encoded by the *PTGER1-4* genes. Produced throughout the nephron and renal vasculature, PGE2 simultaneously regulates numerous physiological and pathophysiological responses [65, 67]. For example, tubular sodium and water handling as well as regulation of renal vascular resistance and glomerular hemodynamics are dependent on renal PGE2 signaling. Low-dose infusion of PGE2 in the renal artery leads to vasodilation while counteracting the actions of vasoconstrictive agents such as angiotensin II (AngII), catecholamines and vasopressin [65, 67, 99, 100]. PGE2 can also buffer the vasoconstrictive response AngII in isolated rat pre-glomerular vessels, an effect associated with maintenance of glomerular filtration rate (GFR) and renal blood flow (RBF) [101]. The severe impact brought on by inhibition of COX-2 metabolites on RBF and GFR in susceptible subjects has been well documented [167]. Accordingly,

NSAID-mediated PGE2 inhibition is contraindicated in volume depleted states, in hypertension, edema or congestive heart failure [81]. Therefore, PGE2 acting via its EP receptors is a potent vasoactive agent critical for preserving renal hemodynamics in conditions of physiological stress [168].

Activation of the renin angiotensin aldosterone system (RAAS) via induction of its rate limiting enzyme renin, is affected by the COX/PGE2/EP4 cascade as PGE2-stimulated renin release in isolated kidneys is blunted in mice with global *PTGER4* deletion [71, 74] COX-mediated EP4 receptors participate in AngII-induced pro-renin receptor activation in the rat renal medulla, promoting renal injury [169, 170]. AngII can significantly affect GFR and RBF through its ability to constrict glomerular and medullary blood vessels. AngII's effect on glomerular hemodynamics occurs primarily via afferent and efferent arteriolar constriction, which is enhanced with concomitant NSAID treatment through inhibition of PGE2-mediated afferent arteriole [171, 172]. Prostaglandins also buffer AngII's actions by mitigating elevations in afferent arteriolar vascular smooth muscle cell (VSMC) intracellular calcium levels [173]. Recent studies in rats identified the EP1 and EP4 receptors as being responsible for transient vasoconstriction and sustained vasodilation, respectively, in pre-glomerular afferent arterioles [102]. EP4 receptors are found in podocytes where they may carry out injurious actions as overexpression or deletion exacerbates or mitigates renal injury in the 5/6 nephrectomy model of CKD, respectively [111]. Selective EP-receptor targeting may be beneficial in CKD since mice with global *PTGER1* deletion are partly protected against development of diabetic nephropathy [106, 142, 174]. Additionally, data suggest EP1 targeting may also be beneficial in hypertension, as genetic deletion and pharmacological antagonism reduces

AngII-induced BP elevation and associated end-organ damage [68, 132]. Since NSAIDs can exacerbate the effects of hypertension on kidney health while EP1-selective inhibition is beneficial in this context, we hypothesized that vasodilatory EP4 receptors are tasked with maintaining renal hemodynamics and that targeting of this subtype worsens hypertension-associated renal injury. In the current study, we show that genetic deletion of VSMC *PTGER4* increases the susceptibility of mice to AngII-induced glomerular and tubulointerstitial injury, coupled with vessel rarefaction, reduced GFR and RBF.

Materials and methods

(See online supplement for detailed procedure)

Antibodies and reagents

Polyclonal rabbit anti-HIF1α (Novus biologicals, Littleton, CO.), ImmPress anti-rabbit Ig (Vector, Burlington, ON), Tamoxifen (Sigma-Aldrich, Oakville, ON), FITC-Inulin (Sigma-Aldrich, Oakville, ON), Angiotensin II (Bachem), anti-α-tubulin (Sigma-Aldrich, Oakville, ON), Gadovist (Bayer, Missisauga, ON)

Experimental animals

We generated inducible vascular smooth muscle cell (VSMC)-specific EP4 knockout mice ($EP4^{VSMC-/-}$). These mice were obtained by intercrossing previously characterized [175] and commercially available SMA-Cre-ER^{T2} mice with $EP4^{flox/flox}$ mice (obtained from Dr. Matthew D. Breyer, Vanderbilt University), each on a congenic FVB/N background. $EP4^{flox/flox}$ mice have loxP sites flanking exon 2 of the EP4 gene making it a conditional knockout [176]. $EP4^{flox/flox}$ mice are healthy and fertile, exhibiting no obvious renal or other phenotypes and were backcrossed for 10 generations to obtain congenic mice on the FVB/N

background. SMA-Cre-ER^{T2} mice express Cre-recombinase under control of the smooth muscle actin (SMA) promoter, which itself is activated by the estrogen-receptor agonist tamoxifen. Positive SMA-Cre-EP4$^{flox/flox}$ expression in the progeny was confirmed by genotyping for the presence of a 550 bp product (SMA-Cre forward: 5'-aggtgtagaaggcacttag-3'; SMA-Cre reverse: 5'-ctaatcggcatctcccagcagg-3'). Excision of the exon 2 of the *PTGER4* gene was achieved by treating 4-6 week old animals with tamoxifen (corn oil as vehicle) for 5 days (1mg/ day, i.p.). Knockout efficiency was validated by quantifying EP4 mRNA transcript levels in isolated aortic and renal vascular RNA preparations and by assessing PGE2-induced (10^{-11}M) vasodilation. In order to test whether the vascular EP4 receptor is required to maintain adequate renal function in a hypertensive context, we challenged EP4$^{VSMC+/+}$ and EP4$^{VSMC-/-}$ mice with the AngII-induced model of hypertension. At 6-8 weeks of age, under isoflurane anesthesia, mini-osmotic pumps (Alzet, model 2004, Cupertino, CA) containing sufficient AngII for 4 weeks of drug delivery at a rate of 1000 ng.kg^{-1}.min^{-1} were surgically implanted subcutaneously. Control mice underwent sham operation, omitting pump implantation. Experimental animals were housed and cared for in the Animal Care Facility at the University of Ottawa with free access to food and water. Surgical protocols were approved by the University of Ottawa Animal Care Committee and conducted according to the guidelines of the Canadian Council on Animal Care. In all instances, anesthesia was done using isoflurane.

Physiological parameters and blood pressure

At sacrifice, organs were excised, weighed and normalized to total body weight. Spot urine samples were collected at baseline and at endpoint for measurement of albuminuria and freezing-point depression-based urine osmolality (Advanced Instruments, Norwood,

MA). Throughout the study, systolic blood pressure (SBP) was measured via tail-cuff plethysmography (BP 2000, Visitech systems, Apex, NC) as described previously [177]. Average SBP was calculated from measurements obtained at the same time period each day (5 preliminary, 10 actual BP readings/ day) and, following a five-day training regimen (10 BP readings/ day), bi-weekly BP measurements were obtained.

Albuminuria

Integrity of the glomerular filtration barrier was assessed by measuring urinary albumin levels in spot urine samples collected throughout the study. Albuminuria was measured using the Mouse Albumin Elisa Kit (Bethyl labs, Montgomery, TX.) following manufacturer's protocol using morning spot urine samples. Creatinine concentration was determined by the Creatinine Companion kit (Exocell, Philadelphia, PA). Urine albumin-to-creatinine ratios (*ACR*; μg/mg) were calculated by normalizing albumin concentrations (μg/mL) to creatinine content (mg/dL). Absorbances readings were obtained using a 96-well plate reader (Fluostar).

FITC-inulin based glomerular filtration rate estimation

Fluorescein isothiocyanate-labeled inulin (FITC-Inulin; Sigma-Aldrich, Oakville, ON.) clearance was used to estimate GFR. Briefly, 5% (w:v) FITC-inulin dissolved in 0.9% (w:v) saline was dialyzed (1000 molecular weight cutoff) overnight and sterilized by filtration (0.2 μm). Anesthetized mice received a bolus (3.74 μL/g BW) of FITC-inulin via tail-vein injections. Blood samples (≈20 μL) were collected from the saphenous vein into heparinized capillary tubes, and centrifuged for 10 minutes at 15000 *x*g. Sampling was carried out at 3, 7, 10, 15, 35, 55 and 75 minutes post injection. Plasma samples were buffered in 500 mM Hepes pH 7.4 and fluorescence was read (Excitation 488 nm/

Emission 538 nm). A two-compartment clearance model was used to calculate GFR as previously described [136] using statistical analysis software (Graphpad Prism, San Diego, Ca.).

Quantitative PCR

Cortical and medullary RNA was extracted using the Qiagen RNEasy minikit as per manufacturer's instructions. Extracted RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription kit (Life technologies, Burlington, ON) with 500 ng starting material per reaction. For determination of renal vascular EP4 mRNA levels, kidney vasculature was immediately dissected at sacrifice and snap frozen. RNA was isolated using RNAqueous-Micro Total RNA isolation kit (Ambion, Life technologies, Burlington, ON). Quantitative PCR (qPCR) was performed using an ABI Prism 7000 Sequence Detection System with SYBR Advantage qPCR Premix (Cedarlane, Burlington, ON) according to manufacturer's instructions. Target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Rodent Taqman GAPDH Reagents (Life technologies, Burlington, ON) Analysis was performed using the $2^{-\Delta\Delta CT}$ method. (COX-2: *forward, 5'-ggggtgcccttcacttctttca-3'; reverse, 5'-tgggaggcacttgcattga-3';* EP4: *forward, 5'-atggtcatcttactcatcgcca-3'; reverse, 5'-ctttcaccacgtttggctgat-3'*).

Vessel myography

To functionally verify the loss of EP4 receptor expression, wire myography was used to assess microvascular relaxation of pre-constricted mesenteric arteries in response to PGE2 (10^{-11}M). Second-order branches of mesenteric arteries were dissected from anesthetized and tamoxifen-treated $EP4^{flox/flox}$ ($EP4^{VSMC+/+}$) and SMA-Cre-$EP4^{flox/flox}$ ($EP4^{VSMC-/-}$) mice and placed in Krebs solution for cleaning and removal of connective

tissue. Arteries were mounted onto a Multi Wire Myograph System (DMT, Ann Arbor, MI). Pre-constriction of isolated arteries was achieved using 60 mM potassium chloride. Arteries were then stimulated with PGE2 (10^{-11} M). Data represented as percent (%) of maximal constriction.

Fluorescence microangiography

Renal microvascular damage was assessed using fluorescence-based micro-angiography as previously established [178] and further optimized [179]. Briefly, At sacrifice, mice were anesthetized using isofluorane and were intracardiacally injected with a pre-warmed (42^{o}C) 1 mL solution of 100 IU/mL of heparin/ 0.9% sodium chloride, followed by 1 mL of 3M potassium chloride directly in the left ventricle. Finally a 5 mL slurry composed of 1% low-melting point agarose (Sigma-Aldrich, Oakville, ON) and 10% (w/v) of 0.02 um yellow/green FluoSpheres sulfate (Invitrogen) in distilled water were injected directly into the left ventricle. Upon removal, visual evaluation of kidney appearance was used to omit samples which were deemed incompletely perfused. Kidneys were immediately placed on ice, sectioned in a sagittal manner and fixed in 4% paraformaldehyde for 2 hours, followed by an overnight incubation in 30% sucrose at 4^{o}C in the dark. Samples were embedded in optimal-cutting temperature media (OCT) and sectioned at 20 μm thickness. Representative images of cortex and medullary regions were acquired by fluorescence microscopy (Zeiss, Germany) using 100X and 200X magnification. Images were processed using Image J and run through the provided Matlab script (supplemental data [179]) for analysis of capillary density, functional area and size distribution. We analyzed 10-15 200X fields, representative of inner cortical and outer-medullary regions in each mouse (3 mice/ group).

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI)

Jugular vein cannulation was performed in anesthetized mice for contrast-agent infusion for DCE-MRI. *In vivo* renal perfusion experiments were performed at the University of Ottawa pre-clinical imaging core using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) on a 7 Tesla GE/Agilent MR 901. During DCE, at 2.5 min, a 66:1 saline solution was used to deliver 3 uL of Gadovist intravenously (~ 0.1 mmol/kg). The median calculated contrast agent concentration in each region of interest was used to generate concentration-vs.-time data in whole kidney, medulla, and cortex. The volume transfer coefficient (K_{trans}), which provides an index of renal perfusion, was estimated in each region using the modified Tofts model in the freely available PMI software package developed by Dr. Steven Sourbron, University of Leeds, UK. (https://github.com/plaresmedima/PMI-0.4-Runtime-Ottawa).

Statistical Analysis

The values are presented as means ± SE. Statistical comparisons between two-groups was performed using the unpaired Student's t-test, while analysis of variance (ANOVA) was used for three or more groups, followed by a Newman-Keuls post-test. Statistical significance was achieved when $P \leq 0.05$.

Results

Table 1: Experimental animal physiological data

	$EP4^{VSMC+/+}$	$EP4^{VSMC+/+}$ + *AngII*	$EP4^{VSMC-/-}$	$EP4^{VSMC-/-}$ + *AngII*
Bodyweight (g)	25.6 ± 0.8	26.4 ± 1.6	25.1 ± 2.5	24.5 ± 0.6
Right Kidney / BW (mg/g)	13.3 ± 0.7	12.7 ± 0.7	13.7 ± 0.4	15.1 ± 0.6†
Urine Osmolality (mOsm.kg^{-1})	2742 ± 326	1943 ± 211*	3082 ± 485	837 ± 142*†

(= $P \leq 0.05$ vs. $EP4^{VSMC+/+}$; †= $P \leq 0.01$ vs. $EP4^{VSMC+/+}$ + AngII).*

EP4 mRNA expression, PGE2-induced vasodilation and BP

Vascular smooth muscle *PTGER4* deletion was confirmed by measuring EP4 mRNA levels in aortic and renal vascular preparations via qPCR. The 5-day regimen of intra-peritoneal tamoxifen injections in a mixed male/female population of $EP4^{flox/flox}$ ($EP4^{VSMC+/+}$) and SMA-Cre-$EP4^{flox/flox}$ mice ($EP4^{VSMC-/-}$) mice significantly reduced EP4 mRNA transcripts in aorta (Fig.1A) ($EP4^{VSMC+/+}$, 1.00±0.04 vs. $EP4^{VSMC-/-}$, 0.23±0.1,$2^{-\Delta\Delta CT}$) and primary renal vessels (Fig.1B) ($EP4^{VSMC+/+}$, 1.11±0.3 vs. $EP4^{VSMC-/-}$, 0.34±0.06, $2^{-\Delta\Delta CT}$). Moreover, loss of EP4 receptor was determined functionally as we assessed PGE2-induced vasorelaxation in pre-constricted mesenteric arteries, isolated from a subset of $EP4^{VSMC-/-}$ mice. When treated with a low dose of PGE2 (10^{-11}M), time-dependent vasorelaxation was not observed in isolated mesenteric vessels from $EP4^{VSMC-/-}$ mice in comparison to $EP4^{VSMC+/+}$ counterparts which dilated readily at this dose (Fig.1C).

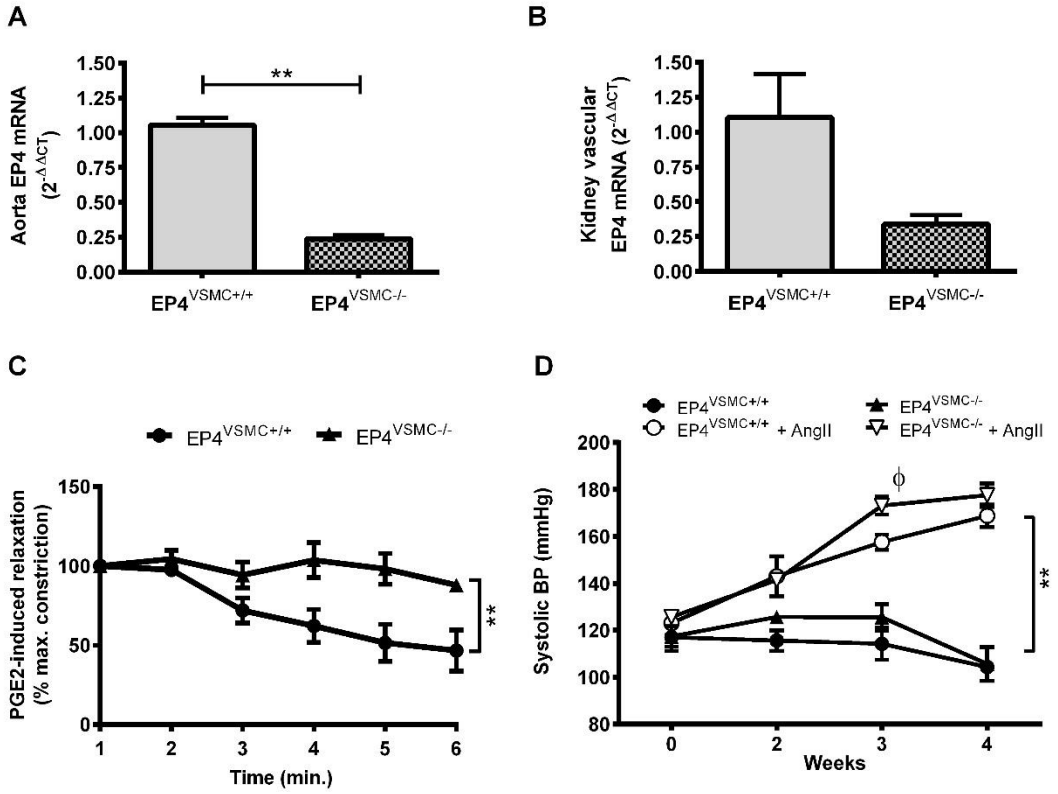

Figure 1: Characterization of tamoxifen-induced decreased EP4 mRNA and PGE2-induced vasorelaxation in EP4$^{VSMC-/-}$ mice and measurement of SBP following AngII-infusion. A-B: Isolated aortic or renal vascular RNA was reverse transcribed and qPCR was performed using SYBR green. Data reported using the delta-delta CT (ΔΔCT) method and normalized to GAPDH. Data are presented as means ± SEM from 4 mice/group assayed in triplicate. ** P≤0.01. C: Isolated mesenteric segments from n=2 mice/group were mounted on a multi wire-myograph system, preconstricted with KCl and stimulated with PGE2 (10^{-11}M). Data represented as percent vasodilation. D: Systolic BP was measured by tail-cuff plethysmography. Data are presented as means ± SEM of weekly measurements, 10 readings/day/mouse. (n=6-8 mice/group). ** P≤0.01 vs. EP4$^{VSMC+/+}$; Φ P≤0.05 vs. EP4$^{VSMC+/+}$ + AngII.

Having confirmed a decrease of EP4 mRNA transcripts and loss of PGE2-induced vasorelaxation in the EP4$^{VSMC-/-}$ group, mice were subdivided into experimental groups, receiving AngII (1000 ng/kg/min) via osmotic minipumps for 4 weeks or subjected to sham operation. Tail-cuff plethysmography was used to measure SBP throughout the study (Fig.1D). Baseline SBP was not affected by VSMC *PTGER4* deletion. Beginning 1

week following minipump implantation, SBP increased progressively over 4 weeks in both $EP4^{VSMC+/+}$ and $EP4^{VSMC-/-}$ AngII-treated groups, while sham-operated mice remained normotensive ($EP4^{VSMC+/+}$, 104±1; $EP4^{VSMC+/+}$ +AngII, 168±4; $EP4^{VSMC-/-}$, 105±7; $EP4^{VSMC-/-}$ + AngII, 177±5 mmHg SBP). A minor but statistically significant difference in SBP at 3 weeks post-AngII was noted for $EP4^{VSMC-/-}$ compared to $EP4^{VSMC+/+}$ mice, but this effect disappeared by week 4. At sacrifice, no significant differences were noted in bodyweight while kidney weights were significantly elevated in AngII treated $EP4^{VSMC-/-}$ mice compared to healthy and AngII treated $EP4^{VSMC+/+}$ groups, indicative of renal hypertrophy (Table 1). Furthermore, chronic AngII administration led to a significant decrease in urine osmolality, an effect which was enhanced in mice lacking VSMC EP4 expression ($EP4^{VSMC+/+}$, 2742±326; $EP4^{VSMC+/+}$ +AngII, 1943±211; $EP4^{VSMC-/-}$, 3082±485; $EP4^{VSMC-/-}$ + AngII, 837±142 mOsm/kg.H_20).

Albuminuria and histological assessment of glomerular and interstitial injury

To assess the role of the EP4 receptor in filtration barrier integrity in this hypertensive model, urine albumin-to-creatinine ratios (ACR) were measured after 4 weeks of AngII administration (Fig.2). AngII treatment raised ACR levels in $EP4^{VSMC+/+}$ mice, an effect that did not reach statistical significance. However, aggravated filtration barrier damage was evident in AngII-treated $EP4^{VSMC-/-}$ mice, as ACR values dramatically increased by nearly10-fold in this group ($EP4^{VSMC+/+}$, 329±70; $EP4^{VSMC+/+}$ +AngII, 917±245; $EP4^{VSMC-/-}$, 253±103; $EP4^{VSMC-/-}$ + AngII, 2907±420, μg albumin/ mg creatinine).

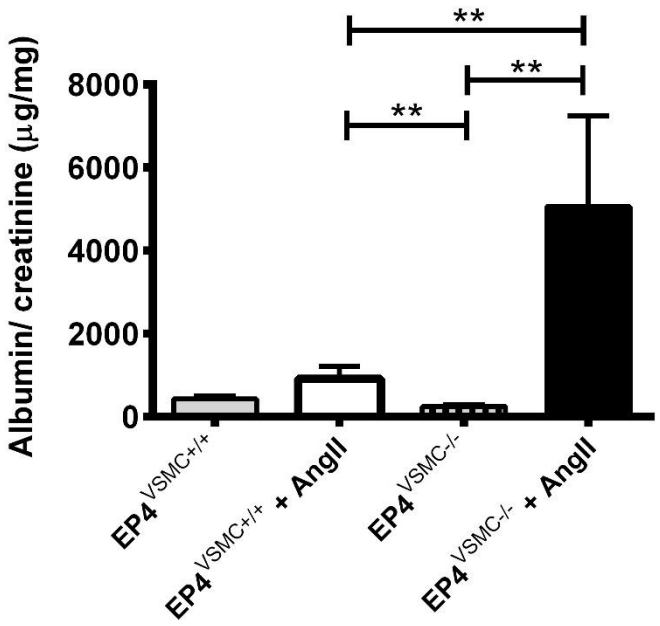

Figure 2: Filtration barrier damage in AngII treated EP4$^{VSMC-/-}$ mice. Spot-urine samples were collected after 4 weeks of AngII-treatment. Urine albumin concentrations were measured by ELISA and normalized to creatinine concentration. Data are presented as means ± SEM of albumin-creatinine ratios (μg albumin / mg creatinine) assayed in duplicate. ** P≤0.01.

We next evaluated renal structural damage in AngII-treated EP4$^{VSMC-/-}$ mice (Fig.3). Increased PAS-positive material in the glomerular tuft, indicative of mesangial matrix expansion, was significantly worsened in AngII-treated EP4$^{VSMC-/-}$ mice, in contrast to EP4$^{VSMC+/+}$ groups (EP4$^{VSMC+/+}$, 28.7±1.2; EP4$^{VSMC+/+}$ +AngII, 32.1±1.2; EP4$^{VSMC-/-}$, 31.2±1; EP4$^{VSMC-/-}$ + AngII, 43.5±2.3, % mesangial matrix/ total glomerular area). Furthermore, proteinaceous casts accumulated in the renal tubules (EP4$^{VSMC+/+}$, N/A; EP4$^{VSMC+/+}$ +AngII, 3.3±2; EP4$^{VSMC-/-}$, 1.3±1; EP4$^{VSMC-/-}$ + AngII, 13.5±3, # casts/ LPF x 10) and abundant PAS-positive material in the interstitium was found exclusively in AngII-treated EP4$^{VSMC-/-}$ mice. EP4 deletion itself, in the absence of AngII treatment had no apparent effect on renal pathology, nor did sham surgery (Fig.3A i, iii, v, vii).

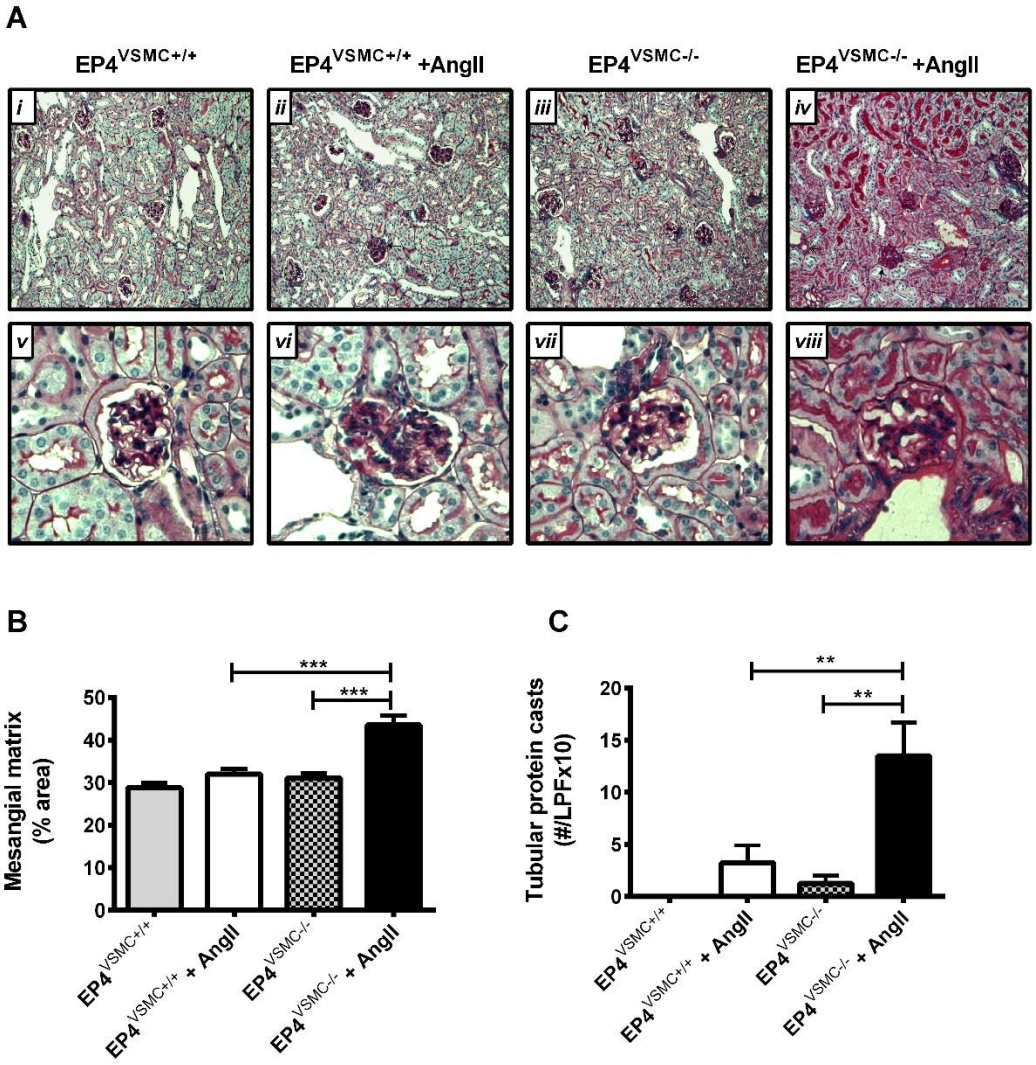

Figure 3: Histological assessment indicates prominent glomerular and interstitial injury after 4 weeks of AngII in EP4$^{VSMC-/-}$ mice. A: Representative interstitial and glomerular profiles of PAS-stained, PFA-fixed paraffin embedded kidney sections at low (10X, *i-iv*) and at high magnification (40X, *v-viii*). B-C: Mesangial matrix and tubular protein cast quantification was performed as described. Data presented as means ± SEM of % matrix to total glomerular surface area in 15-20 glomeruli/mouse (A) or # of proteinaceous casts/ low magnification field (10X) in 10-15 fields/mouse (B). (n=5 mice/group). **P≤0.01, ***P≤0.001.

COX-2 mRNA expression

The existence of a positive feedback loop between PGE2/EP signaling and COX2 induction has been shown in dentritic cells and the renal medulla [180, 181]. Additionally, AngII-induced vasoconstriction is thought to be buffered by increased renal medullary COX-mediated prostanglandin production [182]. We therefore determined whether VSMC EP4 receptor loss would affect COX2 expression in cortical and medullary regions. As shown in figures 4A and B, COX2 mRNA levels were significantly enhanced by approximately 3-fold in the renal medulla but remained unaltered in the cortex of AngII-treated $EP4^{VSMC+/+}$ mice. In contrast, AngII failed to increase medullary COX2 mRNA levels in $EP4^{VSMC-/-}$ mice ($EP4^{VSMC+/+}$, 0.95±0.2; $EP4^{VSMC+/+}$ +AngII, 2.9±0.6; $EP4^{VSMC-/-}$, 1.3±0.5; $EP4^{VSMC-/-}$ + AngII, 0.72±0.3, $2^{-\Delta\Delta CT}$ medullary COX2 mRNA levels/ GAPDH).

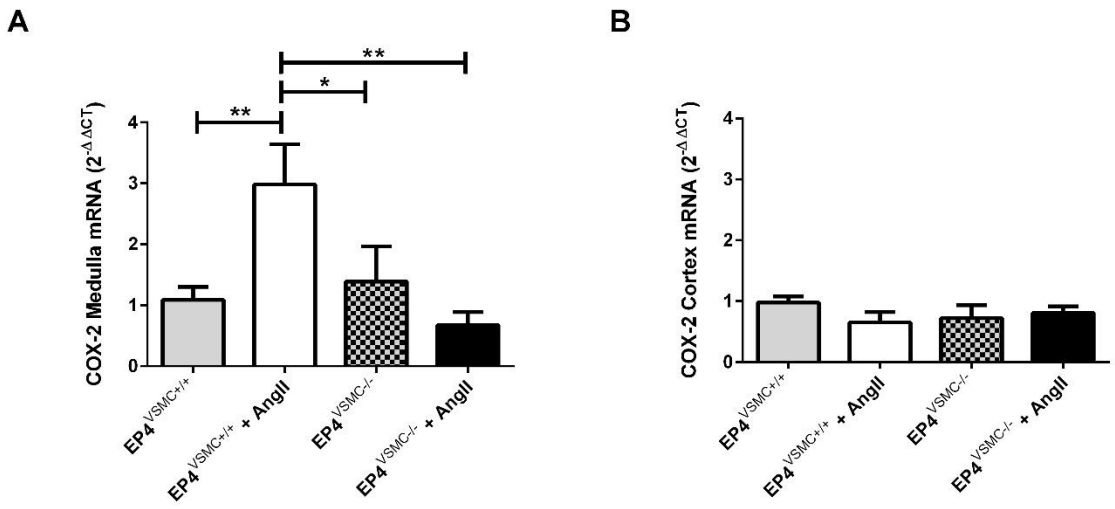

Figure 4: Impaired AngII-induced increased COX-2 mRNA in medulla but not in cortex of EP4$^{VSMC-/-}$ mice. RNA isolated from dissected medulla (A) or cortex (B) samples was reverse transcribed and qPCR was performed using SYBR green. Data reported using the delta delta CT (ΔΔCT) method and normalized to GAPDH. Data are presented as means ± SEM from 4-6 mice/group assayed in triplicate. * P≤0.05, ** P≤0.01.

GFR and renal perfusion

We next assessed renal function by estimation of GFR using FITC-labeled inulin clearance (Fig.5A). Chronic administration of AngII did not significantly affect endpoint GFR in EP4$^{VSMC+/+}$ mice. However when AngII was given to EP4$^{VSMC-/-}$ mice, FITC-inulin clearance was significantly diminished (EP4$^{VSMC+/+}$, 0.26±0.04; EP4$^{VSMC+/+}$ +AngII, 0.23±0.07; EP4$^{VSMC-/-}$, 0.16±0.05; EP4$^{VSMC-/-}$ + AngII, 0.07±0.02 mL/min).

In parallel, we measured RBF (renal perfusion) using contrast-enhanced dynamic magnetic resonance imaging (CE-MRI) to determine whether reduced GFR was associated with reduced RBF. As shown in figure 5C and plotted as GAD signal vs. time in figure 5B, upon contrast-agent (gadovist, GAD) infusion via the cannulated jugular

vein, signal accumulation in the kidney occurred significantly faster and in $EP4^{VSMC+/+}$ than in $EP4^{VSMC-/-}$ mice subjected to AngII. After 5 minutes of imaging, GAD signal accumulated in both left and right kidneys in AngII-treated mice. In contrast, GAD signal remained undetectable in $EP4^{VSMC-/-}$ + AngII mice until 10 and 15 minutes of imaging. Data were used to generate k_{trans} values which relate to renal perfusion, which we used as surrogates for RBF measurements. As shown in figure 5B, k_{trans} values determined for combined left and right kidneys were lower in AngII treated animals, an effect which was significantly more evident in $EP4^{VSMC-/-}$+AngII ($EP4^{VSMC+/+}$, 0.034±0.007; $EP4^{VSMC+/+}$ +AngII, 0.018±0.002; $EP4^{VSMC-/-}$, 0.039±0.007; $EP4^{VSMC-/-}$ + AngII, 0.003±0.0004; $1/k_{trans}$). Thus it appears loss of the vascular EP4 receptor greatly impairs the kidney's ability to withstand AngII-induced vasocontriction, leading to a significant drop in renal perfusion and/or blood flow in these mice.

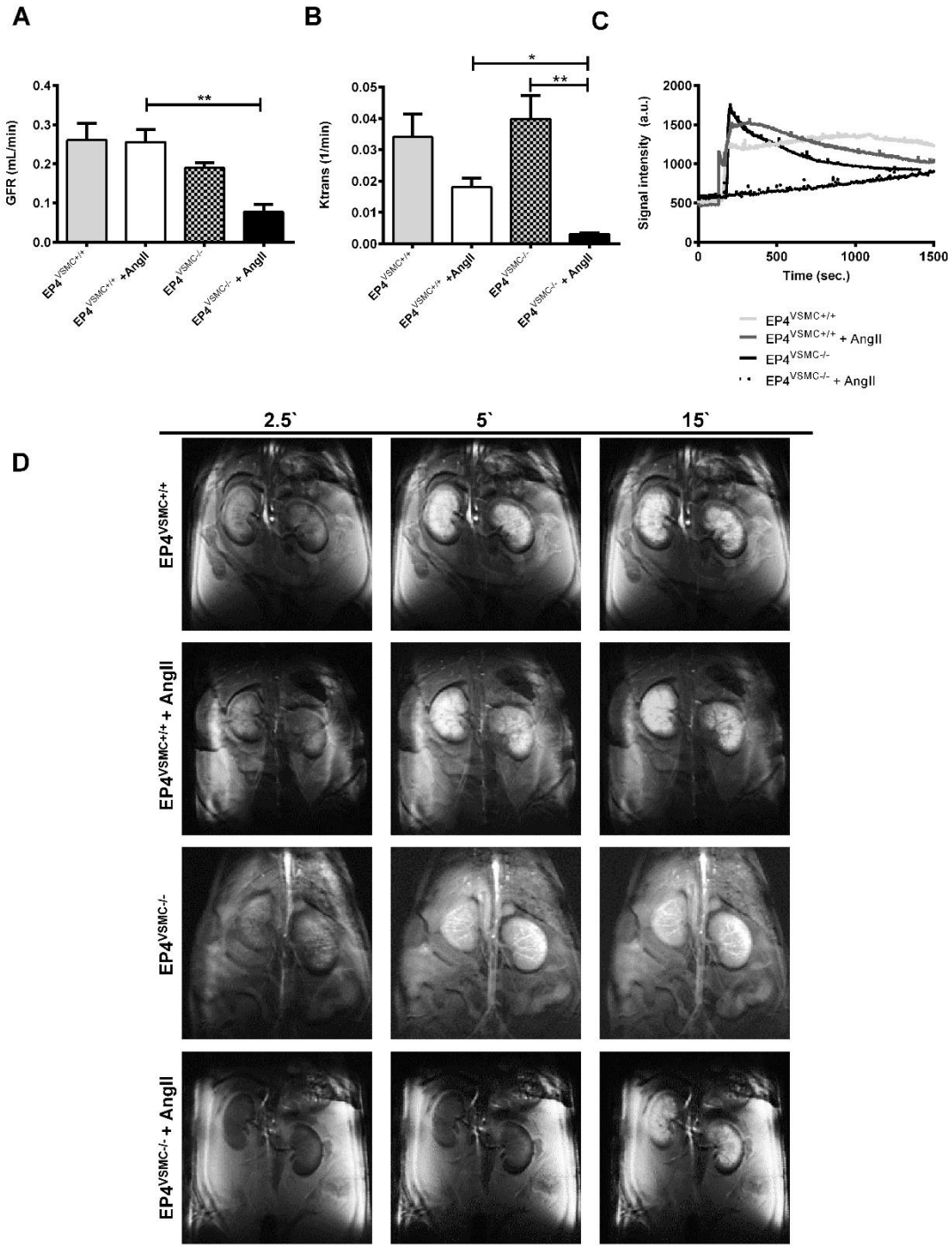

Figure 5: Inability to maintain GFR and RBF in EP4$^{VSMC-/-}$ mice subjected to AngII. A: Mice were subjected to tail-vein FITC-inulin injections for GFR estimation. Data are presented as means ± SEM from 4-6 mice/group. ** P≤0.01. B-D: Dynamic CE-MRI. B: Mean gadolinium K_{trans} values for the right kidney were generated C: Averaged representative signal intensity curves as a function of time and D: representative CE-MRI images at t=2.5, 5 and 15 min post-gadolinium injection. (n=3-4 mice per group).

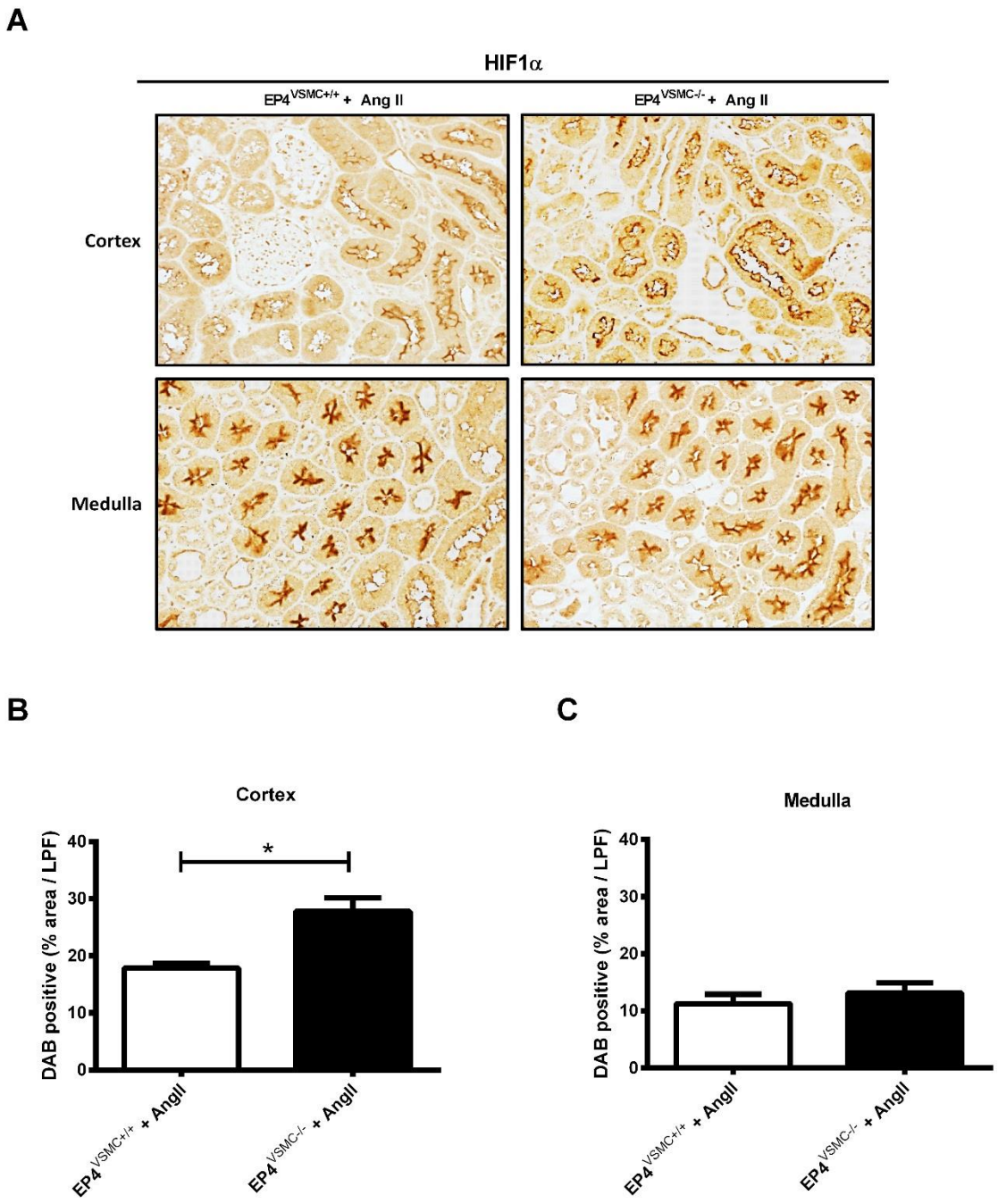

Figure 6: Immunodetectable HIF1α expression is enhanced in cortex but not in medulla of AngII-treated EP4$^{VSMC-/-}$ mice. PFA-fixed paraffin-embedded kidney sections were stained with HIF1α primary antibody. A: Representative immunodetectable HIF1a in cortical and medullary regions. B,C: DAB-positive pixel quantification in cortex (A) and medulla (B) presented as mean DAB-positive staining per low magnification (10X) field. (n=4 mice/group). * P≤0.05.

HIF-1α staining and fluorescence microangiography

Decreases in GFR and RBF were confirmed in AngII-treated mice lacking VSMC *PTGER4*, consistent with the notion that EP4 receptors help preserve renal hemodynamics under pathological conditions (i.e., hypertension). As impaired blood flow and ischemic/ hypoxic injury are often associated, we next examined the impact of EP4 receptor deletion on the induction of hypoxia-inducible factor 1α (HIF1α). As depicted in figure 6A and quantified in figures 6B and C, AngII-treatment in $EP4^{VSMC-/-}$ but not in $EP4^{VSMC+/+}$ mice led to a significant rise in cortical HIF1α expression, while remaining generally unchanged in the medulla (*Cortex*; AngII, 17.8±0.8 vs. $EP4^{VSMC-/-}$ + AngII, 27.7±2.4; *Medulla*; AngII, 11.3±2 vs. $EP4^{VSMC-/-}$ + AngII, 13.1±2, % HIF1α / DAB-positive area). HIF1α staining in the cortex was detected along the proximal tubule brush border, yet absent from the interstitium. No significant changes in HIF1α were noted in sham-operated $EP4^{VSMC+/+}$ and $EP4^{VSMC-/-}$ mice (*data not shown*).

We assessed the degree of microvascular damage in our study groups by fluorescence-based microangiography (FMA) [179]. As depicted in Figure 7A and quantified in figures 7B-D, AngII-treated $EP4^{VSMC-/-}$ mice showed a significant reduction in positive fluorescence signals and distribution in both cortical and medullary capillaries. Average capillary cross-sectional area decreased by approximately 5-8 μm (Fig.7B,C; *Cortex:* $EP4^{VSMC+/+}$ + AngII, 25.3±1.2; $EP4^{VSMC-/-}$ + AngII, 22.1±1.2; *Medulla*: $EP4^{VSMC+/+}$ + AngII, 23.7±1.1; $EP4^{VSMC-/-}$ + AngII, 19.1±1.4, mean cross-sectional area, μm^2, $p \leq 0.05$), while total functional area in the cortex and medulla was lowered by 20 and as much as 40%, respectively (Fig.7D; *Cortex:* $EP4^{VSMC+/+}$ + AngII, 7906±375; $EP4^{VSMC-/-}$ + AngII, 5778±554; *Medulla*: $EP4^{VSMC+/+}$ + AngII, 5659±372; $EP4^{VSMC-/-}$ + AngII, 2926±372,

functional area, μm^2 / hpf, $p \leq 0.01$). We generated frequency distribution histograms of capillary cross-sectional areas for both cortical and medullary regions *(data not shown)*. Of interest, while we observed decreased capillary density in both cortex and medulla, we noted a shift in capillary cross-sectional areas only in medullary regions, where the damage appeared to be more severe. Our data revealed that the medullary microcirculation of $EP4^{VSMC-/-}$ + AngII mice had a 10% increase in the number of capillaries with smaller cross-sectional areas (5-10 μm^2), while the number of larger capillaries ($\geq 10 \mu m^2$) was reduced.

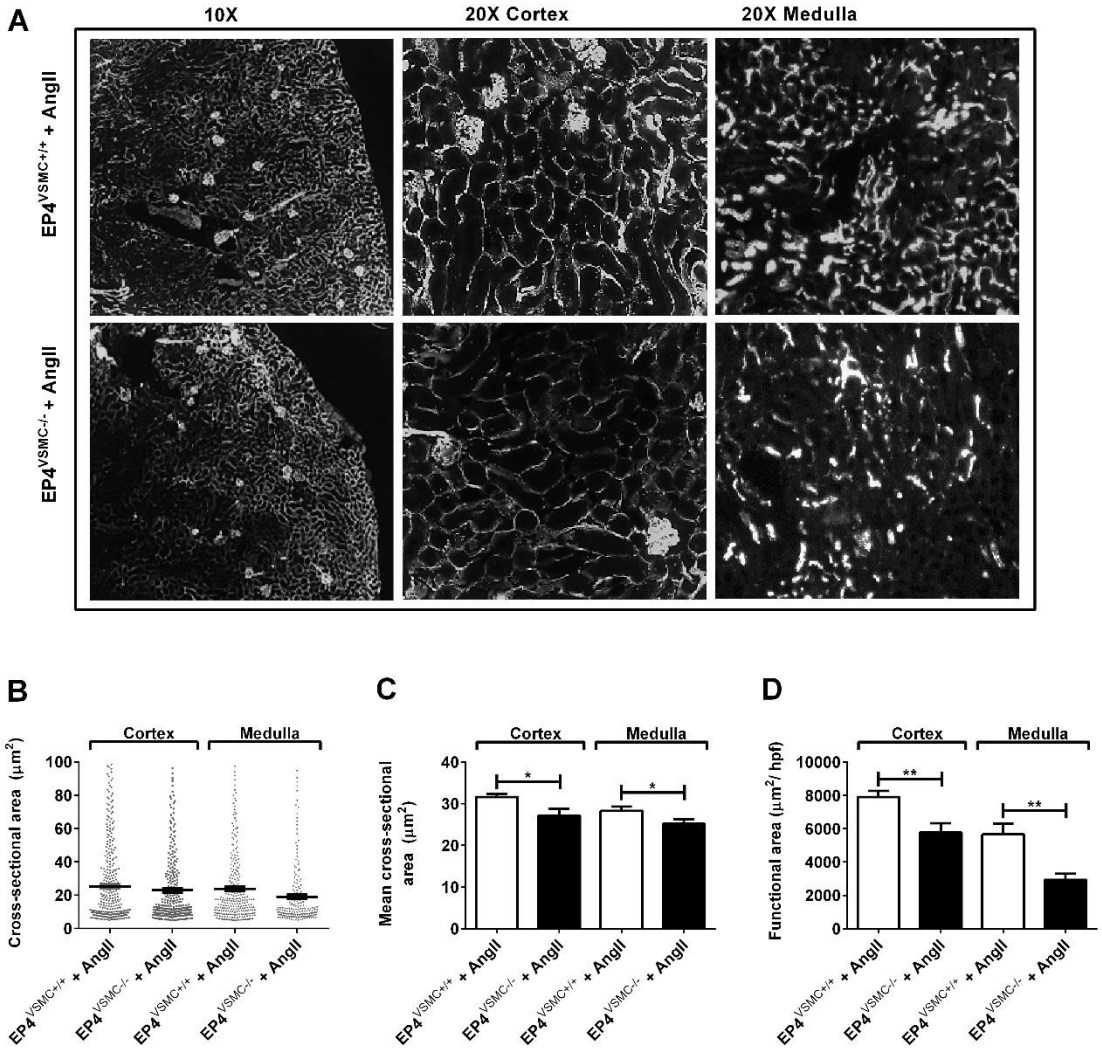

Figure 7: Decreased cross-sectional area and loss of functional area in both cortical and medullary capillary beds analyzed from EP4$^{VSMC-/-}$ mice by FMA. Frozen OCT-embedded kidney-sections obtained from mice subjected to intra-cardiac fluosphere injection were sectioned at 20μm and visualized under fluorescence microscopy. A: Representative low magnification (10X) whole kidney profiles or higher magnification (20X) cortical and medullary regions. Cortical and medullary vascular profiles (7-10/ mouse, 3-4 mice/ group) were analyzed using published MATLAB scripts. B: All capillary cross-sectional area (4 - 100 μm area). C: Mean cross-sectional area. D: Mean functional area (total cross-sectional area) / high magnification field (20X). * P≤0.05, **P≤0.05.

Discussion

Our study determined the role of the VSMC EP4 receptor in preserving renal function in an AngII-dependent hypertensive setting. Our data suggest that VSMC-specific *PTGER4* deletion renders mice susceptible to AngII-induced reductions in GFR and RBF, resulting in increased glomerular, interstitial and microvascular damage, contributing to a hypoxic environment. Our findings are consistent with the notion that COX-derived prostaglandins preserve renal blood flow in hypertension [183].

COX-derived metabolites help maintain renal homeostasis by governing glomerular hemodynamics [125]. Critical roles for PGE2, the primary COX-2 metabolite, in maintaining adequate glomerular perfusion and thus GFR in response to vasoconstrictive agents such as AngII have been well documented. COX-inhibition via NSAIDs potentiates AngII's effect on pre-glomerular but not post-glomerular microvessels [172]. Central to the buffering actions of PGE2 are vasodilatory EP4 receptors, the predominant EP isoform expressed in pre-glomerular VSMC's. While we did not assess renal afferent arteriole responses in the present study, and differences in EP receptor subtype expression may exist between vessels of distinct organs, a prominent vasodilatory role for the EP4 receptor was indicated in that mesenteric arteries isolated from $EP4^{VSMC-/-}$ mice lose their capacity to vasodilate when challenged with a low dose of PGE2 (10^{-11}M).

A four week regimen of AngII infusion led to progressive yet similar increases in SBP in both control and knockout mice. Increased SBP in the AngII model of hypertension is commonly associated with increased NADPH-derived reactive-oxygen species production in the vasculature [184] and enhanced centrally-driven sympathetic tone [185], both of which promote renal vasoconstriction and electrolyte/volume retention. As

for the EP4 receptor, its activation may impact SBP by stimulating the RAAS via increased renin secretion from juxtaglomerular granular cells [72, 125]. Our data suggest that SBP regulation in response to AngII treatment is independent of PGE2/EP4 signaling in the vasculature as similar increases in SBP were obtained irrespective of VSMC EP4 receptor expression. However, we cannot rule out a role for EP4 receptors expressed elsewhere, including the endothelium or along the nephron, in blood pressure regulation [186]. Unlike the EP4, the EP2 receptor, which is likewise Gs-coupled has previously been linked to the regulation of BP as its deletion renders mice susceptible to salt-induced hypertension, and abrogates PGE2-induced hypotension [187]. While other EP receptors, such as the Gq-coupled EP1, can increase vascular tone and BP as in type-2 diabetes, our findings are consistent with previously published data showing unchanged SBP in healthy, or low-salt fed, or diuretic-challenged EP4-/- mice [71, 73].

VSMC EP4 deletion in our mice significantly reduced urine osmolality in response to AngII treatment. The mechanism underlying this effect awaits further investigation. However, studies of mice with global EP4 receptor deletion showed that activation of the RAAS by low salt diet was accompanied by diuresis [73]. The authors speculate that loss of tubular EP4 receptors which were shown to promote AQP2 trafficking in a collecting duct cell line could potentially account for the observed diuresis. Moreover, EP4-selective agonists have been shown to alleviate symptoms associated with X-linked diabetes insipidus, including failure to concentrate urine and polyuria [188]. While the authors speculate these effects are mediated via PGE2/EP4 signaling in the collecting duct, we cannot dismiss the involvement of vascular EP4 receptors expressed in the vasa recta and their role in sodium/water balance along the nephron. Indeed, early studies

identified PGE2 as an inhibitor of sodium reabsorption along the thick ascending limb and collecting duct [189, 190].

A major finding in our study was the significant impact of VSMC-specific *PTGER4* deletion on GFR and RBF, brought on by AngII treatment. GFR is influenced by several factors such as BP along with afferent/efferent arteriolar vasoconstriction or blood colloid osmotic pressure. Additionally, mesangial cell or podocyte injury can also affect GFR by reducing the ultrafiltration coefficient (Kf) [191]. GFR can also be impacted indirectly by tubular damage, leading to activation of the tubuloglomerular feedback mechanism or by impaired blood flow and ischemic injury[192]. Increased intrarenal AngII production typically acts to preserve GFR under conditions of low BP or reduced extracellular fluid volume via efferent arteriolar constriction and increased filtration fraction. However, several studies show that chronic administration of exogenous AngII dose-dependently decreases GFR and RBF via increased afferent and efferent arteriolar tone [18, 193]. In our study, AngII infusion had no effect on GFR in $EP4^{VSMC+/+}$ mice, while loss of the EP4 subtype led to a striking fall in GFR after 4 weeks of chronic AngII infusion. Additionally, our MRI data support the close association between GFR and RBF, revealing that cortical and medullary RBF was markedly decreased in $EP4^{VSMC-/-}$ mice. Our data suggest that under conditions of chronic AngII administration, the COX/PGE2 system may act locally, buffering the vasoconstrictive effects of AngII on glomerular microcirculation through EP4 receptors residing in the afferent arteriole, thereby maintaining vessel patency and preserving GFR. Moreover, as EP4 receptors reside in other renal vascular beds, including the vasa recta and medullary capillaries, its deletion from these locales may also have impaired blood flow via unbuffered cortical or

medullary vascular resistance [17]. Consistent with this notion, PGE2 is effective in maintaining vascular tone in the outer-medullary vasa recta, in response to AngII [194].

Our mouse cohorts were generally resistant to AngII-induced renal structural injury, yet $EP4^{VSMC-/-}$ mice treated with AngII showed significant glomerular scarring and tubular protein cast accumulation along with albuminuria. The mechanism by which decreased GFR led to increased filtration barrier leakage and glomerular scarring awaits investigation. Increased glomerular capillary pressure (i.e, glomerular hypertension) can exacerbate albuminuria by subjecting the filtration barrier components to injurious mechanical forces. For example, glomerular hyperfiltration is an early feature of diabetic nephropathy, and is a major risk factor for albuminuria and subsequent disease progression [195, 196]. Such a mechanism fails to explain the albuminuria observed in AngII-treated $EP4^{VSMC-/-}$ mice where both GFR and RBF are significantly reduced. An alternative mechanism which could explain how hypofiltration would promote albuminuria is the electrokinetic model. Accordingly, glomerular flow creates a local electrical field termed the 'streaming potential', contributing to the glomerular filtration barrier's charge selectivity. Any interruption or decrease in flow would lead to decreases in glomerular streaming capacity, leading to rapid diffusion of negatively charged albumin across the filtration barrier [197]. Alternatively, proximal tubule injury and associated tubulointerstitial damage can promote glomerulosclerosis via interstitial capillary loss resulting in GFR reduction or interglomerular-tubular paracrine signaling has recently been suggested [198]. Our study did not investigate the impact of VSMC-specific EP4 deletion on proximal tubule integrity, which, if affected, could also

participate in increasing albumin excretion through the aforementioned mechanism or through impaired proximal tubule albumin reabsorption [199].

Our $EP4^{VSMC-/-}$ mice challenged with AngII showed evidence of several phenomena involved with CKD progression. Of these, measurable decreases were observed in the size and density of cortical and medullary capillaries, as assessed by FMA. It is conceivable that impaired blood flow may be the primary insult participating in the appearance of microvascular damage in this model. Decreased glomerular blood flow and resulting hypoxic damage may also have contributed to glomerular injury and albuminuria. On the other hand, peritubular capillary rarefaction is primarily governed by injury to the endothelial cell layer, impairing nitric oxide production increasing vascular resistance. Interestingly, a majority of global EP4-knockout (EP4-/-) pups die from patent ductus arteriosus at birth [200] and PGE2/EP4 signaling in endothelial cells (EC) appears to be key as EC-specific EP4-null mice similarly fail to thrive [201]. EP4 activation in cultured pulmonary microvascular EC promotes migration and tubulogenesis in vitro [201] as well as the generation of nitric oxide in aortic preparations, which subsequently stimulates cyclic guanosine monophosphate-induced VSMC relaxation [186]. It is conceivable that in a hypertension setting, loss of EP4 receptors selectively in the vasculature may, through decreased paracrine crosstalk, affect endothelial cell integrity and function, leading to increased medullary capillary damage. Accordingly, medullary infusion of a COX-2 inhibitor led to dysregulated BP in salt-sensitive rats [86]. On the other hand, it is also likely that in AngII-treated $EP4^{VSMC-/-}$ mice, an impaired positive feedback loop led to reduced COX-2 induction in the medulla, predisposing to hypertension-associated microangiopathy in this locale, as COX-2 and EP4 mRNA have

recently been shown to be upregulated in the medullary vasculature [72]. Taken together, our study strengthens the notion that careful consideration should be taken when NSAIDs are prescribed to patients with compromised renal function. COX-derived PGE2 signaling via vascular EP4 receptors is critical in maintaining GFR and RBF when AngII-levels are chronically elevated. Targeting of these vasodilatory receptors in hypertension predisposes the kidney to glomerular, interstitial and vascular injury. Thus pharmacological EP4 selective activation may be a beneficial therapeutic target to preserve blood flow [202].

Perspectives

Our findings reinforce the notion that COX-derived prostaglandin signaling in a predisposed kidney maintains renal function and that NSAIDs and COX-inhibitor use in at risk patients should be avoided or carefully monitored. The physiological and pathophysiological implications of the COX-2 pathway and receptor specific targeting warrant further investigation in order to mitigate unwanted adverse side effects while unleashing potential therapeutic benefits.

Chapter 4: A novel mouse model of advanced diabetic kidney disease

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RESEARCH ARTICLE

A Novel Mouse Model of Advanced Diabetic Kidney Disease

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Description

Studies charged with elucidating new signalling pathways or dissecting new therapeutic avenues associated with DN are hampered by the lack of appropriate mouse models of diabetic kidney disease. Most available rodent models do not fully recapitulate the full spectrum of human disease. We therefore sought to develop a mouse model which would putatively develop signs of advanced DN-induced renal injury with concomitant hypertension. To this end, we bred a type-1 diabetic mouse with a renin-dependent hypertensive mouse yielding ‘HD’ (hypertensive-diabetic) mice. The resulting phenotype is reminiscent of human DN as prominent increases in BP, tubulointerstitial fibrosis and decreases in GFR were observed.

Abstract

Currently available rodent models exhibit characteristics of early diabetic nephropathy (DN) such as hyperfiltration, mesangial expansion, and albuminuria yet features of late DN (hypertension, GFR decline, tubulointerstitial fibrosis) are absent or require a significant time investment for full phenotype development. Accordingly, the aim of the present study was to develop a mouse model of advanced DN with hypertension superimposed (HD mice). Mice transgenic for human renin cDNA under the control of the transthyretin promoter (TTRhRen) were employed as a model of angiotensin-dependent hypertension. Diabetes was induced in TTRhRen mice through low dose streptozotocin (HD-STZ mice) or by intercrossing with OVE26 diabetic mice (HD-OVE mice). Both HD-STZ and HD-OVE mice displayed more pronounced increases in urinary albumin levels as compared with their diabetic littermates. Additionally, HD mice displayed renal hypertrophy, advanced glomerular scarring and evidence of tubulointerstitial fibrosis. Both HD-OVE and HD-STZ mice showed evidence of GFR decline as FITC-inulin clearance was decreased compared to hyperfiltering STZ and OVE mice. Taken together our results suggest that HD mice represent a robust model of type I DN that recapitulates key features of human disease which may be significant in studying the pathogenesis of DN and in the assessment of putative therapeutics.

Introduction

Diabetic nephropathy (DN) is a serious microvascular complication that affects a significant proportion of patients suffering from both type 1 and type 2 diabetes, accounting for over 40% of end-stage renal disease (ESRD) cases in North America [7]. Current interventions that target the renin-angiotensin aldosterone system (RAAS) along with strict glycemic control are associated with a slower deterioration of renal function and delayed ESRD onset in patients with diabetes. However, these therapies only slow progression and do not cure the disease [203]. Thus a pressing issue remains the development of new treatment strategies.

Research focused on novel therapeutic interventions for the treatment of DN has been significantly hindered by the fact that animal models fail to reliably recapitulate the full spectrum of human disease. In 2005 the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) established the Animal Models of Diabetic Complications Consortium (AMDCC) with the objective of developing a list of criteria for validating progressive DN in mouse models [204]. These criteria were further updated in 2009 and provide a benchmark against which current DN models are measured [205]. As reviewed elsewhere, the majority of mouse models currently available develop pathologies reminiscent of early DN provided they are bred onto susceptible backgrounds [206-210]. However changes associated with advanced DN such as tubulointerstitial fibrosis, arteriolar hyalinosis, and >50% decline in GFR over the lifetime of the animal are often absent. A limited number of mouse models do meet the majority of AMDCC criteria, such as the *eNOS*$^{-/-}$ *db/db* and BTBR *ob/ob* models, however the complex breeding strategies and significant time investment required for the pathological changes to

develop are restrictive. Therefore we sought to develop a new mouse model that would rapidly develop pathological changes associated with advanced DN while being tractable to genetic manipulation.

In this study we have employed transgenic mice with the human renin cDNA under the control of the transthyretin promoter (TTRhRen) and induced diabetes either through streptozotocin (STZ)-injections or by crossing with the OVE26 transgenic type 1 diabetes mouse on the susceptible FVB/n background. These mice consistently display features of advanced DN outlined by the Diabetes Complications Consortium including >10-fold increase in albuminuria, mesangial matrix expansion, tubulointerstitial fibrosis, and signs of GFR decline [205]. These animals are amenable to the current array of genetic strategies (i.e., gene-targeting / transgenics) that are used widely to explore the role of any number of putative players in the progression of DN.

Materials and methods

Physiological data

Blood samples were collected via cardiac puncture into heparinized syringes, kept on ice and centrifuged at 5000 g for 10 minutes at 4°C. Collected plasma was immediately frozen at -80°C until subsequent analysis. Plasma glucose levels were determined by glucometry (Bayer Contour). At sacrifice, tibias, kidneys and hearts were removed, individually weighed and organ weights were normalized to tibia length.

Albuminuria

Albuminuria was measured using the Mouse Albumin Elisa Kit (Bethyl labs, Montgomery, TX.) following manufacturer's protocol in spot urine samples. Albumin

levels were determined by normalizing to creatinine concentration, determined by the Creatinine Companion kit (Exocell, Philadelphia, PA).

Animals

Hypertensive TTRhRen mice have been previously described [177, 211]. Briefly, liver-specific expression of a modified human pro-renin cDNA transgene was achieved under control of a 3-kb region of the mouse transthyretin promoter. The synthesis of active human renin was optimized by introducing a furin cleavage site between the pro and active segments of the human renin transgene. Cleavage of the pro segment from the renin transgene occurs by the ubiquitously expressed furin enzyme in cells expressing this construct. Hyperreninemic TTRhRen mice on an FVB/N background display elevated systolic blood pressure (140-150 mmHg) and develop cardiac hypertrophy by 4 months of age [211] that may be attenuated by ACE inhibition or ARBs [177, 211, 212] . Hypertensive TTRhRen mice do not display a renal phenotype.

Hypertensive diabetic mice (HD) were generated using two type 1 diabetic mouse models including the streptozotocin (HD-STZ) and OVE26 (HD-OVE) models. The former was achieved using the low-dose STZ protocol [134]. Briefly, 8-10 week old wild-type (WT) or TTRhRen (H) male mice were subjected to 5-day intraperitoneal injections of STZ (50mg kg^{-1} BW^{-1}; Sigma-Aldrich, Oakville, ON.) or 0.1 M Na-Citrate buffer pH 4.5 as vehicle. The latter mouse model studied was the previously characterized transgenic OVE26 mice on the FVB/N background, which are hypoinsulinemic at birth due to pancreatic beta-cell specific overexpression of a calmodulin mini-gene [135]. HD-OVE mice were obtained by intercrossing OVE26 mice (Male, 2-3 months, Jackson Laboratory, Bar Harbor, ME) with TTRhRen mice (Female, 2-3 months). Experimental

animals (male, 6-20 weeks) were housed and cared for in the Animal Care Facility at the University of Ottawa with free access to food and water. Protocols were approved by the University of Ottawa Animal Care Committee and conducted according to the guidelines of the Canadian Council on Animal Care.

Blood pressure measurement

Throughout the study, systolic BP was measured via tail-cuff plethysmography (BP 2000, Visitech systems, Apex, NC) as described previously [177]. Daily systolic BP was calculated from measurements obtained at the same time period each day (5 preliminary, 10 actual BP readings/ day) and, following a five-day training regimen (10 BP readings/ day), weekly BP measurements were obtained.

FITC-inulin clearance

Fluorescein isothiocyanate-labeled inulin (FITC-Inulin; Sigma-Aldrich, Oakville, ON.) clearance was used to estimate glomerular filtration rate (GFR). Briefly, 5% (w:v) FITC-inulin dissolved in 0.9% (w:v) saline was dialyzed (1000 MWCO) overnight and sterilized by filtration (0.2 μm). Anesthetized mice received a bolus (3.74 μL/g BW) of FITC-inulin via tail-vein injections. Blood samples (≈ 20 μL) were collected from the saphenous vein into heparinized capillary tubes, and centrifuged for 10 minutes at 15,000 *x*g. Blood sampling was carried out at 3, 7, 10, 15, 35, 55 and 75 minutes post injection. Samples were buffered in 500 mM Hepes pH 7.4 and plasma fluorescence was measured (Excitation 488 nm/ Emission 538 nm). A two-compartment clearance model was used to calculate GFR as previously described [136] using statistical analysis software (Graphpad Prism, San Diego, Ca.).

Histology and α-SMA immunofluorescence

At sacrifice, mice were anesthetized (isoflurane), perfused with phosphate buffered saline (PBS) and kidneys were excised, dissected and immediately fixed in 4% paraformaldehyde (PFA). Paraffin-embedded kidney sections (3 μm) were obtained and stained with periodic-acid Schiff (PAS) or Masson's Trichrome reagent. All sectioning, paraffin embedding and PAS-staining were performed by the University of Ottawa's pathology department. Kidney sections were viewed using a light microscope at either 200x or 400x magnification (Axioskop 2 Imager A1, Zeiss, Germany). Representative glomerular (20-25 glomeruli/ mice) areas for each group were analyzed in a blinded manner. Imaging software (Axiovision v4.8, Carl Zeiss, Germany) was used to calculate relative mesangial matrix/ glomerular area, whereby the area of the mesangial scar as a percentage of total glomerular area was determined.

Kidney α-smooth muscle actin (α-SMA; Santa Cruz Biotechnology, Dallas, TX.) immunofluorescence was performed on paraffin-embedded sections mounted on glass slides. Sections were deparaffinized in mixed xylenes (Fisher), and rehydrated through a gradient of ethanol and distilled water. Sections were washed 3x in PBS, boiled for 20 minutes in 0.1 M Na-citrate buffer (pH 6.0) for antigen unmasking. Sections were blocked in PBS containing 10% donkey serum/ 1% BSA for 1 hour and incubated with mouse anti-α-smooth muscle actin (1:200) overnight at 4°C. Slides were washed and treated with a FITC-labelled donkey anti-mouse secondary antibody (1:1000; Molecular Probes, Burlington, ON.) for 1 hour, followed by 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Oakville, ON.) for nuclear localization. Sections were covered with fluorescent mounting medium (Vector laboratories, Burlington, ON.) and coverslips.

Slides were visualized under fluorescence microscopy whereby representative cortical profiles from each group were obtained in a blinded manner.

Western immunoblotting and quantitative PCR

Cortical kidney tissue was homogenized with a COE Capmixer and suspended in RIPA lysis buffer (150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50mM Tris pH 8.0), supplemented with protease inhibitor cocktail 1:100 (Sigma-Aldrich, Oakville, ON). Protein lysates were processed by SDS-PAGE, transferred to nitrocellulose membranes, incubated with appropriate antibodies and processed for chemiluminescence. Primary antibodies, including rabbit anti-fibronectin 1:1000 (Sigma-Aldrich, Oakville, ON) and mouse anti-α-tubulin 1:2000 (Sigma-Aldrich, Oakville, ON) were incubated o/n at 4°C. Secondary antibodies, including HRP-goat anti-rabbit 1:10000 (Jackson ImmunoResearch Laboratories, West Grove, PA) and HRP-goat anti-mouse 1:10000 (Jackson ImmunoResearch Laboratories, West Grove, PA), were incubated for 1 hour at room temperature. For quantitative PCR (qPCR), kidney tissue was homogenized using QIAshredder columns (Qiagen). RNA was extracted using the Qiagen RNEasy minikit as per manufacturer's instructions. Extracted RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) with 500 ng starting material per reaction. Assay was performed using an ABI Prism 7000 Sequence Detection System with SYBR Advantage qPCR Premix (Clontech) according to manufacturer's instructions. Primers used: Collagen-IV sense (5'- ATGGGGCCCCG GCTCAGC -3'), Collagen-IV antisense (5'- ATCCTCTT TCACCTTTCAATAGC -3'); GAPDH sense and antisense were purchased from Invitrogen.

Statistical analysis

The values are presented as means ± SE. Statistical comparisons between two-groups was performed using the unpaired Student's *t*-test, while analysis of variance (ANOVA) was used for three or more groups, followed by a Newman-Keuls post-test. Statistical significance was achieved when $P \leq 0.05$.

Results

Table1: OVE26 study physiological parameters and organ hypertrophy. (*=P≤0.01 vs. WT; †=P≤0.01 vs. H; ‡=P≤0.05 vs. OVE)

	WT	H	OVE	HD-OVE
Plasma glucose (mg/dL)	11.3 ± 0.7	12.3 ± 1	29.9 ± 0.8*	35 ± n/a†‡
Bodyweight (g)	32.4 ± 1.2	32.3 ± 1.1	27.3 ± 0.9*	26.4 ± 1.1†
Right kidney/ tibia (mg/mm)	12.3 ± 1.3	10.8 ± 0.7	17.1 ± 1.4*	25.6 ± 5.6†‡
Heart/ tibia (mg/mm)	8.4 ± 0.1	9.3 ± 0.6	7.3 ± 0.5	8.6 ± 0.7

Table 2: STZ study physiological parameters and organ hypertrophy. (*=P≤0.01 vs. WT; †=P≤0.05 vs. WT; ‡=P≤0.05 vs. STZ)

	WT	H	STZ	HD-STZ
Plasma glucose (mg/dL)	10.5 ± 0.8	12.1 ± 0.5	30.8 ± 1.7	31.2 ± 1.9
Bodyweight (g)	28.8 ± 1.7	32.8 ±1.5	30.7 ± 1.1	33.5 ± 1.1
Right kidney/ tibia (mg/mm)	10.3 ± 0.2	13.1 ± 0.6†	17.4 ± 1.1*	14.5 ± 1.3‡
Heart/ tibia (mg/mm)	7.1 ± 0.3	9.7 ± 0.1	8.7 ± 0.5	9.7 ± 0.6

Systolic BP is progressively increased in HD mice

Two models of HD mice were studied. In the first model, 8-12 week-old male WT and TTRhRen (H) mice were subjected to a low-dose STZ diabetes regimen (HD-STZ) and followed for 18 weeks. For the second model, OVE26 and H mice were intercrossed to obtain HD-OVE mice, the males of which were followed for up to 20 weeks of age. Cardiac and renal hypertrophy were analyzed by normalizing kidney and heart weights to tibia length. (Tables 1 and 2). Similar plasma glucose levels were measured for both HD-STZ and HD-OVE26 models (STZ study: WT, 10.5±1; H, 12.1±1; STZ, 30.7±2; HD-STZ, 31.2±2 and OVE26 study: WT, 11.3±1; H, 12.4±1; OVE, 32.8±2; HD-OVE, 35±0

mM). In addition, decreased bodyweight was noted in OVE26 mice. Characteristic renal hypertrophy accompanied the hyperglycemia in both STZ and OVE cohorts, while HD-OVE blood glucose values were slightly albeit significantly higher than OVE mice. Non-diabetic hypertensive mice did not develop renal hypertrophy, but showed a non-significant trend towards increased heart-to-tibia ratios.

Longitudinal systolic BP was assessed throughout the study in both models (Fig.1a-b). We observed equivalent BP elevations for H and HD-STZ groups 2 weeks post-STZ, (WT, 113±5; H, 140±8; STZ, 120±3; HD-STZ, 140±5 mmHg). These values increased progressively and significantly in the HD-STZ group, and to a lesser degree in the STZ mice, while H mice showed a slight reduction at 18 weeks post-injection (WT, 114±6; H, 137±8; STZ, 135±7; HD-STZ, 161±7 mmHg). In the HD-OVE study (Fig.1b), baseline (6 weeks of age) BP was elevated in H and HD-OVE mice versus WT and OVE mice (WT, 109±9; H, 144±13; OVE, 116±7; HD-OVE, 145±5 mmHg). The combination of both hypertension and diabetes led to a persistent and significant rise in BP that significantly exceeded that of H mice by 20 weeks of age (WT, 112±7; H, 138±3; OVE, 128±9; HD-OVE, 174±7 mmHg).

Exacerbated albuminuria in HD mice

In order to examine the effects of hypertension superimposed upon diabetes on filtration barrier integrity, urine albumin-to-creatinine ratios (ACR; *μg/mg*) were determined (Fig. 1c). Increased ACR levels were observed in STZ-treated mice, while the HD-STZ phenotype exacerbated this parameter. In the HD-OVE model, hypertension alone did not lead to albuminuria, while diabetes led to a significant 3-fold increase in ACR versus WT. Remarkably, at 20 weeks of age HD-OVE mice exhibited a 40-fold increase in ACR

versus OVE mice, suggesting significant glomerular filtration barrier dysfunction (WT, 245±69; H, 504±166; STZ, 1026±204; OVE, 483±81; HD-STZ, 6504±1584; HD-OVE, 22023±4802 μg/mg, ACR μg/mg).

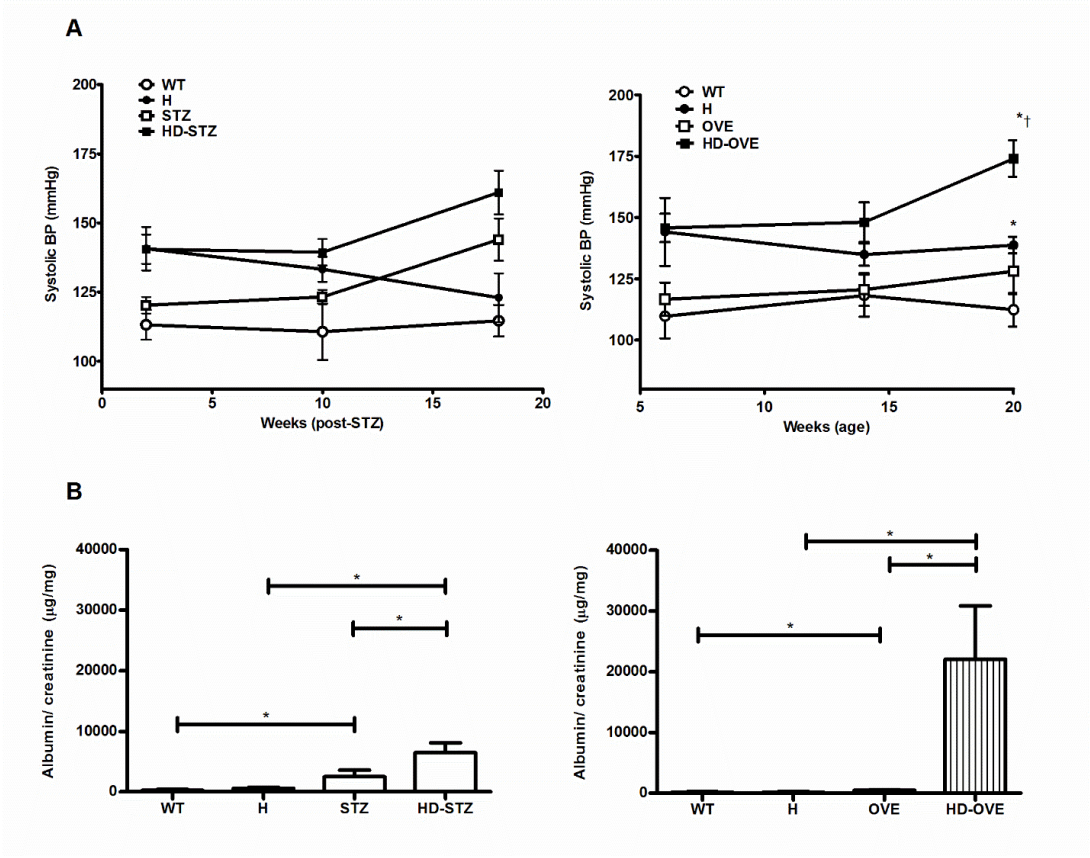

Figure 1: Systolic BP and albuminuria. Longitudinal BP measurements were obtained by tail-cuff plethysmography (A) while urinary ACR levels were measured in urine samples at endpoint (B) using an ELISA-based method (Bethyl) in both the STZ (left; 4-6 mice per group) and OVE26 studies (right; n=4-7 mice per group). Data represented as mean with standard error. (*=P≤0.05; †=P≤0.05 vs. WT control)

Glomerular hypertrophy and mesangial matrix expansion is exacerbated in HD mice

Persistent hyperglycemia leads to glomerular hypertrophy and induces mesangial matrix overproduction. We analyzed glomerular profiles from both HD-STZ and HD-OVE cohorts (Fig. 2). While the onset of hypertension yielded observable increases in glomerular surface area, these levels were significantly surpassed in the HD-STZ mice and greatly exceeded that of STZ mice (WT, 3321±191; H, 3442±370; STZ, 3996±78; HD-STZ, 4281±87 μm^2). Similar findings were obtained for the HD-OVE (WT, 3601±638; H, 4778±201; OVE, 6223±300; HD-OVE, 8235±785 μm^2). Accordingly, mesangial area as a percentage of total glomerular surface area was also increased in diabetic mice from both studies, which was worsened when hypertension was present (STZ study: WT, 32.9±1; H, 33.8±1; STZ, 35.7±1; HD-STZ, 39±1 and OVE26 study: WT, 28.6±3; H, 27.7±2; OVE, 34.5±1; HD-OVE, 44.4±2, % of glomerular area). Furthermore, the presence of proteinaceous material in the tubules of HD-OVE mice is consistent with compromised glomerular structural integrity in this group.

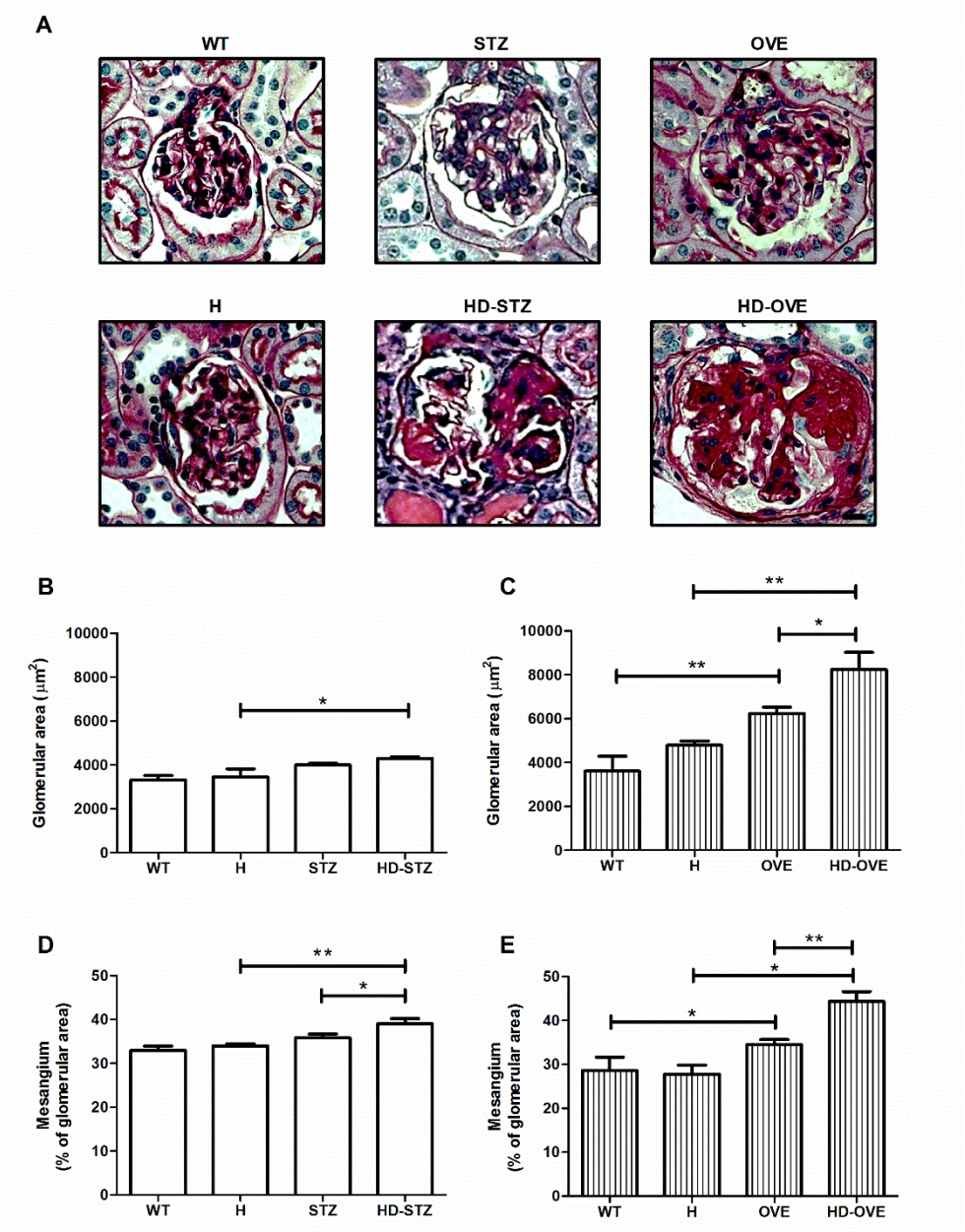

Figure 2: Glomerular pathology. Paraffin-embedded PFA fixed-kidney sections were stained with periodic-acid Schiff. (A) Representative images of glomerular profiles for each group. Glomerular surface area (B, C) and mesangial area (D, E) analysis was performed on 15-25 glomeruli per mouse, 3-5 mice per group. Data represented as means with standard error. *=P≤0.05; **=P≤0.01. (Scale bar = 5 um, 40X Mag.)

Renal tubulointerstitial fibrosis and elevated α-SMA in HD-OVE mice

The impact of the HD phenotype on fibrosis of the kidney's tubulointerstitium was examined in a qualitative manner. Using microscopic examination, increased PAS-positive material was observed in most HD-OVE mice compared to uniquely diabetic counterparts. In contrast to the OVE26 study, while in agreement with the STZ model's characteristic milder phenotype, a portion of HD-STZ mice showed some signs of interstitial damage yet to a lesser extent than the HD-OVE cohort (data not shown). Under immunofluorescence microscopy, enhanced immunodetectable α-SMA was evident in both the interstitium and in peri-glomerular areas (crescentic glomerulosclerosis) for the HD-OVE cohort (*arrows*, Fig. 3), while similar baseline vascular α-SMA staining was observed in all mice *(asterisks*, Fig. 3*).*

Increased collagen and fibronectin production in HD-OVE mice

Further understanding of the HD-OVE cohort's propensity for developing advanced glomerular and tubulointerstitial lesions earlier than their OVE littermates was confirmed using Masson's trichrome staining on kidney sections (Fig. 4a). Positive staining for collagen (in blue) was readily observed in the glomerular tuft and in the tubulointerstitial regions of HD-OVE kidneys, while being minimally increased in OVE mice and absent from H and WT groups. To confirm increased collagen expression, we measured collagen-4 mRNA levels by qPCR of kidney cortex RNA isolates. Accordingly (Fig. 4b), HD-OVE mice harbored a three-fold increase in collagen-4 mRNA levels versus WT, H or OVE alone (WT, 0.99±0.04; H, 0.75±0.11; OVE, 0.96±0.17; HD-OVE, 2.99±0.8, a.u.). Immunoblotting for fibronectin was also performed in cortical lysates from the OVE study (Fig.4c). H and OVE mice exhibited similar fibronectin protein levels as WT

controls. However HD-OVE mice showed greater increases fibronectin production (Fig. 4d.) (WT, 0.93±0.1; OVE, 1.3± 0.2; H, 0.90± 0.2; HD-OVE, 1.9± 0.1, a.u.), corroborating the indications of tubulointerstitial fibrosis and the increases in α-SMA protein observed by immunofluorescence.

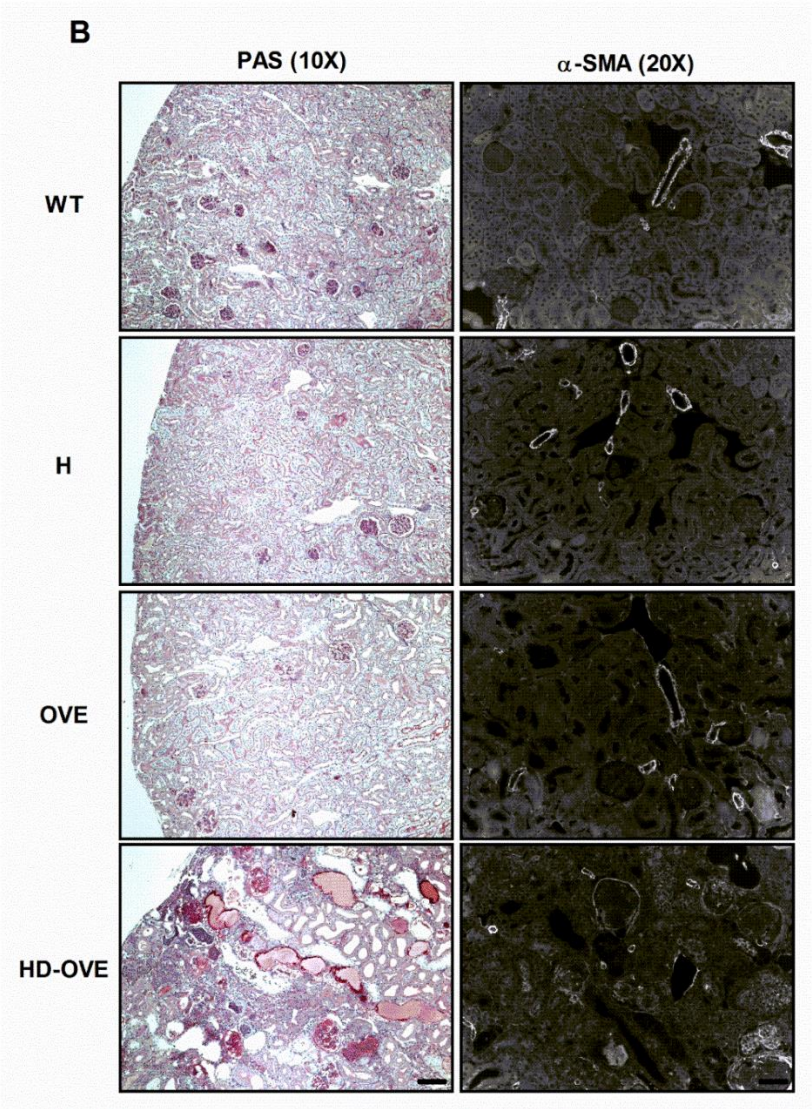

Figure 3: OVE26 study - PAS and α-SMA staining. Paraffin-embedded PFA fixed-kidney sections were stained with periodic-acid Schiff (left) or α-SMA (right) and visualized by either light or fluorescence microscopy at 40X. Representative images. (Scale bar =10 um.)

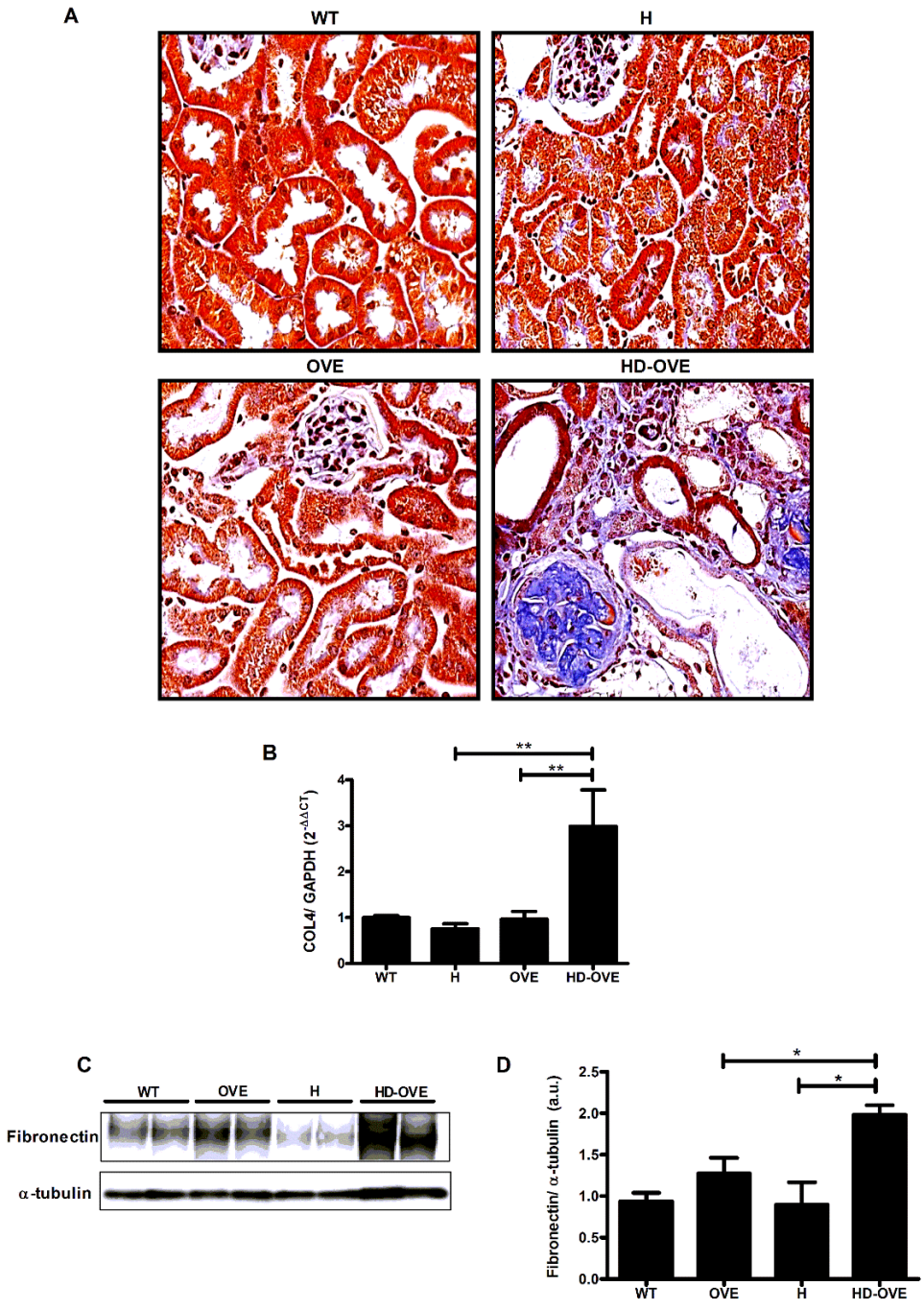

Figure 4: OVE26 study - collagen and fibronectin expression. A) Representative images of paraffin-embedded PFA fixed-kidney sections stained with Masson's trichrome (40X mag.) B) qPCR determination of collagen-4 mRNA expression in kidney cortex normalized to GAPDH (n=3-5 mice/group). C) Representative fibronectin and α-tubulin protein immunoblotting in kidney cortex samples. D) Quantification of fibronectin expression in OVE26 study kidney cortex. (n=4-6 mice/ group; *=P≤0.05; **=P≤0.01)

Decreased GFR in HD mice

As GFR decline is a key feature of late stage DN, we performed FITC-inulin GFR measurements in a subset of HD-OVE mice and at endpoint for the STZ study (Fig. 5A). Type 1 diabetic mouse models rarely show signs of renal function decline, and usually remain in the hyperfiltration stage [204]. HD-OVE mice exhibited hyperfiltration levels of GFR at 12 weeks of age, which were similar to levels seen in 20 week old OVE mice. By 20 weeks, HD-OVE mice showed significant GFR reductions compared to aged matched OVE mice, indicating a decline in renal function as disease progressed (20 weeks: OVE, 0.65±0.04; HD-OVE, 0.26±0.04, $mL.min^{-1}$). Similarly, at 18 weeks post STZ, diabetes led to a 2-fold increase in GFR, while HD-STZ had significantly lower GFR values (WT, 0.31±0.04; H, 0.21±0.02; STZ, 0.75±0.15; HD-STZ, 0.45±0.04 $mL.min^{-1}$).

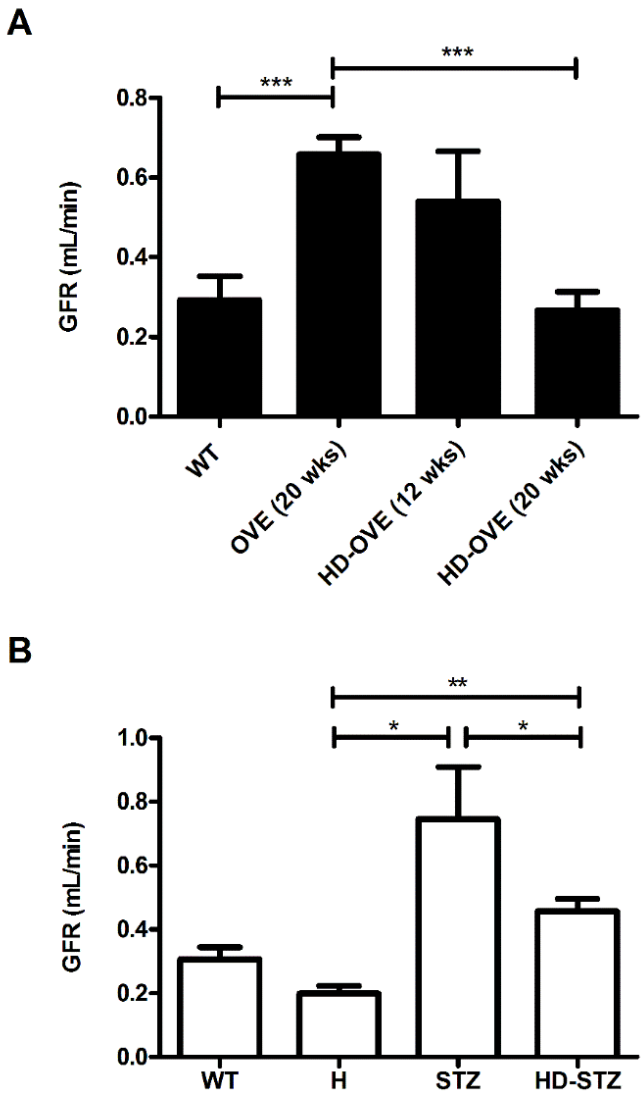

Figure 5: GFR estimation using FITC-inulin clearance. A) GFR was estimated in a subset of mice from the OVE26 study at early (12 weeks) and later (20 weeks) time points (2-6 mice/ group) and B) in the STZ study at 18 weeks post-STZ (n=5-9/ group; *=P≤0.05; **=P≤0.01; ***=P≤0.001).

Discussion

Rodent models have provided important insights into the etiology of DN [50]. However, interpretations are tempered by the lack of an ideal model that reproduces not only early but also late characteristics of human DN [50, 213]. In the current report, we describe the generation of a novel DN model that addresses this concern by combining hypertension and diabetes (HD mice) resulting in an accelerated and robust nephropathy phenotype.

Provided they are bred onto so-called DN susceptible background strains (e.g., DBA/2, FVB/n, BLKS, etc.), the majority of currently available mouse models exhibit many of the characteristics of early DN [50, 213]. These include glomerular hyperfiltration, mesangial expansion, glomerular basement membrane thickening (>50% over baseline), glomerular and renal hypertrophy, arteriolar hyalinosis, and albuminuria. However, one or more key features of late DN are often absent – namely, GFR decline and/or tubulointerstitial fibrosis. Moreover, while hypertension often develops in humans as DN progresses [38], most rodent models exhibit limited increases in blood pressure (e.g., *Ins2*$^{Akita/+}$ mice, systolic blood pressure ~130mmHg [214]). A model that shows evidence of both early and late DN features is the OVE26 type 1 diabetic mouse. This line of transgenic mice was generated on the FVB/n background by Epstein *et al* by overexpressing the calmodulin gene under the control of the rat insulin II promoter to allow for β-cell –specific expression [215]. Due to the destruction of the β-cells, OVE26 mice develop diabetes neo-natally. FVB/n OVE26 mice exhibit many of the hallmarks observed in both early and late stage human DN [48]. These include an initial increase in GFR, accompanied by significant albuminuria. As the animals age, mesangial matrix expands, glomerular basement membrane thickens, tubulointerstitial fibrosis develops

and kidney weight doubles. While GFR increases significantly early on in the OVE26 model, it declines between 5 and 9 months of age. Podocyte loss, a characteristic finding of human DN is evident after 16 months [216]. However, systolic BP changes minimally in OVE26 mice which may partly underlie the length of time needed for the DN phenotype to develop.

A model generated recently that features BP elevation is the *eNOS*$^{-/-}$ mouse [52, 217, 218]. Vascular endothelial nitric oxide synthase (eNOS) dimer formation and phosphorylation are reduced by high glucose in cultured endothelial cells suggesting impaired activity under diabetic conditions [219] - leading to attenuation of NO production and diminished vasodilatation. With increasing age, mice with targeted *eNOS* deletion subjected to low dose STZ-induced diabetes have normalized GFR, presumably due to a progressive decline in hyperfiltration, and exhibit tubulointerstitial fibrosis along with the onset of moderate hypertension [52, 217, 218]. *eNOS*$^{-/-}$ mice bred onto the type 2 diabetes *db/db* line which lack the leptin receptor exhibit even greater DN severity. Interestingly, recent studies by Harris's group have underscored the importance of BP elevation for DN progression, in finding that glomerulosclerosis and albuminuria in *eNOS*$^{-/-}$ *db/db* mice were decreased when BP was lowered independent of RAS inhibition [220].

However with many existing DN models, mice need to be of advanced age, some requiring 6-12 months for a consistent and full development of a DN phenotype [216]. Moreover, such models are limited by logistically challenging breeding strategies to arrive at (in some cases) triple homozygous compound gene-targeted animals. Together, these factors conspire to impede our ability to efficiently study the etiology of the

disease. In light of these limitations, an accelerated and robust mouse model is needed for a more comprehensive understanding of diabetic nephropathy.

Our approach employs mice transgenic for the human renin cDNA under the control of the transthyretin promoter (TTRhRen) on an FVB/n background previously developed by Dr. Timothy Reudelhuber (U. of Montreal) [29]. Similar approaches have been realized by others using a variety of transgenes (i.e, RenTgARE, RenTgKC, and RenTgMK) on the 129S6/SvEvTac background [221-223]. A similar model was recently generated in rats, wherein the murine *renin-2* gene was driven by the cytochrome P450a1 promoter [224]. These rats become moderately hypertensive in response to indole-3-carbinol. Induction of hypertension along with STZ-induced diabetes produced a 500-fold increase in albuminuria, glomerulosclerosis and tubular interstitial fibrosis, while GFR tended to be lower in both diabetic and non-diabetic transgenic rats, but did not reach statistical significance. By translating a similar approach to mice using either STZ-induced or OVE26 type 1 diabetic mice, we have generated a model amenable to the current array of genetic strategies (i.e., gene-targeting / transgenics) that are used widely to explore the role of any number of putative players in the progression of DN. One caveat of the current approach is that unlike human diabetic nephropathy, where hypertension typically develops after indications of nephropathy have emerged, the HD model involves diabetes-induced renal injury with a concurrent elevation in blood pressure. Moreover, the HD mice do not represent non-proteinuric subsets of DN. Nevertheless, the HD mice developed in the present study fulfill much of the criteria set out by the Diabetes Complications Consortium [50], Specifically, both HD-STZ and HD-OVE mice have >10-fold increase in albuminuria, show evidence of widespread mesangial matrix

expansion, and tubulointerstitial fibrosis. While tubular lesions appeared significantly more severe in HD-STZ vs. STZ mice, those which developed in HD-OVE mice represented even greater progression, perhaps due to the fact that the latter mice develop diabetes from a very early age. Following an initial period of hyperfiltration GFR declined progressively (by 50% of peak values) to levels within the 'normal' range for both HD-STZ and HD-OVE models. Given the extensive glomerular/tubular damage, it is likely that such a filtration rate represents hyperfiltration at the single nephron GFR level derived from residual glomerular function. Despite the presence of chronic hypertension, extensive glomerular and tubulointerstitial lesions in the HD models, we were unable to detect arteriolar hyalinosis. It remains possible that the relatively short duration of our models (<20 weeks) could account for the lack of this late human DN characteristic. We cannot therefore rule out whether arteriolar hyalinosis would have emerged if the mice were allowed to age beyond this time period. Additionally, while our model was successful on the FVB/n strain, whether it is amenable to more resistant strains (e.g., C57BL/6, which also become hypertensive with the TTRhRen transgene [212]) remains to be determined.

The accelerated phenotype of the HD model is likely due to superimposition of elevated blood pressure on a diabetic state. Both clinical and experimental data consistently show that interventions which reduce blood pressure are effective in mitigating renal disease progression in diabetes [225-227]. Indeed, blood pressure of HD-STZ mice was elevated in comparison to STZ mice alone, which did not differ from that of non-diabetic controls. In contrast, HD-OVE mice developed profound hypertension from 16-20 weeks of age (>180 mmHg) that dramatically exceeded that of non-diabetic renin-expressing mice.

The underlying mechanism accounting for this difference is unclear. Despite these observations, one cannot discount blood pressure-independent effects of angiotensin II [228]. While we did not measure circulating or renal AngII in our HD models, previous studies showed plasma AngII in TTRhRen mice are 1-2 times normal [29] while renal levels are similarly elevated [177]. Such elevated AngII could exert damage-inducing effects directly upon the renal vasculature [220], glomerular filtration barrier [229-231] and tubular segments [214, 232]. Other transgenic models of hepatic renin overexpression, such as the RenTgMK mice (which show AngII levels 4-6-fold above wild type mice) exhibit glucose intolerance with normal fasting glucose levels and insulin sensitivity, suggesting that either circulating renin or AngII might impact glucose handling [233]. While we did not perform glucose tolerance tests on either TTRhRen or HD mice, blood glucose levels were invariably similar within non-diabetic or diabetic groups, suggesting that diabetes was induced equivalently irrespective of transgenic renin expression.

In summary, we have developed a mouse model of diabetic nephropathy with superimposed hypertension that recapitulates many key features of both early and late human disease over a relatively short timeframe. The HD model requires minimal breeding of readily available mouse lines and thus represents an attractive choice to study pathogenic mechanisms underlying diabetic nephropathy progression.

Chapter 5: General discussion

CKD currently affects an estimated 3 million Canadians, making it a major health and economic burden. The major cause of mortality in CKD patients is due to cardiovascular disease. COX-derived prostaglandins maintain renal function and perfusion when effective circulating volume is low, in part by activating and buffering the RAAS. It is well established that use of NSAIDs is associated with increased risk of AKI and progression of CKD, especially in the elderly population and other high risk patients [234].

5.1 EP1 receptor in diabetic nephropathy

In the last 20 years, there have been several studies aimed at elucidating the COX/PGE2 and EP receptor-specific signaling pathway in the pathogenesis of CKD, especially in DN, its primary cause. COX-2 derived PGE2 production is enhanced in diabetic kidneys [89, 90] and inhibition of COX-2 production lowers proteinuria, glomerulosclerosis and markers of fibrosis in animal models [93, 94, 235], giving it some therapeutic potential. In DN, glomerular hyperfiltration is thought to be due in part to increased PGE2-mediated vasodilation of the pre-glomerular vasculature, contributing to enhanced physical stress in resident cells (podocytes, EC, mesangial cells). However other factors such as NO or VEGF play important roles in this regard as well. Moreover, as overexpression of COX-2 specifically in podocytes renders them increasingly susceptible to Adriamycin-induced injury, the involvement of PGE2 signaling a specific, locally expressed detrimental EP receptor is likely [158]. Seemingly of therapeutic interest, discrepant results regarding COX-inhibition in human DN- patients [154] preclude its use

as effective anti-proteinuric target, which may implicate a genetic component. Accordingly, the presence of COX-2 polymorphisms have recently been described in type-1 diabetic patients [236]. Also, the lack of conclusive data surrounding COX-2 inhibition in DN may be due to the non-specific nature of this therapeutic strategy, as beneficial prostanoids and signaling pathways downstream of COX-2 induction may be affected. Along these lines, Makino and colleagues were the first to speculate a detrimental role for the EP1 receptor in the pathogenesis of DN [106]. These authors showed that an orally-active pharmacological antagonist could prevent the development of DN in STZ-treated rats with noticeable improvements in renal and glomerular hypertrophy, scarring and suppression of proteinuria and fibrosis. While the specific cell-type which benefitted from EP1-blockade remained in question, these results were the first to suggest a potentially detrimental role for the EP1 in DN. We took a different, while similar, approach to test the aforementioned hypothesis. By subjecting EP1-/- mice to two distinct models of type-1 diabetes, we were able to corroborate the original findings using a genetically-based approach, in a different rodent [174]. Limitations of our study included the non-specific nature of our EP1 knockout mice which hampered our ability to fully understand the cell-specific impact underlying the protective nature of EP1-targeting in diabetes *in vivo*. However using EP1-selective antagonists in human podocyte and PT cell lines, we identified the EP1 signaling cascade as promoting AngII-induced ROS production and PGE2-mediated fibronectin upregulation respectively. Furthermore, AngII-dependent vasoconstriction of isolated mesenteric arteries was mitigated in diabetic EP1-/- mice, suggesting that vascular EP1 receptors may contribute to AngII-dependent vasoconstriction in diabetes. Our conclusions were that in the

diabetic kidney, the COX/PGE2/EP1 signaling cascade may be detrimental to various cell-types and locales. Our findings also reinforced data from studies implicating the EP1 receptor in increasing vascular tone and BP in type-2 diabetic mice, and promoting AngII-dependent vascular injury in various experimental models of hypertension [104, 132, 165]. NSAIDS and COX-2 inhibition are contraindicated in hypertension patients as they may hasten the development of renal injury through loss of hemodynamic and electrolyte balance control. However data suggests that EP1-selective targeting is beneficial in this context, which may be due to a synergistic relationship existing between the AT1 (AngII) and EP1 (PGE2) receptors, both of which activate Gq-coupled downstream signaling pathways. This relationship has been shown in the cerebral vasculature, promoting oxidative stress in this locale [140, 165]. More work is required to further elucidate whether a synergistic link exists between these receptors (*see: Section 5.4*), possibly through receptor heterodimerization as is the case for EP1 and beta adrenergic receptors in lung smooth muscle [237].

5.3 Vascular EP4 in hypertension

Control of glomerular hemodynamics is dependent on a variety of hormones and signaling cascades which either directly or indirectly affect the tone of pre and post-glomerular arterioles. Indeed, the potent vasoconstrictor AngII exerts its GFR regulating actions primarily via post-glomerular (efferent) vascular- AT1 receptors, with moderate effects on the afferent AT1 receptors as well. AngII-actions on the kidney are primarily encountered in conditions of low effective circulating volume, due to extreme blood loss, dehydration and/ or pre-existing renal/cardiovascular disease. In this context of low BP, macula densa renin activity is stimulated, which leads to local production of AngII,

which preserves GFR by constricting the glomerular vasculature to maintain filtration fraction. It is well established that the COX-PGE2 system is also activated in this context. Recent findings identify the COX-PGE2 pathway as critical in the activation of the RAAS pathway via stimulation of renin exocytosis from juxtaglomerular cells via the EP2 and EP4-mediated cAMP production [72-74]. Local RAAS activation is also dependent on this pathway as AngII-induced increased prorenin receptor expression in the medulla and subsequent increased renal renin activity is dependent on EP4 activation [169]. While RAAS activation rests upon prevailing COX-2 activity, the latter pathway is also involved in buffering the actions of the former. Renal physiology pioneers Baylis and Brenner were amongst the first to demonstrate a direct effect of COX-inhibition on renal function in response to AngII. They found that AngII-dependent vasoconstriction, decreased GFR and glomerular capillary flow rate was exacerbated when the rats were pretreated with indomethacin, a non-selective COX inhibitor. Thus prostaglandin synthesis is critical in maintaining renal function in conditions of elevated AngII [238]. The role of the EP4 receptor in mediating these actions has been confirmed in isolated glomerular arterioles, confirming its predominantly vasodilatory effect [172].

Our study was designed to test the hypothesis that the VSMC EP4 receptor maintains renal hemodynamics in AngII-dependent hypertension. By deleting the EP4 receptor specifically in VSMC's ($EP4^{VSMC-/-}$) we identified the EP4 as being critical in maintaining glomerular hemodynamics and overall renal function and integrity in a context of elevated levels of AngII. AngII-dependent tubulointerstitial, glomerular and microvascular damage was significantly enhanced when EP4 signaling was lost in VSMC's, and virtually undetectable in WT mice. These structural changes were

associated with enhanced renal hypoxia and decreased renal blood flow. In our manuscript, we highlight increased albuminuria in this model as an interesting finding. Albuminuria can occur as a consequence of injury to various components of the glomerular filtration barrier including the endothelium, mesangial cells and primarily podocytes. A classic notion is the detrimental effect that increased filtration pressure aka hyperfiltration can have on glomerular architecture thus promoting loss of albumin in the urine. Glomerular hypofiltration as seen in our AngII treated $EP4^{VSMC-/-}$ mice is not usually associated with increased albuminuria [195, 196]. However a recent hypothesis known as the electrokinetic model suggests that decreased or loss of flow could affect the charge selectivity of the filtration barrier which participates in the repulsion of negatively charged albumin [197]. Alternatively, we also cannot dismiss the possibility that increased damage to the tubulointerstitum brought on by hypoxia due to decreased RBF and GFR, could subsequently promote glomerular structural injury including proteinuria and scarring, as previously suggested by Grgic and colleagues [198].

EP4 receptor activation has shown to be either beneficial or detrimental to renal integrity, and appears to be disease/ context dependent. Pharmacological EP4 agonists reduce serum creatinine levels and maintain glomerular, tubular and vascular integrity in both acute and chronic models of renal failure in rats [108]. While in DN, inconsistent data regarding the EP4 receptor's beneficial role make it an unattractive target in this context. In as much as PGE2/EP4-mediated vasodilation is a positive effect in some circumstances, including GFR and RBF regulation when AngII levels are elevated, glomerular hyperfiltration which is associated with DN progression, is also thought to be due to local PGE2/EP4 signaling. Results generated by this study reinforce the idea that

caution should be taken when NSAIDs or COX-2 inhibitors are prescribed to patients with impaired or compromised renal function, as it may oppose the beneficial impact brought on by kidney EP4 activation by blocking PGE2 synthesis. Antagonism of these vasoactive receptors in hypertension predisposes the kidney to glomerular, interstitial and vascular injury, therefore pharmacological EP4 selective activation may be a potential therapeutic target to preserve blood flow in this context [202].

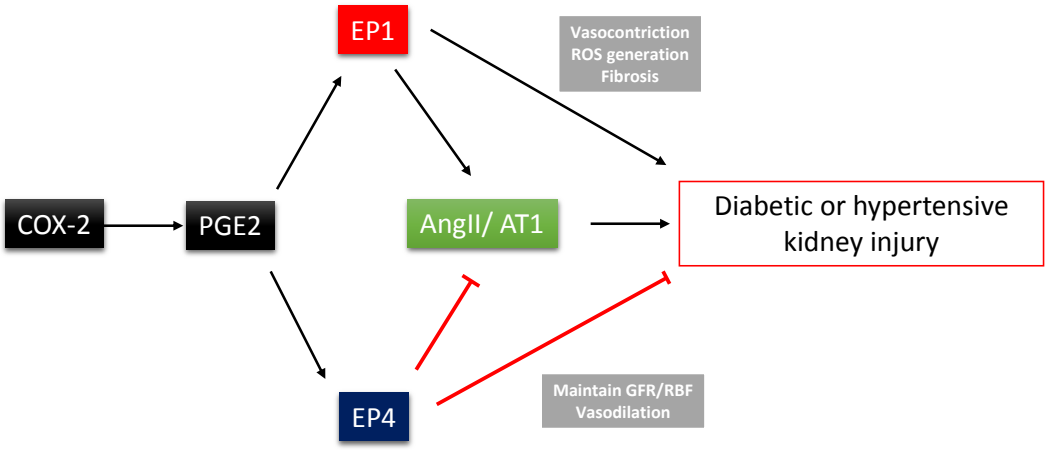

Figure 1: Thesis summary figure. COX-derived PGE2 production is elevated in DN and participates in the promotion of renal injury as its inhibition confers renal protection. PGE2 acting via its EP1 receptor is detrimental in this context as it promotes vasoconstriction, podocyte and proximal tubule injury and renal interstitial fibrosis. Possible cross-talk or synergism between the EP1 and AT1 receptors may exist in the kidney as observed in other organs. EP4 activation in diabetes has more often than not shown to be involved in altering renal hemodynamics early in disease. However, in a context of AngII-dependant hypertension, the EP4 buffers the vasoconstrictive effects of AngII, maintaining adequate renal perfusion (i.e. GFR and RBF) and renal functional and structural integrity.

5.4 Novel model of DN

The final chapter of this doctoral thesis represents the culmination of a study in which the objective was to generate a mouse model of diabetic nephropathy which we believed would develop advanced features of disease, mimicking human DN pathogenesis. The rationale behind this study was that adequate mouse models of DN which fully recapitulate the full spectrum of disease are generally difficult to obtain, requiring complex breeding schemes or long study duration. Following the establishment of the Animal Models of Diabetic Complications Consortium (AMDCC) in 2005, criteria for validating progressive DN mouse models were devised and updated in 2009 [204] which provide a benchmark against which current DN models are measured.

Based on this rationale, we and others believed that the lack of available mouse models of DN may be due to their resistance to developing diabetes-induced hypertension [204, 207, 209]. Also some evidence suggest a strain-dependent susceptibility to DN-induced renal injury as most T1DM and T2DM models are more effective when used on Fvb versus C57BL/6J mouse strains [51, 239]. Therefore we sought to develop a mouse model of DN which would be generated by intercrossing commercially available OVE26 type-1 diabetic mice with TTRhRen mice, developed by Dr. Reudelhuber's group in Montreal several years ago [212]. This group developed a genetically-hypertensive mouse in which the transthyretin promotor drives liver-specific expression of a human form of the pro-renin gene in which a furin-cleavage site has been introduced between the pro and active renin segments. These mice have elevated levels of circulating renin, enhancing AngII production thus leading to hypertension [212]. We obtained progeny which was diabetic and hypertensive at birth by breeding these mice with the

aforementioned OVE26 mice. We reinforced our study by including a second model of T1DM by subjecting hypertensive TTRhRen mice to STZ injections which rendered them hyperglycemic after a couple of weeks. Both OVE and STZ diabetic TTRhRen mice displayed markers of advanced DN including markedly high albumin-to-creatinine ratios, elevated systolic BP and evidence of GFR decline. Consistent with model-specific differences, hypertensive OVE mice displayed significantly worse renal phenotypes compared to hypertensive STZ mice. An unfortunate finding in this study was that these mice failed to develop indices of arteriolar hyalinosis or advanced tubulointerstitial fibrosis. We speculate that both features may have appeared if these mice had been followed for a longer time period, especially in the OVE cohort.

The rationale behind superimposing a T1DM mouse onto a hypertensive phenotype governed by renin-mediated AngII production is clearly warranted as RAAS inhibition is a primary target in treating DN. Also, several studies have confirmed a synergistic relationship between hyperglycemia and hypertension in the progression of DN. Of interest, two rat models have been used in conjunction with established T1DM models to obtain advanced renal injury, the mRen2 and Cyp1a1mRen2 rats [224, 240, 241] both of whom display advanced markers of renal injury and pronounced albuminuria, which increases when BP reaches hypertensive levels.

A potential benefit of using our novel mouse model of DN is that it displays markers of disease that usually do not appear until later stages in humans. As most patients do not begin to be treated for DN at the early stages of malady, since clinically measurable signs are rarely evident at this stage, our mouse model may be more representative of the state at which treatment would commence in humans. Most available mouse models of DN

rarely progress to that extent, therefore testing new compounds to halt or prevent disease progression may be increasingly relevant in this model as it may better resemble the human context. A potential caveat from our study is that our mice were hypertensive and diabetic from birth while in human DN, markers of renal injury are usually apparent prior to the onset of hypertension. Nevertheless, we believe this mouse model may be amenable to future studies in which new therapeutic compounds or gene-targeted deletions may be warranted to study the etiology and/or pathogenesis of DN.

5.5 Future studies

We found that deletion of the EP1 receptor was beneficial in that it reduced markers of DN-induced renal injury. However the EP1 receptors contribution to cell-type specific injury remains incompletely understood. We have in vitro data suggesting EP1 activation promotes AngII-induced ROS generation in cultured podocytes, which may underlie the beneficial effects associated with EP1-deletion. Furthermore, cultured PT-cells showed less fibronectin upregulation in response to PGE2 when the EP1 was antagonized. Thus, a logical extension of this study would be to generate either podocyte or PT-specific EP1 knockout mice and to challenge them with T1DM to determine whether the beneficial impact of EP1 deletion predominates in a certain renal locale versus the other. In parallel, we observed maintained PT-megalin expression in diabetic EP1-/- mice, yet we were unable to establish a causal relationship between PGE2/EP1 activation in PT-cells, and megalin downregulation. This interesting finding could be addressed by studying the effect of PT-specific EP1-deletion on megalin expression. It is conceivable that activation of the Gq-coupled EP1 receptor triggered a signaling cascade involved in downregulating PT megalin gene or protein expression, as has been previously observed in a AT1-

dependent manner [163]. Alternatively, the reduction in immunodetectable megalin may have been due to enhanced albuminuria, which in itself may damage downstream tubules, leading to megalin shedding loss of megalin into the urine. Our study did not however further investigate this possibility.

A mechanistic explanation as to how the EP1 receptor promotes renal injury in a diabetic context remains incompletely understood thus far, yet we and others speculate it may be due to a maladaptive synergism between the EP1 and AT1 receptors as previously hypothesized. We have begun characterizing the contribution of the EP1 receptor to AngII/AT1 mediated renal injury by breeding EP1-/- mice with hypertensive TTRhRen (aka LinA3) mice, which have an intrinsically activated RAAS system. Although this model is not ideal in that AT1 activation relies on the enhanced production of AngII due to high active levels of circulating renin, we nevertheless expect that by deleting the EP1 in these mice, we may be able to attenuate increases in BP. Preliminary BP obtained by tail-cuff plethysmography indicate that TTRhRen/EP1-/- mice have similarly elevated systolic and diastolic BP in comparison with their TTRhRen counterparts at 4 and 8 months of age (data not shown). Further work will be required to properly assess the impact, if any, of this genotype.

Our second study clearly identifies a beneficial role for the vascular EP4 receptor on renal injury in AngII-induced hypertension. Our findings were however limited to kidney structure and function. Since our EP4 knockout mice are VSMC-specific, this genotype and its impact on cardiovascular health in this model may be underappreciated. Of importance, the notion that injury to the kidney can in turn affect cardiovascular integrity is well documented and is termed the cardio-renal syndrome, whereby acute or chronic

injury to one of these organs may lead to acute or chronic injury to the other. It is thereby conceivable that loss of the EP4 receptor in the vasculature may not only predispose to hypertension-induced renal injury, but also increase the susceptibility of cardiovascular injury in CKD. A more in depth analysis of cardiac tissue and the assessment of peripheral vascular reactivity in these mice may shed some light on these questions. Furthermore, the model of hypertension which was exploited for this study does not accurately reflect what occurs in human disease. To this end, different models of hypertension may be employed to validate this study's findings.

In addition to CKD, the use of NSAIDS has been associated with increased susceptibility to developing AKI. It would be therefore worthwhile to conduct experiments in which $EP4^{VSMC-/-}$ mice were subjected to the renal ischemia reperfusion model of AKI. If the EP4 receptor is critical in maintaining blood flow and tissue perfusion, vascular injury in this model would be expected to be severely enhanced when this receptor is lost. Lastly, the impact brought on by VSMC-specific deletion of the EP4 receptor on AngII-dependent increases in arterial BP was difficult to interpret due to a lack of sensitivity of the tail-cuff method. The tail-cuff method is an accepted method to determine large changes, while it may not be amenable to detect small differences in BP. Therefore the use of implantable telemetric devices may be of interest for future studies.

The last study focused on generating a mouse model of advanced diabetic nephropathy by intercrossing two previously established mouse models of T1DM and hypertension respectively. We observed an advanced phenotype which included injurious markers such as severe glomerulosclerosis and proteinuria as well as the presence of interstitial fibrosis and declining GFR with age. Future studies will be focused on employing this mouse as

our DN-model, to test new hypothesis and or treatment options. This mouse model may be a useful tool to test new therapeutic avenues in more advanced stages of DN-pathogenesis, when some form of renal injury is already present.

5.6 Conclusions and perspectives

In summary, COX-derived prostaglandins are a family of vasoactive hormones which regulate a variety of physiological processes. PGE2-dependent maintenance of renal hemodynamics and electrolyte handling is essential, especially when renal function is compromised. COX-inhibition is an interesting target to mitigate DN-associated albuminuria, yet unwanted side effects preclude their widespread clinical use in this regard, due to their effect on BP and GFR regulation. EP-selective targeting represents a more elegant approach to increase the beneficial effects while avoiding unwanted side effects. Clearly, EP1 receptor activation participates in the pathogenesis of DN and hypertension, while EP4 receptor activation appears to be beneficial in a hypertension setting, while detrimental in DN. Our studies support the notion that avoiding COX-inhibition in certain contexts may offer renoprotection by maintaining the activation of beneficial receptors (i.e. EP4), while this therapeutic strategy may be detrimental in other contexts. Thus, great care should be taken prior to prescribing NSAIDS and COX-2 inhibitors for the treatment of CKD.

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