



## Review

# Coagulation, Protease-Activated Receptors, and Diabetic Kidney Disease: Lessons from eNOS-Deficient Mice

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Endothelial nitric oxide synthase (eNOS) dysfunction is known to exacerbate the progression and prognosis of diabetic kidney disease (DKD). One of the mechanisms through which this is achieved is that low eNOS levels are associated with hypercoagulability, which promotes kidney injury. In the extrinsic coagulation cascade, the tissue factor (factor III) and downstream coagulation factors, such as active factor X (FXa), exacerbate inflammation through activation of the protease-activated receptors (PARs). Recently, it has been shown that the lack of or reduced eNOS expression in diabetic mice, as a model of advanced DKD, increases renal tissue factor levels and PAR1 and 2 expression in their kidneys. Furthermore, pharmaceutical inhibition or genetic deletion of coagulation factors or PARs ameliorated inflammation in DKD in mice lacking eNOS. In this review, we summarize the relationship between eNOS, coagulation, and PARs and propose a novel therapeutic option for the management of patients with DKD.

**Keywords:** diabetic glomerulosclerosis; factor Xa; inflammation; tissue factor  
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## Introduction

The number of patients with diabetic kidney disease (DKD) is increasing worldwide (de Boer et al. 2011; Kainz et al. 2015; Ogurtsova et al. 2017). DKD is one of the major causes of mortality in patients with diabetic complications (Afkarian et al. 2013). Furthermore, DKD is progressive and is the main cause of end-stage kidney disease requiring renal replacement therapy (Gregg et al. 2014; Liyanage et al. 2015). In Japan, diabetic nephropathy accounts for more than 39% of new dialysis patients (Nitta et al. 2020). Therefore, the development of novel therapeutic options is required to manage patients with DKD.

There is increasing interest in the role of the coagulation system in DKD pathogenesis. Hypercoagulability is associated with DKD (Goldberg 2009; Domingueti et al. 2016). An elevated fibrinogen level in blood was shown to be associated with low estimated glomerular filtration rate, high proteinuria, and severe histological injury, and it was a predictor of the progression to end-stage kidney disease (Dalla Vestra et al. 2005; Pan et al. 2018; Zhang et al. 2018). Some studies have shown a correlation between the

level of D-dimer, a fibrin degradation product, and renal dysfunction in DKD (Domingueti et al. 2018; Pan et al. 2018). Hypercoagulability in DKD is likely determined by multiple factors. For example, renin-angiotensin-aldosterone system activates tissue factor (TF, factor III) and increases thrombotic events (Dielis et al. 2005). Otherwise, hyperglycemia, dyslipidemia, inflammation, or endothelial dysfunction are involved in the pro-thrombotic state under DKD pathogenesis (Goldberg 2009).

Endothelial dysfunction is present in DKD that progresses to microvascular complications (Goldberg 2009; Nakagawa et al. 2011). Impaired endothelial nitric oxide synthase (eNOS) production or reduced eNOS activity (e.g., impaired eNOS phosphorylation) is a hallmark of endothelial dysfunction in DKD (Nakagawa et al. 2011; Cheng et al. 2012). We have shown that a loss of eNOS expression was linked to the elevation in the TF level and the extrinsic coagulation system activity (Li et al. 2010; Wang et al. 2011a), which is closely associated with thrombotic events in patients with chronic kidney diseases (Kolachalama et al. 2018).

In addition to the elevated risk of thrombotic events,

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coagulation proteases, such as active factor FVII (FVIIa), active factor (FXa), and thrombin, which are present in the extrinsic coagulation cascade, mediate tissue injury through a protease-activated receptor (PAR)-dependent mechanism (Madhusudhan et al. 2016; Posma et al. 2019). In this review, we summarize the findings obtained from diabetic mice lacking eNOS and discuss the relationship between the coagulation-PAR pathway, eNOS levels, and DKD pathogenesis.

### Tissue Factor/Protease-Activated Receptors Pathway

Tissue factor (TF), known as factor III, is a 47 kDa transmembrane protein that interacts with factor VII (FVII) (Grover and Mackman 2018). The TF/FVIIa complex is an activator of the extrinsic coagulation cascade and catalyzes the activation of FX and FIX. FXa and activated co-factor V (FVa) form a prothrombotic complex that generates thrombin. Finally, thrombin converts fibrinogen to fibrin, resulting in the formation of thrombi (Grover and Mackman 2018). TF is expressed in both vascular smooth muscle cells and adventitial fibroblasts. Under inflammatory conditions, its expression is induced in endothelial cells or circulating cells such as monocytes (Østerud and Bjørklid 2006).

Protease-activated receptors (PARs) are members of the G-protein-coupled receptor superfamily, comprising four PAR proteins (PAR1-4). PARs undergo cleavage by proteases at the N-terminal end and are activated upon binding to a new N-terminus containing a tethered ligand. The four members of PARs (PAR1-4) are activated by specific coagulation proteases: the TF and FVIIa complex activates PAR2, factor Xa activates both PAR1 and PAR2, and thrombin activates PAR1, PAR3, and PAR4 (Camerer et al. 2000; Coughlin 2005; Rothmeier and Ruf 2012; Zhao et al. 2014). The relationship between TF, coagulation proteases, and PARs is shown in Fig. 1.

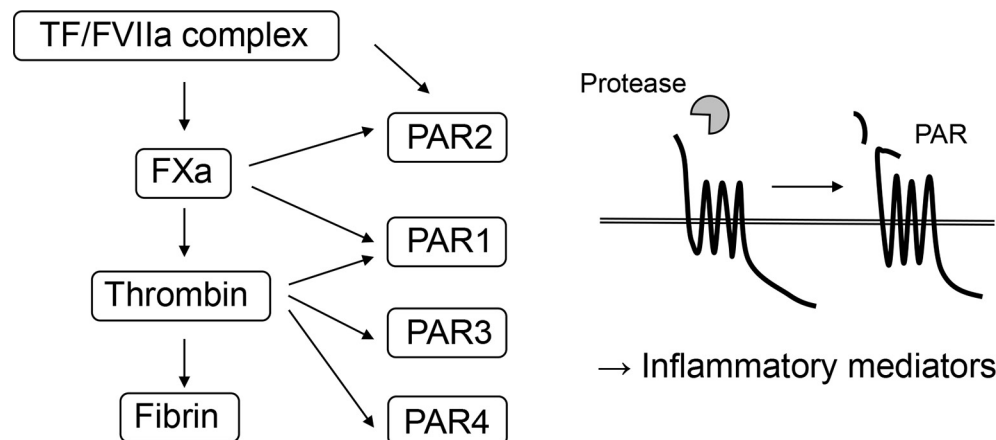


Fig. 1. Relationship between coagulation factors and protease-activated receptors (PARs).

In the extrinsic coagulation cascade, the tissue factor (TF) and active FVII (FVIIa) complex activates protease-activated receptor 2 (PAR2), active FX (FXa) activates both PAR1 and PAR2, and thrombin targets PAR1, PAR3, and PAR4. Cleavage of the N-terminal sequence of PARs by coagulation proteases reveals a new N-terminal sequence that acts as a tethered ligand and promotes inflammation.

PARs are known to be widely expressed in renal cells and are involved in the pathophysiology of kidney injury. Both PAR1 and PAR2 are expressed in glomerular endothelial cells, mesangial cells, and kidney tubular cells derived from human or mouse; PAR2, PAR3, and PAR4 are expressed in human podocytes; PAR1, PAR3, and PAR4 are expressed in murine podocytes (Tanaka et al. 2005; Vesey et al. 2005; Madhusudhan et al. 2012; Dong et al. 2015; Madhusudhan et al. 2016).

Although the harmful or protective effects of PARs in kidney injury have been demonstrated, accumulating data suggest that PARs exacerbate inflammation by stimulating the production of cytokines and chemokines (Rothmeier and Ruf 2012; Isermann 2017; Posma et al. 2019). Consistent with this finding, PAR1 and PAR2 agonists promote the expression of inflammatory mediators, such as monocyte chemoattractant protein 1 (MCP1) and plasminogen activator inhibitor-1 (PAI-1), and pro-fibrotic molecules in endothelial, mesangial, and kidney tubular cells (Vesey et al. 2005; Vesey et al. 2013; Ellinghaus et al. 2016; Waasdorp et al. 2016; Oe et al. 2019). In *in vivo* studies, the lack of PAR1 or the presence of PAR1 inhibitors reduced inflammation in models of crescentic glomerulonephritis or obstructive kidney injury (Cunningham et al. 2000; Waasdorp et al. 2019; Lok et al. 2020). Similarly, the therapeutic effects of PAR2 inhibition in kidney injuries were accompanied by a reduction in inflammation in the kidneys (Hayashi et al. 2016; Du et al. 2017; Han et al. 2019; Watanabe et al. 2019).

### Endothelial Nitric Oxide Synthase Polymorphisms in DKD

Endothelial nitric oxide synthase (eNOS) is one of the three NOS isoforms, and it contributes to the production of NO in the vascular endothelium (Walford and Loscalzo 2003). NO produced by eNOS in the vascular endothelium

plays a crucial role in regulating vascular relaxation, anti-inflammation, and prevention of thrombus formation (Walford and Loscalzo 2003). Impaired eNOS expression is associated with the development of DKD. Recently conducted meta-analyses on humans have revealed that G894T (rs1799983), C-786T (rs2070744), and intron 4b/4a (rs869109213) in eNOS (*NOS3*) genes are closely associated with the development of DKD (Dellamea et al. 2014; Zhang et al. 2015; Dong et al. 2018). Of these variants, the role of the G894T (Glu298Asp) polymorphism in eNOS function has been well characterized. The reduced production of NO or nitrite accumulation was indicated in transfected CHO cells with 298Asp compared to those with 298Glu (Noiri et al. 2002). Collectively, these results indicate that the reduced production of NO by eNOS dysfunction is important for the progression of DKD in humans.

### Diabetic Mice Lacking eNOS as a Model of Human Diabetic Nephropathy

Establishment of reliable preclinical models resembling human DKD is essential for the study of novel therapeutic options. Based on the evidence showing an association between eNOS polymorphism and DKD, a number of studies have demonstrated that eNOS knock-out models of type I and type II DM are some of the successful models mimicking human DKD (Brosius et al. 2009; Azushima et al. 2018). It was demonstrated that streptozotocin-induced diabetic mice lacking eNOS develop severe albuminuria, mesangial expansion, thickening of the glomerular basement membrane, and arteriolar hyalinosis, resembling human DKD (Nakagawa et al. 2007). Similarly, type II diabetic mice (db/db) lacking eNOS showed severe glomerulosclerosis and albuminuria (Zhao et al. 2006).

### Elevated TF in Diabetic Mice Lacking eNOS

NO inhibits thrombus formation and platelet aggregation (Walford and Loscalzo 2003). Because glomerular thrombus formation was observed in diabetic mice lacking eNOS (Nakagawa et al. 2007), impaired eNOS expression is likely associated with the increase in TF-dependent coagulation. The association between eNOS and TF in DKD has been addressed in our previous reports (Li et al. 2010; Wang et al. 2011a; Oe et al. 2016) (Fig. 2).

#### *Reduced or a lack of eNOS expression increases renal TF activity in diabetic Akita mice*

We characterized TF expression in diabetic Akita (*Ins2<sup>Akita/+</sup>*) mice, a model of type I DM, with various expression levels of eNOS (*eNOS<sup>+/+</sup>*, *eNOS<sup>+/-</sup>*, and *eNOS<sup>-/-</sup>*) (Wang et al. 2011a). We found that the severity of DKD was associated with reduced eNOS expression. Urinary albumin excretion, glomerulosclerosis, and reduction in glomerular filtration rate were exacerbated in the following order: *eNOS<sup>+/+</sup>; Ins2<sup>Akita/+</sup> < eNOS<sup>+/-</sup>; Ins2<sup>Akita/+</sup> < eNOS<sup>-/-</sup>; Ins2<sup>Akita/+</sup>*. Interestingly, kidney TF expression and activity were increased in *eNOS<sup>+/-</sup>; Ins2<sup>Akita/+</sup>* and *eNOS<sup>-/-</sup>; Ins2<sup>Akita/+</sup>* mice compared with those in *eNOS<sup>+/+</sup>; Ins2<sup>Akita/+</sup>* mice. Glomerular fibrin deposition was also remarkable in *eNOS<sup>+/-</sup>; Ins2<sup>Akita/+</sup>* and *eNOS<sup>-/-</sup>; Ins2<sup>Akita/+</sup>* mice. Furthermore, renal *Tf* mRNA expression was correlated with disease severity, urinary albumin excretion, and renal inflammatory cytokine expression.

#### *High-fat diet and lack of eNOS synergistically increase TF in diabetic mice*

Many studies have demonstrated a connection between obesity and thrombosis (Samad and Ruf 2013). For exam-

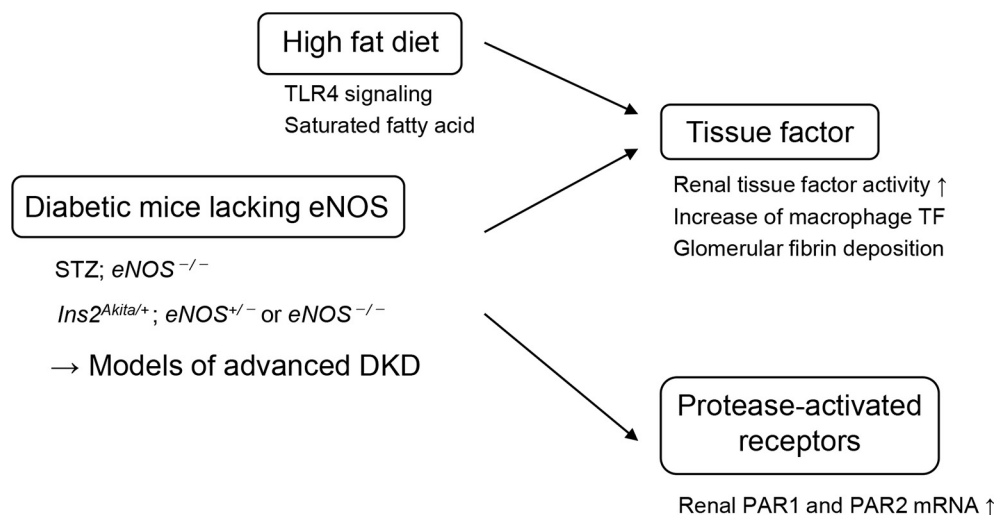


Fig. 2. Lack of eNOS expression is associated with an increase in tissue factor (TF) and protease-activated receptor (PAR) expression in diabetic kidneys.

Lack or reduced expression of eNOS in diabetic mice, models of advanced diabetic kidney disease (DKD), increases renal tissue factor (TF) activity. Furthermore, a high-fat diet synergistically increases TF levels. Similarly, the renal expression levels of *Par1* and *Par2* mRNA were increased in diabetic mice lacking eNOS.

ple, upregulation of the TLR4-NF- $\kappa$ B pathway under metabolic disease conditions increases TF levels (Lv et al. 2009; Owens et al. 2012; Rogero and Calder 2018). Furthermore, saturated fatty acid palmitate induces histone H3 release via ROS and JNK pathway-dependent mechanisms and extracellular histones, which increase TF expression in monocytes (Shrestha et al. 2013). Focusing on the relationship between coagulation, eNOS, and dyslipidemia, our previous study has demonstrated the combined effects of eNOS deficiency and a high-fat diet on TF expression (Li et al. 2010). In the study, eNOS-null mice were treated with low-dose STZ to develop DM and were fed a high-fat diet. The lack of eNOS expression increased urinary albumin excretion and histological injuries, which were further exacerbated by a high-fat diet. The expression and activity of TF were increased in mouse kidneys because of a lack of eNOS expression and a high-fat diet in a synergistic manner. Interestingly, an increase in the level of immunoreactive TF was demonstrated by colocalization experiments with a glomerular monocyte/macrophage marker.

### Neutralizing Antibodies against TF Ameliorate Inflammation in Diabetic Kidneys in eNOS-Knockout Mice

An increase in TF-dependent coagulation is associated with increased inflammation (Witkowski et al. 2016). Inhibition of the TF-dependent coagulation system ameliorated inflammatory diseases, such as LPS-induced sepsis (Pawlinski et al. 2010). To elucidate the causal link between inflammation and TF in DKD, we examined the short-term effect of neutralizing antibodies against TF on inflammation of diabetic kidneys in eNOS-deficient mice (Li et al. 2010). The results showed that administration of anti-TF antibodies markedly reduced the renal expression levels of inflammation- and fibrosis-related genes such as

*Tnfa*, *Ccl2*, *Tgfb*, and *Col4* mRNA in mouse kidneys four days after TF neutralization.

### Coagulation FXa Inhibitor Ameliorates Kidney Injury in Diabetic Mice Lacking eNOS

Coagulation FXa, located downstream of TF/VIIa, also contributes to inflammation through PAR-dependent pathway. Its role in DKD has been demonstrated in several animal models. Sumi et al. (2011) demonstrated that fondaparinux, an FXa inhibitor, reduced urinary protein levels, glomerular hypertrophy, and fibrin deposition in db/db mice. The therapeutic effect of FXa inhibition in DKD mice lacking eNOS was demonstrated by our group (Oe et al. 2016). Edoxaban (50 mg/kg/day), an oral FXa inhibitor, was administered to *eNOS*<sup>-/-</sup>; *Ins2*<sup>Akita/+</sup> mice for three months, and the results showed amelioration of histological injury, such as mesangial matrix proliferation. The expression levels of inflammatory genes in the kidneys were reduced by edoxaban (Table 1). FXa activates both PAR1 and PAR2. To elucidate the mechanism of FXa-mediated kidney injury, we demonstrated that the anti-inflammatory effects of FXa inhibitors are similar to those found in *PAR2*<sup>-/-</sup> mice and *PAR2*<sup>-/-</sup> mice with FXa inhibitors. These findings suggested that FXa likely caused inflammation through a PAR2-dependent mechanism in DKD. In contrast, edoxaban did not improve glomerular injury in *eNOS*<sup>+/+</sup>; *Ins2*<sup>Akita/+</sup> mice, suggesting that the therapeutic effect of FXa inhibition was associated with eNOS-dependent hypercoagulability in type I diabetic mice.

### Other Coagulation Factors in DKD

Because thrombin targets PAR1, which progresses vascular inflammation (Chen and Dorling 2009), thrombin likely exacerbates DKD. However, the protective or harmful roles of thrombin in DKD pathogenesis have been dem-

Table 1. Effects of tissue factor (TF), coagulation factor Xa (FXa), and protease-activated receptor (PAR) inhibition in diabetic kidney disease (DKD) mice with reduced or lacking eNOS expression.

Diabetic model	eNOS genotype	Therapeutic intervention	Outcomes	Reference
STZ	<i>eNOS</i> <sup>-/-</sup>	Anti-TF antibody	Cytokines/chemokines ↓	Li et al. (2010)
<i>Ins2</i> <sup>Akita/+</sup>	<i>eNOS</i> <sup>-/-</sup>	Edoxaban (FXa inhibitor)	Mesangial matrix area ↓ Cytokines/chemokines ↓	Oe et al. (2016)
<i>Ins2</i> <sup>Akita/+</sup>	<i>eNOS</i> <sup>+/-</sup>	PAR2 knock-out	Urinary albumin excretion ↓ Mesangial matrix area ↓ Cytokines/chemokines ↓	Oe et al. (2016)
<i>Ins2</i> <sup>Akita/+</sup>	<i>eNOS</i> <sup>+/-</sup>	E5555 (PAR1 antagonist)	Mesangial matrix area ↓ Glomerular collagen IV expression ↓	Mitsui et al. (2020)
<i>Ins2</i> <sup>Akita/+</sup>	<i>eNOS</i> <sup>+/-</sup>	FSLRLY (PAR2 antagonist peptide)	Mesangial matrix area ↓ Glomerular collagen IV expression ↓	
<i>Ins2</i> <sup>Akita/+</sup>	<i>eNOS</i> <sup>+/-</sup>	E5555 + FSLRLY	Urinary albumin excretion ↓ Mesangial matrix area ↓ Glomerular collagen IV expression ↓ Glomerular macrophage infiltration ↓ Cytokines/chemokines ↓	

DKD, diabetic kidney disease; eNOS, endothelial nitric oxide synthase; TF, tissue factor; FXa, coagulation factor Xa; PAR, protease-activated receptor; STZ, streptozotocin.

onstrated; low-dose thrombin (50 pM) prevented, whereas high-dose thrombin (20 nM) aggravated, glucose-induced apoptosis in podocytes (Wang et al. 2011b). The effects of thrombin inhibitors such as dabigatran on diabetic mice lacking eNOS should be elucidated in the future.

Fibrinogen is involved in various inflammatory statuses. Interestingly, partial reduction or the absence of fibrinogen was beneficial to renal ischemia-reperfusion models or obstructive kidney fibrosis (Sørensen et al. 2011; Craciun et al. 2014). Because glomerular fibrin deposition is increased in diabetic mice lacking eNOS (Nakagawa et al. 2007; Li et al. 2010; Wang et al. 2011a), elucidating its role in DKD pathogenesis deserves further research.

### Expression of PAR1 and PAR2 in Diabetic Mice Lacking eNOS

Increased expression of PARs is associated with kidney injury. We and others have demonstrated that the expression levels of *Par1* and/or *Par2* mRNA were increased in animal models with diabetic nephropathy, adenine-induced kidney injury, obstructive renal fibrosis, and cisplatin-induced kidney injury (Chung et al. 2013; Hayashi et al. 2016; Oe et al. 2016; Watanabe et al. 2019). Furthermore, glomerular PAR2 protein levels were increased in db/db mice, a model of type II DM (Sumi et al. 2011). We demonstrated the association between PAR expression and eNOS deficiency in diabetic mice (Oe et al. 2016). The expression level of *Par1* mRNA was significantly higher in *eNOS*<sup>-/-</sup>; *Ins2*<sup>Akita/+</sup> mice than in *eNOS*<sup>-/-</sup>; *Ins2*<sup>Akita/+</sup> and non-DM mice. Similarly, the expression of *Par2* was significantly higher in *eNOS*<sup>-/-</sup>; *Ins2*<sup>Akita/+</sup> mice than in non-DM mice. In contrast, *Par4* mRNA levels did not differ between the genotypes. A lack of eNOS exacerbates inflammation in DKD (Wang et al. 2011a). Because pro-inflammatory cytokines reportedly increase PARs (Nystedt et al. 1996), elevated inflammation caused by a lack of eNOS likely induces renal *Par*s expression in DKD. Collectively, the results of our studies suggested that the expression levels of PAR1 and PAR2, similar to the case of TF, were increased in diabetic kidneys when eNOS was lacking (Fig. 2).

### PAR2 Deletion Ameliorated Diabetic Kidney Injury in Mice with Reduced eNOS

We and others have addressed the role of PAR2 in DKD in previous studies. *Par2* deletion did not affect glomerular injury in diabetic wild-type Akita mice (*Ins2*<sup>Akita/+</sup>) with eNOS (Oe et al. 2016). Furthermore, STZ-induced diabetic mice lacking PAR2 showed reduced albuminuria compared to diabetic wild-type mice, but increased mesangial expansion (Waasdorp et al. 2017). Collectively, these results demonstrate that there are no or mild therapeutic effects of PAR2 inhibition on kidney injury in the early and mild models of DKD. In contrast, we have demonstrated the effects of PAR2 deficiency on DKD in diabetic Akita mice with reduced expression of eNOS (*eNOS*<sup>+/-</sup>; *Ins2*<sup>Akita/+</sup>)

(Oe et al. 2016). The lack of PAR2 significantly reduced the levels of urinary albumin excretion, mesangial expansion, and thickness of the GBM. The expression levels of pro-inflammatory and fibrosis-related genes, including *Tnfa*, *Tgfb*, and *Col4*, were also reduced in mouse kidneys. These findings suggest that PAR2 is pathogenic not in early, but in the relatively advanced diabetic glomerular injury caused by eNOS deficiency (Table 1).

### Dual Blockade of PAR1 and PAR2 in Diabetic Mice with Reduced eNOS

We have demonstrated that PAR1 and PAR2 cooperatively contribute to DKD pathogenesis (Mitsui et al. 2020). In this study, male type I diabetic Akita mice heterozygous for eNOS (*Ins2*<sup>Akita/+</sup>; *eNOS*<sup>+/-</sup>) were used as a model of DKD. These mice were treated with vehicle, PAR1 antagonist (E5555, 60 mg/kg/day), PAR2 antagonist (FSLRY, 3 mg/kg/day), or E5555 + FSLRY for 4 weeks. Administration of the PAR1 or PAR2 antagonist alone attenuated glomerular injury, such as mesangial expansion and collagen IV deposition, compared to administration of vehicle. Synergistic therapeutic effects of both PAR1 and PAR2 inhibition were observed, and the urinary albumin to creatinine ratio was significantly reduced when both PAR1 and PAR2 were blocked with E5555 + FSLRY compared with the vehicle administration. Furthermore, dual blockade of PAR1 and PAR2 by E5555 + FSLRY synergistically ameliorated histological injury, including mesangial expansion, glomerular macrophage infiltration, and deposition of type IV collagen. The expression levels of inflammation- and fibrosis-related genes in the kidneys were also reduced (Table 1).

We focused on the pro-inflammatory effects of PAR1 and PAR2 agonists on human endothelial cells (Mitsui et al. 2020). The results showed that stimulation with both PAR1 and PAR2 agonists synergistically increased the expression levels of *MCPI* and *PAII* mRNA. The effect of the PAR1 agonist was blocked by an NF- $\kappa$ B inhibitor, whereas that of the PAR2 agonist was blocked by NF- $\kappa$ B and MAPK inhibitors. Collectively, PAR1 and PAR2 cooperatively contribute to vascular inflammation and DKD through different signaling pathways (Fig. 3).

### Conclusion

In this review, we focused on the relationship between the coagulation protease-PAR pathway and eNOS deficiency in diabetic mice. Low production of eNOS is linked to hypercoagulability and increased PAR signaling, which are harmful in DKD. These findings may indicate their pathological roles in advanced or later phase of DKD (e.g., with renal failure and/or massive proteinuria). Oral FXa inhibitors are widely used to prevent thrombosis (Patel et al. 2011; Robertson et al. 2015). Their use in the treatment of DKD is a promising option. Furthermore, some PAR1 antagonists, including atopaxar and vorapaxar, can be used in antiplatelet therapy to prevent acute coronary syndrome

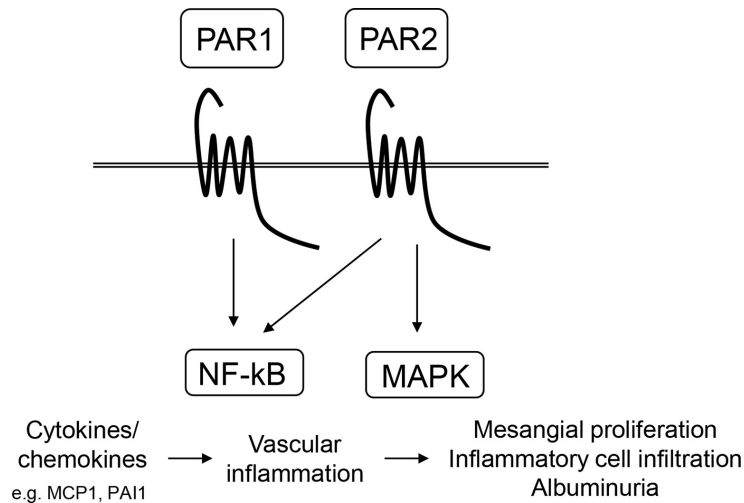


Fig. 3. Distinct roles of PAR1 and PAR2 in vascular inflammation and diabetic kidney disease (DKD).

In human endothelial cells, the PAR1 agonist increases chemokine and cytokine expression via the NF- $\kappa$ B signaling pathway, whereas the PAR2 agonist increases them via the NF- $\kappa$ B and MAPK signaling pathways. PAR1 and PAR2 synergistically cause vascular inflammation via distinct pathways in DKD.

(Goto et al. 2010; Tricoci et al. 2012). In addition, there is remarkable progress in the development of PAR2 antagonists (Lim et al. 2013; Cheng et al. 2017; Jiang et al. 2018), and they can be novel therapeutic options to treat patients with DKD.

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### Conflict of Interest

The authors declare no conflict of interest.

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