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REVIEW

Role of upstream stimulatory factor 2 in diabetic nephropathy

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Abstract **Diabetic nephropathy (DN) is the most common cause of end-stage renal disease (ESRD). About 20%–30% of people with type 1 and type 2 diabetes develop DN . DN is characterized by both glomerulosclerosis with thickening of the glomerular basement membrane and mesangial matrix expansion, and tubulointerstitial fibrosis . Hyperglycemia and the activation of the intra-renal renin-angiotensin system (RAS) in diabetes have been suggested to play a critical role in the pathogenesis of DN. However, the mechanisms are not well known.**

Studies from our laboratory demonstrated that the transcription factor—upstream stimulatory factor 2 (USF2) is an important regulator of DN. Moreover, the renin gene is a downstream target of USF2. Importantly, USF2 transgenic (Tg) mice demonstrate a specific increase in renal renin expression and angiotensin II (AngII) levels in kidney and exhibit increased urinary albumin excretion and extracellular matrix deposition in glomeruli, supporting a role for USF2 in the development of diabetic nephropathy. In this review, we summarize our findings of the mechanisms by which diabetes regulates USF2 in kidney cells and its role in regulation of renal renin-angiotensin system and the development of diabetic nephropathy.

Keywords USF2, renin-angiotensin system, TGF-β, renal fibrosis

Diabetic nephropathy

Diabetic nephropathy (DN) is one of the most prevalent diabetic microvascular complications and characterized by both glomerulosclerosis with thickening of the glomerular basement membrane and mesangial matrix expansion, and tubulointerstitial fibrosis (Lim, 2014). A relationship between hyperglycemia and DN has been established in both animal and clinical studies. DN starts with microalbuminuria through proteinuria, azotaemia, and culminates in ESRF. Before the onset of overt proteinuria, renal function changes include renal hyperfiltration, hyperperfusion, and increasing capillary permeability to macromolecules. During this early stage, the hypertrophic glomeruli exhibit normal structure. After several years of diabetes, the increased thickness of the glomerular basement membrane, as well as mesangial expansion with the accumulation of matrix are the fundamental changes . These changes correlate strongly with the clinical onset of proteinuria, hypertension and kidney failure. Production of the extracellular matrix by mesangial cells contributes to progressive glomerulosclerosis.

The mechanisms of DN are complicated. Both metabolic and hemodynamic factors are involved in the development of DN (reviewed by Cooper(2001) and Forbes et al. (2007)). Metabolic factors include advanced glycated end products (AGE) (Petrica et al., 2015), oxidative stress (Dabhi and Mistry, 2015), aldose reductase/polyol pathway flux and hexosamine flux (Giacco and Brownlee, 2010). Hemodynamic factors include the renin-angiotensin system (RAS), endothelin and nitric oxide etc. These factors act independently or coordinately to activate intracellular second messengers such as PKC, NF-κB, and MAPK or various cytokines including TGF-β, connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and inflammatory cytokines (IL-1, IL-6 or TNF-α) (Navarro-González and Mora-Fernández, 2008) leading to renal functional and structural changes as described above. Among the many mediators of DN, TGF-β has been considered to be an important cytokine in mediating renal fibrosis in diabetes.

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The renin angiotensin system (RAS) and DN

Systemic RAS (classic RAS) and its role in DN

The RAS is a multi-step enzymatic cascade to produce a family of angiotensin peptides that control blood pressure and fluid homeostasis. The RAS has been acknowledged as an endocrine, paracrine, autocrine, and intracrine system (Navar et al., 2002; Kobori et al., 2007; Re, 2007). The components of the RAS, including angiotensinogen, renin, and ACE are present systemically for endocrine production of AngII, termed the classic RAS. Angiotensinogen, the only known precursor to AngII, is constitutively secreted from liver hepatocytes as well as other cell types and generally considered to be non-rate limiting in the production of systemic AngII (Peach, 1977). Angiotensinogen is cleaved by the aspartyl protease renin to generate the decapeptide angiotensin I (AngI). For systemic production of AngII, renin synthesis and release from the kidney juxtaglomerular (JG) cells of the afferent arteriole is the rate limiting step. Hydrolysis of the C-terminal amino acids of AngI by the dicarboxypeptidase angiotensin converting enzyme (ACE) results in the formation of AngII, the primary peptide of the RAS with biological activity. A homolog of ACE, termed ACE-2, has been recently discovered, which hydrolyzes AngI to Ang(1-9) and AngII to Ang(1-7) (Donoghue et al., 2000). ACE2 has been suggested as the catabolic arm of the RAS (Ferrario et al., 2005), blunting the actions of AngII and favoring effects of the vasodilator Ang(1-7). AngII exhibits the majority of its well-known physiological effects via the angiotensin type 1 receptor (AT1 receptor).

Accumulating evidence suggests that the RAS plays an important role in the pathogenesis of diabetic renal disease (Carey and Siragy, 2003; Gurley and Coffman, 2007; Yamout et al., 2014). Studies shows that the renin inhibitor (aliskiren) ameliorates renal damage in diabetic TG (mREN-2) rats (Kelly et al., 2007; Feldman et al., 2008) or in type 1 or 2 diabetics (Parving et al., 2008; Wang et al., 2014). ACE inhibitors (ACEI) or AT1 receptor antagonists reduce mesangial sclerosis and proteinuria in animal models of both type 1 and type 2 diabetes (Andersen et al., 2000; Chan et al., 2000; Lewis et al., 2001; Brenner et al., 2001; Lewis, 2002; Carey and Siragy, 2003) . A large scale clinical trial (RENNAL) demonstrated that losartan, an AT1 receptor antagonist, delayed the progression to proteinuria and end stage renal disease in type 2 diabetics (Brenner et al., 2001; Zhang et al., 2005). These beneficial effects of RAS inhibitors in the prevention of diabetic renal disease suggest that AngII, acting through the AT1 receptor, is a major mediator of progressive renal injury. AngII has many actions that might contribute to DN (Carey and Siragy, 2003), most prominently stimulating extracellular matrix protein synthesis in the kidney through induction of TGF-β expression (Kagami et al., 1994; Sharma et al., 1999; Singh et al., 1999; Erman et al., 2004). In addition to the contribution of AngII to DN, recent studies have suggested that ACE2 (Wong et al., 2007) or the newly described prorenin/renin receptor (Ichihara et al., 2006) plays a role in DN.

Intrarenal RAS and its role in DN

In addition to the systemic RAS, the kidney contains a complete intrarenal RAS (Carey and Siragy, 2003). Each RAS component has been localized to kidney (Navar et al., 1996; Miyata et al., 1999; Carey and Siragy, 2003). The intrarenal RAS is regulated independently from the systemic RAS. In general, circulating components of the RAS are normal or suppressed in diabetic patients (Burden and Thurston, 1979; Price et al., 1999), suggesting that a local RAS in the kidney is the primary contributor to AngII effects in the kidney with diabetes (Anderson et al., 1993; Border and Noble, 1998b; Ruiz-Ortega et al., 1998; Leehey et al., 2000; Zhang et al., 2002; Tamura et al., 2005). Accumulating evidence suggests that the intrarenal RAS is activated under diabetic conditions. Studies have shown that STZ-induced diabetes in rats activates the intrarenal RAS (Anderson et al., 1993), including activation of a glomerular RAS (Singh et al., 2005; Leehey et al., 2008). Diabetes also alters the distribution of RAS components within the various kidney compartments. In experimental diabetes, redistribution of ACE to vascular and glomeruli sites has been demonstrated by Anderson et al. (1993).

It has been demonstrated that glomerular mesangial cells express mRNA for renin, AGT, and ACE (Andrade et al., 2002). Moreover, mesangial cells in culture are able to synthesize, store, and secrete both renin and prorenin (Andrade et al., 2002) as well as AngII, and contain both the AT1 receptor (Miyata et al., 1999) and prorenin/renin receptor (Nguyen et al., 1996; Nguyen et al., 1998). Previous studies demonstrated that high glucose stimulates intracellular renin activity and AngII generation in mesangial cells (Vidotti et al., 2004; Wang et al., 2013). In addition to mesangial cells, podocytes and proximal tubular cells have been shown to express all components of the RAS, and interestingly RAS components in these cells are also stimulated by high glucose exposure (Zhang et al., 2002; Velez et al., 2007; Yoo et al., 2007; Durvasula and Shankland, 2008; Sonneveld et al., 2014), and have been suggested to contribute to the development of diabetic nephropathy (Phillips et al., 1999; Hong et al., 2001; Hsieh et al., 2003; White, 2006; Phillips, 2007; Kanwar et al., 2008; Liu et al., 2008; Niranjan et al., 2008; Márquez et al., 2015).

TGF-β and DN

TGF-βs are a family of cytokines that influence cell growth, differentiation, apoptosis, inflammatory processes, and gene expression. TGF-β is synthesized and secreted in a latent

complex (latent TGF-β) (Gleizes et al., 1997). It must be converted to the active state before binding to its receptors and eliciting cellular functions. Latent TGF-β can be activated by a number of factors, including heat, extreme pH, plasmin, integrin binding, reactive oxygen species, and thrombospondin 1 (Lyons et al., 1990; Rifkin et al., 1993; Schultz-Cherry and Murphy-Ullrich, 1993; Schultz-Cherry et al., 1994; Taipale et al., 1995; Daniel et al., 2007).

The importance of TGF-β in the development of DN is well established. Increased expression of both active and total TGF-β in glomeruli occurs in both patients and in animal models of diabetes (Park et al., 2014; Shankland et al., 1994; Sharma and Ziyadeh, 1994; Young et al., 1995; Bertoluci et al., 1996; Sharma et al., 1997; Border and Noble, 1998a; Hong et al., 2001; Vasanthakumar et al., 2015). TGF-β is a major mediator of extracellular matrix deposition in the diabetic kidney (Border et al., 1995; Chen et al., 2001; Ziyadeh, 2004). The fibrogenic effects of TGF-β are due to its upregulation of extracellular matrix components, including collagen, fibronectin, osteopontin, and the downregulation of matrix degrading enzymes (Ziyadeh et al., 1994; Oh et al., 1998; Isono et al., 2002). TGF-β1-specific neutralizing antibodies diminish overproduction of extracellular matrix proteins secreted by mesangial cells incubated with high glucose (Sharma et al., 1996).

Upstream stimulatory factors (USFs) in DN

USFs, including USF1 and USF2, were initially characterized as transcription factors implicated in the regulation of the adenovirus major late promoter. In mammals, USF1 and USF2 are ubiquitously expressed with a molecular weight of 43 kDa and 44 kDa, respectively. They predominantly localize in nuclei (Qyang et al., 1999) and belong to the Myc family of transcription factors characterized by a basic/helix loop helix/leucine zipper domain responsible for dimerization and DNA binding. These ubiquitous factors form homo- and hetero-dimers and recognize *in vitro* a CACGTG core sequence termed E box . Through binding to E boxes of target genes, USF factors regulate the gene expression (Rippe et al., 1997; Vallet et al., 1997; Qian et al., 1999; Kingsley-Kallesen et al., 2001; Nicolas et al., 2001; Bidder et al., 2002; Zhu et al., 2005; Chen et al., 2006). The USF2 structure and its regulated genes are illustrated in Fig. 1. USF1 and USF2 null mice have been generated and their phenotypes have been described previously. An embryonic lethal phenotype was observed with the double null mouse mutants . For USF1 deficient mice, no physical abnormalities have been reported in adult mice, except for the occasional occurrence of epileptic seizures in female USF1 null mice. USF2 null mice, however, show a severe phenotype including growth defects, abnormalities in fertility, mammary gland malfunction, an impaired transcriptional response to glucose in liver, and multivisceral iron overload.

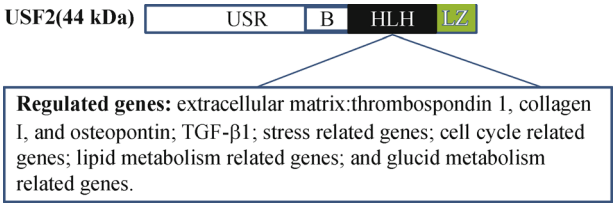

Figure 1 Schematic illustration of USF2 structure and its regulated genes. USR: USF-specific region; B: basic region; HLH: helix–loop–helix domain; LZ: leucine zipper domain.

Although the pups display an obvious growth defect, and have an increased prenatal mortality rate (40%–50%), the surviving pups subsequently develop in an apparently normal fashion. USF2 null mice have a decreased lifespan (2.5 to 4.5 months in males; 10 months in females).

High glucose or glycated albumin upregulates USF2 expression in mesangial cells at the transcriptional level

Studies from our laboratory demonstrated that USFs are transcription factors involved in glucose mediated upregulation of thrombospondin 1 (TSP1) gene expression and TGF-β activity in glomerular mesangial cells and that these effects of USF2 contribute to diabetic renal complications (Wang et al., 2004). We also showed that treatment of rat mesangial cells (RMCs) with high glucose (30 mM) upregulates USF2 but not USF1 protein accumulation in mesangial cells through the activation of PKC, ERK, and p38 MAPK pathways (Wang et al., 2004). Furthermore, high glucose exposure stimulated USF2 gene transcription. Using the luciferase-promoter deletion assay, site-directed mutagenesis, and transactivation assay, we identified a glucose-responsive element in the USF2 gene promoter (−1740 to −1620, relative to the transcription start site) and demonstrated that glucose-induced USF2 expression is mediated through a cAMP-response element binding protein (CREB)-dependent transactivation of the USF2 promoter.

In addition to hyperglycemia, glycated proteins have been shown to accumulate in the kidneys of diabetic patients and contribute to DN. We found that glycated albumin upregulated USF2 expression (mRNA and protein) in a dose- and time-dependent manner. We also demonstrated that glycated albumin stimulated USF2 gene expression at the transcriptional level. By using the luciferase-promoter deletion assay, site-directed mutagenesis, and transactivation assay, we identified a glycated albumin-responsive region in the USF2 gene promoter (−837 to −430, relative to the transcription start site) and demonstrated that glycated albumin-induced USF2 expression was mediated through NF-κB-dependent transactivation of the USF2 promoter. Furthermore, glycated albumin increased nuclear NF-κB subunit-p65 protein levels. siRNA-mediated p65 knockdown prevented glycated albumin-induced USF2 gene expression (promoter activity, mRNA, and protein levels). Taken together, these data

suggest that glycated albumin upregulated USF2 gene transcription in MCs through NF-κB-dependent transactivation of the USF2 promoter (Li and Wang, 2010).

High glucose upregulates USF2 in renal proximal tubular cells through angiotensin II-dependent activation of CREB

In addition to glomerular mesangial cells, our studies found that high glucose upregulated USF2 expression and increased extracellular matrix accumulation in human renal proximal tubular cells (HK-2 cells); both were inhibited by siRNA-mediated USF2 knockdown. In addition, high glucose stimulated angiotensinogen and renin expression, increased renin activity, and resulted in increased angiotensin II formation. Treatment of HK-2 cells with an angiotensin II receptor 1 (AT1) blocker- losartan- prevented high-glucose-induced USF2 expression and high-glucose-enhanced phosphorylation of CREB (cAMP response element binding protein). These data established that high glucose stimulated USF2 expression in HK-2 cells, at least in part, through angiotensin II-AT1-dependent activation of CREB, which can contribute to diabetic tubulointerstitial fibrosis (Visavadiya et al., 2011).

USF2 regulates glucose-induced TGF-β levels in mesangial cells

It has been shown that USF2 can bind to the TGF-β gene promoter and regulates its expression (Riccio et al., 1992; Kingsley-Kallesen et al., 2001; Weigert et al., 2004; Zhu et al., 2005). To determine the role of USF2 in glucose-mediated stimulation of TGF-β production, we utilized USF2-siRNA (from Santa Cruz) to knock down USF2 gene in mouse mesangial cells and then examined the effect of high glucose (30 mM glucose) on TGF-β levels in these cells using the method as described previously (Wang et al., 2004). As shown in Fig. 2, siRNA-USF2 transfection significantly inhibited USF2 expression under both normal and high glucose conditions (around 80% inhibition). Importantly, high glucose stimulated increases in active and total TGF-β levels were inhibited by siRNA-USF2 transfection. siRNA-USF2 transfection had no effect on basal TGF-β production (under 5mM glucose conditions).These data are consistent with that from Weigert et al. (2004), indicating thatUSF2 regulates glucose-induced TGF-β production in mesangial cells.

Overexpression of USF2 accelerates diabetic kidney injury

To define the functional role of USF2 in the development of diabetic nephropathy (DN) *in vivo*, we generated the global USF2 transgenic mice (Liu et al., 2007). Type 1 diabeteswas induced in USF2 (Tg) and their wild-typelittermates (WT) by injection of streptozotocin. Control USF2 (Tg) mice (~6 months old) exhibited increased urinary albumin excretion. Thesemice also exhibited glomerular hypertrophy, accompanied by increasedTSP1, active TGF-β, fibronectin accumulation in the glomerulicompared with control WT littermates. Type 1 diabetes onsetfurther augmented glomerular USF2 protein levels, the urinary albumin excretion and glomerularhypertrophy and fibrosis in the USF2 (Tg) mice. Together, these data indicate that functional changes (albuminuria) and structural changes (mesangial matrix expansion) exhibited in kidneys from USF2 (Tg) mice under basal conditions, which were significantly augmented by diabetes onset, suggesting that overexpression of USF2 accelerates diabetic kidney injury.

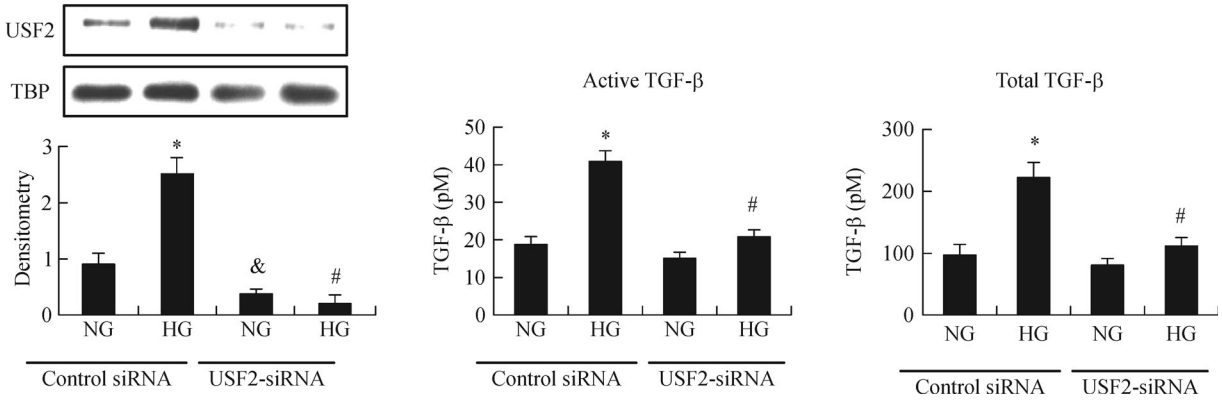

Figure 2 siRNA-USF2 transfection inhibits high glucose levels-induced TGF-β production. Mouse mesangial cells were transfected with siRNA-USF2 or control siRNA for 48 h. Then cells were treated with normal or high glucose media for 24 h. Conditioned media were collected and used for measuring active and total TGF-β levels by PAI-1/lucifersae assay. Cells were harvested to determine the USF2 protein levels in nuclear extracts by immunoblotting. TBP (anti-TATA binding protein) was used as internal nuclear loading control. Data are represented as mean of 3 replicates±S.D. *, $p < 0.05$ vs. NG. &, $p < 0.05$ vs. NG of control siRNA. # $p < 0.05$ vs. HG of control siRNA.

Activation of renal RAS in USF2 transgenic mice

It has been shown that USF1/2 binds to the mouse renin promoter and regulates renin gene expression in a kidney-derived renin expressing cell line (As4.1) (Pan et al., 2001). Therefore, the regulation of the RAS in USF2 (Tg) mice were determined. Our data demonstrated that renal renin expression (mRNA and protein levels), renin activity, and glomerular AngII levels were upregulated in USF2 transgenic mice, but plasma renin concentration was not altered. Systolic blood presser was not altered in USF2 (Tg) mice as compared to littermates control. Moreover, renin receptor protein levels in kidney homogenates or the mRNA levels of angiotensinogen, ACE, ACE2, AT1a and AT2 receptors (determined by real-time PCR) were not changed in kidneys from USF2 transgenic mice as compared to control littermates (Shi et al., 2009). Collectively, these results suggest that USF2 represents an important mechanism of intra-renal RAS activation and local AngII formation.

Model of the role of USF2 in the development of diabetic nephropathy

Summary

Underdiabetic conditions USF2 expression is increased in the kidneys. Increased glomerular USF2 levels correlates with the glomerular mesangial matrix expansion. Furthermore, overexpression of USF2 accelerates the development of diabetic nephropathy in a type 1 diabetic mouse model, supporting the role of this transcription factor in the development of diabetic nephropathy. Our studies describe a novel effect of USF2 to promote renin gene expression and AngII formation in mesangial cells. This effect of USF2 was linked to glucose-induced regulation of TGF-β production, suggesting that this pathway may contribute to the development of diabetic nephropathy (Fig. 3).

Future directions

Our studies demonstrated that USF2 transgenic mice exhibited increased renal renin gene expression, renin activity and AngII levels without alteration of other components of the renin-angiotensin system (RAS) including angiotensinogen, ACE, ACE2, renin receptors, AT1 or AT2 receptors (Liu et al., 2007). Moreover, plasma renin concentration was not altered in USF2 transgenic mice. Together, these results suggest that increased renin enhances AngII synthesis, leading to the activation of the renal RAS in USF2 (Tg) mice, and contributing to the development of nephropathy. Therefore, the mechanistic role of RAS in USF2-mediated acceleration of diabetic nephropathy warrants future investigation.

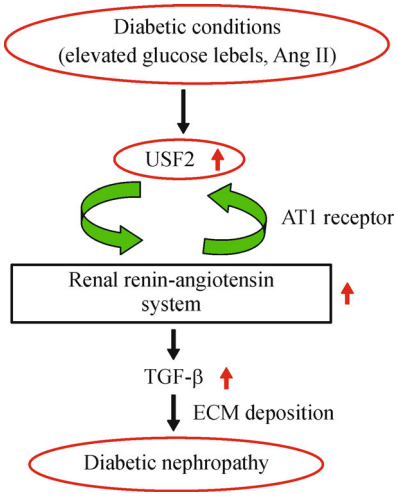

Figure 3 Model of the role of USF2 in DN. Diabetic conditions upregulate kidney USF2 levels. USF2 binds to renin promoter and increases renin gene expression and stimulates angiotensin II production in kidney cells. Increased angiotensin II increases TGF-β levels and then extracellular matrix production in kidney cells, which leads to renal fibrosis.

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Compliance with ethics guidelines

Shuxia Wang declares that there is no conflict of interest. This article does not contain studies with human subjects. It contains studies with animal subjects, which conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

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