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Sugar, Sex, and TGF- β in Diabetic Nephropathy

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Summary

TGF- β is well known to play a critical role in diabetic kidney disease, and ongoing clinical studies are testing the potential therapeutic promise of inhibiting TGF- β production and action. An aspect of TGF- β action that has not received much attention is its potential role in explaining sex-related proclivity for kidney disease. In this review, we discuss recent studies linking TGF- β signaling to sex-related effects in diabetic kidney disease and suggest targets for future studies.

Keywords

Sex difference; kidney; diabetes

Diabetic nephropathy is a major complication of diabetes mellitus, affecting approximately one third of diabetic patients,¹ and diabetes accounts for approximately 44% of all cases of end-stage renal disease in the United States.² Diabetic nephropathy is characterized by declining glomerular filtration rate and albuminuria, owing in part to thickening of glomerular and tubular basement membranes, podocyte dysfunction, and expansion of the mesangial and tubulointerstitial matrix.³ Proliferation of mesangial cells and increased mesangial cell production of extracellular matrix proteins lead to expansion of the mesangial matrix.^{4–6} Although clinical markers of overt kidney damage often become apparent 15 to 20 years after the onset of type 1 diabetes,^{7,8} expansion of the mesangial matrix and thickening of the basement membrane are observed as early as the first 10 years after the diagnosis of type 1 diabetes.⁹ Much of the diabetic renal pathology is due to overproduction and activity of the transforming growth factor- β (TGF- β) system.^{10,11}

TGF- β IN DIABETIC NEPHROPATHY

TGF- β is a master regulator of extracellular matrix production in the diabetic kidney. Urinary excretion and tissue expression levels of TGF- β 1 are increased in patients with diabetes^{12,13} and in experimental models of diabetic kidney disease.^{14–16} TGF- β is known to stimulate the production of several of the extracellular matrix proteins that accumulate in the diabetic kidney, including type IV collagen, fibronectin, and laminin in podocytes,^{17,18} and overexpression of TGF- β by glomerular cells, including podocytes and mesangial cells, in response to high glucose levels leads to increased deposition of matrix proteins, resulting in glomerulosclerosis.^{19–21} High glucose-induced expression and activation of TGF- β also contributes to podocyte apoptosis, which in turn leads to disruption of the filtration barrier

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and impaired kidney function.²¹ Disruption of TGF- β signaling, by knockout of TGF- β type 2 receptor (T β R-2), by administration of anti-TGF- β antibodies, or by knockout of the downstream signaling molecular Smad3, prevents mesangial matrix expansion and deterioration of renal function in mice.^{22–24} Studies in human beings thus far have revealed that anti-TGF- β antibodies may be delivered safely in patients with kidney disease and results of phase II trials are anxiously awaited.

TGF- β SIGNALING

Previous reviews have focused on regulation of TGF- β expression by glucose and activation by the mitogen-activated protein kinases, latency-associated peptide, thrombospondin, and decorin.^{25,26} Once TGF- β is released, it binds T β R-2, which binds and phosphorylates and activates T β R-1/ALK5 (TGF- β type 1 receptor/activin-receptor-like kinase). Activated T β R-1 phosphorylates serine residues on Smad2 and Smad3. These phospho-Smads form heteromers with Smad4 and are transported to the nucleus where they regulate TGF- β target genes.²⁶

Signal transduction is negatively regulated by Smad7, Ski, and SnoN, among others. Smad7 competitively binds activated T β R-1/ALK5 and prevents phosphorylation of Smad2 and Smad3.^{26,27} Two related nuclear proteins, Ski and SnoN, are other repressors of TGF- β signaling. SnoN binds Smad4 and recruits the nuclear transcription co-repressor N-CoR to Smad-responsive genes when Smad4 is present.²⁸ Interestingly, Smad3 overexpression causes down-regulation of SnoN protein expression, thus allowing TGF- β signal transduction.²⁸ In different models of fibrotic kidney disease Ski and SnoN protein expression are decreased owing to TGF β -induced ubiquitin-mediated degradation, and overexpression of SnoN or Ski prevents TGF- β -induced expression of fibrosis-promoting genes.^{29–31}

SEX DIFFERENCES AND TGF- β

Sex Difference in Diabetic Nephropathy

A sex difference exists in susceptibility to diabetic kidney disease, with women showing protection against the development and progression of the disease compared with men. Male sex is an independent risk factor for the rate of decline in glomerular filtration rate,³² and women with type 1 diabetes develop microalbuminuria at a slower rate and have a lower incidence of overt nephropathy than men.³³ The sex-related difference between men and women is true only if type 1 diabetes is first diagnosed during adolescence (not earlier), suggesting a role for sex hormones in mediating the difference.³⁴ 17 β -estradiol (E2) is the predominant sex hormone in females, and testosterone is the predominant sex hormone in males. Thus, the prevailing hypothesis to explain sex differences in susceptibility to kidney disease was that E2 is good and testosterone is bad. Studies in human beings, animal models, and renal cell culture have provided evidence in support of this hypothesis. In addition, these studies have suggested that E2 does indeed protect females from diabetic kidney disease in part by regulating TGF- β expression and signaling. However, the underlying pathways by which E2 and testosterone confer their effects are far more complex.

Estrogen and TGF- β

Serum levels of TGF- β are higher in men than in women.³⁵ Knockout of estrogen receptor- β (ER α -/-) in nondiabetic mice³⁶ and ovariectomy in diabetic rats³⁷ were associated with increased renal expression of TGF- β , and E2 supplementation to ovariectomized (OVX) rats normalized TGF- β expression.³⁷ Similarly, treatment of intact diabetic (db/db) mice with E2 decreased TGF- β expression in podocytes compared with db/db mice not treated with E2.³⁸

In contrast, in diabetic mice with ovarian failure caused by injection of 4-vinyl-cyclohexene diepoxide, renal TGF- β messenger RNA expression was significantly lower than in diabetic females with normal hormonal cycling.³⁹ Interestingly, despite differences in the effect of E2 on TGF- β expression, in all of the earlier-described models a lack of E2 was associated with worse kidney disease (Table 1). Furthermore, in female mice overexpressing TGF- β , treatment with E2 ameliorated TGF- β -induced progressive kidney disease without decreasing TGF- β expression; in fact, E2-treated mice had higher levels of TGF- β than untreated mice.⁴⁰ This suggests that the more important effect of E2 is to disrupt TGF- β signaling, rather than to regulate its expression. Cell culture studies support this hypothesis. E2 inhibited TGF- β signaling, suppressing TGF- β -induced expression of collagens I and IV in cultured rat and mouse mesangial cells^{41,42} and preventing TGF- β -induced apoptosis of cultured podocytes,⁴³ without altering TGF- β messenger RNA expression.⁴¹

MECHANISMS BY WHICH E2 REGULATES TGF- β

There are several mechanisms by which E2 may interfere with TGF- β signaling (Fig. 1). ER α , the predominant ER isoform in the kidney, binds Smad2 and Smad3 and targets them for degradation.⁴⁴ This was shown in a breast cancer cell line and transfected human embryonic kidney 293 (HEK293) cells. However, E2 supplementation decreased expression of pSmad2/3 and total Smad2/3 in kidneys from OVX diabetic rats,⁴⁵ suggesting a similar mechanism may regulate TGF- β signaling in the kidney.

Regulation of casein kinase II (CK2) is another mechanism by which E2 may regulate TGF- β activity. CK2 is a serine/threonine protein kinase that, when active, phosphorylates early growth response-1 (Egr-1). Egr-1 normally complexes with Sp1, preventing Sp1 from binding to target sequences. CK2-induced Egr-1 phosphorylation in response to TGF- β prevents Egr-1/Sp1 complex formation, increasing unbound Sp1. Sp1 in turn binds its target sequence in the collagen IV promoter, increasing collagen IV synthesis. In murine mesangial cells E2 treatment prevented the TGF- β -induced increase in CK2 expression and activity, thus inhibiting TGF- β signaling and collagen IV up-regulation.⁴⁶

Previous reviews have discussed upstream regulators of TGF- β , including the renin-angiotensin-aldosterone system (RAAS), upstream stimulating factor 1/2 (USF1/2), decorin, and thrombospondin.^{25,47} TGF- β regulation by the RAAS previously has been reviewed extensively.^{47,48} Sex hormone-related differences in the RAAS also previously have been reviewed,⁴⁹ and the data suggest that E2 may protect the kidney against diabetic nephropathy in part through decreased RAAS stimulation of TGF- β . USF1 is a transcription factor that regulates gene expression of TGF- β in the diabetic kidney,^{25,50} and E2 modulation of USF1 activity could lead to decreased TGF- β expression in females. No studies have directly investigated regulation of USF1 by E2 in the kidney. However, there is evidence in breast cancer cells that USF1 and ER α interact⁵¹ and that E2 stimulates recruitment of USF1 to the promoter of an E2-responsive gene.⁵² Interestingly, genetic variants in the USF1 gene were associated with altered risk of type 2 diabetes⁵³ and cardiovascular disease⁵⁴ in women but not men, and with increased body mass index in premenopausal women but not postmenopausal women.⁵⁵ These data suggest that E2 regulation of USF1 may be an important modulator of metabolic risk factors and complications. Further studies to determine if there are sex differences in USF1 regulation in the diabetic kidney are warranted.

Several studies have examined regulation of decorin and thrombospondin by E2. For example, OVX led to decreased expression of decorin in skin, suggesting decorin may be regulated by E2.⁵⁶ However, to date none of these studies has been performed in kidney or kidney cells, making extrapolation of the results to diabetic kidney disease difficult.

In addition to regulation of TGF- β expression and activity in kidney cells, E2 may indirectly regulate TGF- β in the kidney by regulating macrophage infiltration. Macrophages are a key source of TGF- β production in the diabetic kidney.²⁵ To the best of our knowledge there has not been a direct comparison of macrophage infiltration in the diabetic kidney between males and females. However, in the spontaneously hypertensive rat model of kidney disease males had higher levels of macrophage infiltration of the kidney than females, and OVX in females increased macrophage number.⁵⁷ Similarly, OVX in diabetic female rats increased macrophage infiltration, and this was normalized by E2 treatment.⁴⁵ These data suggest that E2 may suppress macrophage infiltration, thus preventing a major source of TGF- β production and possibly protecting the kidney from damage.

TESTOSTERONE AND TGF β

Although males are more susceptible to kidney damage, recent data challenge the simplistic paradigm that E2 is good and testosterone is bad (Table 2). In diabetic men, progression from microalbuminuria to macroalbuminuria is associated with decreased testosterone levels, although progression to end-stage renal disease is associated with an increase in testosterone.⁵⁸ Furthermore, E2 levels increased in men that progressed from macroalbuminuria to end-stage renal disease.⁵⁸ Similarly, in male rats diabetes was associated with increased E2 levels.⁵⁹ Furthermore, in male diabetic rats castration, which decreased serum testosterone, increased expression of TGF- β and downstream matrix proteins.⁵⁹ Supplementation with low doses of dihydrotestosterone (DHT) to castrated diabetic male rats decreased TGF- β expression, but supplementation with high doses increased TGF- β expression to levels higher than in castrated diabetic rats without DHT supplementation.⁶⁰ Testosterone did not affect TGF- β expression in cultured podocytes⁴³ or mesangial cells isolated from male rats.⁴¹ Instead, testosterone levels appear to affect macrophage infiltration, which paralleled TGF- β expression levels in diabetic males.^{59,60}

STEROID PRODUCTION AND METABOLISM BY THE KIDNEY

Most studies examining sex differences in diabetic kidney disease measure serum hormone concentrations. However, it should be noted that the kidney is also a steroid hormone-producing and -metabolizing organ,^{61,62} raising the possibility of sex differences in local concentrations of E2 and testosterone, which could affect the kidney. Furthermore, there is evidence that diabetes results in altered hormone synthesis and metabolism in the kidney. Aromatase, which converts testosterone to E2, is expressed in kidneys, and its expression is increased in kidneys of diabetic male rats relative to nondiabetic males.⁶³ Concordant with this, direct measurement of renal E2 levels showed that renal E2 was increased in diabetic male rats relative to nondiabetic male rats.⁶³ Castration further increased aromatase expression levels in the kidney, and renal E2 remained increased in castrated males despite lower serum E2 levels in these animals.⁶³ In another example, expression of 3 β -hydroxysteroid dehydrogenase, isoform 4 (3 β -HSD4) negatively correlates with and predicts the degree of glomerulosclerosis in diabetic human beings and mice.⁶⁴ 3 β -HSD4 is a 3-ketosteroid reductase, and as such reduces progesterone and testosterone to inactive metabolites. Normal activity of 3 β -HSD4 may protect the kidney from possible negative effects of testosterone,⁶⁵ and loss of 3 β -HSD4 in diabetes may increase kidney susceptibility to testosterone-induced damage. However, there is no direct evidence to support this hypothesis at this time. The cause of the reduction in 3 β -HSD4 expression also is unclear. 3 β -HSD4 expression was decreased in diabetic mice with ovarian failure relative to normally cycling diabetic mice, suggesting that E2 may regulate 3 β -HSD4.³⁹ In male mice, 48-hour water restriction increased expression of 3 β -HSD4 in the renal medulla, suggesting it is regulated by vasopressin, which is increased in diabetes.⁶⁶ Further data regarding the regulation and function of this important protein are largely absent. Given that expression

levels of 3β -HSD4 are predictive of the degree of the development of glomerulosclerosis,⁶⁴ further research into the interaction of 3β -HSD4 with TGF- β signaling is warranted.

HORMONE THERAPY TO TREAT DIABETIC NEPHROPATHY?

Given the preponderance of evidence showing that E2 protects the kidney from diabetic damage by interfering with TGF- β signaling, an obvious extension of these studies would be to administer E2 to diabetic patients to prevent or ameliorate diabetic kidney damage. To date, no large-scale clinical trials have been designed to examine hormone replacement therapy interventions for diabetic renal disease.

Estrogen is considered protective against the development and progression of cardiovascular disease as well,⁶⁷ and in the 1990s two large studies, the Heart and Estrogen/progestin Replacement Study trial and the Women's Health Initiative trial, directly examined the role of hormone replacement therapy in the treatment of cardiovascular disease in women.^{68–72} In these trials renal disease and its markers were not a primary outcome, limiting the usefulness of these trials for understanding the impact of sex and hormone replacement therapy on renal disease. In addition, although E2 supplementation may be beneficial to women, it is unlikely to serve as an effective treatment strategy for men. Diabetic men already have higher-than-normal levels of E2,⁵⁸ and men on E2 therapy experience negative sexual side effects. Further research that goes beyond differences in E2 and testosterone levels into the physiology that underlies the sex differences in susceptibility to diabetic kidney disease is needed. This research can lead to the development of therapeutics that could be personalized for both sexes.

TGF- β AND GLUCOSE TRANSPORTERS

High intracellular glucose concentrations are a prime stimulator of TGF- β expression in the kidney.²⁵ Glucose is taken up into cells via the glucose transporters (GLUTs) on the basolateral side and the sodium/glucose co-transporters (SGLTs) on the luminal side. Interestingly, the path by which glucose enters the cell may be critical when it comes to stimulating TGF- β expression. Application of glucose to the basolateral side of cultured polarized tubular epithelial cells primed the cells to respond to platelet-derived growth factor with an increase in TGF- β synthesis.⁷³ However, no effect on TGF- β expression was observed if the glucose was applied to the apical side of the cells.⁷³ Uptake of the glucose through GLUT1 on the basolateral membrane and metabolism of the glucose was required to achieve the priming effect. This may be of great importance because agents to block luminal entry of glucose via SGLT2 may soon be used as a means of lowering blood glucose level. The polarity of the response to glucose application suggests that reducing glucose uptake from the luminal side will not decrease expression of TGF- β in the proximal tubules. However, the main goal of anti-SGLT2 therapies is to lower blood glucose level. This would decrease the amount of glucose taken into the tubular epithelial cells through basolateral GLUT1 and thus reduce TGF- β production.

Although glucose taken up through these transporters increases TGF- β expression, TGF- β in turn regulates expression of the glucose transporters. Specifically, in cultured rat⁷⁴ and human⁷⁵ mesangial cells, TGF- β increased expression of GLUT1. In contrast, TGF- β treatment of primary cultures of rat proximal tubule cells decreased expression of SGLT1 and SGLT2 and glucose uptake into the cells.⁷⁶

TGF- β , AMPK, AND MAMMALIAN TARGET OF RAPAMYCIN

Apart from its matrix-stimulating effects, TGF- β also has been implicated in the organ and cell hypertrophy associated with diabetes. Neutralizing antibodies to TGF- β partially reverse

the renal hypertrophy and increase in glomerular volume in models of type 1 diabetes in the mouse and rat.^{77,78} Recent studies also have implicated the master energy sensor AMPK and mammalian target of rapamycin (mTOR) in playing critical roles in organ enlargement with diabetes.^{79,80} There is inhibition of AMPK activity and an up-regulation of mTOR activity with diabetes in the kidney, and this is associated with proteinuria.⁸¹ Stimulation of AMPK and inhibition of mTOR block the albuminuria and renal hypertrophy in these models.^{80,81} The cross-talk between AMPK, mTOR, and TGF- β is an area of active investigation (Fig. 2). At present, it appears that AMPK inhibits TGF- β signaling, although phosphorylation of Smad2/3 does not appear to be affected.^{82,83} In one clinical study, treatment with metformin, a known stimulator of AMPK, did not improve serum TGF- β levels in diabetic patients.⁸⁴ Activation of mTOR in response to high glucose levels in cultured proximal tubule epithelial cells correlated with increased apoptosis of these cells, and addition of rapamycin, an inhibitor of mTOR, prevented the increase in mTOR activation and the downstream effects on apoptosis.⁸⁵ In this study, TGF- β expression was not measured, however, it is known that high glucose levels can induce apoptosis of kidney cells via increased expression of TGF- β .⁸⁶ Thus, these data suggest a role of the AMPK/ mTOR pathway in inhibition of TGF- β signaling.

Other proteins in the AMPK/mTOR pathway also interact with the TGF- β signaling pathway. LKB1, an upstream kinase of AMPK, phosphorylates Smad4 on Thr77, inhibiting the ability of Smad4 to bind to promoters, thus inhibiting TGF- β signaling.⁸⁷ Furthermore, in addition to AMPK regulation of TGF- β , the reverse also may be true. TGF- β stimulates TGF- β -activated kinase 1 (TAK1) in mesangial cells,⁸⁸ and TAK1 is a known upstream kinase of AMPK.⁸⁹ These data suggest that the coordination of the two signaling pathways occurs at multiple levels.

CONCLUSIONS

The full implication of these data on the development of diabetic nephropathy is not clear, however, the data suggest TGF- β remains an important player in diabetic nephropathy worthy of further study. Examination of sex differences in diabetic nephropathy, the role TGF- β plays in these differences, and the regulation of TGF- β by sex hormones clearly is warranted. In addition, the relationship between the TGF- β and AMPK signaling pathways, both master regulators involved in diabetic kidney disease, needs to be better understood. Future studies in these areas will further our understanding of the role of TGF- β signaling in diabetic nephropathy and possibly contribute to sex-specific and sex-independent treatments for both men and women.

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Figure 1. Mechanisms of prevention of diabetic kidney disease by estrogen. ECM, extracellular matrix; ?, hypothesized effect.

Figure 2.

Interactions between TGF- β , AMPK, and mTOR in diabetic kidney disease. TAK1, TGF- β -activated kinase 1,?, hypothesized effect; dashed line, protein-protein interaction.

Table 1**E2 Disruption of TGF- β Signaling Ameliorates Diabetic Kidney Damage**

	TGF-β Expression	Markers of Diabetic Kidney Damage
Female ER α $-/-$ mice	↑	Made worse
OVX female mice	↑	Made worse
VCD-treated female mice	↑	Made worse
Db/Db mice + E2 treatment	↓(in podocytes)	Improved
Mice overexpressing TGF- β + E2 treatment	↑	Improved
Cultured mesangial cells + E2	↔	Improved
Cultured podocytes + E2	↔	Improved

Abbreviations: OVX, ovariectomized; VCD, 4-vinylcyclohexene diepoxide.

Table 2Testosterone and Estrogen Levels in Diabetic Males and the Impact of Testosterone on TGF- β Expression

Condition	Effect
Progression from microalbuminuria to macroalbuminuria in men	↓ T
Progression from macroalbuminuria to end-stage renal disease in men	↑ E2
Male rats with diabetes	↑ E2
Castrated male diabetic rats	↓ T, ↑ TGF- β
Castrated male diabetic rats + low levels of DHT	↓ TGF- β
Castrated male diabetic rats + high levels of DHT	↑ TGF- β
Cultured mesangial cells + E2	↔ TGF- β
Cultured podocytes + E2	↔ TGF- β