RAS and sex differences in diabetic nephropathy

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Clotet S, Riera M, Pascual J, Soler MJ. RAS and sex differences in diabetic nephropathy. Am J Physiol Renal Physiol 310: F945–F957, 2016. First published March 9, 2016; doi:10.1152/ajprenal.00292.2015.—The incidence and progression of diabetic nephropathy (DN) (21). The renin-angiotensin system (RAS) is an important regulator of cardiovascular and renal function. Sex differences in the renal response to RAS blockade have been demonstrated. Circulating and renal RAS has been shown to be altered in type 1 and type 2 diabetes; this enzymatic cascade plays a critical role in the development of diabetic nephropathy (DN). Angiotensin-converting enzyme (ACE) and ACE2 are differentially regulated depending on its localization within the diabetic kidney. Furthermore, clinical and experimental studies have shown that circulating levels of sex hormones are clearly modulated in the context of diabetes, suggesting that sex-dependent RAS regulation may also be affected in these individuals. The effect of sex hormones on circulating and renal RAS may be involved in the sex differences observed in DN progression. In this paper we will review the influence of sex hormones on RAS expression and its relation to diabetic kidney disease. A better understanding of the sex dimorphism on RAS might provide a new approach for diabetic kidney disease treatment.

angiotensin-converting enzyme 2; angiotensin-converting enzyme; gender

DIABETES IS THE LEADING CAUSE of end-stage renal disease (ESRD) in the Western world, responsible for nearly one-half of all new ESRD cases in the United States (21). In addition, hypertension is a major risk factor for the development and progression of diabetic nephropathy (DN) (21). The renin-angiotensin system (RAS) is an important regulator of cardiovascular and renal function (100), and its blockade using angiotensin-converting enzyme inhibitors (ACEi) or angiotensin-receptor blockers (ARB) is the cornerstone in the treatment of DN (100). Interestingly, sex differences in the renal response to RAS blockade have been demonstrated (115). Clinical and experimental studies have also shown sex differences in RAS components in several tissues and in the circulation (33, 94, 100, 140). In addition, chemical and surgical castration modulate the expression of different RAS components such as angiotensinogen (AOGEN), renin, angiotensin-converting enzyme (ACE), and ACE2 (24, 57, 59).

Sexual dimorphism on the progression of renal disease has become an area of active investigation (83, 110). The mechanisms responsible for sexual dimorphism in diabetic pathology represent an area of investigation (81). In diabetic people, men are at higher risk than premenopausal women for microvascular complications, such as nephropathy (2). The relationship between RAS and the progression of diabetic renal disease has been widely studied. However, the specific mechanisms in which sex hormones such as dihydrotestosterone (DHT) and 17β-estradiol (E2) modulate RAS expression in DN remain unclear. For this reason, we aimed to review the more relevant clinical and experimental studies focused on the sex differences in DN, the influence of sex hormones in RAS regulation, and the effect of diabetes on androgen and estrogen levels.

SEX DIFFERENCES IN DIABETIC NEPHROPATHY

Clinical Studies

Sex differences in diabetic nephropathy progression in type 1 diabetic patients. Several studies suggest that males with type 1 diabetes mellitus (T1DM) have significantly higher rates of decline in glomerular filtration rate (GFR), and an increased risk of developing microalbuminuria and progressing to macroalbuminuria than women (84). In particular, a large national-wide prospective study from Germany in 27,805 type 1 diabetic patients reported that the male sex was associated with the development of macroalbuminuria. This study was conducted in children, adolescents, and adults with a follow-up time of 2.5 yr. Interestingly, childhood diabetes onset was found to be protective against the development of micro- or macroalbuminuria while the majority of the patients showing macroalbuminuria or ESRD at the last visit were adults (mean age 37.2 yr). These observations suggest that high male sex hormone levels in the onset of diabetes predispose the patients to a worsened outcome in terms of renal disease (97). In concordance, Orchard et al., in a large epidemiological study of 657 type 1 diabetic subjects diagnosed in childhood, observed a higher risk of nephropathy in men coupled with increased progression from microalbuminuria to macroalbuminuria compared with female subjects (86). Subsequently, in their study of predictors of macroalbuminuria in 340 normotensive patients with T1DM, Villar et al. (129) found male sex to be a predictor of progression to microalbuminuria, independent of glycated hemoglobin levels. In a population of subjects with insulin-requiring diabetes, some of whom had T1DM, men had sig-

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significantly more microalbuminuria than women. This study found that hypertension and obesity were associated with an increase in albumin excretion rate (UAE) (91). In addition, the incidence of ESRD in people with diabetes in the United States was studied (47). Among Caucasians younger than 45 yr old, the progression to ESRD was significantly increased in men compared with women (7.3/100,000 vs. 2.8/100,000, average annual increments in risk of ESRD) (47). However, the protective effect of the female sex with T1DM in the progression to ESRD was lost after menopause (47). One may surmise that, when women lose female hormones, the positive effect disappears, and the progression of diabetic kidney disease is not favorable (Fig. 1).

**Sex differences in diabetic nephropathy progression in type 2 diabetic patients.** Male sex has also been associated with higher rates of albuminuria compared with females in the context of type 2 diabetes mellitus (T2DM) (90, 98, 107). A prospective and cross-sectional study of the prevalence and causes of persistent albuminuria (>300 mg/24 h) conducted in 224 males and 139 females with T2DM, age <66 yr, revealed a higher prevalence of albuminuria in males (19%) than in females (5%) (90). To further examine the risk factors associ-

Fig. 1. Schematic representation depicting the evolution of sex hormone levels throughout a lifespan under type 1 (A) or type 2 (B) diabetic conditions, and its corresponding effect on sex-specific susceptibility to develop diabetic nephropathy (DN). Under physiological conditions, male and female sex hormones dramatically increase at puberty, remain elevated during adulthood, and slightly decrease until the onset of andropause or menopause, when such decrease is clearly accentuated. During puberty, high levels of estrogens in female adolescents are protective against DN while high androgen levels predispose male adolescents to albuminuria (area depicted in red). A: after the onset of type 1 diabetes mellitus (T1DM), sex hormone levels are reduced, increasing the susceptibility of women to develop DN compared with men (area depicted in blue). When androgen levels are diminished below a certain threshold due to age and diabetes progression, susceptibility to DN in men increases again. B: type 2 diabetes mellitus (T2DM) is characterized by very low levels of androgens and higher levels of estrogens compared with T1DM. As a consequence, the incidence and severity of DN is clearly augmented in men throughout life. After menopause, the decrease of estrogen levels in type 2 diabetic women is accompanied by a rise in the progression of DN, attenuating the difference between the sexes at later stages in life. SH, sex hormone.
lated with UAE in T2DM, Savage et al. recruited 933 patients with T2DM from the appropriate blood pressure control in diabetes trial and classified them according to urinary albumin excretion (UAE) status: normoalbuminuria (<20 pg/min), microalbuminuria (20–200 pg/min), and macroalbuminuria (>200 pg/min). Using univariate analyses, it was found that the male sex significantly correlated with microalbuminuria and macroalbuminuria, together with Hispanic ethnicity, African-American race, poor glycemic control, insulin use, long duration of diabetes, dyslipidemia, diastolic and systolic hypertension, smoking, and obesity. However, sex differences were lost in the multivariate analysis. It is worth noting that in this study the mean age was 59 yr (107). Another prospective long-term follow-up study conducted on 574 patients, aged 40–60 yr, with recent onset of T2DM showed that male sex was associated with DN according to the final value of UAE, together with low levels of high-density lipoprotein, body mass index, cigarette smoking, and low socioeconomic status (98). Altogether these results suggest that the deleterious effect of the male gender in the development and progression of DN are related to the patient age and subsequently the hormonal changes that are observed with aging (Fig. 1). In a study of national US and United Kingdom heart disease mortality for three birth cohorts (1916-25, 1926-35, and 1936-45), all birth cohort’s linear heart disease mortality rates peaked in men around age 45, with slower age-related increases thereafter. Conversely, in women there was no accelerated increase in heart disease mortality rate at age 50 (menopause). In both sexes, proportional increases fit the data better than absolute increases, presumably reflecting competing risks with aging. The authors concluded that deceleration of the age-related increase in male heart disease mortality in midlife explained sex differences in cardiovascular mortality better than postmenopausal estrogen deficiency in women. Thus, at a younger age, diabetic men have an increased risk in cardiovascular diseases and DN compared with women, but once the disease is present and progresses over the years, it seems that renal- and cardiovascular-related mortality tend to equilibrate (8).

Effect of estrogen replacement therapy and oral contraceptives in DN. Estrogen replacement therapy (ERT) in postmenopausal women has shown to exert beneficial effects by attenuating diabetic complications within the kidney. Short-term administration of estrogen alone or together with norgestrel (a synthetic progestin) has been shown to reduce proteinuria and improve creatinine clearance in diabetic and hypertensive postmenopausal women (117). In addition, short-term treatment with raloxifene [a selective estrogen receptor (ER) modulator] limited the progression of albuminuria in 39 postmenopausal women with T2DM included in a 6-mo double-blind placebo-controlled trial (40). Interestingly, ERT has shown to improve not only renal function but also metabolic control in postmenopausal women with diabetes mellitus. In a systemic review and meta-analysis of 16 studies comprised of 17,971 cases, postmenopausal women taking low-dose combined ERT (estrogen and progestrone) showed a decreased risk of developing diabetes and better diabetic control. Specifically, ERT significantly reduced the incidence of diabetes and the levels of fasting plasma glucose, hemoglobin A1c (HbA1c), total cholesterol, and low-density lipoprotein (139). In contrast, ERT had no significant effect on microalbuminuria, glucose levels, or lipid profile in 60 healthy postmenopausal patients receiving E2 in a prospective randomized double-blind placebo-controlled study (64). In summary, the protective role of estrogens within the kidney seems to be more clinically relevant in the context of diabetes, suggesting that restoring female sex hormone levels in diabetic women may attenuate the effect of proinflammatory and proinflammatory factors such as high glucose and ANG II (49, 56).

Hormonal contraceptives treatments, in particular combined oral contraceptives (OC), are well known to increase the cardiovascular risk and affect the metabolic system by inducing changes in lipids, lipoproteins, carbohydrate metabolism, and hemodynamic factors (108). In concordance, several authors have reported that the use of OC at a high dose is associated with elevated glucose and insulin levels, higher rates of impaired glucose tolerance, and adverse effects on lipid profile and blood pressure (36, 43, 130, 135). As a consequence, OC can alter RAS and exert a detrimental effect in diabetic kidneys. Ahmed et al. reported in a prospective observational study with type 1 diabetic women a strong association between OC use and the angiotensin-dependent control of renal circulation in addition to the development of macroalbuminuria, highlighting OC use as a risk factor for DN (3). These adverse effects, however, are almost absent in clinical trials using progestin-only (27) and low-dose contraceptives (124). The use of high-dose OC for a prolonged period may cause hyperestrogenicity and lead to an adaptation state in which the beneficial actions of estrogens are downregulated. In addition, high doses of OC may overactivate estrogen-regulated cellular pathways in a detrimental manner (72). The differences observed between the estrogen hormone replacement regarding DN development and progression may be related to the baseline hormonal status of the women. When given in a deficient status (as it is in menopause), their effects are clearly beneficial, whereas in fertile women as anticonceptive therapy, they exert a deleterious effect. Thus, it seems that a hormonal balance is needed to maintain a decreased DN progression.

Effect of androgen replacement therapy in DN. Testosterone replacement therapy (TRT) in men with T2DM improved some of the key parameters associated with metabolic syndrome in several observational, retrospective, and prospective trials (38, 55, 79, 119). In particular, body weight, waist circumference, blood pressure, heart rate, fasting blood glucose and insulin sensitivity, as well as HbA1c, triglycerides, cholesterol, and low-density lipoprotein levels were significantly improved in type 2 diabetic men receiving TRT for at least 24 mo (34, 123, 141). In concordance, TRT in nine men with T1DM, erectile dysfunction, and hypogonadism improved in glycemic control, lipid profiles, and erectile function (104). Note that some observational studies have reported that TRT increases cardiovascular events in patients with metabolic syndrome (119). However, the Food and Drug Administration in the US has reviewed these reports and found them to be seriously flawed (22). Although the beneficial effects of TRT in diabetic men have been widely demonstrated in terms of metabolic parameters, whether they are accompanied by an improvement in kidney function in patients with renal complications remains unclear. To our knowledge, interventional clinical trials evaluating the influence of TRT in albuminuria and other typical alterations of DN have not been conducted.
Experimental Studies

Sex differences in experimental type 1 diabetes. Different studies in experimental models of diabetes have been performed to analyze the role of sex hormones on DN (6, 116, 138). Sex differences in several hallmarks of diabetic kidney disease have been assessed in the streptozotocin (STZ) model. In STZ-induced 6-wk-old Sprague-Dawley rats, 12 wk of T1DM led to significantly higher albuminuria and systolic blood pressure in diabetic males compared with females. In addition, diabetic males, but not females, showed increased renal collagen I and fibronectin mRNA levels compared with controls (24). In contrast, when STZ was administrated to 11-wk-old mRen2.Lewis hypertensive rats, diabetic females exhibited a marked increase in the inflammatory marker C-reactive protein that was not evident in the diabetic males. This alteration observed in females was associated with an increase in proteinuria and albuminuria after 4 wk of follow-up. Diabetic and hypertensive females also exhibited greater glomerular vascular endothelial growth factor staining and higher levels of inflammation in terms of tubulointerstitial CD68+ cells within the kidney (140). Of note, the onset and duration of diabetes in these studies were clearly different (24, 140), which probably determined a different hormonal status at the end of each follow-up. These data suggest that sex-specific susceptibility to develop certain features of DN can vary according to different factors, such as age, diabetes duration, and the presence of hypertension.

Sex differences in experimental type 2 diabetes. Few studies have been focused on the study of sex differences in experimental T2DM. Slyvka et al. demonstrated that female obese Zucker rats (fa/fa) showed better renal function than males at 13 wk of age. In addition, males exerted higher levels of eNOS and nNOS mRNA (cortex) and higher protein levels of eNOS (cortex and medulla), nNOS (medulla), and iNOS (cortex) than females. These differences observed may indicate upregulation of NOS isoforms in males compared with females in an attempt to increase NO levels and vasodilation (111). In another murine model of T2DM, the high-fat diet model, males showed increased blood glucose, UAE, and kidney weight compared with females. However, GFR was unchanged (85). To our knowledge, no other studies on DN and sex differences have been performed in models of T2DM.

Experimental studies with androgen supplementation or deprivation. Testosterone administration promotes tubular damage in STZ-induced rats. Sun et al. demonstrated that testosterone worsens tubular damage in diabetic rats in terms of increased fibrotic markers, such as α-smooth muscle actin and fibroblast-specific protein, two markers of cell damage and potential epithelial mesenchymal transition (116). In concordance, Xu et al. demonstrated that the administration of a high dose of DHT also exacerbated the development of albuminuria, index of glomerulosclerosis, and tubulointerstitial fibrosis associated with diabetes. However, a lower dose of DHT attenuated renal injury in castrated diabetic rats. DHT may play an important role in the pathophysiology of diabetic renal disease, and these effects are dose-dependent (138). Thus, a dual and dose-specific effect of DHT in the diabetic kidney has been observed; while the administration of low doses of DHT is renoprotective, higher doses are damaging. The determinants of these seemingly opposing effects of DHT remain unclear.

There are several potential explanations for this apparent paradox, including dose-dependent expression and activation of androgen receptor (AR) interacting with transcriptional coactivator proteins; however, the most likely explanation is that of the indirect effect of E2 rather than the direct effects of DHT. Interestingly, while diabetic male rats previously presented a reduction in the ratio of AR to ER protein expression in the renal cortex compared with nondiabetic animals (67, 96), the dual treatment with DHT and anastrozole restored this ratio (66). Considering that changes in sex hormone receptor expression in the diabetic kidney may reflect altered levels of circulating androgens and estrogens, it is conceivable that the diminished renal alterations observed after this dual treatment were achieved by restoring relative balance between sex hormones.

The effect of surgical androgen depletion by castration is controversial. While Xu et al. showed that castration worsens albuminuria as a marker of renal function in type 1 diabetic rats (138), in the Otsuka-Long Evans-Tokushima fatty (OLETF) rat, a model of T2DM (121), and the Cohen diabetic rat, a genetically selected sucrose-fed rat, castration attenuated proteinuria (20). In addition, in the STZ model of T1DM, castration was shown to have neither a detrimental nor a protective effect on the progression of diabetic renal disease (116). These apparent discrepancies in the effects of castration on diabetic renal disease may be ascribed to the duration and model of diabetic renal disease.

Experimental studies with estrogen supplementation or deprivation. Supplementation with E2 has shown to exert a protective effect on the development of functional and structural kidney damage by reducing albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis after several weeks of untreated diabetes (26, 68, 69). These effects have been attributed to different cellular mechanisms, including reduction of TGF-β synthesis, decreased accumulation of collagen type IV, laminin, and fibronectin, and increased expression of matrix metalloproteinases (MMP) (31, 82, 95). In this sense, studies in knockout of estrogen receptor-α (ERα−/−) in nondiabetic mice and ovariectomy in diabetic rats were associated with increased renal expression of TGF-β, and E2 supplementation to ovariectomized rats normalized TGF-β expression. 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. 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. This suggests that the most important effect of E2 is to disrupt TGF-β signaling, rather than to regulate its expression (25). Raloxifene, a selective ER modulator, has also been shown to diminish these renal alterations via similar mechanisms (19, 26).

The protective effects of estrogens have also been described in podocytes. E2 treatment protected nondiabetic podocytes from apoptosis induced in vitro by TGF-β and TNF-α (29). Such effect may be mediated by activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-protein kinase B (AKT) signaling cascade, since podocytes isolated from E2-treated db/db mice presented increased levels of AKT phosphorylation. Activation of extracellular signal-regulated kinases (ERKs), another downstream path-
way of TGF-β signaling, was decreased in these E2-treated podocytes. Lower activation of ERK may lead to increased expression of MMP2 and MMP9, which could explain the amelioration of extracellular matrix (ECM) accumulation and glomerular basement membrane thickening observed in these mice. In this work, tamoxifen also modulated podocyte signaling pathways via upregulation of ERβ (15). In addition, estrogens are thought to potentially decrease reactive oxygen species (ROS)-induced events by regulating podocyte antioxidant markers, such as Mn-superoxide dismutase and glutathione (17).

In contrast, ovariectomy caused a significant decrease in the incidence of nephropathy (20) while E2 administration exacerbated renal disease (101) in Cohen sucrose-fed diabetic rats. In the diabetic OLETF rat, treatment with E2 had no effect on albuminuria, although it diminished mesangial expansion and glomerulosclerosis (121). Once again, different hormone circulating levels and ER expression may represent a reasonable explanation for these discrepancies. In this sense, it has been demonstrated that, after experimental induction of menopause by selectively killing the small primordial and primary ovarian follicles through 4-vinylcyclohexene diepoxide (VCD) administration, renal damage develops more rapidly and severely in diabetic postovarian failure female mice compared with cycling females (50). In this study, cortical mRNA abundance of MMP9 was decreased after menopause, strengthening the results from previous in vivo studies in which MMP9 protein expression and activity level were decreased in ovariectomized diabetic (69) and Dahl salt-sensitive (71) rats. In concordance, in vitro experiments in mesangial cells in nonhyperglycemic diabetic postovarian failure female mice compared with cycling females (50). In this study, cortical mRNA abundance of MMP9 was decreased after menopause, strengthening the results from previous in vivo studies in which MMP9 protein expression and activity level were decreased in ovariectomized diabetic (69) and Dahl salt-sensitive (71) rats. In concordance, in vitro experiments in mesangial cells in nonhyperglycemic diabetic postovarian failure female mice compared with cycling females (50).

In the diabetic OLETF rat, treatment with E2 had no effect on albuminuria, although it diminished mesangial expansion and glomerulosclerosis (121). Once again, different hormone circulating levels and ER expression may represent a reasonable explanation for these discrepancies. In this sense, it has been demonstrated that, after experimental induction of menopause by selectively killing the small primordial and primary ovarian follicles through 4-vinylcyclohexene diepoxide (VCD) administration, renal damage develops more rapidly and severely in diabetic postovarian failure female mice compared with cycling females (50).

**SEX DIFFERENCES ON RAS IN DIABETIC NEPHROPATHY**

Clinical studies have shown that inhibiting the ACE-ANG II-ANG II type 1 receptor (AT1R) axis through the action of ACEIs or ARBs slows the progression of chronic kidney disease (CKD), especially when the renal disease is associated with proteinuria (11, 56, 118, 142). Thus, clinical guidelines recommend RAS blockade in patients with diabetic kidney disease (4). Interestingly, it has been demonstrated that renal and peripheral hemodynamic responses to RAS activation may vary according to sex (18, 76). To design more specific and efficient treatments for DN, understanding this sex effect has become a critical issue during the last decades. For this purpose, a large number of studies assessing sex differences at different levels of renal and circulating RAS in the context of diabetes have been performed.

**Sex Differences on RAS Activation: Angiotensinogen and Renin**

Renal AOGEN expression was increased in males (but not in females), showing strong association with albuminuria and renal fibrosis in STZ diabetic Sprague-Dawley rats. On the contrary, the plasma renin activity and renal renin mRNA levels were decreased in both diabetic males and females. Androgen blockade by flutamide administration decreased UAE only in diabetic males without affecting the endocrine or renal RAS (24). In concordance to cortical data, diabetes increased the medullary AOGEN content in male STZ Wistar rats. In addition, a diabetes-induced increase of urinary AOGEN was also found (92) (Fig. 2). Thus, the increase of renal AOGEN in the setting of diabetes is mainly observed in males, whereas the AOGEN decrease in plasma from diabetic animals is observed in both males and females. Again, these results suggest that there is a difference in the regulation of intrarenal and circulating RAS depending on the sex. These differences between the tissue and circulating RAS combined with the tissue sex divergences add complexity to the system (see Fig. 2). In rodent models (13, 46) and in patients with diabetes (48), it has been reported that ROS are important for intrarenal AOGEN augmentation in the progression of DN, highlighting the importance of the activated oxidative stress-AOGEN-RAS axis in the pathogenesis of DN. In addition, the redox-responsive transition of AOGEN to a form that preferentially interacts with receptor-bound renin has been demonstrated by crystallography and kinetic analysis (143). Clinical and experimental studies have provided evidence that oxidative damage parameters in renal tissue may vary according to sex (23, 126). It is conceivable that sex differences on renal RAS hyperactivation are due not only to genomic actions of sex hormones directly on AOGEN and renin genes, but also to sex-specific modulation of the oxidative stress status within the diabetic kidney (Fig. 2).

In contrast, in the context of diabetes and hypertension, circulating AOGEN is decreased also in both males and females. Interestingly, diabetic males showed higher plasma AOGEN compared with females (Fig. 2). Surprisingly, hyperglycemia was also associated with increased renal AOGEN and renin expression only in females, whereas the urinary excretion of AOGEN was similarly increased in both sexes (140). In this study, T1DM was accompanied with renal inflammation. It has been demonstrated that inflammatory cytokines, IL-1 and IL-6, can inhibit renin promoter activity via ERKs and signal transducer and activator of transcription 3 (STAT3) (60, 89). Both ERK and STAT3 are involved in DHT-independent AR activation and translocation to the nucleus (62, 93). Briefly, activation of the PI3K/Akt pathway results in phosphorylation and activation of AR (58). Activated STAT3 can form a heterologous complex with the phosphorylated AR (STAT3-AR) by interacting directly with amino acids 234–558 in the NH2-terminal domain of the receptor (125). This interaction takes place whenever both STAT3 and AR are activated, for example, as a response to epidermal growth factor or IL-6 (1), resulting in enhanced AR-mediated transcriptional activity (74) (Fig. 3). Diabetes is associated with lower levels of androgens (53, 70); thus, decreased male sex hormones in the context of diabetes and hypertension may favor the ERK/STAT3-mediated inhibition of renin expression and explain the lack of renin upregulation within the male diabetic kidney. Taken together, these results suggest that lower levels of renal AOGEN in diabetic hypertensive males may be ascribed, at least in part, to a higher ANG II-mediated turnover of renal AOGEN compared with diabetic females (140). In summary, sex-dependent effect of diabetes on renal AOGEN and renin expression may vary in the context of hypertension.
ACE2 mRNA levels were significantly increased in females patients with CKD (78). Surprisingly, when analyzing urine, dimorphism in urinary ACE2 activity or protein levels in hemodialysis patients (99). Mizuiri et al. did not find sexual compared with females after kidney transplant (112) and in activity of circulating ACE2 has also been detected in males factors, namely older age, diabetes, and male sex (7). Increased sity directly correlated with the classical cardiovascular risk increases in circulating ACE and consequently ANG II are not always accompanied by elevated blood pressure, they seem to lead to hemodynamic alterations reflected in glomerular hypelfiltration and albuminuria. In diabetic females, the more pronounced increase in circulating ACE2 may result in protection against hypertension. At the kidney level, however, diabetes-associated decrease in estrogen levels results in RAS hyperactivation and stimulation of the most relevant ANG II-mediated effector mechanisms such as TGF-β1. HG, hyperglycemia; HT; hypertension; GFR, glomerular filtration rate.

Sex Differences on RAS Regulatory Arms: ACE and ACE2

Circulating ACE activity is increased in diabetic mRen2.Lewis rats. Interestingly, this increase was more pronounced in males compared with females (140) (Fig. 2). In contrast, a decrease in renal ACE expression has been described in both male and female mice (88, 134, 136). However, studies focused on the assessment of renal ACE expression in the context of diabetes and sex differences are lacking.

Different groups have studied sex differences regarding ACE2 activity. Soro-Paavonen et al. found that males had significantly higher ACE2 activity than females, both among patients with T1DM and healthy individuals (113). For this reason, the analysis of their study was performed separately for males and females. In concordance, in CKD patients without previous history of cardiovascular disease, our group recently reported that ACE2 activity from human EDTA-plasma samples is significantly increased in males compared with females. In addition, in a multivariate analysis, circulating ACE2 activity directly correlated with the classical cardiovascular risk factors, namely older age, diabetes, and male sex (7). Increased activity of circulating ACE2 has also been detected in males compared with females after kidney transplant (112) and in hemodialysis patients (99). Mizuiri et al. did not find sexual dimorphism in urinary ACE2 activity or protein levels in patients with CKD (78). Surprisingly, when analyzing urine, ACE2 mRNA levels were significantly increased in females compared with males in renal transplant patients with diabetes (137). These results may be ascribed to an increased ACE2 shedding from female diabetic proximal tubules. Taken together, these data reinforce the idea that separate analyses for males and females should be considered when measuring ACE2 activity in future studies. The increase of circulating ACE2 activity in males may be an early marker of increased risk of cardiovascular disease in CKD patients. Oudit et al. studied the effect of ACE2 deletion in nondiabetic kidneys from male and female mice. Their data showed that loss of ACE2 in male (but not female) C57BL/6 mice is associated with the development of age- and ANG II-dependent glomerular damage (87). Gupta et al. also used ACE2-deficient mice to investigate the mechanistic role of ACE2 on the development of obesity-associated hypertension in males vs. females. They observed that male high-fat-fed ACE2−/− mice had significantly greater systolic blood pressure compared with high-fat-fed ACE2−/− females (39). These data suggest that males have a higher dependence on ACE2-mediated renoprotection.

In experimental studies with hypertensive and diabetic ani-
mRen2.Lewis diabetic rats were not protected from vascular damage, renal inflammation, and kidney injury in this model of early STZ-induced diabetes.

Sex Differences on RAS Effector Mechanisms: Angiotensin Peptides and Their Receptors

Experimental studies demonstrated that males have greater expression of “classical” components of the RAS, including ANG II and AT₁R, whereas females have greater expression of “non-classical” components of the RAS, including ANG II type 2 receptor (AT₂R) and ANG-(1–7) (12, 122). To our knowledge, only one experimental study has assessed sex differences on renal and circulating ANG II levels in DN. In this work, STZ-induced mRen2.Lewis rats presented augmented plasma levels of angiotensin peptides compared with controls, with no significant variations within the kidney. Diabetic and hypertensive males showed increased circulating and renal ANG II as well as decreased ANG-(1–7) in plasma compared with females (140) (Fig. 2). It is well accepted that ANG II mediates progressive diabetic kidney injury by enhancing renal fibrosis and inflammation (102, 103) via...
stimulation of growth factor TGF-β expression (9, 133). Interestingly, in vitro experiments revealed that E2 is capable of inhibiting TGF-β-mediated upregulation of α1 type IV collagen gene transcription in murine mesangial cells (109). Thus, decreased estrogen levels in the context of aging or diabetes progression might be responsible, at least in part, for the increased susceptibility to ANG II-induced renal alterations in postmenopausal or diabetic women. Further investigations are needed to confirm if the sex differences on angiotensin peptide levels observed under physiological and hypertensive conditions are changed or maintained in the presence of diabetes.

Sex Differences on RAS Blockade in Diabetic Nephropathy

Clinical studies. Renal hemodynamic responses to RAS blockade differ between men and women. Miller et al. evaluated the effects of 8 wk of ARB irbesartan administration on the pressor response to ANG II (3 ng·kg⁻¹·min⁻¹) in young healthy men (n = 15; mean age = 27 yr) and women (n = 15; mean age = 28 yr). In this study, lower dosages of irbesartan in women achieved significantly reduced ANG II sensitivity compared with men. Interestingly, after 8 wk of irbesartan administration, AT1R gene expression was decreased in women but not in men skin biopsies compared with baseline levels, indicating that blocking the ANG II-AT1R interaction can result in decreased expression of the receptor, enhancing the favorable effects of the compound in a sex- and dose-dependent manner (76). In turn, Cherney et al. studied sex differences in the renal response to hyperglycemia and ACE inhibition after 21 days of treatment with enalapril in young adolescents with uncomplicated T1DMD. During clamped hyperglycemia, only females exhibited reductions in renal plasma and blood flow, as well as increased renal vascular resistance and filtration fraction. After ACE inhibition treatment, both sexes exhibited significant declines in arterial pressure, but only females displayed a reduction in GFR and filtration fraction (18). In both studies, the female sex was associated with a major sensitivity to RAS blockade, suggesting that renal alterations due to RAS dysregulation in T1DMD may be more relevant in women. Accordingly, clinical trials studying the effects of irbesartan (56) or losartan (49) on the progression of nephropathy in T2DM found a protective role of male sex, with albuminuria progressing more rapidly in women. Once again, the inclusion in these studies of many postmenopausal women could have played a significant role in the outcome of these results.

Experimental studies. At an experimental level, both ACE inhibitors and ARBs have been shown to block the development of renal injury in both type 1 (61) and type 2 (42, 77, 144) diabetic male rodents. However, less is known regarding the effects of RAS inhibition on kidney disease in females, especially in the context of DN. To our knowledge, only Kelly et al. compared the effect of valsartan treatment with the endothelin receptor blockade in the STZ-induced diabetic female Ren-2 rat (51). They found that the administration of the AT1R antagonist valsartan reduced systolic blood pressure, ameliorated kidney lesions, and improved the renal function in female rats overexpressing ANG II.

In recent years, many authors have examined the impact of compound C21 (C21), a selective AT1R agonist, on DN. In STZ-induced type 1 diabetic male mice, C21 treatment showed a renoprotective effect by significantly attenuating renal hypertrophy and levels of cystatin C, albuminuria, mesangial expansion, and glomerulosclerosis, in association with inhibited expression of various proteins implicated in oxidative stress, inflammation, and fibrosis (54). In concordance, C21 improved albuminuria through the prevention of renal inflammation and production of NO and cGMP in STZ-induced male Sprague-Dawley rats (73). In T2DM, C21 treatment in combination with losartan showed an additive effect on reducing albuminuria and slowing the progression of nephropathy in male Zucker diabetic fatty rats (14). Acute C21 administration improved renal function in female, but not in male, spontaneously hypertensive (SHR) rats. Considering that AT1R are expressed to a greater extent in the kidney of female SHR rats (44), this effect of C21 in DN prevention seems to be more effective in female rats than in males.

From our perspective, there is a lack of clear evidence regarding sex differences in experimental RAS blockade or modulation in DN. For this reason, male and female animals should be simultaneously included in future studies evaluating the potential effect of a specific compound on RAS activation and DN. Despite the increasing number of publications demonstrating sex differences in RAS expression in DN, the complexity of this pathology and the fact that sex hormones are altered in the setting of hyperglycemia make it more challenging to elucidate the specific molecular mechanisms by which androgens and estrogens modulate RAS expression.

EFFECT OF DIABETES ON ANDROGEN AND ESTROGEN LEVELS

Effect of Type 1 Diabetes on Androgen and Estrogen Levels

The relationship between T1DM and serum androgen levels is controversial. In some studies, men with T1DM do not appear to have a high prevalence of androgen deficiency (45, 52). However, Maric et al. demonstrated that diabetes without renal disease was associated with decreased testosterone and estrogen levels compared with healthy nondiabetic adult men. In this study, progression of renal disease from micro- to macroalbuminuria accentuated the decrease in serum total testosterone (70). Renal complications derived from T1DM are rarely observed before puberty (75). Interestingly, when studying females, Amin et al. found that testosterone levels were increased in T1DM patients with microalbuminuria compared with the normoalbuminuric ones (5). These results suggest that high androgen levels predispose T1DM females to the development of microvascular disease such as DN (Fig. 1).

In experimental models of T1DM, assessment of the effect of hyperglycemia on male fertility in rats revealed that animals injected with STZ also showed significant decrease in serum testosterone levels, which were accompanied by diminished testicular and epididymal weight (80). Interestingly, elevation of circulating testosterone by aracoline in rats with established T1DM was associated with increased levels of serum insulin and upregulation of critical genes related to β-cell regeneration, such as glucose transporter 2 (105). In experimental models, castration in T1DM male rats worsened renal injury accompanied by a reduction of serum testosterone and kidney AR expression. Interestingly, circulating E2 levels and kidney aromatase activity remained increased after removal of male sex hormones, providing stronger evidence for extrastellar...
sources of E₂, while the expression of kidney ERα was not altered (96, 138). These results may suggest that testosterone-E₂-ERα, rather than the testosterone-DHT-AR axis, plays a role in the development of nephropathy in T1DM males. Of note, proinflammatory cytokines are known to upregulate the activity of aromatase, to effectively reduce testosterone levels, and increase the intracellular concentration of E₂ (132). Thus, the particular inflammatory status in type 1 diabetic males might explain the divergences in E₂ patterns observed in different studies.

Whether the estrogen signaling pathway is also detrimental for diabetic females is controversial. Several authors have reported that T1DM is associated with decreased E₂ levels in human (28, 106, 114) and animal female subjects (63, 131). In the context of type 1 DN, decreased E₂ levels have been associated with an imbalance in the expression of renal ERs. Specifically, female diabetic kidney exhibited increased protein expression of ERα, but not ERβ (96, 131) (Fig. 3). While ovariectomy increased renal ERα and reduced ERβ expression in these diabetic females, E₂ administration caused the opposite effect (131). In this sense, it has been reported that the deletion of ERα in STZ-induced females attenuated the development of albuminuria and glomerular hypertrophy, suggesting a role of ERα on promoting harmful events in the kidney (63). Interestingly, the absence of ERα in nondiabetic mice was not protective and led to the development of glomerulosclerosis, probably due to accumulation of endogenous testosterone (30). Thus, despite that ERα-mediated actions may be beneficial under physiological conditions, it is presumable that decreased estrogen levels in females with T1DM promote pathological overexpression and hyperactivation of renal ERα that, together with a downregulation of the protective effects of ERβ, may contribute to a more severe progression of DN.

Effect of Type 2 Diabetes on Androgen and Estrogen Levels

Grossmann et al. demonstrated in a cross-sectional survey that testosterone deficiency is common in men with diabetes, regardless of the type (38). However, clinical evidence supports that low testosterone levels are more strongly associated with T2DM rather than T1DM. This tendency has been observed when studying either young or old patients with diabetes (16, 120). Poor glycemic control in Korean men with T2DM resulted in increased levels of fasting plasma glucose and HbA1c values, the major markers of diabetes, which appeared to be associated with testosterone deficiency (53). In addition, diabetic men had also lower levels of sex hormone-binding globulin (SHBG) compared with nondiabetic men (10, 52). In fact, several prospective studies have shown that diabetes and metabolic syndrome are more strongly predicted by low SHBG than by low testosterone (41, 55, 128).

T2DM is associated with augmented E₂ levels in men (123, 124). These increased E₂ levels are associated with complications, such as atherosclerosis, in men with T2DM and metabolic syndrome (35, 65). The activation of G protein-coupled estrogen receptor (GPER) in isolated rat Leydig cells and adult human testis downregulates testosterone production (127). It is conceivable that these E₂-mediated mechanisms exacerbate the reduction on circulating testosterone levels in T2DM, conferring a reasonable explanation to the fact that type 2, but not type 1, diabetic men show a clearly increased susceptibility to develop DN than women (Fig. 1). These findings suggest that in T2DM males there is an imbalance between sex hormones that exacerbates DN.

When studying ERs, Doublier et al. found that the beneficial effects of E₂ treatment on attenuating DN in type 2 diabetic female mice were accompanied by increased ERβ but not ERα protein expression within the podocyte (29). While interaction between E₂ and ERα seems to be detrimental in T2DM, activation of ERβ can be considered renoprotective. Interestingly, it has been found that aldosterone activates GPER30 and induces rapid vascular effects (Fig. 3). Under physiological conditions, these GPER-mediated nongenomic effects are considered beneficial in the vasculature (37). However, T2DM in female db/db mice increased expression of GPER30 in mesenteric resistance arteries and impaired the vascular effects of aldosterone (32). Thus, hyperactivation of GPER30 also plays a role in the pathophysiology of type 2 DN, at least at the vascular level. These vascular alterations can be attributed to the hyperactivation of circulating RAS in diabetes, which may lead to higher ANG II levels and, in consequence, increased stimulation of aldosterone secretion by adrenal glands and further GPER30 activation.

CONCLUDING REMARKS

RAS and sex differences in DN progression are observed and seem to be related to differences in the hormone levels through the development of the disease. While the progression of DN is accentuated in young males, with aging, and the subsequent estrogen deprivation in women, these sex differences are lost. As a consequence, ERT has been shown to exert beneficial effects by attenuating diabetic complications within the kidney. Therefore, testosterone administration worsens tubular damage in diabetic rats in terms of increased fibrotic markers and cell damage. RAS modulation is crucial in the development and progression of diabetic kidney disease. Interestingly, sex differences on RAS in DN have been observed in terms of expression in RAS compounds and RAS blockade response. In fact, diabetes per se alters sex hormone levels in males and females. These deregulations of sex hormones may lead to sex-dependent imbalance of ACE/ACE2 that, in turn, can vary between the different compartments and segments within the kidney, adding more complexity to the understanding of DN. To assess this issue, further studies focused on the sexual dimorphism in tubular and glomerular ACE and ACE2 localization and expression at the gene, protein, and activity level are required. Sex-specific modulation of RAS cascade can lead to different degrees of intrarenal ANG II accumulation according to the sex and the etiology of the disease. In conclusion, understanding the hormonal alterations coupled with RAS differences that take place at every stage of DN and sex dimorphisms may be helpful in designing a specific therapeutic approach for delaying the progression of DN.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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Diabetes mellitus is a chronic disease that affects many aspects of health, including cardiovascular disease. The development of diabetes is influenced by genetic, environmental, and lifestyle factors.


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RAS AND SEX DIFFERENCES IN DIABETIC NEPHROPATHY


