Imbalance in Sex Hormone Levels Exacerbates Diabetic Renal Disease

Qin Xu, Corinne C. Wells, Joseph H. Garman, Laureano Asico, Crisanto S. Escano, Christine Maric

Abstract—Studies suggest that the presence of testosterone exacerbates, whereas the absence of testosterone attenuates, the development of nondiabetic renal disease. However, the effects of the absence of testosterone in diabetic renal disease have not been studied. The study was performed in male Sprague-Dawley nondiabetic, streptozotocin-induced diabetic, and streptozotocin-induced castrated rats (n = 10 to 11 per group) for 14 weeks. Diabetes was associated with the following increases: 3.2-fold in urine albumin excretion, 6.3-fold in glomerulosclerosis, 6.0-fold in tubulointerstitial fibrosis, 1.6-fold in collagen type I, 1.2-fold in collagen type IV, 1.3-fold in transforming growth factor-β protein expression, and 32.7-fold in CD68-positive cell abundance. Diabetes was also associated with a 1.3-fold decrease in matrix metalloproteinase protein expression and activity. Castration further exacerbated all of these parameters. Diabetes was also associated with a 4.7-fold decrease in plasma testosterone, 2.9-fold increase in estradiol, and 2.1-fold decrease in plasma progesterone levels. Castration further decreased plasma testosterone levels but had no additional effects on plasma estradiol and progesterone. These data suggest that diabetes is associated with abnormal sex hormone levels that correlate with the progression of diabetic renal disease. Most importantly, our results suggest an important role for sex hormones in the pathophysiology of diabetic renal complications. (Hypertension. 2008;51:1218-1224.)

Key Words: diabetes ■ kidney ■ sex hormones ■ glomerulosclerosis ■ tubulointerstitial fibrosis

Epidemiological studies show that the incidence and the rate of progression of nondiabetic renal disease are greater in men compared with age-matched women.1,2 These observations lead to the belief that the male sex is a risk factor and/or that the female sex is a protective factor against the development of renal disease. In the setting of diabetes, however, this relationship is not so clear. Although some studies indicate that the male sex is still a risk factor for the development of diabetic nephropathy and progression to end-stage renal disease,3,4 other studies suggest either no difference5,6 or that females progress at a faster rate.7,8 The actual truth is that the existing data are inadequate for the precise determination of whether sex differences, which clearly exist in nondiabetic renal disease, exist or “disappear” in the setting of diabetes.

Our previous studies have shown that diabetes is associated with reduced plasma levels of estradiol in the female streptozotocin (STZ)-induced diabetic rat.9 Supplementation of estradiol in these animals either from the onset10,11 or 2 months after the induction of diabetes12 attenuates the development of renal disease. Clinical studies indicate that diabetes is associated with decreased testosterone levels in men with diabetes,13,14 suggesting that testosterone deficiency may contribute or at least be associated with the development of diabetic renal disease. Similarly, hypertension and associated nondiabetic renal disease in men are associated with reduced testosterone levels.15,16 These observations may indicate that testosterone supplementation would be beneficial in attenuating hypertension and renal disease. However, testosterone supplementation in experimental models exacerbates, whereas castration attenuates, both hypertension and associated nondiabetic renal disease.17 These observations suggest that the simple assumption that absolute levels of testosterone or changes in testosterone levels are not a reliable predictor of the disease. Our hypothesis is that it is more likely the relative balance rather than the absolute levels of sex hormones that correlates with and plays a key role in the pathophysiology of renal disease. The aim of the present study was to examine the effects of longer-term (14 weeks) diabetes on the relative balance of sex hormone levels and their contribution to the pathophysiology of diabetic renal disease.

Methods

Animals
The study was performed in male Sprague-Dawley rats (Harlan, Madison, Wis; 12 weeks of age). All of the animals were given standard rat chow and tap water ad libitum. The animals were randomly divided into 3 treatment groups: nondiabetic (ND; n = 10),...
STZ-induced diabetic shams (D; n = 11), and STZ-induced diabetic castrated (Dca; n = 10). Diabetes was induced by a single IP injection of 55 mg/kg of STZ (Sigma) in 0.1 mol/L of citrate buffer (pH 4.5) after an overnight fast. Nondiabetic animals were injected with 0.1 mol/L of citrate buffer only. All of the D animals were given subcutaneous injections of insulin every 3 days (2 to 4 U, Lantus, Aventis Pharmaceuticals Inc) to maintain blood glucose levels between 300 and 450 mg/dL, as measured using a OneTouch Ultra glucometer. During the treatment period (14 weeks), the animals were placed in metabolic cages for 24 hours every 4 weeks for measurement of urine output and albumin concentration.

Blood Pressure
After 14 weeks of treatment, the animals were anesthetized with sodium pentobarbital (40 mg/kg IP) and their femoral vessels catheterized. Systemic blood pressure was monitored electronically using the Cardiomax-II blood pressure analyzer (Columbia Instruments). After blood pressure measurements, the animals were weighed and blood collected (via cardiac puncture) for the measurement of plasma testosterone, estradiol, and progesterone. The kidneys were weighed and then either snap frozen in liquid nitrogen for protein analysis or immersion fixed with HistoCHoICE (Amresco) for immunohistochemical analysis. All of the experiments were performed according to the guidelines recommended by the National Institutes of Health and approved by the Georgetown University Animal Care and Use Committee.

Castration and Sex Hormone Levels
At 12 weeks of age, rats were subjected to sham operation or castration. Briefly, the testes were exposed via a midline scrotal incision, the vascular supply ligated, and the organs were removed. The scrotal sac was sutured and closed. Sham operation consisted of exposing but not removing the testes. Plasma testosterone, estradiol, and progesterone levels were measured by ELISA (Assay Designs), according to the manufacturer’s protocol.

Urine Albumin Excretion and Creatinine Clearance
Urine albumin concentration was measured using the Nephrat II albumin kit (Exocell, Inc), according to the manufacturer’s protocol. The rate of urine albumin excretion (UAE) was calculated based on urine albumin concentration and 24-hour urine output.

Urine and plasma creatinine concentrations were measured using a kit (BioAssay Systems), according to the manufacturer’s protocol. Creatinine clearance (CrCl) was calculated based on plasma and urine creatinine concentrations and 24-hour urine output.

Glomerulosclerosis and Tubulointerstitial Fibrosis
Indices of glomerulosclerosis (GSI) and tubulointerstitial fibrosis (TIFI) were assessed in periodic acid Schiff and Masson’s trichrome-stained paraffin sections (4 μm) using a semiquantitative method as described previously. GSI was defined as mesangial expansion and TIFI as tubularatrophy or dilatation, deposition of extracellular matrix (ECM) proteins, and the presence of inflammatory cells. The analyses were performed with the observer masked as to the treatment group.

Immunohistochemistry
Paraffin-embedded (collagen type IV, matrix metalloproteinase-9 [MMP-9], transforming growth factor-β [TGF-β], and CD68) or frozen sections (collagen type I) were incubated with 0.1% albumin (for collagen type I and type IV) or with 10% nonimmune goat serum (MMP-9, TGF-β, and CD68) in PBS (pH 7.4) to block nonspecific immunolabeling. Sections were then incubated with antisera against collagen type I (1:200, mouse monoclonal, Sigma), collagen type IV (1:400, goat polyclonal, Southern Biotech), MMP-9 (1:1000, mouse monoclonal, Oncogene), and TGF-β (1:400, rabbit polyclonal, R&D Systems), or CD68 (1:400, mouse monoclonal, Serotec) at 4°C overnight. After washing with PBS, sections were incubated with biotinylated anti-rabbit, anti-mouse, or anti-goat IgG ( Dakopatts) diluted 1:200 in PBS for 1 hour at room temperature, followed by incubation with the avidin-biotin complex (Vector) diluted 1:200 with PBS for 1 hour at room temperature. Positive immunoreaction was detected after incubation with 3, 3-diaminobenzidine for 2 minutes at room temperature and counterstaining with Mayer’s hematoxylin. Sections incubated with 0.1% albumin and 10% goat serum instead of the primary antisera were used as negative controls. Macrophage number was assessed by counting the number of CD68-positive cells in 6 sections per animal from each group and expressed per millimeter squared.

Zymography
MMP activity was measured by zymography as described previously. Briefly, homogenized renal cortical samples were loaded onto a 10% SDS acrylamide gel containing 1 mg/mL of gelatin (BioRad). Gelatinolytic activity of MMP-9 was visualized as clear bands against a blue background after staining with Coomassie blue. The densities of specific bands were quantified by densitometry using the Scion Image beta (version 4.02) software.

Statistical Analysis
All of the values are expressed as means±SEMs and were analyzed using a 1-way ANOVA. Posthoc comparisons were performed with a Tukey’s test (Prism 4, Graph Pad Software). Analysis of correlation between sex hormone levels and parameters of renal function and pathology were performed by the Pearson product moment correlation tests (Prism 4). Significance for all of the analyses was accepted at P<0.05.

Results
Blood Glucose, Body and Kidney Weight, and Food Intake
The D animals exhibited a 5.0-fold increase in blood glucose levels, 1.5-fold decrease in body weight despite a 1.5-fold increase in food intake, and a 1.9-fold increase in kidney/body weight ratio compared with ND animals (Table). Castration had no further influence on these parameters in the diabetic animals (Table).

UAE, CrCl, and Mean Arterial Pressure
The D animals exhibited a 3.2-fold increase in UAE compared with ND animals (Table). Dca was associated with a 5.0-fold increase in UAE compared with the ND group and a 1.8-fold increase in UAE compared with the D group (Table). No differences in CrCl or mean arterial pressure were observed between the treatment groups (Table).
Sex Hormone Levels
Diabetes was associated with a 4.7-fold decrease in plasma testosterone, 2.9-fold increase in estradiol, and a 2.1-fold decrease in plasma progesterone levels compared with ND animals (Table). Although Dcas had no further effect on plasma estradiol and progesterone levels, Dcas was associated with a 210-fold and 45-fold decrease in plasma testosterone levels compared with ND and D animals, respectively (Table).

GSI and TIFI
The D animals exhibited moderate glomerular and tubulointerstitial changes characterized by mild mesangial expansion, accumulation of ECM proteins, and presence of inflammatory cells (Figure 1A and 1B). These changes were more pronounced in the Dcas animals (Figure 1A and 1B). The semiquantitative analysis of the degree of renal pathology showed a 6.3-fold increase in GSI (Figure 1C) and a 6.0-fold increase in TIFI (Figure 1D) in the D compared with the ND group. GSI was further increased by 1.7-fold (Figure 1C) and TIFI by 1.5-fold (Figure 1D) in the Dcas compared with the D group.

Collagen Protein Expression
In the ND renal cortex, collagen type I was immunolocalized to tubulointerstitial spaces, whereas collagen type IV was immunolocalized to basement membranes of proximal and distal tubules and the mesangial areas in the glomerulus (Figure 2A). D was associated with an overall increase in the intensity of immunostaining for both collagen types I and IV, and castration increased the intensity of immunostaining even further (Figure 2A). Quantitative analysis of collagen type I and IV protein expression by Western blotting confirmed the immunohistochemical studies. In the renal cortex of D animals, collagen type I and collagen type IV protein levels were increased by 1.6-fold and 1.2 fold, respectively, compared with ND animals (Figure 2B and 2C). Both collagen type I and type IV protein levels were increased further by 1.2-fold in the Dcas compared with D animals (Figure 2B and 2C).

MMP-9 Protein Expression and Activity
In the ND kidneys, MMP-9 was immunolocalized to proximal and distal tubules and mesangial cells (Figure 3A). The

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Table. Effects of Diabetes and Castration on Metabolic and Renal Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ND</th>
<th>D</th>
<th>Dcas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mg/dL</td>
<td>86.3±4.5</td>
<td>426.7±13.8*</td>
<td>433.9±13.6*</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>459.1±9.1</td>
<td>298.7±11.4*</td>
<td>287.1±13.1*</td>
</tr>
<tr>
<td>Kidney/body weight, g/kg</td>
<td>2.96±0.05</td>
<td>5.63±0.25*</td>
<td>5.50±0.20*</td>
</tr>
<tr>
<td>Food intake, g/d</td>
<td>24.8±1.3</td>
<td>37.3±1.2*</td>
<td>34.3±1.7*</td>
</tr>
<tr>
<td>UAE, mg/d</td>
<td>15.4±1.6</td>
<td>49.5±4.9*</td>
<td>49.2±12.9*</td>
</tr>
<tr>
<td>CrCl, mL/min per kidney weight</td>
<td>0.73±0.07</td>
<td>0.56±0.07</td>
<td>0.62±0.09</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>100.3±6.4</td>
<td>115.5±5.9</td>
<td>113.1±5.8</td>
</tr>
<tr>
<td>Plasma testosterone, ng/mL</td>
<td>4.2±0.6</td>
<td>0.9±0.3*</td>
<td>0.02±0.004†</td>
</tr>
<tr>
<td>Plasma estradiol, pg/mL</td>
<td>5.9±0.3</td>
<td>17.2±3.7*</td>
<td>15.3±3.2*</td>
</tr>
<tr>
<td>Plasma progesterone, ng/mL</td>
<td>16.0±6.4</td>
<td>7.5±4.2*</td>
<td>8.6±3.5*</td>
</tr>
<tr>
<td>Data are expressed as means±SEMs. Statistical significance was accepted at P&lt;0.05. MAP indicates mean arterial pressure. *P&lt;0.05 vs ND. †P=0.05 vs D.</td>
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</table>
overall intensity of immunolocalization was decreased in the D animals compared with ND animals, whereas Dcas decreased the intensity of immunostaining even further (Figure 3A). Western analysis and zymography confirmed the immunohistochemical findings. Diabetes was associated with a 1.3-fold decrease in both MMP-9 protein levels (Figure 3B) and MMP-9 activity (Figure 3C). Both MMP-9 protein levels and activity were decreased further by 1.3-fold in the Dcas compared with D animals (Figure 3B and 3C).

**TGF-β Protein Expression**

Although TGF-β was not detectable in the ND kidneys by immunohistochemistry (Figure 4A), TGF-β immunoeexpression was evident predominantly in the glomerular mesangial areas of the D kidneys (Figure 4A). The overall intensity of immunostaining was increased in the Dcas group compared with the D group. Western analysis showed a 1.3-fold increase in TGF-β protein levels in the D compared with the ND group, whereas the Dcas animals showed a further 1.2-fold increase in renal cortical TGF-β protein levels compared with D (Figure 4B).

**CD68-Positive Cell Abundance**

Diabetes was associated with a 32.7-fold increase in the abundance of CD68-positive cells, indicating the presence of macrophages (Figure 5A and 5B). Dcas animals showed a further 1.3-fold increase in CD68-positive cell abundance compared with D animals (Figure 5A and 5B).

**Correlation Between Sex Hormone Levels and Parameters of Renal Function and Pathology**

Decreases in testosterone levels associated with diabetes and castration showed an inverse correlation with all of the markers of renal function and pathology except for MMP-9. Correlation coefficients and P values are as follows (UAE, P = 0.001; GSI, P = 0.001; TIFI, P = 0.001; collagen type I, P = 0.001; collagen type IV, P = 0.001; MMP, P = 0.001; TGF-β, P = 0.001; CD68, P = 0.001). The ratio of testosterone/estradiol (UAE, P = 0.001; GSI, P = 0.001; TIFI, P = 0.001; collagen type I, P = 0.001; collagen type IV, P = 0.001; MMP, P = 0.001; TGF-β, P = 0.001; CD68, P = 0.001) correlated with all of the changes in renal function and pathology, including MMP-9. Testosterone/progesterone ratio, similar to testosterone levels, correlated with all of the parameters except MMP-9 (UAE, P = 0.001; GSI, P = 0.001; TIFI, P = 0.001; collagen type I, P = 0.001; collagen type IV, P = 0.001; MMP, P = 0.001; TGF-β, P = 0.001; CD68, P = 0.001).

**Discussion**

The major finding of the present study is that the male STZ-induced diabetic rat is characterized by abnormal sex hormone levels; in particular, a reduction in testosterone and an increase in estradiol and progesterone levels. The resultant decreases in testosterone/estradiol and testosterone/progesterone ratios correlate with the increase in albuminuria and renal pathology associated with diabetes, suggesting that sex hormones may play an important role in the pathophysiology of diabetic renal disease.

Although there is still much controversy regarding whether sex differences in the incidence and progression of diabetic renal disease exist or not, several studies suggest that the male sex is a risk factor for the progression of diabetic nephropathy. Interestingly, diabetes and end-stage renal disease have been reported to be associated with decreased circulating levels of testosterone. These observations seem somewhat contradictory, because they suggest that male sex is a risk factor for disease development, yet these males exhibit reduced levels of testosterone. The most likely explanation for these contradictory observations is that it is not the absolute levels of testosterone that renders the male sex as a risk factor for the development and progression of renal disease but rather a relative balance of testosterone and other...
hormones such as estradiol and progesterone. Our study in the male STZ-induced diabetic rat supports the clinical observations that diabetes is associated with low plasma levels of testosterone. In addition, we show that diabetes is also associated with elevated levels of plasma estradiol and progesterone in male rats. Our previous studies reported that the female STZ-induced diabetic rat exhibits low levels of circulating estradiol and elevated levels of progesterone and testosterone. These observations strongly support the concept that diabetes is characterized by abnormal sex hormone levels, and this may be sex specific.

The present study shows that the decreases in testosterone levels and concomitant increases in estradiol and progesterone with diabetes (ie, decreases in testosterone/estradiol and testosterone/progesterone ratios) correlate with the development of albuminuria, a hallmark of diabetic renal disease. Interestingly, castration in diabetic rats, which reduced testosterone levels even further than that in intact diabetic rats (but had no additional effect on either estradiol or progesterone), was associated with a more severe albuminuria than in the intact diabetic rats. This was a surprising finding given the wealth of evidence suggesting that castration ameliorates hypertension and most nondiabetic renal diseases in experimental models. The increased severity of albuminuria associated with castration in diabetes observed in the present study was not related to changes in CrCl, suggesting that the complete absence of testosterone may unmask the mechanisms that promote the hemodynamic changes, alterations in glomerular basement membrane composition, and podocyte, all of which are mediators of albuminuria. In models of nondiabetic renal disease, castration reduces albuminuria by downregulating the activity of the renin-angiotensin system. It is conceivable that, in the setting of diabetes, castration is insufficient to reduce renal activity, which is upregulated in the face of persistent hyperglycemia, leading to the development of albuminuria. Further studies are needed to examine the effects of castration on the renin-angiotensin system expression and activity. In support of our findings on the detrimental effect of castration on renal disease progression is a recent report showing that gonadectomy exacerbates albuminuria and decreases CrCl in experimental models of chronic renal failure. In contrast to the findings of our study, castration attenuates proteinuria in the Otsuka-Long-Evans-Tokushima-Fatty rat, a model of type 2 diabetes, and the Cohen diabetic rat, a genetically selected sucrose-fed rat. In a similar model of diabetic renal disease used in our study, castration was shown recently to have neither a detrimental nor a protective effect on the progression of diabetic renal disease. One of the likely explanations for these apparent discrepancies in the effects of castration on diabetic renal disease is the duration and model of diabetic renal disease.

Interestingly, whereas castration reduced testosterone levels, it had no effect on plasma estradiol or progesterone. The lack of an effect of castration on plasma estradiol and progesterone levels in diabetic animals has also recently been reported by others. These observations suggest that estradiol and progesterone in diabetic subjects may be of extratesticular origin, such as the adrenal gland or even the kidney itself. Despite the fact that previous studies from this and other laboratories demonstrated renoprotective effects of estradiol in female diabetic rats, the present study does not support the concept of the beneficial effects of estradiol in the diabetic males, because estradiol levels were elevated with diabetes, coinciding with increases in albuminuria and renal pathology. What the present study demonstrates is that it is not the absolute level of sex hormones that correlates with the severity of the disease but rather the ratio of testosterone/estradiol and testosterone/progesterone.

In addition to correlating with the development of albuminuria, decreases in testosterone levels and testosterone/estradiol and testosterone/progesterone ratios correlated with the severity of GSI and TIFI. Furthermore, expression of profibrotic and proinflammatory markers, including collagen type I and type IV, TGF-β, and CD68, was exacerbated with both decreases in testosterone and the testosterone/estradiol ratio, especially with castration. Interestingly, a recent study by Sun et al examining the effects of castration on TGF-β and ECM proteins in the renal cortex of diabetic male rats showed no effect of castration on these parameters, despite observed decreases in testosterone levels and increases in estradiol. The most likely explanation for this discrepancy is the duration of diabetes: 6 weeks in the study by Sun et al versus 14 weeks in the present study.

Because our studies indicate that the severity of diabetic renal disease inversely correlates with testosterone levels, it is conceivable that testosterone supplementation could be renoprotective. Although the present study did not examine the effects of testosterone supplementation, experimental studies have shown that testosterone supplementation exacerbates hypertension and associated nondiabetic renal disease. These observations suggest that it is most likely that beneficial effects of hormone therapy lie in supplementation of not just one, but all sex hormones to physiological levels observed in healthy subjects. Further studies are needed to evaluate this hypothesis in models of diabetic renal disease.
The present study showed that diabetes was associated with an increase in blood glucose levels, kidney/body weight ratio, and food intake and a decrease in body weight, whereas castration had no additional effect on any of these parameters. These findings suggest that the high levels of estradiol and progesterone in the castrated rats may be protective from a further increase in kidney/body weight ratio, possibly through regulating blood glucose levels.

Consistent with the previous reports, STZ-induced diabetic rats, at least during a short and moderate duration of diabetes, do not exhibit hypertension.36 These findings, whereas disappointing in that they did not allow us to examine the effects of sex hormones on blood pressure in these rats, did allow us to analyze the data independent of blood pressure.

**Perspectives**

Our study demonstrates a correlation between changes in sex hormone levels and the severity of albuminuria, GSI, TIFI, regulation of ECM metabolism, and expression of profibrotic and proinflammatory markers including collagens, TGF-β, and CD68 with the reduced testosterone levels in the male STZ-induced diabetic rat. These studies underscore the importance of further examining the mechanisms by which sex hormones regulate renal function and contribute to the pathophysiology of diabetic renal disease.

**Sources of Funding**

This work was supported by an RO1 grant (DK075832) from the National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases and the research award from the American Diabetes Association (to C.M.).

**Disclosures**

None.

**References**


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Hypertension. 2008;51:1218-1224; originally published online February 7, 2008;
doi: 10.1161/HYPERTENSIONAHA.107.100594
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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