Inhibition of estradiol synthesis attenuates renal injury in male streptozotocin-induced diabetic rats

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The rate of progression of diabetic renal disease is greater in men compared with age-matched women (21, 29). Based on this trend, one would expect that testosterone, being the predominant sex hormone in males, would be the underlying cause of this risk. Surprisingly, the opposite appears to be the case; both type 1 and type 2 diabetic male patients exhibit low circulating testosterone levels along with increased circulating estradiol levels (8, 9, 18, 33), suggesting that diabetes is a state of an imbalance in sex hormone levels. Experimental and clinical studies showed that this imbalance in sex hormone levels was associated with the progression of diabetic renal disease. Similar to males, diabetic females also exhibit an imbalance in sex hormone levels, albeit in the opposite direction: decreased estradiol and increased testosterone levels (19, 27). The changes in the balance in sex hormone levels in both males and females support that concept that sex hormones may play a role in the pathogenesis of diabetic renal disease. Based on these observations, it is conceivable that restoring the balance in sex hormone levels in diabetics to that observed in nondiabetics may prevent or attenuate the progression of diabetic renal disease. Indeed, studies showed that supplementation with estradiol in female STZ-induced diabetic rats partially attenuated diabetes-associated renal injury (16). In males, supplementing dihydrotestosterone (DHT), the nonaromatizable and biologically more potent androgen, to STZ-induced diabetic rats attenuates diabetic renal disease (34). However, this therapy only provided partial renoprotection. Since the STZ-induced diabetic rat shows not only reductions in testosterone levels but also increases in estradiol levels, the aim of the present study was to examine whether inhibiting estradiol synthesis, via preventing aromatization of testosterone into estradiol, would afford renoprotection in the male STZ-induced diabetic rat.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Harlan, Madison, WI; 12 wk of age) were maintained on regular rat chow and water ad libitum. The rats were randomly divided into three groups: nondiabetic (ND; n = 8), STZ-induced diabetic (D; n = 11), and STZ-induced diabetic that were treated with 0.15 mg-kg⁻¹-day⁻¹ of anastrozole (AstraZeneca, Pharmaceuticals, Wilmington, DE) by oral gavage (Da; n = 10). Nondiabetic and untreated diabetic animals were orally gavaged daily with equivalent volumes of 0.9% NaCl. After an overnight fast, diabetes was induced by a single intraperitoneal injection of STZ as described previously (35). Throughout the length of the study (12 wk), all diabetic rats received 2–4 U of insulin, every 3 days (Lantus, Aventis Pharmaceuticals, Kansas City, MO) by subcutaneous injection to maintain blood glucose levels between 300 and 450 mg/dl, to promote weight gain and to prevent mortality. Blood glucose levels were monitored throughout the study and measured using a FreeStyle Lite glucometer (Abbott Diabetes Care, Alameda, CA). All rats were placed into metabolic cages every 4 wk for 24 h and food intake, water intake, and urine output were recorded. At the end of the study, the rats were anesthetized with isoflurane and blood was collected via cardiac puncture for measurement of circulating plasma testosterone and estradiol levels. The kidneys were dissected, weighed, and immersed in either Histochoice (Amresco, Solon, OH) or snap-frozen in liquid nitrogen for Western blotting. All experiments were approved by the University of Mississippi Medical Center Animal Care and Use Committee.

Urine albumin excretion. Urine albumin concentration was measured every 4 wk using the NeprHIT II albumin kit (Exocell, Philadelphia, PA) according to the manufacturer’s protocol. The rate of urine albumin excretion (UAE) was calculated based on the measured urine albumin concentrations and 24-h urine output.

Measurement of plasma hormone levels. Plasma testosterone and estradiol levels were measured by radioimmunoassay (testosterone: Siemens Med. Solutions Diagnostic; cat. no.TKTT2; Los Angeles, CA; estradiol: Diagnostic System Labs; cat. no. DSL-4800; Webster, TX), according to the manufacturer’s protocol.

Glomerulosclerosis and tubulointerstitial fibrosis. To assess markers of renal pathology, indexes of glomerulosclerosis (GSI) and tubulointerstitial fibrosis were evaluated by light microscopy. Each kidney was immersion fixed in either Histochoice (Amresco, Solon, OH) or snap-frozen and embedded in OCT compound. 5 μm sections were cut and stained with H&E, Masson’s trichrome, and immunostaining with CD68 (1:500, Abcam, Cambridge, MA). The density of renal cortical CD68-positive cells was calculated using an automated CAS-system (Leica, Bannockburn, IL).

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tubulointerstitial fibrosis (TIFI) were evaluated using a semiquantitative scoring method as previously described (16).

Immunohistochemistry. Paraffin-embedded sections (4 μm) were incubated with 10% nonimmune goat or 0.1% bovine serum to block nonspecific immunolabeling. Sections were then incubated with antisera against CD68 (1:200; mouse monoclonal; cat. no. MCA341R; Serotec, Oxford, UK), collagen IV (1:800; goat polyclonal; cat. no. MAB1910; Millipore, Billerica, MA), androgen receptor (AR; 1:500; rabbit polyclonal; cat. no. sc-816; Santa Cruz Biotechnology), estrogen receptor α (ERα; 1:500; rabbit polyclonal; cat. no. ab18995; Abcam), aromatase (1:500; rabbit polyclonal; cat. no. ab18995; Abcam), transforming growth factor-β (TGF-β; 1:500; rabbit polyclonal; cat. no. sc-146; Santa Cruz Biotechnology), podocin (1:2,000; rabbit polyclonal; cat. no. sc-1265; Santa Cruz Biotechnology), or tumor necrosis factor-α (TNF-α; 1:500; mouse monoclonal; cat. no. sc-1265; Santa Cruz Biotechnology) and appropriate secondary antibodies conjugated to horseradish peroxidase. Proteins were visualized by enhanced chemiluminescence (KPL, Gaithersburg, MD) and the densities of specific bands were quantitated by densitometry using the Scion Image (version alpha 4.0.3.2) software and normalized to the total amount of protein loaded in each well following densitometric analysis of gels that were stripped and reprobed with an antibody against β-actin (1:2,000; mouse monoclonal; cat. no. sc-4970; Cell Signaling, Danvers, MA) and actin (1:2,000; mouse monoclonal; cat. no. MAB1910; Millipore, Temecula, CA). Statistical analysis. All values are expressed as means ± SE and were analyzed using a one-way ANOVA (Prism 4, Graph Pad Software, San Diego, CA). Post hoc comparisons were performed using Tukey’s test.

### Table 1. Metabolic parameters and steroid hormone levels

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ND</th>
<th>D</th>
<th>Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mg/dl</td>
<td>82 ± 2</td>
<td>378 ± 13</td>
<td>387 ± 14</td>
</tr>
<tr>
<td>Kidney wt, g</td>
<td>1.3 ± 0.1</td>
<td>1.6 ± 0.0</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>442 ± 12</td>
<td>331 ± 9</td>
<td>347 ± 12</td>
</tr>
<tr>
<td>Kidney/body weight, g/kg</td>
<td>2.9 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>Urine output, ml/day</td>
<td>30 ± 4</td>
<td>207 ± 15</td>
<td>227 ± 11</td>
</tr>
<tr>
<td>UAE, mg/day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 wk</td>
<td>4.3 ± 0.9</td>
<td>6.7 ± 1.6</td>
<td>5.8 ± 1.1</td>
</tr>
<tr>
<td>8 wk</td>
<td>5.4 ± 1.0</td>
<td>14.0 ± 3.3</td>
<td>7.1 ± 1.7</td>
</tr>
<tr>
<td>12 wk</td>
<td>5.8 ± 1.4</td>
<td>30.1 ± 4.3</td>
<td>17.5 ± 3.2</td>
</tr>
<tr>
<td>Plasma testosterone, ng/ml</td>
<td>3.9 ± 0.8</td>
<td>0.8 ± 0.1</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>Plasma estradiol, pg/ml</td>
<td>22.8 ± 1.1</td>
<td>90.1 ± 14.7</td>
<td>54.7 ± 7.3</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. Statistical significance was accepted at *P* < 0.05. *a P* < 0.05 vs. nondiabetic (ND). *b P* < 0.01 vs. ND. *c P* < 0.001 vs. ND. *d P* < 0.05 vs. diabetic (D). Da, diabetic treated with anastrozole.
the Newman-Keuls Multiple Comparison Test. Differences were considered statistically significant at $P < 0.05$.

**RESULTS**

**Metabolic parameters.** As expected, urine output and blood glucose levels were higher in both diabetic groups (D and Da) compared with ND animals, with no observed differences in either urine output or glucose levels between D and Da. While there were no differences in food intake between any of the treatment groups, D and Da animals had reduced body weight and an increased kidney-to-body weight ratio compared with ND (Table 1).

**UAE.** All three groups showed similar levels of UAE after 4 wk of diabetes. By 8 wk of diabetes, the D group had doubled the amount of albumin excreted while Da animals had similar values to ND. After 12 wk of diabetes, D animals had a 419% increase in UAE compared with the ND group and treatment with anastrozole resulted in a 42% reduction in UAE compared with D animals (Table 1).

**Sex hormone levels.** Plasma estradiol levels were elevated by 295% in the D compared with ND. Treatment with anastrozole was associated with a 39% reduction in circulating estradiol levels compared with D. Conversely, plasma testosterone was decreased by 80% in D animals compared with ND. Treatment with anastrozole in a 187% increase in circulating serum testosterone levels compared with D (Table 1).

**GSI and TIFI.** Glomerular and tubulointerstitial injury, as defined by mesangial expansion, inflammatory cell infiltration, and deposition of extracellular matrix, were assessed in renal sections using a semiquantitative scoring method. D animals were characterized by a 1.558% increase in GSI (Fig. 1, A and C) and by 287% in TIFI (Fig. 1, B and D) compared with ND, which was attenuated by 30 and 32%, respectively, following treatment with anastrozole.

**Aromatase, AR, and ERα protein expression.** While diabetes was associated with a 44% increase in aromatase protein expression, no further effect was observed after inhibition of aromatase activity (Fig. 2A). Diabetes was associated with a 43% reduction in the AR/ERα protein expression compared with ND. While not statistically significant, treatment with anastrozole resulted in a 35% increase in AR/ERα protein expression compared with D (Fig. 2B).

**Podocyte structural marker.** Podocin is an integral membrane protein located at the insertion site of the slit membrane and is thought to act as a scaffold protein necessary for the maintenance and regulation of the structural integrity of the slit diaphragm (10, 32). The D animals had an 18% reduction in podocin protein expression compared with ND that was attenuated following treatment with anastrozole (Fig. 3).

**Collagen type IV protein expression.** Immunohistochemistry showed collagen type IV to be localized in the extracellular space in ND. In the D animals, in addition to being present in the extracellular space, collagen type IV was also prominent in the expanded mesangial areas in the glomerulus, as well as in the tubulointerstitium. However, the pattern of staining in the Da group was more similar to that of the ND group (Fig. 4A). Quantitative analysis by Western blot confirmed these observations, with collagen type IV protein expression increasing by 69% in D compared with ND, while treatment with anastrozole resulted in a decreased collagen type IV protein expression by 29% compared with D (Fig. 4B).

**Inflammatory markers.** TGF-β protein expression, as measured by Western blotting, was increased by 53% in D compared with ND. However, treatment with anastrozole resulted in a 20% decrease in TGF-β protein expression compared with D. These results were confirmed with immunohistochemistry, which showed TGF-β protein to be immunolocalized to proximal tubules. The intensity of immunostaining was greater in D compared with ND and treatment with anastrozole partially reduced this (Fig. 5, A and B).

CD68-positive cells (indicating presence of activated macrophages) were abundantly present in the renal cortex of D animals, resulting in a 757% higher density of positive cells in D compared with ND, while treatment with anastrozole resulted in the density of positive cells by 50% compared with D (Fig. 6, A and B).

D animals had a 60% increase in TNF-α protein compared with ND animals, while treatment with anastrozole decreased TNF-α protein expression by 28% (Fig. 7A). IL-6 is secreted by multiple cell types, including fibroblasts, monocytes, and endothelial cells, as a 23- to 30-kDa phosphorylated and

![Fig. 2. Renal aromatase, androgen receptor (AR), and estrogen receptor α (ERα) protein expression. A: renal aromatase protein expression. Top: representative immunoblot of renal aromatase protein expression. Bottom: densitometric scans in relative optical density (ROD) expressed as a ratio of renal aromatase/β-actin. B: renal AR and ERα protein expression. Top: representative immunoblots of renal AR and ERα protein expression. Bottom: densitometric scans in ROD expressed as a ratio of AR/ERα.](http://ajprenal.physiology.org/10.1152/ajprenal.00307.2011)
variably glycosylated molecule (20). Analyzing the 28-kDa product of IL-6 by Western blot showed that D animals had a 40% increase in the amount of IL-6 protein compared with ND. However, treatment with anastrozole resulted in a 25% reduction in IL-6 protein expression in the Da group compared with the D group (Fig. 7B).

DISCUSSION

The present study confirms our previous report that diabetes in males is associated with decreased testosterone and increased estradiol levels (35), thus providing the rationale for blocking estradiol synthesis to prevent diabetes-associated renal injury. We demonstrate that inhibiting estradiol synthesis by treatment with an aromatase inhibitor partially attenuates the progression of diabetic renal disease by preventing the development of albuminuria, GSI, TIFI and reducing markers of inflammation. Since treatment with anastrozole did not alter blood glucose levels, this suggests that the beneficial effects of the treatment were not attributable to altered glycemic control, but are more likely due to lowering circulating estradiol levels. Therefore, these observations indicate that, unlike in diabetic females in which estradiol is renoprotective, in diabetic males estradiol may contribute to the development of renal injury associated with diabetes. Thus, blocking estradiol synthesis may be beneficial in the prevention or treatment of diabetic renal disease in males.

Aromatase is the rate-limiting enzyme in the conversion of androgens to estrogens and is found both in reproductive and nonreproductive tissues, including the kidney (1, 4, 14, 15, 23, 25, 30). We show that aromatase protein expression is increased in the diabetic animals, explaining the increase in estradiol levels. We thus used a nonsteroidal aromatase inhibitor, anastrozole, to suppress aromatase activity in the STZ-induced diabetic rat. Anastrozole is highly specific and able to inhibit estradiol synthesis without altering any other enzymes involved in steroid biosynthesis, has no intrinsic hormonal activity (5), and was originally developed for the treatment of postmenopausal women with breast cancer (22). However, to our knowledge, no other study has investigated the use of anastrozole for the treatment of inhibiting the diabetes-associated upregulation of estradiol synthesis. While treatment with anastrozole, and thus inhibition of estradiol synthesis, did reduce circulating plasma...
estradiol levels in the present study, it did not reduce it to levels observed in nondiabetic animals. One possible explanation for this incomplete suppression of estradiol synthesis is that the selected dose of 0.15 mg·kg\(^{-1}\)·day\(^{-1}\) may not have been sufficient for the complete suppression. However, previous reports showed that even a lower dose (0.1 mg·kg\(^{-1}\)·day\(^{-1}\)) of anastrozole given to female rats on day 2 or 3 of the estrus cycle was sufficient to block ovulation (5) and the use of higher doses did not further decrease plasma estradiol levels (6, 13). Additionally, these studies showed that while circulating

estradiol levels in the present study, it did not reduce it to levels observed in nondiabetic animals. One possible explanation for this incomplete suppression of estradiol synthesis is that the selected dose of 0.15 mg·kg\(^{-1}\)·day\(^{-1}\) may not have been sufficient for the complete suppression. However, previous reports showed that even a lower dose (0.1 mg·kg\(^{-1}\)·day\(^{-1}\)) of anastrozole given to female rats on day 2 or 3 of the estrus cycle was sufficient to block ovulation (5) and the use of higher doses did not further decrease plasma estradiol levels (6, 13). Additionally, these studies showed that while circulating
plasma estradiol levels may only appear to be suppressed by 50 to 60%, whole body aromatase activity was blocked by 97% with this same dose (5). It is important to note that while we observed no effect of anastrozole on aromatase protein expression, this has no bearing on estradiol levels, since anastrozole blocks aromatase activity, not protein expression. We thus conclude that the chosen dose of anastrozole also completely suppressed renal aromatase activity, as evidenced by the beneficial effects observed following treatment.

Results from our study show that blocking estradiol synthesis, using an aromatase inhibitor, in diabetic males decreases UAE throughout the development of the disease. In addition, treatment with anastrozole prevents the development of GSI and TIFI. Some of the mechanisms by which anastrozole mediates these effects is by decreasing extracellular matrix deposition (collagen type IV) and inflammatory markers (TGF-β, IL-6, and macrophage infiltration), which are key regulators of renal injury in diabetes (24). Several studies showed that podocyte injury plays a key role in the development of albuminuria and GSI (26, 31) and our data show that treatment with anastrozole prevented the diabetes-associated reduction in podocin protein, one of the components of the slit diaphragm (32). Collectively, these data suggest that the presence of estradiol may have a profibrotic and proinflammatory effect in the diabetic kidney and that inhibition of estradiol synthesis protects from diabetes-associated renal injury.

The fact that inhibition of estradiol synthesis is beneficial in diabetic renal disease in males suggests that estradiol may be contributing to the development of renal injury in diabetic males. This is in contrast to the effects of estradiol observed in experimental models of diabetic renal disease in females, in which the majority of studies show renoprotective effects of estradiol. Supplementation of estradiol to STZ-induced diabetic female rats either from the onset of diabetes or after 2 mo of untreated disease attenuates UAE, GSI, and TIFI via promoting extracellular matrix degradation and reducing inflammation (16, 17). Similar renoprotective effects of estradiol have been observed in the female Wistar rat remnant kidney model (2) and female Sprague-Dawley rats after 5/6 nephrectomy (12). These observations strongly support an anti-fibrotic and anti-inflammatory effect of estradiol in females. However, it is conceivable that estradiol may have opposing effects in males, as observed in the STZ-induced diabetic rat. Indeed, in male hyperlipidemic albuminuric rats, estradiol supplementation accelerates the development of renal disease by promoting proteinuria and GSI (11). Supplementation of estradiol in castrated male MF-1 mice slows and delays epithelialization in wound healing (7). Thus, estrogens appear to exert opposing effects in diabetic males and females; however, the mechanisms by which these opposing effects are mediated remain to be elucidated.

Confirming our previous report, diabetes in males is associated with a reduction in renal AR/ERα protein expression compared with ND and this correlates with increases in UAE, GSI, and TIFI (23). These observations suggest that the actions of estrogens in the diabetic kidney via the ERα may overpower the actions of testosterone via the AR and that this imbalance in the activation of ERα and ARs may contribute to the development of diabetic renal injury. While treatment with anastrozole tended to restore this imbalance in the AR/ERα, this was not statistically significant. We therefore conclude that the beneficial effects of anastrozole may not be mediated via regulation AR and ERα protein expression, but more likely via reducing estradiol levels.

To our knowledge, this is the first report describing the effects of an aromatase inhibitor in an experimental model of renal disease. However, in other disease models, such as traumatic hemorrhage, which is associated with elevated estradiol levels in males, treatment with an aromatase inhibitor restores the depressed immune response (3, 28). These data suggest that in disease processes in males characterized by elevated estradiol levels, such as diabetes, aromatase inhibition may provide an effective therapeutic treatment. It should be noted however that aromatase inhibition in the present study only resulted in partial renoprotection. Given that the STZ-induced male diabetic rat exhibits an imbalance in sex hormones, namely reduced testosterone and elevated estradiol levels, it may be the relative balance of both androgens and estrogens that determines the degree of renal damage. Indeed, our previous studies showed that low or no testosterone, as in castration, is detrimental to the diabetic kidney (35). Furthermore, a similar partial renoprotection in the castrated STZ-induced diabetic rat was also previously observed following supplementation with DHT (34). Based on these findings, it is conceivable that restoring both androgens and estrogens to their physiological levels by simultaneously treating with DHT...
and aromatase inhibition could afford full renoprotection in diabetes. Future studies are warranted to examine the renoprotective effect of this combined treatment.

In summary, the present study demonstrates that inhibition of estradiol synthesis, using an aromatase inhibitor, attenuates diabetic renal disease by decreasing markers of fibrosis and inflammation. Most importantly, these observations underscore the importance of sex hormones in the pathophysiology of diabetic renal disease and the need to further examine the mechanisms by which sex hormones regulate cellular processes in health and disease.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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