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Sex dependent differences in renal angiotensinogen as an early marker of diabetic nephropathy

Debora de Alencar Franco Costa[1*], Mihail Todiras[2*], Luciana A. Campos[1], Jose Cipolla-Neto[3], Michael Bader[2], Ovidiu C. Baltatu[1]

[1] Center of Innovation, Technology and Education—(CITE), University Camilo Castelo Branco, Sao Jose dos Campos, Brazil

Technology Park, Sao Jose dos Campos, Brazil

[2] Max-Delbrueck Center for Molecular Medicine, Berlin, Germany

[3] Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil

* contributed equally to the study

Corresponding author:

Ovidiu Constantin Baltatu, MD PhD

Center of Innovation, Technology and Education—(CITE), Camilo Castelo Branco University (UNICASTELO), Sao Jose dos Campos Technology Park, Avenida Doutor Altino Bondensan 500, Sao Jose dos Campos – SP, 12247-016, Brazil

E-mail: ocbaltatu@gmail.com

Running Title:

Renal angiotensinogen in diabetic nephropathy

Abstract

AIM. The renal renin-angiotensin system (RAS) has been implicated in the pathogenesis of diabetic nephropathy. The aim of this study was to investigate sex differences in renal renin-angiotensin system (RAS) and the roles of androgens in diabetes-associated renal injury.

METHODS. Renal injury and fibrosis was studied in streptozotocin-induced diabetic rats by albuminuria and by gene expression of collagen-I and fibronectin. RAS was investigated by analyzing the plasma angiotensinogen (AOGEN) and renin activity (PRA) and their renal gene expression. Also, a group of diabetic rats was treated with the antiandrogen flutamide.

RESULTS. Albuminuria was significantly lower in diabetic females than in males (1.2 [0.8-1.5] vs. 4.4 [2.2-6.1] mg/24h, data are median [IQR] values, $p<0.05$). Renal AOGEN mRNA levels were increased by diabetes in males (8.1 ± 0.8 % in diabetes vs 0.8 ± 0.2 % in control, $p< 0.001$) but not in females (1.0 ± 0.1 % in diabetes vs 0.8 ± 0.1 % in control, $p>0.05$), as were collagen-I and fibronectin mRNAs. Furthermore, AOGEN mRNA levels were strongly correlated with albuminuria (Spearman r = 0.64, 95%[CI] 0.36 to 0.81, $p< 0.0001$). Diabetes decreased PRA, renal renin mRNA and plasma AOGEN in both females and males. Antiandrogen treatment decreased albuminuria only in diabetic males without affecting the endocrine or renal RAS.

CONCLUSIONS. These data indicate that renal but not hepatic AOGEN or renin is positively associated with diabetic albuminuria and contribute to the sex-dependent differences in renal injury. Androgens may contribute to albuminuria in male independently of the RAS.

Keywords: diabetes; renal angiotensinogen; albuminuria; androgens; sexual dimorphism

Introduction

The leading causes of chronic kidney disease are hypertensive nephropathy and diabetes. Novel therapeutic strategies are being explored to delay progression of chronic nephropathy to end-stage renal disease. Current knowledge indicates that an effective therapy would not only target a blood pressure lowering effect but also would have a direct tissue protective effect (Rashid and Mende, 2011). The generally accepted surrogate marker for chronic nephropathy is albuminuria. However, accumulating evidence is puzzling the predictive value of albuminuria in nephropathy development (Dronavalli et al., 2008). In fact, one third of patients with microalbuminuria have already renal functional decline (Perkins et al., 2007). Therefore, characterizing biomarkers that are implicated in the pathogenesis of chronic kidney disease is subject of contemporary investigations (Shlipak and Day, 2013). The relationship between the renin-angiotensin system (RAS) and the progression of diabetic renal disease has been a subject of large investigation. A chronic overexpression of endocrine or paracrine RAS, localized in several cardiovascular organs, is considered to be an important contributor to the pathophysiology of chronic kidney disease (Campos et al., 2011, Baltatu et al., 2011, Skov et al., 2014). RAS inhibitors are utilized as first-line agents for hypertensive or

diabetic patients with progressive chronic kidney disease (Steckelings et al., 2011). Therapeutic efficacy of RAS inhibitors may importantly depend on the stage of disease progression, conditional on the pathological activation of endocrine and/or tissue RAS (Goncalves et al., 2011). Also, sexual dimorphisms have been observed in pathogenesis and treatment of chronic kidney disease induced by diabetes or hypertension (Kautzky-Willer et al., 2013, Baltatu et al., 2003).

In the present study we aimed at investigating sex dependent differences in RAS associated with renal injury in diabetes. Possible correlations between RAS components and albuminuria, as surrogate marker of diabetic nephropathy were studied. Also, roles of androgens in diabetes-associated renal injury were explored through anti-androgen treatment.

Methods

All procedures complied with guidelines from the American Physiological Society, and institutional review board approved the study.

Experimental diabetes-associated renal injury was studied in female and male rats Sprague-Dawley rats (6 weeks old) for 12 weeks after the injection of streptozotocin (65mg/kg, ip). DM was considered when glucose reached levels greater than 270 mg/dL. Streptozotocin-diabetic rats may develop chronic kidney injury, while they are normotensive. To study the involvement of androgens in the development of diabetic renal injury, we treated them with flutamide (specific nonsteroidal competitive

antagonist of the androgen receptor, 30 mg/kg/day subcutaneously). Each investigational rat group comprised 6 rats. Rats were housed in groups of three in standard cages, synchronized to a 12-hour light–dark cycle, at ambient temperature 23 ± 2°C. A standard rat diet and tap water were supplied ad libitum.

Renal endpoints studied were albuminuria and gene expression of collagen I and fibronectin. Albuminuria was determined in 24 hours collected urine by Immundiagnostics (Bensheim, Germany) using a specific ELISA (Baltatu et al., 2014). To evaluate kidney fibrosis, collagen I and fibronectin mRNA was determined by real-time RT-PCR SYBR-Green assay on 7500HT Fast Real-Time PCR System, using Power SYBR Green (Applied Biosystems, Foster City, California, USA). The variation in the amount of samples was normalized by parallel amplification with the target samples with ß-actin gene. Primer sequences were:

collagen I F 5´-CTAGCCTCACACACTTAGTGATCTGC-3´;

collagen I R 5´-CTAGGTGTGTGGGTGGCTTT-3´;

fibronectin F 5'-CCCTCCATTTCTGAGTGGTC-3';

fibronectin R 5'-GACAGTGAGTCCTGTGGGGT-3';

ß-Actin F CCTCTGAACCCTAAGGCCAA;

ß-Actin R AGCCTGGATGGCTACGTACA.

The cycling conditions utilized in SYBR-Green assay were: 15 min at 95^0C of polymerase activation followed by 40 cycles of 3-step PCR consisting of 15 s at 95^0C of denaturation, 30 s for both 60^0C of annealing and 72^0C of extension.

Systolic blood pressure (SBP) was measured in conscious rats by a non-invasive tail-cuff method using automated IN125/R in conjunction with PowerLab data acquisition systems and MLA5024 Rodent Restrainer (ADInstruments, NSW, Australia).

Plasma renin activity (PRA) and angiotensinogen were determined in plasma obtained from trunk blood as previously described (Baltatu et al., 2002). The PRA was determined using a trapping immunoassay while AOGEN through radioimmunoassay. The renal gene expressions of renin and angiotensinogen were determined through real-time RT-PCR SYBR-Green assay. Primer sequences used were:

Renin F 5´-GCTACATGGAGAATGGGACTGAA-3´;

Renin R 5´-ACCACATCTTGGCTGAGGAAAC-3´;

AOGEN F 5´-AGCACGGACAGCACCCTATT-3´;

AOGEN R 5´-AGAACTCATGGAGCCCAGTCA-3´.

Statistical Analysis

Two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test was performed using GraphPad Prism version 6.0e for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com. Comparisons between control female and male groups were by unpaired T test. $P < 0.05$ was considered to be statistically significant.

Results

Renal end-points of diabetic renal injury and fibrosis.

In experimental DM, while the levels of albuminuria were not significantly different within the female groups, those in male diabetic group were significantly higher than male control, and the Flutamide-treated male diabetic group was significantly different from the male control or diabetic groups (Figure 1.A.). Renal levels of collagen I (Figure 1.B.) and fibronectin (Figure 1.C.) mRNAs were not significantly different within the female groups, while those in male diabetic-untreated and Flutamide-treated diabetic groups were significantly higher than male control. Systolic blood pressure was significantly increased in male but not female diabetic rats in comparison with respective control groups (Figure 2).

Plasma renin and angiotensinogen.

Females had significantly lower PRA than males (Figure 3.A.). The PRA in both female and male diabetic-untreated and Flutamide-treated diabetic groups were significantly lower than respective control groups (Figure 3.A.).

Females had significantly lower plasma angiotensinogen levels than males (Figure 3.B.). The plasma angiotensinogen levels in both female and male diabetic-untreated and Flutamide-treated diabetic groups were significantly lower than respective control groups (Figure 3.A.).

Plasma renin activity or angiotensinogen levels in Flutamide-treated diabetic groups were not significantly different from the respective diabetic-untreated groups, neither in females nor in males.

Renal renin and angiotensinogen.

The renal renin mRNA levels in both female and male diabetic-untreated and Flutamide-treated diabetic groups were significantly lower than respective control groups (Figure 4.A.).

Renal AOGEN mRNA levels were significantly increased by diabetes in males but not in females in comparison with the respective control non-diabetic groups (Figure 4.B.).

Correlations between renin-angiotensin system and the surrogate marker of diabetic nephropathy.

Figure 5 shows correlations of albuminuria with renal AOGEN mRNA, renal renin mRNA, plasma AOGEN or PRA. Renal AOGEN mRNA levels were strongly correlated with albuminuria (Spearman r = 0.64, 95% confidence intervals [CI] 0.36 to 0.81, $p < 0.0001$). On the opposite, plasma AOGEN had a weak negative correlation, while PRA and renal renin mRNA were significantly negative correlated with albuminuria.

Discussion

The present findings provide evidence that renal but not hepatic AOGEN or renin is positively associated with diabetic albuminuria and may contribute to the sex-dependent differences in diabetes-associated renal injury and fibrosis. Androgens may contribute to the aggravated diabetes-associated renal injury in males independently of the RAS.

The pathogenesis of diabetic nephropathy is directed by a chronic activation of several local and systemic factors including RAS that are responsible for multiple mechanisms related to kidney injury (Dronavalli et al., 2008). A recent individual patient meta-analysis has challenged early changes in proteinuria as a surrogate end point for kidney disease progression (Inker et al., 2014). Moreover, MacIsaac et al. contested the usefulness of microalbuminuria in diabetic nephropathy and call for the development of "risk markers apart from those related to the traditional 'albuminuric pathway' to renal impairment" (MacIsaac et al., 2014). Considering that the renal RAS plays a central role in the pathogenesis of chronic nephropathy such as induced by diabetes (Van Buren and Toto, 2013) and in consequence albuminuria, it appears reasonable to argue that an increase in renal angiotensinogen may precede albuminuria. Indeed, research from the Navar group indicates that urinary angiotensinogen may provide index of the activated intrarenal RAS as a predictive marker of chronic nephropathy (Kobori et al., 2013, Mills et al., 2012). Saito et al. have further demonstrated that increased urinary angiotensinogen is precedent to increased urinary albumin in patients with type 1 diabetes (Saito et al., 2009, Kamiyama et al., 2012). In a study with patients with type 2 diabetes,

hypertension, and albuminuria, Persson et al. have concluded that urinary angiotensinogen is a marker of filtration barrier injury rather than intrarenal RAS activity (Persson et al., 2013). Besides, van den Heuvel et al. indicated that urinary renin more closely reflects renal RAS activity than urinary angiotensinogen in diabetes (van den Heuvel et al., 2011). This apparent contradiction may be because RAS components have been determined at different stages of the diabetic pathology. In the present study we determined both circulating levels of angiotensinogen, renin and their renal levels of gene expression. The renal angiotensinogen gene expression levels were strongly correlated with the levels of albuminuria. This was not the case for plasma angiotensinogen. On the opposite, the plasma renin activity and renal renin mRNA levels were inversely correlated with albuminuria. Hence, the present study provides further support for renal angiotensinogen as a good predictor of diabetic nephropathy. Further studies should investigate the evolution of endocrine vs renal RAS associated with the progression of disease, when complications such as hypertension occur (Persson et al., 2013, van den Heuvel et al., 2011).

A sexual dimorphism on the progression of renal disease has become an area of active investigation (Silbiger and Neugarten, 2008, Neugarten, 2002). In diabetic people, men are at higher risk than women for microvascular complications, such as nephropathy (Abbate et al., 2012). The mechanisms responsible for this sexual dimorphism in diabetic pathology represent a subject of actual investigation (Nedungadi and Clegg, 2009). In our study, renal angiotensinogen synthesis was increased by diabetes in male but not female rats and was associated with

albuminuria and renal fibrosis only in males. Thus considering renal angiotensinogen as a marker of renal RAS activation (Kobori et al., 2013, Mills et al., 2012), it indicates that the renal RAS contribute to the sexual dimorphism met in diabetic renal injury.

Since renin is downregulated in this incipient phase of diabetes, alternative pathways for angiotensinogen cleavage may be considered. Enzymes other than the juxtaglomerular renin may provide alternative pathways for angiotensin II, such as prorenin from collecting duct (Kang et al., 2008) and chymase (Park et al., 2013) (angiotensin I-degrading enzyme (Nishimura et al., 1996)), whose synthesis is also increased in diabetes. Another candidate is cathepsin L that belongs to the cathepsin family able to generate angiotensin II from angiotensinogen (Legedz et al., 2004, Watanabe et al., 1989) and that is localized in the proximal tubule (Bauer et al., 2011). Besides, the Bauer group found cathepsin L strongly associated with the degree of renal injury and proposed it as a potential sex-specific biomarker for renal damage (Bauer et al., 2011).

In the present study, androgen receptor blockade attenuated diabetes-induced albuminuria but not renal fibrosis. Besides androgens, female sexual hormone may also contribute to the development of diabetic end-organ injury because blocking estradiol synthesis in male diabetic rats lowers albuminuria and renal fibrosis (Manigrasso et al., 2011). Since estradiol is also natural ligand for androgen receptor (Yeh et al., 1998), the antialbuminuric effects of the receptor blockade in our study may inhibit the effects of both androgens and estrogens in males. The fact that the

renal anti-fibrotic effect was observed after inhibition of estradiol synthesis and not androgen receptor blockade may suggest that estrogen receptors localized in the mesangial cells of males (Irsik et al., 2013) may contribute to this effect. This appears to be in contrast to the renoprotective effects of estradiol in diabetic renal disease in females (Mankhey et al., 2005). Estrogen receptor could therefore count for the protection against end-organ injury in female diabetic rats. A possible interference between androgens and RAS was also investigated. In the present study, androgen blockade did not decrease an already declined renin activity and synthesis in diabetes, contrarily from malignant hypertension with overactive RAS and consequent renal end-organ injury (Baltatu et al., 2002, Baltatu et al., 2003).

In summary, our study provides evidence for a sexual dimorphism in renal RAS activated in diabetes and further support the concept of renal angiotensinogen as an early biomarker in diabetic nephropathy. We envisage that further translational studies will advance our knowledge to further characterize urinary angiotensinogen in chronic nephropathy as a predictive biomarker.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1: Renal injury in experimental diabetes. (A) Albuminuria; (B) Renal collagen I mRNA levels; (C) Renal fibronectin mRNA levels. DM = diabetes mellitus group, DM_FLU = diabetes mellitus treated with flutamide group. Data are mean with

SEM; * $p < 0.05$ compared with the respective control group; # $p < 0.05$ compared with the DM_FLU group.

Figure 2: Systolic blood pressure in experimental diabetes. DM = diabetes mellitus group, DM_FLU = diabetes mellitus treated with flutamide group. Data are mean with SEM; * $p < 0.05$ compared with the respective control group.

Figure 3: Plasma renin activity (A) and angiotensinogen (B) in experimental diabetes. DM = diabetes mellitus group, DM_FLU = diabetes mellitus treated with flutamide group. Data are mean with SEM; * $p < 0.05$ compared with the respective control group and between the control groups of females and males.

Figure 4: Renal renin (A) and angiotensinogen (B) gene expression in experimental diabetes. DM = diabetes mellitus group, DM_FLU = diabetes mellitus treated with flutamide group. Data are mean with SEM; * $p < 0.05$ compared with control group.

Figure 5: Spearman's rank correlations of albuminuria with: plasma renin activity (PRA), plasma angiotensinogen (AOGEN), renal renin mRNA levels and renal AOGEN mRNA levels; plotted data are Spearman's ρ coefficient with 95% confidence intervals. * p (two-tailed) < 0.05.