Acta Histochemica 118 (2016) 183–193

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Antioxidant diet and sex interact to regulate NOS isoform expression and glomerular mesangium proliferation in Zucker diabetic rat kidney

Yuriy Slyvka[a,c], Ramiro Malgor[a,b,c], Sharon R. Inman[a,b,c], Julia Ding[e], Victor Heh[d], Felicia V. Nowak[a,b,c,*]

[a] Department of Biomedical Sciences, Ohio University, Athens, OH 45701, USA
[b] Program in Molecular and Cellular Biology, Ohio University, Athens, OH 45701, USA
[c] The Diabetes Institute at Ohio University, Ohio University, Athens, OH 45701, USA
[d] OUHCOM Office of Research & Grants, Ohio University, Athens, OH 45701, USA
[e] College of Natural Sciences, School of Biological Sciences, University of Texas at Austin, TX 78713, USA

ARTICLE INFO

Article history:
Received 29 September 2015
Received in revised form
30 December 2015
Accepted 31 December 2015

Keywords:
Diabetic nephropathy
Obesity
Antioxidant diet
Sex difference
Nitric oxide synthases
Mesangial matrix

ABSTRACT

Oxidative stress contributes substantially to the pathophysiology of diabetic nephropathy (DN). Consumption of an antioxidant-fortified (AO) diet from an early age prevents or delays later development of DN in the Zucker rat female with type 2 diabetes. We hypothesize this is due to effects on mesangial matrix and renal nitric oxide synthase (NOS) distribution and to sex-specific differences in NOS responses in the diabetic kidney. Total glomerular tuft area (GTA) and PAS-positive tuft area (PTA), endothelial (e), neuronal (n) and inducible (i) NOS were quantified in males and females on AO or regular (REG) diet at 6 and 20 weeks of age. eNOS was observed in glomeruli and tubules. nNOS predominantly localized to tubular epithelium in both cortex and medulla. iNOS was expressed in proximal and distal tubules and collecting ducts. Sex, diabetes duration and AO diet affected the distribution of the three isoforms. GTA and PTA increased with duration of hyperglycemia and showed a negative correlation with renal levels of all NOS isoforms. AO diet in both genders was associated with less PAS-positive staining and less mesangial expansion than the REG diet, an early increase in cortical iNOS in males, and sex-specific changes in cortical eNOS at 20 weeks. These effects of AO diet may contribute to sex-specific preservation of renal function in females.

1. Introduction

Diabetic nephropathy (DN) develops in up to 40% of patients with type 2 diabetes mellitus (T2DM) and is the leading cause of end stage renal disease (ESRD) in the United States. Obesity is a contributing factor to the debilitating pathologies associated with T2DM (Collins and Foley, 2009; Harvey, 2003; Schena and Gesualdo, 2005; Sego et al., 2007), including DN. DN that develops in individuals with both obesity and diabetes (T2bDM) may have pathological features not seen with diabetes alone.

On the structural level, DN is characterized by progressive expansion of the mesangial matrix associated with glomerular hypertrophy, thickening of glomerular basement membrane with later mesangiolysis and formation of Kimmelstiel–Wilson nodules (Abrass, 1995; Kanetsuna et al., 2007; Nakagawa et al., 2007). Progressive expansion of the mesangium ultimately occludes the glomerular capillaries, a central mechanism in the development of ESRD (Steffes et al., 1989). DN is also characterized by tubular dysfunction and primary tubulointerstitial injury may play a role in initiating loss of renal function (Bonventre, 2012; Najafian et al., 2011; Phillips and Steadman, 2002). High glucose and production of advanced glycation end products stimulate proinflammatory cytokines and these, in turn, contribute to increases in intracellular reactive oxygen species (ROS) in renal tubular epithelial cells (Han et al., 2005; Tang et al., 2011). This has considerable impact as the tubulointerstitium accounts for more than 90% of kidney volume (Bonventre, 2012).

Abbreviations: AO, antioxidant; DN, diabetic nephropathy; ECM, extracellular matrix; Enos, endothelial nitric oxide synthase; ESRD, end stage renal disease; GTA, glomerular tuft area; IHC, immunohistochemistry; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PAS, periodic acid Schiff stain; PTA, PAS-positive glomerular tuft area; T2DM, type 2 diabetes mellitus; T2bDM, type 2 diabetes associated with obesity.

* Corresponding author at: Department of Biomedical Sciences, 228 Irvine Hall, Ohio University, Athens, OH 45701, USA.
E-mail address: nowak@ohio.edu (F.V. Nowak).

http://dx.doi.org/10.1016/j.acthis.2015.12.011
0065-1281/

Multiple mechanisms contribute to the development of DN. One that is supported by numerous studies is the role of impaired nitric oxide (NO) synthesis in the development of renal dysfunction (Baylis, 2008a,b; Komers and Anderson, 2003). In the normal kidney, this vasodilator helps regulate renal hemodynamics and maintain diuresis and natriuresis (Eppel et al., 2003; Majid and Navar, 2001; Mount and Power, 2006; Wilcox, 1998).

The obese Zucker rat (*fa*/*fa*) model of DN is characterized by a gene mutation (*fa*/*fa*) that results in lack of leptin receptors and development of T2bDM with DN (Chander et al., 2004; Coimbra et al., 2000; Ionescu et al., 1985; Zucker and Antoniades, 1972). At 6 weeks of age hyperglycemia develops and by 20 weeks the rats exhibit impaired renal function and glomerulosclerosis (Chander, et al., 2004; Coimbra et al., 2000; Ionescu et al., 1985). Sexual dimorphism in development of DN has been described in patients (Baylis, 2008b, 2009; Denton and Baylis, 2007) and has also been described in the obese diabetic Zucker rat (Slyvka et al., 2009).

We have previously shown that an antioxidant-fortified (AO) diet is associated with preservation of renal function in the obese female Zucker rat and that this correlates with effects on protein levels of endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Slyvka et al., 2009; Slyvka et al., 2011), the three NOS isoforms that are responsible for NO production in kidney (Alderton et al., 2001). In addition, multiple metabolic parameters were studied in obese diabetic Zucker rats and compared between those on regular (REG) and AO-fortified diets. Beneficial differences that were found include: lower body weight of males at 6 weeks of age (AO < REG), higher glomerular filtration rate (GFR), with less glomerular and tubulo-interstitial pathology in females at 20 weeks, and lower blood glucose of females at 6 and 13 weeks (AO < REG). However the relationship between glomerular matrix expansion and NO synthase (NOS) isoform expression and distribution in both glomeruli and tubules has not previously been examined.

The goal of the present study is to measure glomerular mesangial matrix proliferation and observe the distribution of eNOS, nNOS, and iNOS in the kidney cortex and medulla of obese Zucker rats and to characterize the effects of sex, age and AO diet on these parameters. The findings will be interpreted in light of previously reported effects of the AO diet on renal function and structure, metabolic profile and NO levels in these rats (Slyvka et al., 2009, 2011) and will contribute to our understanding of the effects of sex and AO on the pathophysiology of DN in T2bDM.

2. Materials and methods

2.1. Animals and diets

Studies were conducted on obese (*fa*/*fa*) male ($n = 24$) and female ($n = 22$) Zucker rats (Table 1) (Harlan-Sprague Dawley, Indianapolis, IN) obtained at four weeks of age. Animals were housed under controlled conditions of lighting, temperature and humidity. Rats were fed ad libitum the REG rat diet 5012 or AO fortified diet, (Purina Mills, Inc., St. Louis, MO). The REG diet contained 32 IU α-tocopherol, 0.23 ppm selenium, 71 ppm zinc, 12 ppm copper, 69 ppm manganese, and 4.3 ppm β-carotene per kg food, and plain tap water. The AO diet consisted of rat diet 5012 supplemented with increased levels of several substances with known antioxidant effects (Vega-Lopez et al., 2004; Wiernsperger, 2003): 160 IU α-tocopherol/kg food, 1.15 ppm selenium, 150 ppm zinc, 60 ppm copper, 150 ppm manganese and 21.5 ppm β-carotene per kg food, and ascorbic acid fortified water, 1000 U/l.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of Ohio University (Permit Number: 27-2956). Rats were euthanized with 100 mg/kg i.v. pentobarbital and all efforts were made to minimize suffering. Kidneys were harvested at two time points: 6 weeks of age when hyperglycemia is first evident, and 20 weeks of age when there is significant nephropathy (Coimbra et al., 2000; Ionescu et al., 1985; Slyvka et al., 2009).

2.2. Tissue preparation

The kidneys were rapidly removed and half of one kidney was fixed in 10% buffered formalin overnight at 4C, and embedded in paraffin. Samples were prepared from 8 groups of animals as detailed in Table 1.

2.3. Mesangial matrix

To quantify the glomerular mesangial matrix area, 4-μm thick sections were prepared, PAS-stained and examined at 400× magnification. Fifteen randomly selected consecutive glomeruli of good quality with centralized polarity were analyzed in each kidney section. All sections were examined and scored by two independent observers with excellent correlation between the two observers. Image-Pro Plus 5.1 software was used to measure the area of the glomerular tuft and quantify the PAS positive area within the tuft. The level of mesangium expansion was assessed blindly by two independent observers. The ratio of PAS-positive tuft area (PTA) (μm^2) to the total glomerular tuft area (GTA) (μm^2), PTA/GTA (%) was calculated.

2.4. Immunohistochemistry (IHC)

For IHC, sections of 4-μm thickness were pretreated for 60 min at 60C after de-paraffination, and hydration antigen retrieval was performed in a crock pot at 90C for 40 min in 10 mM sodium citrate buffer, pH 6.0. Slides were cooled to room temperature, then treated with 3% H_2O_2 in PBS (Fisher Scientific, Pittsburg, PA), pH 7.4, for 30 min to block endogenous peroxidase. To block nonspecific binding, sections were incubated for 30 min at room temperature in 1% bovine serum albumin (Sigma–Aldrich, St. Louis, MO) in PBS, pH 7.4, followed by streptavidin and biotin blocking according to the manufacturer's protocol (#SP-2002, Vector Laboratories, Burlingame CA). Sections were incubated overnight at 4C with one of the following isoform specific primary rabbit polyclonal IgG antibodies: anti-eNOS 1.5 μg/ml, SC-654 (Santa Cruz Biotech, Santa Cruz, CA), anti-nNOS 1 μg/ml, #7155, (Sigma–Aldrich) and anti-iNOS 2 μg/ml, ab3523, (Abcam Inc., Cambridge, MA). All primary antibodies were validated by their manufacturers for use in rats and were certified by the manufacturers to exhibit no cross reactivity among the different isoforms. Isotype negative controls were performed on a consecutive section using isotypic normal rabbit IgG at equivalent concentration for each primary antibody (#10500C, Invitrogen Corporation, Camarillo, CA). Slides were washed and 0.5 μg/ml of the biotinylated secondary goat anti-rabbit antibody (# B-2770, Thermo Fisher, Rockford, IL) was added for 1 hour at room temperature. Immunohistochemical reactivity was localized by reaction with diaminobenzidine (Sigma-Aldrich, St. Louis, MO) using DAB Enhanced Liquid Substrate System for Immunohistochemistry (D 3939, Sigma Aldrich). Following deposition of the oxidized insoluble brown DAB end-product, the immunostained sections were counterstained with hematoxylin for 15 s, dehydrated and mounted.

Slides from both cortex and medulla from each animal were examined blindly by light microscopy at 200-fold magnification. The numbers of NOS isoform positive stained tubules and percentage of positively stained glomeruli (glomerular score) were counted