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Short Communication

Renal disease in rats with Type 2 diabetes is associated with decreased renal nitric oxide production

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Abstract

Aims/hypothesis. In several other models of chronic renal disease, decreases in renal nitric oxide activity and nitric oxide synthase (NOS) protein abundance have been demonstrated. Here, we studied diabetic obese Zucker (ZDF Gmi *fa/fa*) rats that develop severe hyperglycaemia and renal disease, together with their lean control animals, to determine if renal nitric oxide deficiency also occurs in this model.
Methods. Obese Zucker rats aged 10 to 12 weeks were maintained on Purina 5008 diet until 4, 8, or 11 months of age and compared with similarly maintained, 4- and 11-month-old lean Zucker rats. NOS activity and abundance of endothelial NOS (eNOS) and neuronal NOS (nNOS) were measured on homogenates of kidney cortex. Blood was analysed for glucose, lipids, creatinine, and blood urea nitrogen and kidney tissue was obtained for histology.
Results. Obese rats exhibited severe hyperglycaemia from 4 months of age and developed increasing hyperlipidaemia, proteinuria, and decreasing renal function with age compared to lean counterparts. At 4 months cortical NOS activity and nNOS abundance were lower in obese rats than in lean ones. At 11 months NOS activity remained depressed and nNOS abundance had declined further in obese rats. Glomerulosclerosis in the obese rats was mild at 4 months, becoming severe by 11 months. Lean rats had only mild age-dependent increases in glomerular injury.
Conclusions/interpretation. The chronic renal disease that occurs in hyperglycaemic, obese Zucker rats is associated with decreased renal cortical nitric oxide production and increasing renal injury, although the changes do not resemble those of diabetic nephropathy in man.

Keywords Clearance · Creatinine · Endothelial nitric oxide synthase · Glomerulosclerosis · Hyperglycaemia · Kidney cortex · Neuronal nitric oxide synthase · Proteinuria · Type 2 diabetic nephropathy · Western blot

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Abbreviations: eNOS, endothelial nitric oxide synthase · ESRD, end-stage renal disease · nNOS, neuronal nitric oxide synthase · NOS, nitric oxide synthase · NO_X, nitrite + nitrate · $U_{NOX}V$, urinary NO_X excretion

Introduction

Clinical and animal studies have shown reduced nitric oxide production in chronic renal disease and end-stage renal disease (ESRD) [1, 2, 3]. Diabetes is the leading cause of renal disease in man, with about 30% of diabetic patients developing nephropathy, most of whom will progress to ESRD. We therefore decided to investigate the nitric oxide system in a rat model of diabetes.

The most commonly studied model of diabetes is streptozotocin-induced Type 1, but approximately 90% of diabetes in man is Type 2 diabetes. Interest has focused on the obese Zucker *fa/fa* rat, which develops obesity, early hyperinsulinaemia, hyperlipidaemia, hy-

perfiltration and significant renal injury [4, 5]. However, blood glucose levels are variable, and when elevated, never achieve the values of 19–22 mmol/l seen with streptozotocin-induced diabetes [4]. Furthermore, the primary causes of renal disease in outbred Zucker rats appear to be severe dyslipidaemia and hyperlipidaemia [4]. The inbred obese Zucker rat, ZDF Gmi-*fa/fa*, has similar characteristics, but also develops severe hyperglycaemia (when maintained on Purina 5008 diet) and renal disease [6]. In this study we evaluated and compared the renal nitric oxide system in these rats with that of their lean control counterparts.

Materials and methods

Our studies on inbred obese male Zucker and lean rats (Genetic Models, Indianapolis, Ind., USA) aged 10 to 12 weeks were conducted in agreement with and approved by the Animal Care and Use Committee at West Virginia University. Obese rats were aged 4 (n=5), 8 (n=8) and 11 (n=8) months. Lean rats were aged 4 (n=5) and 11 months (n=5). All rats were fed Purina 5008 diet to maintain hyperglycaemia in obese animals. One week before being killed, rats were put on to low NO_X diet for 36 to 48 h, placed without food in metabolic cages for an 8-h urine collection, then returned to the Purina 5008 diet. Tissues were removed and terminal blood was collected for measurement of NO_X, creatinine, blood urea nitrogen, HDL, LDL, total cholesterol and triglycerides. Blood glucose was measured from tail bleeds before anaesthesia. Urine was assayed for creatinine, total protein and NO_X concentrations.

In vitro nitric oxide synthase (NOS) activity and abundance was measured as described [7], except that for NOS inhibition a combination of *N*-monomethyl-L-arginine (5 mmol/l) and trifluoperazine (2 mmol/l) was employed in the NOS activity assay. Final NOS activity was expressed as total activity minus activity not inhibitable with *N*-monomethyl-L-arginine + trifluoperazine.

A pathologist ranked kidney sections on a blinded basis, for overall injury (scale: 1–31, with 1 reflecting least severe injury). The percentage of glomeruli showing segmental or global sclerosis was determined. Glomerular volume was estimated on the basis of the mean glomerular cross-sectional surface area determined for 50 to 100 glomeruli and calculated using established formulas [5].

Results

Obese Zucker rats were severely hyperglycaemic by 4 months and remained so at 8 and 11 months (Table 1). At 4 months of age they had very high levels of triglycerides, HDL, LDL and total cholesterol compared to lean counterparts. In obese rats, LDL cholesterol increased progressively with age while plasma HDL cholesterol remained constant (Table 1), increasing the LDL : HDL ratio. Creatinine clearance was lower in obese than in lean rats at 4 months with a further decline by the 11th month (Table 2). At 4 months proteinuria was minimal in lean rats but moderate in obese ones, increasing progressively with age (Table 2).

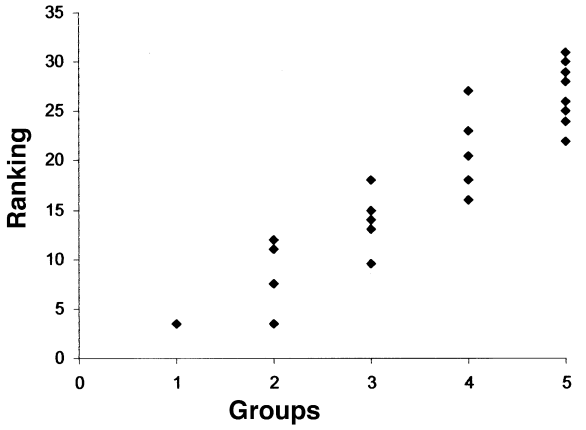

Fig. 1. Overall renal injury ranking with 1 reflecting the least severe injury and 31 the most severe damage. Groups 1 and 2: 4- and 11-month-old lean rats respectively; groups 3, 4, and 5: 4-, 8- and 11-month-old obese rats respectively. No glomerular injury was seen in rats with a rank of 14 or less

The appearance of pelvic dilation (hydronephrosis) was highly variable with a similar incidence in both rat groups. Histological ranking showed that glomerular and interstitial injury increased with age in lean and obese rats (Fig. 1). Lean rats showed no evidence of glomerular or tubular disease at 4 months, while by 11 months a few had tubular injury manifested as tubular dilatation, small hyaline casts, or focal mild interstitial nephritis. Lean rats also showed minimal glomerular sclerosis (Fig. 2a).

At 4 months, one-third of obese Zucker rats showed mild glomerular injury (Fig. 2b), which included focal, segmental glomerular sclerosis with hyalinosis lesions and occasional adhesions of the tuft to Bowman's capsule. By 8 months, all obese rats showed glomerular injury but five of six still had only focal damage, although more severe than at 4 months (Fig. 2c). All obese rats had greater glomerular injury at 10 to 11 months, with more global sclerosis and severe involvement of individual glomeruli (Fig. 2d); several had large dilated tubules with prominent hyaline casts. Quantitative focal and segmental glomerulosclerosis is shown in Table 2. The glomerular volume of undamaged glomeruli was higher in 4-month-old obese rats than in lean rats, with further increases with age (Table 2).

Urinary NO_X excretion ($U_{NOX}V$) (Table 2) was unchanged with age in lean rats. At 4 months obese rats had similar $U_{NOX}V$ to lean ones, becoming significantly greater at 11 months. Also, the plasma NO_X : creatinine ratio was always higher in obese rats (Table 2). Renal in vitro NOS activity of the cortex was lower in the obese than in lean rats at 4 months and remained depressed at 11 months (Table 2). Protein abundance of renal cortical neuronal nitric oxide synthase (nNOS) was lower in 4-month-old obese rats than in lean rats and fell further by 11 months (Table 2). Renal cortical endothelial nitric oxide synthase (eNOS) abundance

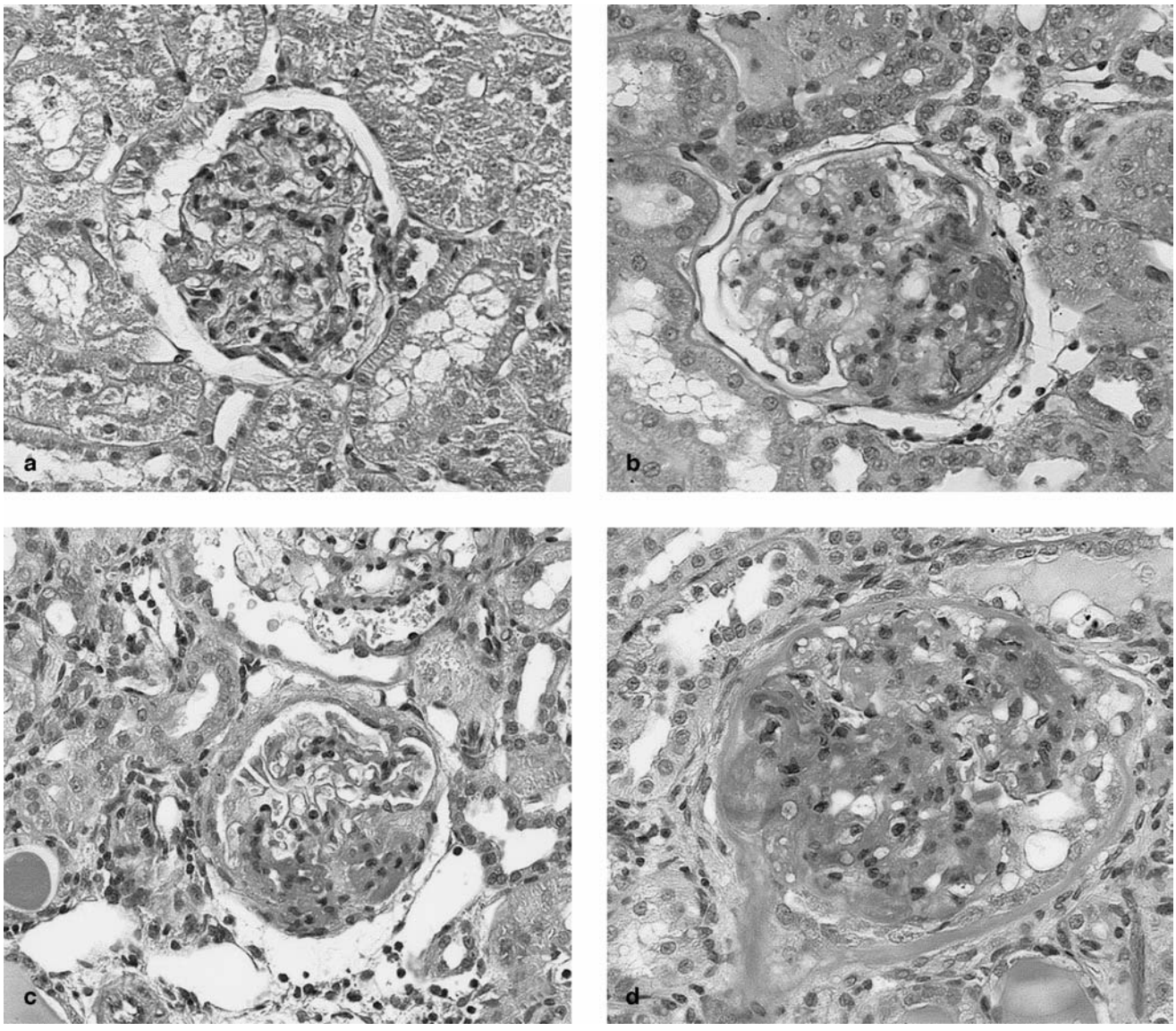

Fig. 2. Photomicrographs showing representative sections of kidneys. **a.** Sections from 4-month-old lean rats with normal glomeruli. **b.** Sections from 4-month-old obese rats with mild focal segmental glomerular sclerosis. **c.** Sections from 8-month-old obese rats with more extensive focal segmental glomerular sclerosis as well as interstitial nephritis and some hyaline casts. **d.** Sections from 10–11-month-old obese rats with extensive glomerular injury and global sclerosis. Magnification 375×

Table 1. Plasma chemistry and body weight in lean and obese Zucker rats

	Plasma glucose (mmol/l)	HDL chol (mmol/l)	LDL chol (mmol/l)	LDL/HDL	Total chol (mmol/l)	Plasma Trigly (mmol/l)	BUN (mmol/l)	Plasma creatinine (μmol/l)	Body weight (grams)
Lean									
4 months	6±1	0.89±0.02	0.69±0.10	0.8±0.1	1.58±0.11	0.3±0.1	5.7±0.1	42±3	344±7
11 months	7±1	1.18±0.05#	0.73±0.08	0.6±0.1	1.91±0.13#	0.7±0.1#	4.9±0.2	47±2#	471±8#
Obese									
4 months	32±2*	2.35±0.18*	3.67±0.59*	1.7±0.4*	6.02±0.41*	2.3±0.6*	7.6±0.2*	33±3*	390±12*
8 months	32±4	2.87±0.41	6.22±0.57	2.5±0.5	9.08±0.83	2.3±0.2	7.9±0.5	38±2	434±35
11 months	28±5*	3.31±0.82*	8.43±1.29*#	3.5±0.7*#	11.74±1.6*#	2.6±0.4*	10.4±1.0*#	59±5*#	394±12*

All data are represented as means ± SE. * $p<0.05$ obese vs lean; # $p<0.05$ 4 months vs 11 months. BUN, blood urea nitrogen; chol, cholesterol; Trigly, trigylcerides

Table 2. Renal characteristics in lean and obese Zucker rats

	Ccr (ml/min)	UpV (mg/24 h)	Damaged glomeruli (%)	Glom vol ($\mu m^3 \times 10^6$)	Plasma NO_X (μmol/l)	Plasma NO_X : creat	UNO_XV (μmol·24 h^{-1}· 100 g^{-1} BW)	KC NOS activity (pmol cit·mg prt^{-1}·min^{-1})	KC nNOS (IOD/Ponc ×100)	KC eNOS (IOD/Ponc ×100)
Lean										
4 months	1.9±0.2	9±1	3.4±0.4	0.70±0.05	15±1	0.36±0.04	0.94±0.06	2.86±1.05	4.4±0.2	1.7±0.4
11 months	2.5±0.5	12±1	8.4±1.6#	0.80±0.02	13±1	0.28±0.01	0.93±15	2.54±0.16	3.8±1.0	1.7±0.3
Obese										
4 months	1.3±0.1*	77±12*	8.6±0.7*	1.00±0.05*	18±2	0.55±0.06*	0.84±0.07	1.08±0.09*	3.3±0.1*	1.5±0.2
8 months	1.2±0.1	188±39	23.5±3.5	1.40±0.04	20±5	0.54±0.12	1.13±0.18			
11 months	0.7±0.1*#	384±35*#	45.4±5.1*#	1.50±0.08*#	42±13	0.77±0.19*	1.77±0.22*#	0.99±0.13*	2.6±0.3#	2.2±0.2#

All data are represented as means ± SE. * $p<0.05$ obese vs lean; # $p<0.05$ 4 months vs 11 months. Ccr, creatinine clearance; UpV, urinary protein excretion; Glom vol, glomerular volume; NO_X, nitrite + nitrate; creat, creatinine; UNOXV, urinary NOX excretion; BW, body weight; KC, kidney cortex; cit, citrulline; prt, protein; IOD/Ponc, integrated optical density/ Ponceau red; NOS, nitric oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS

was similar between lean and obese rats at 4 months, while a small increase was seen in 11-month-old obese rats. Thoracic aorta eNOS abundance was lower in obese than in lean rats at 11 months (0.053±0.009 vs 0.091±0.016, $p<0.05$), while nNOS was similar. There was no difference in nNOS abundance or activity in cerebellum and eNOS abundance in liver (nNOS undetectable) between obese and lean rats (data not shown).

Discussion

The novel findings of this study are reduced renal clearance and increased proteinuria with decreased renal nitric oxide production by 4 months of age in inbred, severely hyperglycaemic obese Zucker rats. At this age mild glomerular injury is already present and by 11 months severe nephropathy exists with compromised renal function, massive proteinuria, structural damage and persistent renal nitric oxide deficiency.

The inbred Zuckers used in this study reproducibly develop and maintain severe hyperglycaemia, as well as hyper- and dyslipidaemia and increased glomerular volume, all of which are risk factors for progression of renal disease [4, 6]. Some endothelial dysfunction may also be present, as aortic eNOS abundance was reduced in 11-month-old obese rats. Neither outbred nor inbred obese Zucker rats develop severe systemic hypertension, a clinical symptom of diabetic nephropathy [4, 6]. Most importantly, glomerular hypertension was absent in the inbred Zucker rat despite significant injury [8]. Metabolic disturbances probably cause most cases of renal injury, since chronic treatment with the insulin-sensitising drug rosiglitazone markedly lowered proteinuria and structural damage [8]. Additionally, inbred Zucker rats (obese and lean) have a variable tendency to develop hydronephrosis and non-diabetic lesions making interpretation of renal morphology complicated [6]. Unaffected glomeruli were unremarkable except for hypertrophy. Importantly, diffuse mesangial sclerosis was not identified, nor were glomerular capillary walls thickened. Tubular injury was present in areas of interstitial inflammation but tubular basement membranes were not thickened. Mild medial hypertrophy was seen in arterioles but hyaline arteriolosclerosis was mild even in rats with the most severe glomerular and interstitial changes. Changes in these rats are typical of those seen in focal segmental glomerulosclerosis but not in human diabetic nephropathy.

Though multiple factors are probably involved, this study investigated whether renal nitric oxide deficiency might also contribute to the progression of chronic renal disease. In rat models of primary renal disease renal NOS activity decreased [2, 3, 7, 9]. It appears that the nNOS isoform is the most sensitive to chronic renal disease as its activity and abundance were reduced in models leading to glomerulonephritis, ablation/infarction and puromycin aminonucleoside [3, 7, 9]. This reduction, moreover, paralleled the development of glomerulosclerosis. In support of a causal link, the renal nitric oxide system was preserved following ablation/infarction in the Wistar Furth rat, which does not develop chronic renal disease [7], while low-level chronic NOS inhibition rendered the Wistar Furth rat susceptible to progression of chronic renal disease [7].

In the present study, the obese Zucker rat exhibited decreased cortical NOS activity and nNOS abundance by 4 months of age, a time when the glomerular filtration rate (creatinine clearance) was substantially reduced. Nitric oxide production remained depressed at 11 months as injury became severe and renal function fell further. Overall, the declines in renal NOS activity and nNOS abundance may reflect the developing chronic renal disease, irrespective of the primary initiating event, in this case the metabolic disturbances seen in the inbred obese Zucker.

Our models of chronic renal disease show declines in $U_{NOX}V$ (total nitric oxide production) paralleling observations of reduced renal nitric oxide, but in the present study the $U_{NOX}V$ and plasma NO_X : creatinine ratios were higher in the diabetic rats with chronic renal disease. As previously discussed by Baylis and Vallance [10], $U_{NOX}V$ cannot be used as an index of renal nitric oxide production, and since the kidney and vasculature only account for a few per cent of the total nitric oxide made in the body, parallel changes in $U_{NOX}V$ and renal nitric oxide production are not inevitable. Major sources of nitric oxide synthesis within the body include cerebellum (high output), liver and skeletal muscle (large organs) and adipose tissue [11]. The increased total nitric oxide generated in the obese rats may thus reflect the high levels of adipose tissue (despite wasting of lean body mass), with widespread stimulation of nitric oxide production resulting from the inflammatory processes underway in this metabolic disease.

In conclusion, obese hyperglycaemic, hyperlipidaemic inbred Zucker rats develop severe kidney disease with an associated reduction in renal NOS activity and nNOS protein abundance. The renal lesions do not resemble those of human diabetic nephropathy.

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