In a type 2 diabetic nephropathy rat model, the improvement of obesity by a low calorie diet reduces oxidative/carbonyl stress and prevents diabetic nephropathy

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Abstract

Background. The present study has been undertaken to unravel the critical factors involved in the progression of diabetic nephropathy (DN).

Methods. A unique type 2 diabetic rat model with a wide range of metabolic derangements and hypertension has been utilized, the spontaneously hypertensive/NIH-corpulent rat SHR/NDmcr-cp(cp/cp). It develops histologically evident glomerular injury and tubulointerstitial damage, including mesangial activation, podocyte injury, and inflammatory cell infiltration in the tubulointerstitium.

Results. A low calorie diet for 22 weeks significantly improves obesity, proteinuria and renal morphological alterations. The correction of renal injury is independent of blood pressure control. Obesity correction, although partial, normalizes the renal content of pentosidine taken as a marker of oxidative stress and advanced glycation end products (AGEs). This occurs despite the fact that, in this model, improvement of glucose control and hyperlipidaemia is limited. Proteinuria and body weight are highly correlated with renal pentosidine content, while proteinuria and body weight are also correlated with each other. Diabetic renal injury is thus inhibited by a low calorie diet with an attendant reduction of oxidative stress and AGE formation, despite sustained hypertension.

Conclusion. The present findings suggest a direct role of obesity in the generation of a localized oxidative stress and AGE formation, directly responsible for DN.

Keywords: advanced glycation end product; multiple risk factor intervention; pentosidine; proteinuria; renoprotection

Introduction

Prevention or retardation of diabetic nephropathy (DN) has become a major goal in biomedical research [1,2]. Several pathways have been implicated in the progression of DN, e.g. systemic and glomerular hypertension, metabolic derangements (hyperglycaemia, hyperlipidaemia, hyperinsulinaemia), oxidative stress and advanced glycation end products (AGEs). Multi-targeted therapeutic interventions include strict control of prevailing blood glucose level, anti-hypertensive treatment by agents with anti-proteinuric action [e.g. angiotensin converting enzyme inhibitors and angiotensin II type 1 receptor blockers (ARB)], lipid lowering strategies and correction of insulin resistance.

Among animal models of type 2 diabetes with nephropathy, we have chosen the spontaneously hypertensive/NIH-corpulent rat [SHR/NDmcr-cp(cp/cp)] to unravel the mechanisms leading to DN [3]. This strain has the same genetic background as SHR/(cp/+) but has a genetic mutation in the leptin receptor gene, and exhibits hyperphagia with an attendant wide range of metabolic abnormalities similar to those of human type 2 diabetes (obesity, hyperglycaemia, hyperlipidaemia and hyperinsulinaemia). Obesity and diabetes combined with dyslipidaemia, increased insulin resistance and increased arterial blood pressure results in a heterogenous entity identified...
as the metabolic syndrome [4–6]. In this syndrome, several risk factors for organ damage operate at a high level of intensity. Due to an increased calorie intake, the accelerated fuel metabolism generates an excess of glucose, fatty acids and reactive oxygen species, which produces oxidative stress and AGEs, and triggers chronic inflammatory changes (reviewed in [7]). This model appears thus suited to assessing the renal changes induced by broad metabolic abnormalities as well as by haemodynamic alterations.

In the present study, we characterize biochemical and morphological changes in SHR/NDmcr-cp(cp/cp) rats. This model develops glomerular and tubulointerstitial damage characteristic of human type 2 DN, e.g.

focal and segmental glomerular sclerosis, mesangial expansion, thickening of basement membrane, inflammatory cell infiltration and tubulointerstitial damage. In order to unravel the respective roles of obesity with its associated metabolic abnormalities and that of hypertension in the development of renal injury, we fed the animals with a low calorie diet. Obesity was significantly reduced. Proteinuria and morphological alterations of the kidney were prevented despite persistent hypertension. Renoprotection was accompanied by a limited though not statistically significant improvement of metabolic abnormalities. In contrast, the renal content of pentosidine, taken as a marker of oxidative stress and AGEs, returned to normal, suggesting a crucial role for localized oxidative stress in the generation of DN.

Subjects and methods

Animals

Spontaneously hypertensive/NIH-corpulent rats [SHR/NDmcr-cp (cp/cp)] established in Disease Model Cooperative Research Association (Kyoto, Japan), and Wistar-Kyoto rats (WKY) were purchased from SLC (Shizuoka, Japan). Preliminary studies demonstrated that renal morphological alterations and proteinuria are more severe in males than in females, so that only male rats were utilized. WKY rats were given a normal diet (Group 1) (n = 5). Heterozygotes of SHR/NDmcr-cp (cp/+) with normal diet, which do not develop diabetes mellitus, served as another control as a wild phenotype (Group 2) (n = 5). SHR/NDmcr-cp (cp/cp), aged 5 weeks, were randomly divided into three groups and allocated to various dietary regimens: five rats on free dietary access (control dietary intake; Group 3), five rats on low calorie diet (30% reduction of control dietary intake; Group 4) and five rats on very low calorie diet (50% reduction of control dietary intake; Group 5). Indeed, preliminary studies disclosed that a 50% reduction of spontaneous dietary intake of SHR/NDmcr-cp (cp/cp) rats is equivalent to that of WKY and SHR rats without hyperphagia. A 30% reduction was used to evaluate the benefits of a partial correction of food intake. Animals were euthanized before tissue sections were taken. The protocol was in accordance with the Animal Experimentation Guidelines of Tokai University School of Medicine.

Blood pressure, urine collection and blood sampling

Systolic blood pressure was determined in conscious rats by the tail-cuff method at the beginning of the study, at week 2, and every 4 weeks thereafter until euthanization. At the end of the study, each rat was weighed and placed in a metabolic cage for a 24 h urine collection. Blood samples were obtained prior to death.

Biochemical measurements in blood and urine

Total cholesterol, triglycerides, creatinine and urea nitrogen (BUN) concentrations were determined in plasma with an automatic analyzer (DRICHEM 3000V, Fuji Photo Film Co., Ltd, Tokyo, Japan); protein and creatinine concentrations were determined similarly in urine with an automatic analyzer (Hitachi Automatic Clinical Analyzer 7170, Hitachi Science Systems, Ibaraki, Japan). Plasma insulin was measured with a commercially available kit (Morinaga Biochemistry Lab, Tokyo, Japan). Taking into account the variability of serum glucose levels in rats, HbA1c plasma levels were used as an index of glucose control. HbA1c was measured using the DCA2000 (Bayer Diagnostics, Pittsburgh, PA), a portable device that uses an immunoassay technique with a monoclonal antibody directed against a sequence of the HbA1c molecule [8].

Morphological analysis

Coronal sections of renal tissue (3–4 μm thick) were stained with periodic acid-Schiff (PAS) and examined by light microscopy in a blinded fashion. Glomerulosclerosis was semiquantitatively evaluated according to criteria developed by Uehara et al. [9]. Briefly, 50 glomeruli were selected randomly in each animal for morphometric analysis. Glomerulosclerosis, defined as synchiae formation by PAS staining with focal or global obliteration of capillary loops, was graded as follows: 0, normal; 1, 0–25% of glomerular area affected; 2, 25–50% affected; 3, 50–75% affected; and 4, 75–100% affected. An overall glomerular sclerosis score per animal was obtained by multiplying each severity score (0–4+) with the percentage of glomeruli displaying the same degree of injury and summing these scores.

Quantification of tubulointerstitial injury was performed in a blinded manner using more than 15 randomly selected fields of cortex per cross section. Tubulointerstitial injury was graded (0–4+) on the basis of the percentage of tubular cellularity, basement membrane thickening, cell infiltration, dilation, atrophy, sloughing or interstitial widening as follows: 0, no change; 1, <25% tubulointerstitial injury; 2, 25–50% injury; 3, 50–75% injury; and 4, 75–100% injury.

Immunohistological analyses

Tissues were fixed in methyl Carnoy’s solution and paraffin-embedded. An indirect immunoperoxidase method was used to identify the following antigen: desmin with monoclonal antibody D33 (Dako, Carpinteria, CA), α-smooth muscle actin with monoclonal antibody asm-1 (Boehringer-Mannheim), type IV collagen with goat polyclonal antibody (Southern Biotechnology Associates), monocytes/macrophages with murine monoclonal IgG1 antibody ED-1 (Chemicon, Temecula, CA) and vimentin with murine monoclonal IgG antibody V9 (Dako).
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For semi-quantitative analysis, desmin staining was graded as follows: 0, no staining; 1+, 1–25% of the glomerular tufts positive; 2+, 25–50%; 3+, 50–75%; 4+, 75–100%. Fifty glomeruli were selected randomly in each animal, and an overall score per animal was obtained by multiplying each score (0–4+) with the percentage of glomeruli displaying the same degree of injury and summing these scores. Vimentin-positive tubules were counted in 10 randomly selected cortical fields with a ×10 objective, and ED1-positive infiltrating cells in the tubulointerstitium were counted in 20 randomly selected cortical fields with a ×20 objective.

Semi-quantitative PCR analyses

Total RNA of the kidney was isolated using ISOGEN (WAKO Chemical, Osaka, Japan) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA). Samples were analysed with an amplification cycle of 35 cycles for RAGE and that of 20 cycles for actin, respectively. Primer sets for RAGE were 5′-CAGGGTACAGAAACCCGG-3′, 5′-ATTACAGCTCTGCAGTTCCTC-3′, and those for actin were 5′-CTTCTTCTCAATGAGCTGCGTG-3. 5′-TCATGAGGTAGTC after the amplification, samples were applied for electrophoresis, followed by staining of the gel with ethidium bromide. The densities of the specific bands were measured by a densitometry utilizing ATTO lane analyser (ATTO, Tokyo, Japan), and relative values of RAGE were obtained by normalization against those of actin.

AGE measurements

Kidney tissue (100 mg) was minced, rinsed with 10% trichloroacetic acid, dried under vacuum, and acid hydrolyzed in 500 μl of 6 N HCl for 16 h at 110°C under nitrogen. Its pentosidine content was measured with a reverse-phase high-performance liquid chromatography (HPLC) as previously described [10]. In brief, a 20 μl aliquot of the acid hydrolysate diluted by PBS was injected into an HPLC system and separated on a C18 reverse-phase column (Waters, Tokyo, Japan). The effluent was monitored with a fluorescence detector (RF-10A Shimadzu, Kyoto, Japan) at an excitation-emission wavelength of 335/385 nm. Synthetic pentosidine was used as a standard.

Protein-bound and free form pentosidine in the plasma was also determined by HPLC according to our previous method [11].

Statistical analysis

All data are reported as the mean±SE. The significant differences among the SHR/NDmcr-cp(cp/cp) group with WKY vehicle and with SHR/NDmcr-cp(cp/+) were determined by oneway ANOVA. Similarly, the significant differences among SHR/NDmcr-cp(cp/cp) were determined by one-way ANOVA. Multiple comparisons were performed among the SHR/NDmcr-cp(cp/cp) group vs the SHR/NDmcr-cp(cp/+) group, the SHR/NDmcr-cp(cp/cp) group vs the WKY and normal diet vs others in the SHR/NDmcr-cp(cp/cp) group by Dunnett’s t-test. A linear regression analysis was performed to study the relationships between renal contents of pentosidines and body weight or urinary protein.

Results

SHR/NDmcr-cp(cp/cp) rats develop metabolic syndrome and a nephropathy similar to type 2 diabetes

Physical (body weight, blood pressure, renal weight) and biochemical (blood and urine) data in the experimental animals are summarized in Table 1. SHR/NDmcr-cp(cp/cp) rats without caloric restriction (Group 3) exhibited hypertension, a wide range of metabolic abnormalities derived from hyperphagia (obesity, hyperglycaemia, hyperlipidaemia, hyperinsulinaemia) and marked proteinuria.

On light microscopy, SHR/NDmcr-cp(cp/cp) rats without caloric restriction (Group 3) developed glomerular damage, characterized by glomerular hypertrophy, mesangial expansion, and focal and segmental

Table 1. Physiological and biochemical data of the experimental rats at the end of the study (mean±SE)

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR/NDmcr-cp(cp/cp)</th>
<th>Normal diet (Group 3)</th>
<th>SHR/NDmcr-cp(cp/cp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle (Group 1)</td>
<td>Vehicle (Group 2)</td>
<td>Normal diet (Group 3)</td>
<td>(Group 4)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>481±11</td>
<td>471±9</td>
<td>697±38h,k</td>
<td>568±7l,0</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>134±3</td>
<td>202±6</td>
<td>182±7h</td>
<td>120±8h,b</td>
</tr>
<tr>
<td>Hba1c (%)</td>
<td>2.5±0.0</td>
<td>2.5±0.0</td>
<td>3.8±0.4h,k</td>
<td>3.5±0.1h,j,m,o</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.8±0.2</td>
<td>3.6±0.7</td>
<td>7.3±1.11h,k</td>
<td>59.7±5.2h,k,p</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>148±6.0</td>
<td>106±4.0</td>
<td>203±6.0h,k</td>
<td>152±9.0h,0-a</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>48±2.0</td>
<td>83±5.0</td>
<td>591±90h,k</td>
<td>285±22h,0,0</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>15.0±1.4</td>
<td>17.3±1.0</td>
<td>24.7±1.5h,d</td>
<td>20.5±1.6h</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dl)</td>
<td>0.36±0.02</td>
<td>0.32±0.02</td>
<td>0.36±0.02</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>Urinary protein (mg/kg/day)</td>
<td>14.1±0.9</td>
<td>44.5±4.4</td>
<td>176.0±49.4h</td>
<td>32.8±10.2l,0</td>
</tr>
</tbody>
</table>

*P<0.001, **P<0.01 by ANOVA (Groups 1, 3, 4 and 5).

*P<0.001, **P<0.01, ***P<0.05 by ANOVA (Groups 2, 3, 4 and 5).

*P<0.001, **P<0.01, ***P<0.05 by ANOVA (Groups 3, 4 and 5).

*P<0.001, **P<0.01, ***P<0.05 vs Group 1 by Dunnett’s t-test.

*P<0.001, **P<0.01, ***P<0.05 vs Group 1 by Dunnett’s t-test.
glomerular sclerosis (Figure 1). Synechiae formed by the attachment of parietal epithelial cells to denuded glomerular basement membrane (BM) were observed, but nodular lesions reminiscent of Kimmelstiel–Wilson nodules were absent at 35 weeks after birth. Interstitial damage was also prominent: tubular hypertrophy and atrophy, inflammatory cell infiltration and thickening of tubular BM. Neither heterozygotes of SHR/NDmcr-cp(cp/+) (except for hypertension) nor WKY rats of corresponding age exhibited similar physical, biochemical and pathologic findings (Table 1).

On electron microscopy, glomerular damage in SHR/NDmcr-cp(cp/cp) rats was characterized by mesangial expansion, thickening of glomerular BM, glomerular epithelial injury such as pseudocyst formation, vacuolization, detachment from the glomerular BM, podocyte depletion, foot process effacement and interstitial damage (data not shown).

Podocyte damage and mesangial activation were confirmed by immunohistochemistry [12,13]. Desmin and α-smooth muscle actin demonstrated by specific antibodies (Figure 2) were expressed respectively in podocytes and mesangial cells. By contrast, neither desmin nor α-smooth muscle actin were found in glomeruli of heterozygotes of SHR/NDmcr-cp(cp/cp) or of WKY rats at the corresponding age. Mesangial matrix proteins such as type 4 collagen accumulated in sclerotic areas of glomeruli of SHR/NDmcr-cp(cp/cp) (data not shown).

Tubulointerstitial injury was characterized in SHR/NDmcr-cp(cp/cp) rats by markers such as vimentin [14], and by monocyte/macrophage infiltration identified by ED-1 staining. Tubular injury was clearly associated with monocyte/macrophage infiltration (Figure 3).

Low calorie diet prevents the development of nephropathy despite the absence of a significant improvement of glucose control and limited correction of lipid disorders

Two levels of caloric restriction (30–50% of control intake) were investigated in SHR/NDmcr-cp(cp/cp) rats. This resulted in a similar improvement in metabolic parameters. Obesity correction was largely but not completely achieved. Hyperlipidaemia was partially but significantly corrected (Table 1). Caloric restriction was associated with a mild, not significant fall in levels of HbA1c ($P = 0.3$).

Renal injury was prevented in both groups: SHR/NDmcr-cp(cp/cp) rats on a restricted calorie intake (Groups 4 and 5) did not develop proteinuria (Table 1) and failed to develop histological damage in the kidney (Table 2 and Figure 1), a finding confirmed by immunohistochemical analysis (Figures 2 and 3). Podocyte injury, mesangial activation or tubulointerstitial injury and inflammatory cell infiltration were not observed in Groups 4 and 5. While three to five glomeruli per section showed α-smooth muscle actin positive mesangial cells in SHR/NDmcr-cp(cp/cp) rats, no α-smooth muscle actin positive mesangial cells were detected in animals on a restricted calorie intake.
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Fig. 2. Immunohistochemical demonstration of podocyte damage and mesangial injury. Glomeruli in SHR/NDmcr-cp(cp/cp) rats with normal diet showed expression of desmin, a marker of podocyte injury (A), while expression of desmin was minimal in SHR/NDmcr-cp(cp/cp) rats with 30% calorie off diet or 50% calorie off diet (B). Staining of desmin was also negligible in WKY rats and heterozygotes with normal diet (C). Expression of α-smooth muscle actin, a marker of mesangial activation and injury (indicated by an arrow), in mesangial cells was observed exclusively in SHR/NDmcr-cp(cp/cp) rats with normal diet (D) in contrast to other groups such as SHR/NDmcr-cp(cp/cp) rats with 50% calorie off diet (E) and heterozygotes with normal diet (F).

Fig. 3. Tubulointerstitial injury. Expression of vimentin, a marker of tubular injury was observed in tubules of SHR/NDmcr-cp(cp/cp) rats (A). Tubular cells of SHR/NDmcr-cp(cp/cp) rats with 30 or 50% calorie off diet (B), WKY rats with normal diet, and heterozygotes of SHR/NDmcr-cp(cp/+) with normal diet (C) did not show expression of vimentin. Tubulointerstitial injury in SHR/NDmcr-cp(cp/cp) rats was associated with infiltration of ED-1 positive cells (D). Inflammatory cells were absent in SHR/NDmcr-cp(cp/cp) rats with 30 or 50% calorie off diet (E), WKY rats with normal diet, or heterozygotes of SHR/NDmcr-cp(cp/+) with normal diet (F).
intake (Groups 4 and 5). Semiquantitative analysis of desmin staining as a marker of podocyte injury as well as counting the number of vimentin positive tubules and ED-1 positive infiltrating cells confirmed these observations (Table 3). In contrast, caloric restriction failed to improve hypertension (Table 1). Our semi-quantitative PCR analysis revealed that different dietary regimens did not change expression of RAGE in the kidney (Table 4).

**Caloric restriction and its attendant body weight control decrease oxidative stress and AGE formation in diabetic kidney**

We previously investigated pentosidine localization using an immunohistochemical approach [10]: pentosidine was detected in the arterial walls of SHR/NDmc-r-cp but not in glomeruli of any group examined. In contrast to human diabetic kidneys [15,16], pentosidine was not detectable by immunohistochemistry in glomeruli of SHR/NDmc-r-cp(cp/cp), or in WKY rats. Furthermore, immunohistochemistry does not allow sensitive, precise quantification of AGE protein modification. The contents of pentosidine, a marker of advanced glycation and oxidative stress, in the kidney were therefore measured chemically by HPLC analysis.

The renal content of pentosidine was significantly higher in the SHR/NDmc-r-cp(cp/cp) group with unrestricted food intake than in both SHR/NDmc-r-cp(cp/cp) and WKY control groups. The low calorie diets returned renal pentosidine contents to the levels observed in both control groups (Table 5). Body weight and tissue pentosidine levels showed a significant correlation (pentosidine \(= 0.161 \div \text{body weight}, r^2 = 0.312 \) \((P < 0.005)\) (Figure 4A). Urinary protein excretion and renal pentosidine contents in the kidneys of SHR/NDmc-r-cp(cp/cp) Group 3–5 (normal feed or low calorie diet) were highly correlated (pentosidine \(= 0.295 \div \text{urinary protein} + 59.672, r^2 = 0.676 \) \((P < 0.0001)\) (Figure 4B). We also determined the content of circulating pentosidine, both in protein-bound and in free forms, by HPLC. Free-form pentosidine was not detectable in any group (below detection limit, <0.4 pmol/ml of plasma). The contents of protein-linked pentosidine in WKY, SHR/NDmc-r-cp(cp/cp), SHR/NDmc-r-cp(cp/cp) given vehicle, SHR/NDmc-r-cp(cp/cp) given a 30% diet reduction, SHR/NDmc-r-cp(cp/cp) given a 50% diet reduction were 16.3 ± 1.1, 15.5 ± 1.6, 18.0 ± 1.4, 13.9 ± 1.9, 16.7 ± 3.0 pmol/mg of protein, respectively, and there was no statistically significant difference among the groups. Thus, the contribution of circulating/filtered pentosidine to

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**Table 2. Morphological evaluation of glomerular sclerosis and tubulointerstitial injury in the experimental rats at the end of the study (mean ± SE)**

<table>
<thead>
<tr>
<th></th>
<th>WKY Vehicle (Group 1)</th>
<th>SHR/NDmc-r-cp(cp/cp) Vehicle (Group 2)</th>
<th>SHR/NDmc-r-cp(cp/cp) Normal diet (Group 3)</th>
<th>SHR/NDmc-r-cp(cp/cp) 30% cut off (Group 4)</th>
<th>SHR/NDmc-r-cp(cp/cp) 50% cut off (Group 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular sclerosis score</td>
<td>0.12 ± 0.08</td>
<td>0.32 ± 0.06</td>
<td>1.96 ± 0.29&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.48 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.24 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tubulointerstitial injury score</td>
<td>0.04 ± 0.04</td>
<td>0.04 ± 0.04</td>
<td>2.60 ± 0.19&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.15 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> \(P < 0.01\).
<sup>b</sup> \(P < 0.01\).
<sup>c</sup> \(P < 0.01\) vs SHR/NDmc-r-cp(cp/cp) (normal diet).

**Table 3. Quantitative immunohistochemical analysis of kidney damage (mean ± SE)**

<table>
<thead>
<tr>
<th></th>
<th>WKY Vehicle (Group 1)</th>
<th>SHR/NDmc-r-cp(cp/cp) Vehicle (Group 2)</th>
<th>SHR/NDmc-r-cp(cp/cp) Normal diet (Group 3)</th>
<th>SHR/NDmc-r-cp(cp/cp) 30% cut-off (Group 4)</th>
<th>SHR/NDmc-r-cp(cp/cp) 50% cut-off (Group 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podocyte damage (desmin; scores 0–4)</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Vimentin positive tubules (number per field ×100)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>5.5 ± 0.4</td>
<td>0.3 ± 0.2</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>ED-1 positive cells (number per field ×200)</td>
<td>8.0 ± 0.9</td>
<td>7.6 ± 0.9</td>
<td>36.4 ± 2.7</td>
<td>9.6 ± 0.9</td>
<td>6.8 ± 1.1</td>
</tr>
</tbody>
</table>

**Table 4. Relative expression of RAGE in the kidney calibrated by actin (mean ± SE)**

<table>
<thead>
<tr>
<th></th>
<th>WKY Vehicle (Group 1)</th>
<th>SHR/NDmc-r-cp(cp/cp) Vehicle (Group 2)</th>
<th>SHR/NDmc-r-cp(cp/cp) Normal diet (Group 3)</th>
<th>SHR/NDmc-r-cp(cp/cp) 30% cut-off (Group 4)</th>
<th>SHR/NDmc-r-cp(cp/cp) 50% cut-off (Group 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative density</td>
<td>1.13 ± 0.40</td>
<td>1.01 ± 0.16</td>
<td>1.33 ± 0.45</td>
<td>1.34 ± 0.21</td>
<td>1.42 ± 0.71</td>
</tr>
</tbody>
</table>
increased renal pentosidine content may be marginal at best.

**Discussion**

The present data demonstrate the possibility of preventing diabetic nephropathy in a rat model of type 2 diabetes. The mere restriction of caloric intake prevents proteinuria and histologic damage of the kidney. Although hypertension persists, glucose control is not significantly improved and lipid disorders only partially corrected. Interestingly, a 50 and 30% reduction of caloric intake seem to be equally effective.

The most significant observation is that renoprotection induced by a restricted caloric intake is associated with the normalization of the renal pentosidine content. Pentosidine is a surrogate marker of AGEs, oxidative stress and carbonyl stress [17], which are implicated in the genesis of DN [18,19]. We previously demonstrated that the plasma pentosidine level was closely linked to the level of renal function in uraemic patients [20,21]. This is not the case in the present study as the renal function of the present diabetic rat model is not impaired: the values of creatinine clearance are 2.69 ± 0.80 ml/min in SHR/NDmcr-cp(cp/cp) rats and 3.01 ± 0.69 ml/min in WKY rats, respectively, and there is no statistical difference in these values. Thus, the present renal function does not influence the retention of filtration of precursors of pentosidine and pentosidine itself.

The close link between diabetic renal damage and oxidative/carbonyl stress observed in the present study is supported by the highly significant correlation existing between proteinuria and renal pentosidine content. In a previous study in diabetic obese rats [10], we demonstrated a similar association between renoprotection and renal pentosidine content in rats given ARB. The beneficial effects of ARB in diabetic rat kidneys were attributed to their anti-oxidative/carbonyl stress effects as well as to their receptor-mediated suppression of the renin-angiotensin system [22]. Obesity is associated with activation of the intrarenal renin-angiotensin system [23], preventable by caloric restriction [24]. It is thus possible that renoprotection observed in this study implicates a caloric restriction-induced inhibition of the renin-angiotensin system.

Other factors should be considered to understand the renoprotection afforded by caloric restriction. Hypertension was unchanged by caloric restriction, probably because the genetic background of our SHR animals destined them to develop hypertension. It was therefore not primarily relevant in this model.

Hyperglycaemia is undoubtedly the ‘conditio sine qua non’ of renal damage in diabetes [25]. In a...
multivariate analysis of diabetic patients with the ‘metabolic syndrome’, disturbances of glucose metabolism proved closely correlated with creatinine clearance [26]. However, in our model at least, caloric restriction afforded renoprotection despite the absence of a significant improvement of glucose control. Although necessary, hyperglycaemia alone does not result in progressive renal disease, emphasizing multifactorial causes of DN [27].

Other elements of the metabolic syndrome were improved by dietary restriction. Hyperlipidaemia has been indicated as the major causal factor of podocyte injury, with eventual renal failure in obese Zucker rats with type 2 diabetes [28]. Similarly, dietary induced hypercholesterolaemia generates glomerular lesions in non-diabetic rats [29]. In the present study, however, caloric restriction only partially returned cholesterol and triglyceride levels to normal, despite full correction of proteinuria and histologic renal damage. Hyperinsulinaemia has also been invoked in the development of DN [30]. Renoprotection observed in the present study is clearly not mediated by changes in insulin levels, which remained elevated in all experimental groups.

In our study design, protein intake was not adapted so as to remain equal among the different groups. We cannot thus exclude the possibility that a low diet protein intake associated with caloric restriction might have contributed to renoprotection. However, it is unlikely that low protein intake played a major role in protecting the kidney in our study because previous studies showed that a low-calorie diet prevented renal damage in a model of uninephrectomized hypertensive rats regardless of protein ingestion [31]. Another component that should be taken into account is the possible dietary contribution to the reduction of renal AGEs. Various kinds of food contain a significant amount of AGE (e.g. pentosidine). Several lines of evidence have implicated the dietary contribution of in vivo AGE accumulation. Previous studies showed that dietary caloric intake restriction reduces the accumulation of tissue AGEs without affecting survival of the animals [32,33].

Finally, obesity itself should be considered independently of the components of the metabolic syndrome, such as glucose, lipid and insulin abnormalities. Caloric restriction in overweight patients with diabetic or nondiabetic proteinuric nephropathies decreases proteinuria, the fall in proteinuria being correlated with weight loss [34]. Also, in experimental animals, caloric restriction prevents renal damage in obese as well as in non-obese rats (reviewed in [35]). The pathogenic mechanisms through which obesity could induce proteinuria are largely unknown, but several data point to a pathogenic role of glomerular hyperfiltration and hypertension secondary to vasodilatation of afferent arterioles [36,37]. In the present study, a specific role of obesity correction in renoprotection was supported by its correlation with renal pentosidine contents.

The lesions observed in the kidneys of SHR/NDmcr-cp (cp/cp) rats given free access to food are characterized by marked glomerular and tubulointerstitial lesions, characteristic of human DN. Our studies confirmed previous reports by Gross et al. [38,39] and extended their findings utilizing various immunohistochemical markers of cell injury. Focal and segmental glomerulosclerosis was indeed associated with podocyte damage demonstrated by the expression of desmin. Mesangial expansion resulted from mesangial cell activation illustrated by the presence of α-smooth muscle actin and the deposition of type 4 collagen. Conspicuous tubulointerstitial damage was characterized by tubular injury witnessed by the expression of vimentin and by an infiltrate of inflammatory cells of the monocyte/macrophage type identified by ED-1 staining. Caloric restriction prevented these lesions, the mild persisting abnormalities being identical with those of the heterozygote control rats, probably resulting from hypertension.

The demonstration of a central role of reduced oxidative stress and AGE formation in the prevention of renal lesions identifies a key factor in the genesis of DN. It is likely that the increased dietary intake accelerates fuel metabolism and releases a number of compounds able to produce, locally at least, oxidative stress and advanced glycation of proteins which, in turn, damage the kidneys. If hypertension and the components of metabolic syndrome do not appear to be prerequisites in this model, there is little doubt that most of these factors are also able to contribute, at least under some circumstances, to the onset of local oxidative stress. Within this framework, a multitargeted approach including rigorous control of blood pressure and plasma glucose levels to prevent clinical DN is certainly needed. The suggestion emerging from this study, that local oxidative stress is a central feature of DN, calls for a special attention to therapies directed towards its prevention.

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References

A role of obesity and oxidative stress in DN


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