Food Restriction Prevents Advanced Glycation End Product Accumulation and Retards Kidney Aging in Lean Rats

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Abstract. Tissue content of advanced glycation end products (AGE) increases with age and contributes to the changes in structure and function of the renal and cardiovascular systems. The effect of chronic food restriction on this AGE accumulation was investigated in lean WAG/Rij rats. A 30% food restriction performed from 10 to 30 mo in female rats reduced their mean body weight from 240 ± 7 to 160 ± 12 g, but did not modify their survival. AGE collagen content increased from 14.3 ± 5.5 to 104.7 ± 13.0 arbitrary units per microgram (AU/μg) of hydroxyproline (OHPro) in kidney between 10 and 30 mo, and from 9.7 ± 1.2 to 310.6 ± 34.6 AU/μg OHPro in the abdominal aorta. Food restriction reduced AGE accumulation to 21.4 ± 3.3 and 74.6 ± 16.5 AU/μg OHPro in kidney and aorta of 30-mo-old animals. Similar results were found for collagen prepared from isolated glomeruli (7.8 ± 1.2, 81.2 ± 16.1, and 10.3 ± 4.3 AU/μg OHPro in 10-mo, 30-mo, and restricted 30-mo-old rats). Reduction of intrarenal and arterial AGE accumulation by food restriction was confirmed by immunostaining in optical microscopy. Age-related changes in arterial and kidney structures as polyuria and proteinuria were mainly prevented by food restriction. These data indicate that chronic food restriction reduces the accumulation of AGE and preserves the structure and function of the renal and cardiovascular systems in lean rats, although it did not affect survival of the animals between 10 and 30 mo.

Chronic food restriction usually slows the aging process and prevents age-related diseases reported in most species. In rodents, as a rule, it lowers body weight of the animals, increases mean and maximal survival, and postpones the morphologic and functional changes observed in the course of aging. Food restriction is efficient when started early in life or after the growing period, and its effects are more or less proportional to the extent of the restriction. Selective reduction of different diet components indicates that this beneficial effect is related to caloric restriction rather than to any constituent, although the underlying mechanisms are not firmly established. Several hypotheses have been proposed to explain the effects of food restriction, including prevention of overeating, reduction of oxidative stress, and decreased accumulation of advanced glycation end products (AGE).

The influence of chronic food restriction on kidney structure and function has been documented in many studies. Caloric restriction prevents the glomerulosclerosis and proteinuria commonly reported in senescent rats. Protein restriction or substitution of animal for vegetable proteins in the diet also has a protective effect on age-related renal diseases in rodents. However, these studies have usually been performed in rats that gain weight throughout their life, and it was suggested that part of the beneficial effect of food restriction was due to the prevention of overeating frequently observed in rodents. Such a role for overeating in accelerated aging and diseases has been documented from comparison of survival in different strains of rodents. Sprague Dawley rats, which may reach 1000 to 1200 g at 24 mo, or Fisher 344 rats, which can grow up to 600 g when fed ad libitum, are short-living animals that develop severe renal diseases with age. Conversely, rats from the Brown-Norway, WAG/Rij, or Wistar/Lou strains, which remain lean, even when fed ad libitum, are long-living animals that are nearly free of glomerulosclerosis throughout their life (1–6). Although food restriction may play a role in reducing overeating-related diseases, it also slows the aging process of lean rats, indicating that the restriction affects some specific molecular mechanisms (2,7,8). AGE accumulation is one of these mechanisms corresponding to the prolonged exposure of proteins to glucose due to changes in glucose tolerance and reduced protein turnover with age (9–11). The consequences of AGE accumulation on renal and cardiovascular systems have recently been documented by aminoguanidine administration in rodents. Treatment of Fischer 344 or Sprague Dawley rats from 6 to 24 mo reduced the AGE content of kidney, heart, and arteries (12). Concomitantly, age-related cardiac hypertrophy, vascular vasodilatory response, proteinuria, and glomerular sclerosis were all improved by aminoguanidine, suggesting a
causal link between AGE production and alterations in renal and cardiovascular systems. This hypothesis was strengthened by the prevention of arterial stiffening and cardiac hypertrophy in aging rats treated with aminoguanidine in the last part of life, when AGE accumulation is maximal (13).

Several experiments have shown that food restriction by lowering plasma glucose may reduce the formation of intermediate glycation products and collagen fluorescence, which is considered an index of AGE content (10.14–19). This would serve to prevent the cross-linking of extracellular matrix and the development of glomerulosclerosis. In the present study, we tested whether diet restriction also aids in the prevention of AGE accumulation and kidney aging in lean rats, as observed in overeating animals. A 30% restriction was performed from 10 to 30 mo in female WAG/Rij rats. AGE accumulation in extracellular matrix was assessed by competitive enzyme-linked immunosorbent assay (ELISA) with anti-AGE antibodies and visualized by immunolocalization in confocal and electron microscopy. The structural consequences of food restriction on kidney and arteries were determined by quantitative morphometry.

Materials and Methods

Animals

Experiments were performed on female WAG/Rij rats that were born and raised in the animal care facilities of the Commissariat à l’Energie Atomique, Centre d’Etudes de Saclay, Gif-sur-Yvette, France. Survival, growth rate, and tumor incidence of the inbred WAG/Rij strain have been published previously by Bureck (1). Mean survival of female rats fed ad libitum is close to 30 mo.

Rats were maintained on a 12/12 light-dark cycle at 50% humidity and a temperature of 21°C. From weaning to 3 mo, they were fed a commercial diet (DO3; UAR, Villemoisson, France) composed of 9% protein, 10% fish, 16% vegetable proteins, and a total of 3500 kcal/kg. At the age of 3 mo, they were switched to a diet containing 2% fish and 15% vegetable proteins, 0.71% phosphate, 0.78% calcium, 0.62% potassium, 0.27% sodium, 0.22% magnesium, and a total of 2900 kcal/kg (DO4; UAR).

Restriction Protocol

Mean food intake measured in female WAG/Rij rats fed ad libitum was 10.7 ± 1.3 g (n = 10) per day and was unchanged until 30 mo (10.3 ± 1.1 g, n = 10). A 40% food restriction, the usual protocol for optimal survival in most experiments, was tested in 10-mo-old female rats that were singly housed or grouped four per cage. In the two conditions, body weight progressively decreased to 110 g, faster in the singly housed than in the grouped rats, and the animals died within 7 to 8 mo. In a second preliminary study, single or grouped rats were food-restricted by 30%, i.e., 7 g/d per rat. The singly housed animals reached a body weight equilibrium close to 130 g and survived. The grouped animals also lost weight, but to a lesser extent. Within a cage, individual body weights were very similar, indicating that each rat ate a comparable amount of food. This latter protocol, a 30% restriction in animals grouped four per cage, was followed from 10 to 30 mo, the time at which rats fed ad libitum or those that were food-restricted were sacrificed for morphologic and biochemical determinations. Animals (44 in control and 44 in restricted groups) were weighed weekly, and individual life spans were recorded to plot survival curve.

BP and Kidney Function

Systemic BP and heart rate were measured by the tail-cuff method in conscious 10-mo-old, 30-mo-old control, and 30-mo-old restricted female rats. Urine volume was collected in metabolic cages for a 24-h period after a 2-d equilibration period. Urine osmolality was measured with a Roebling Automatik Osmometer (Berlin, Germany). Protein concentration in urine was assessed by the Bradford method with fraction V bovine serum albumin as a standard. Urine samples were further submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine the contribution of albumin to whole proteinuria.

Insulin Response to Glucose Administration

Insulin secretion induced by a glucose load was determined in 10-mo-old, 30-mo-old ad libitum, and 30-mo-old restricted animals. The rats were fasted for 4 h, and a bolus of 0.54 g of glucose per 100 g body wt was injected in the saphenous vein of anesthetized animals. Blood samples were sequentially collected on aprotinin at the caudal vein before glucose administration and at 5, 10, 15, 20, and 30 min after glucose injection. Glycemia was immediately determined (One-Touch; Lifescan, Neckargemünd, Germany), and the remaining samples were stored at −20°C. Plasma insulin was measured in these samples by RIA with the INSIK-5 kit (Sorin, Cedex, France).

Collagen Extraction

Collagen extracts were prepared from whole kidney, abdominal aorta, and glomerular basement membranes. Rats were sacrificed by cervical dislocation. Lower abdominal aorta and kidneys were rapidly removed, frozen in liquid nitrogen, and stored at −80°C. Aorta and kidneys were further homogenized with a Polytron (Ultra-Turrax; IKA Labortechnik) and suspended in phosphate-buffered saline, pH 7.4. The resulting suspension was centrifuged at 40,000 × g for 30 min at 4°C. Lipid extraction of the pellet was performed by addition of 5 ml chloroform/methanol (2:1 vol/vol) followed by gentle shaking, and left to stand overnight at 4°C. The upper layer was removed and the pellet was washed three times with methanol and distilled water. Then the pellets were resuspended in 0.5 M acetic acid and 1 mg/ml pepsin, incubated for 18 h at 4°C to remove noncollagen proteins, and washed three times with 0.1 M CaCl₂, 0.02 M Tris-HCl (pH 7.5), and 0.05% toluene. The pellets were digested with type VII collagenase (0.1 mg/ml; Sigma, St. Louis, MO) by gentle shaking at 37°C for 24 h and centrifuged at 40,000 × g for 30 min, 4°C. The resulting collagen supernatant was quantified from its hydroxyproline content according to Bergman and Loxley (20).

In a second series, the glomeruli were isolated from kidney cortex by the sieving method, as described by Krakower and Greenspon (21), and their basement membranes were prepared according to Meezan et al. (22). Collagen was extracted from the obtained glomerular basement membranes as described previously and quantified by hydroxyproline determination (23).

Quantification of Renal and Arterial AGE Content by Competitive ELISA

The used AGE polyclonal antibodies were raised against bovine pancreatic ribonuclease A (AGE-RNase) incubated for 60 d with 0.5 M glucose in phosphate-buffered saline, pH 7.5, according to the protocol of Makita et al. (24). The specificity of the obtained antibody has been tested previously with soluble and structural glycoproteins, fibronectin, type IV collagen, laminin, and AGE-bovine serum albumin (BSA) (23). ELISA was set up according to Papanastasiou et al. (25). The standard curve was established with AGE-BSA dilutions (1
to 5000 ng) and AGE-RNase polyclonal antibody (1/500). The reaction was quantified by goat antibody directed against rabbit IgG coupled to peroxidase (1/1000) (Sigma). The data were expressed as arbitrary units (AU)/μg hydroxyproline, with 1 AU corresponding to the reactivity of 1 ng of AGE-BSA.

**Immunofluorescence**

Intrarenal and vascular localization of AGE was performed by immunocytochemistry, as described previously (23). Rats were anesthetized with a 10 mg/100 g body wt intraperitoneal injection of Inactin (Byk-Gulden, Constance, Germany). A catheter, connected to a warmed tank (37°C) containing the Dubosq-Brazil solution (formaldehyde, acetic acid, alcohol 80%, 4/1/10 vol/vol, and picric acid 0.36%), was inserted in the abdominal aorta below the renal arteries. The vena cava was incised, and 50 to 100 ml of the fixative solution were perfused to the animal at a pressure of 120 mmHg. Kidney and aorta were embedded in paraffin and sliced in 5-μm-thick sections. These were incubated with anti-AGE antibodies (1/20) for 1 h and with the fluorescence antibody FITC-labeled goat anti-rabbit IgG 1/20 for an additional hour (Cappel, Cochranville, PA). The sections were examined with a confocal Argon-Krypton laser microscope Leica TCS NT (Leica Microsystems, Rueil-Malmaison, France), using an excitation wavelength at 488 nm, and an emission wavelength band from 500 to 540 nm. The same intensity of laser energy and the same photomultiplier setting was used to allow comparison between section staining.

**Electron Microscopy and AGE Localization by Immunogold Labeling**

Small pieces of renal cortex were fixed by immersion in 4% paraformaldehyde/0.1 M sodium cacodylate buffer for 24 h, dehydrated, and embedded in LR White. Ultrathin 60 Å sections were processed for immunogold labeling with anti-AGE antibody (1/20) and protein A-gold complex (diameter, 15 nm) (1/80) (Biol Cell, Cardiff, United Kingdom) (23). The resulting preparations were examined on a Zeiss 912 transmission electron microscope.

**Renal and Arterial Quantitative Morphometry**

Kidneys and abdominal aorta of 10-mo-old, 30-mo-old *ad libitum*, and 30-mo-old restricted rats were fixed by perfusion as described for immunofluorescence. The left kidney and the abdominal aorta were excised, stored for 24 h in the Dubosq-Brazil solution, and embedded in paraffin. Sections of kidneys and aorta were stained by Marinozzi silver staining or elastic stain, respectively.

For each rat, quantitative morphometry analyses of the different glomerular domains were performed as described previously (26). A 5-μm-thick section was obtained from left kidney transversely cut through the hilus in each rat. On this section, 30 different superficial glomeruli were randomly sampled for morphometric analysis as follows: The microscopic slide was scanned clockwise along the superficial cortex, and one glomerulus out of three was analyzed. Preliminary experiments indicated that convergence of the running mean and variance of every tested parameter was reached for 30 glomeruli measured in each animal. For every investigated glomerulus, the following measurements were obtained with an automated image analysis system: (1) the total glomerular profile area limited by the internal edge of Bowman’s capsule; (2) the glomerular tuft area; (3) the mesangial domain area defined as glomerular tuft area minus areas of the capillary lumens and the capillary-free walls; (4) the total area of the capillary lumen profiles; and (5) the number of capillary lumen profiles (26). Mean glomerular volume was calculated in each rat from the glomerular section area according to Gil and Barba (27) and Weibel (28). In their model, the true mean glomerular diameter \( \bar{D} = (4/3) \times \pi \times (\bar{d}/2)^3 \). Although the sphere hypothesis used for volume calculation is not an unbiased method, a possible error would most likely affect all groups equally. The volumes of the different domains were calculated from this mean glomerular volume and the area fraction of mesangial, capillary, and whole tuft sections. For abdominal aorta, circumference of the vessels, thickness of the arterial wall, and total area surface were determined on transverse sections (29). Total cross-sectional parietal area corresponded to the section area of the vessel minus the luminal area.

**Renal Histology**

Paraffin sections of the kidney were stained with Masson’s trichome. The percentage of glomeruli with focal and segmental sclerosis or total sclerosis (obsolecent glomeruli) was determined on 100 glomeruli randomly sampled in a transverse renal section for each rat.

**Statistical Analyses**

Results are presented as means ± SEM and were statistically analyzed by ANOVA. Survival rates were compared with the log-rank test. Significance was set at \( P < 0.05 \).

**Results**

**Body Weights and Survival**

Body weights of female WAG/Rij rats fed *ad libitum* and restricted by 30% from 10 to 30 mo are shown in Figure 1. Mean values were 213 ± 3 g (\( n = 44 \)) for the 10-mo-old rats and 240 ± 7 g (\( n = 28 \)) for the 30-mo-old *ad libitum* rats. In restricted female rats, body weight decreased during the first 3 mo of treatment and reached a steady state close to the 160 ± 12 g value recorded at 30 mo (\( n = 28 \)).

At 30 mo, corresponding to the end of the experiment, 28 *ad libitum* and 28 food-restricted animals were still alive. When compared with the initial cohorts of 44 animals in each group,
mo-old rats (270
respectively), but was significantly lowered in restricted 30-
mo-old animals food-restricted by 30%. Results are given as mean ± SEM of six animals in each group.

d this corresponded to 64% surviving in the two groups. The two survival curves were not statistically different.

Physiologic Parameters
Systolic BP measured in conscious animals was comparable in 10- and 30-mo-old rats (128 ± 3 mmHg, n = 12 and 129 ± 4 mmHg, n = 12, respectively), and was not significantly modified by the restriction (137 ± 2 mmHg, n = 12). Heart rate was similar in 10- and 30-mo-old animals fed ad libitum (348 ± 6 beats/min, n = 12 and 361 ± 7 beats/min, n = 12, respectively), but was significantly lowered in restricted 30-mo-old rats (270 ± 7 beats/min, n = 12). Heart weight increased in proportion to body weight from 10 to 30 mo (0.67 ± 0.01 g, n = 7 and 0.80 ± 0.02 g, n = 7, respectively) in ad libitum fed rats and was reduced in the restricted animals (0.54 ± 0.02 g, n = 7).

Plasma insulin concentration measured before glucose ad-
ministration was not statistically different in 10- and 30-mo-old control animals, but was significantly lowered in the 30-mo-old restricted rats (Figure 2). Injection of similar glucose load with respect to body weight increased plasma glucose concentration to the same extend in the three groups of rats (data not shown). This increase in plasma glucose concentration resulted in a transient release of insulin, the amplitude of which was significantly larger in the ad libitum 30-mo-old rats than in the 10-mo-old animals (Figure 2). The glucose-induced insulin secretion in 30-mo-old restricted animals was significantly lower than that obtained in the 10- and 30-mo-old rats fed ad libitum (Figure 2).

Values for daily water intake, urine volume, and osmolality are shown in Table 1. Urine volume was larger in the 30-mo-old than in the 10-mo-old animals, and urine osmolality was decreased in proportion. Chronic food restriction prevented this age-related change in water homeostasis. To check that the maintained urine osmolality in the restricted animals was not just related to the reduced daily food intake, the effect of an acute 30% food restriction on urine volume and osmolality was investigated in control 30-mo-old rats. In these additional series, urine volume and osmolality were 7.7 ± 1.5 ml/24 h and 1361 ± 210 mosmol/kg H₂O (n = 10) in 30-mo-old ad libitum-fed animals and 4.9 ± 0.7 ml/24 h and 1321 ± 131 mosmol/kg H₂O (n = 10) in the same animals acutely restricted by 30% for 1 wk.

Proteinuria was significantly increased from 10 to 30 mo in ad libitum-fed rats (Table 1). Electrophoresis of the urine indicated that proteinuria was mainly due to an increase in albumin excretion (data not shown). Food restriction prevented the age-related increase in protein and albumin excretions, which were comparable in 10-mo-old and restricted 30-mo-old rats (Table 1).

AGE Accumulation
AGE content of collagen extracted from abdominal aorta and whole kidney markedly increased from 10 to 30 mo in rats

Table 1. Kidney weight, water homeostasis, and proteinuria

<table>
<thead>
<tr>
<th>Group</th>
<th>Right Kidney (g)</th>
<th>Right Kidney (g/100 g body wt)</th>
<th>Left Kidney (g)</th>
<th>Left Kidney (g/100 g body wt)</th>
<th>Water Intake (ml/24 h)</th>
<th>Urinary Volume (ml/24 h)</th>
<th>Urine Osmolality (mosmol/kg H₂O)</th>
<th>Proteinuria (mg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-mo-old</td>
<td>0.65 ± 0.02</td>
<td>0.318 ± 0.004</td>
<td>0.64 ± 0.02</td>
<td>0.314 ± 0.005</td>
<td>13.2 ± 0.7</td>
<td>3.7 ± 0.4</td>
<td>2389 ± 112</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>30-mo-old</td>
<td>0.80 ± 0.02</td>
<td>0.326 ± 0.007</td>
<td>0.77 ± 0.02</td>
<td>0.316 ± 0.009</td>
<td>16.3 ± 1.6</td>
<td>8.3 ± 1.7</td>
<td>1339 ± 157</td>
<td>13.8 ± 2.4</td>
</tr>
<tr>
<td>30-mo-old restricted</td>
<td>0.55 ± 0.01</td>
<td>0.368 ± 0.013</td>
<td>0.55 ± 0.02</td>
<td>0.360 ± 0.012</td>
<td>16.3 ± 1.6</td>
<td>8.3 ± 1.7</td>
<td>1960 ± 154</td>
<td>3.4 ± 0.6</td>
</tr>
</tbody>
</table>

- Kidney weight (n = 7 in each group), daily water intake, urinary volume, urine osmolality, and proteinuria (n = 10 in each group) in 10- and 30-mo-old female WAG/Rij rats fed ad libitum and 30-mo-old animals food-restricted by 30%. Results are given as mean ± SEM.
- p < 0.05, significantly different from 10-mo-old rats.
- p < 0.05, significantly different from 30-mo-old rats fed ad libitum.
chronic food restriction mostly prevented this accumulation of AGE in both aorta and kidneys of 30-mo-old animals (Figure 3, A and B). Comparable determinations performed on collagen prepared from isolated glomerular basement membranes confirmed the rise of AGE in ad libitum-fed animals and the beneficial effect of diet restriction on intrarenal accumulation of AGE (Figure 3C).

Immunolocalization of AGE in renal cortex by confocal microscopy showed a weak labeling in peritubular matrix and Bowman’s capsule of 10-mo-old animals (Figure 4A). This immunofluorescent staining of AGE was greatly increased in basement membranes and Bowman’s capsule of 30-mo-old rats fed ad libitum (Figure 4B). Accumulation of AGE was also evident on capillary walls and mesangium within the glomeruli of 30-mo-old rats. Food restriction markedly reduced the immunofluorescent staining of AGE in the extracellular peritubular and glomerular matrix (Figure 4C). Immunolocalization of AGE in abdominal aorta is shown in Figure 5. In 10-mo-old animals, AGE were localized in the extracellular matrix of the adventitia, whereas the media matrix was mostly negative. At 30 mo, AGE accumulated in the matrix of both the adventitia and the media. Food restriction reduced this accumulation (Figure 5).

Ultrastructural localization of AGE in the renal cortex was performed by immunogold staining. As shown in Figure 6, specific immunogold labeling of AGE was evident on tubular basement membranes in 10- and 30-mo-old animals fed ad libitum, as well as in animals on a restricted diet. Within the glomerulus, AGE accumulation was localized mainly in the glomerular basement membrane in every group (data not shown).

**Morphometry**

Kidney weight increased by 20% between 10 and 30 mo in ad libitum-fed rats, as did body weight (Table 1). In 30-mo-old restricted animals, kidney weight and body weight were reduced, respectively, by 15 and 25% compared with 10-mo-old animals, and by 30 and 38% compared with 30-mo-old rats fed ad libitum. The kidney to body weight ratio was significantly greater in 30-mo-old restricted animals than in adult and senescent rats fed ad libitum (Table 1). Examination of kidney sections failed to detect interstitial fibrosis, inflammation, or marked vascular alterations whatever the age of the animals or the diet. The proportion of glomeruli with evidence of segmental or total sclerosis did not exceed 2% of the nephrons in 10-mo-old rats. This proportion was not significantly modified in senescent rats fed ad libitum or in those that were restricted. The lack of glomerulosclerosis in senescent WAG/Rij rats is consistent with the previous observations performed in this strain (1,26).

Quantitative morphometry performed by automated image analysis showed an age-related increase in the volume of the glomeruli and of the glomerular tuft, a mesangial expansion, and a greater capillary surface in senescent than in adult rats (Table 2). The increase in capillary volume was proportional to the glomerular enlargement, whereas the mesangial volume increased by 86% when glomerular volume was raised by 19%.

Chronic food restriction prevented glomerular hypertrophy and the increase in capillary volume, but did not affect mesangial expansion (Table 2). The abdominal aorta was enlarged from 10 to 30 mo, as was the wall thickness and total parietal surface (Table 2). Food restriction partly prevented the increase in size and parietal surface of abdominal aorta (Table 2).

**Discussion**

This study shows that a 30% food restriction initiated at 10 mo in lean female rats prevents intrarenal and vascular accumu-
mulation of AGE, but does not modify survival of the animals until 30 mo. Such restriction improves renal function as judged by proteinuria and urine osmolality, and reduces glomerular hypertrophy. It also limits the age-related enlargement of the abdominal aorta without a change in BP.

The effect of food restriction on rodent survival has been explored previously with different protocols. In most cases, it increased mean and maximal life span of the animals from 10 to 50%, depending on the studies (2,4–6). This differs somewhat from the similar survival curves we found in lean rats fed ad libitum and in those that were food-restricted. Although unusual, this observation may be related to the experimental model and to the extent of the restriction.

Female WAG/Rij rats are animals that remain lean even when they have free access to food, in contrast to many laboratory rats, which gain weight during most of their life. If diet restriction acts partly through prevention of obesity and related diseases, it is expected that its effects on survival will be less pronounced in light than in heavy animals. A relationship between overeating and the effect of food restriction has already been documented by Harisson et al., who found that diet restriction in B6 ob/ob obese mice increased their survival by 56%, to a value comparable to lean B6+/+ mice fed ad libitum (30). In contrast, a 33% food restriction in lean B6+/+ mice has little, if any, effect on their mean life span. The extent of the restriction may also contribute to the comparable survival of female WAG/Rij rats fed ad libitum and those that were food-restricted. Previous experiments have already shown that restriction beginning from 10 to 13 mo in rats and mice either did not modify mean and maximal survival rates or raised mean life span by 50%, compared with the 50% increase observed when the restriction was started from 2 to 6 mo (6,31–34). Taken together, the low spontaneous food intake and body weight of female WAG/Rij rats fed ad libitum,
the present moderate 30% food restriction, and the extent of the treatment from 10 to 30 mo may be responsible for the similar survival curves reported for ad libitum-fed and restricted animals. It is not excluded, however, that an effect on maximal life span should have been evident if the experiment was prolonged over 30 mo.

Although survival is comparable in ad libitum and restricted lean female WAG/Rij rats, the present data indicate that food restriction prevents the age-related accumulation of AGE in collagen extracted from the abdominal aorta and kidney, as in collagen isolated from glomerular basement membrane. Extracellular localization of AGE accumulation was confirmed by immunofluorescence in confocal microscopy and by immunogold labeling in electron microscopy. This prevention of AGE accumulation is in good agreement with the reduced pentosidine or furosine formation—two Amadori-derived products—and the lower fluorescence of skin, aortic, or tail collagen reported in food-restricted rats or mice (10,14,16–18). Different mechanisms have been proposed to explain the age-related accumulation of AGE. A reduced glucose tolerance and an insulin resistance, would tend to increase plasma glucose concentration during the different feeding and fasting periods of the day (9,35). The resulting increase in glucose concentration over time would favor the nonenzymatic reactions between proteins and sugars, and enhance production of AGE (9,11). According to this hypothesis, any sustained decrease in plasma glucose concentration would reduce AGE formation. A lower concentration during the different feeding and fasting periods of the day would favor the nonenzymatic reactions between proteins and sugars, and enhance production of AGE (9,11). Conversely, prevention of AGE accumulation would lower these alterations. This has been recently demonstrated in aging rats using aminoguanidine, a drug that inhibits AGE formation without altering plasma glucose concentration (9,11–13). When administered from 6 to 24 mo or from 24 to 30 mo, aminoguanidine did not modify survival of Sprague Dawley, Fisher 344, or WAG/Rij rats, but prevented heart hypertrophy and arterial stiffening. In rats that exhibited glomerulosclerosis, it also improved the development of chronic progressive nephropathy.

The present food restriction in lean rats prevented AGE accumulation, glomerular hypertrophy, proteinuria, and the reduced urine osmolality commonly reported in aging rats. The enlargement in glomerular volume from 10 to 30 mo is consistent with the determinations already performed in female WAG/Rij rats by automated image analysis or in dissected nephrons (37,38). The effect of food restriction on glomerular morphology may be partly due to a reduction in body weight of the animals, and partly to prevention of the AGE cross-linking of extracellular matrix components and of the AGE-stimulated collagen synthesis by mesangial and interstitial cells (9,39,40). These mechanisms could also apply to the age-related enlargement and thickening of the aorta and its prevention by food restriction. In the absence of any change in BP, a lower AGE accumulation in the arterial media of food-restricted rats may contribute to a decrease in age-related arterial stiffness and cardiac hypertrophy (11,13,26,41).

Protein excretion was increased with age. This was mostly due to albuminuria and usually reflects a change in glomerular hemodynamics or sieving (42). The prevention of albuminuria by diet restriction suggests that either filtration rate and pressure are modified by food intake, or that the molecular structure of the glomerular basement membrane is preserved in 30-mo-old food-restricted animals (43,44). In addition, AGE

Table 2. Glomerular and arterial morphometry

<table>
<thead>
<tr>
<th>Variable and Group</th>
<th>Glomerular Volume ($\mu$m$^3 \times 10^6$)</th>
<th>Glomerular Tuft ($\mu$m$^3 \times 10^6$)</th>
<th>Mesangial Volume ($\mu$m$^3 \times 10^6$)</th>
<th>Capillary Volume ($\mu$m$^3 \times 10^6$)</th>
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</thead>
<tbody>
<tr>
<td>Glomerulus</td>
<td></td>
<td></td>
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<tr>
<td>10-mo-old</td>
<td>1.49 ± 0.05</td>
<td>0.96 ± 0.03</td>
<td>0.14 ± 0.01</td>
<td>0.62 ± 0.02</td>
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<tr>
<td>30-mo-old</td>
<td>1.77 ± 0.07$^b$</td>
<td>1.26 ± 0.05$^b$</td>
<td>0.26 ± 0.02$^b$</td>
<td>0.75 ± 0.03$^b$</td>
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<tr>
<td>30-mo-old restricted</td>
<td>1.49 ± 0.05$^c$</td>
<td>1.01 ± 0.03$^c$</td>
<td>0.23 ± 0.03$^b$</td>
<td>0.58 ± 0.02$^c$</td>
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<tr>
<td>Abdominal aorta</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10-mo-old</td>
<td>4.55 ± 0.05</td>
<td>0.066 ± 0.003</td>
<td>0.34 ± 0.02</td>
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</tr>
<tr>
<td>30-mo-old</td>
<td>5.46 ± 0.08$^b$</td>
<td>0.091 ± 0.003$^b$</td>
<td>0.55 ± 0.02$^b$</td>
<td></td>
</tr>
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<td>30-mo-old restricted</td>
<td>5.04 ± 0.05$^{b,c}$</td>
<td>0.082 ± 0.002$^{b,c}$</td>
<td>0.47 ± 0.01$^{b,c}$</td>
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$^a$ Quantitative morphometry of superficial glomeruli and abdominal aorta of 10- and 30-mo-old female WAG/Rij rats fed ad libitum and 30-mo-old animals food-restricted by 30%. Measurements were performed in 30 superficial glomeruli randomly sampled in transversal sections for each rat. Results are given as means ± SEM of 10 animals per group.

$^b$ $P < 0.05$, significantly different from 10-mo-old rats.

$^c$ $P < 0.05$, significantly different from 30-mo-old rats fed ad libitum.
may have a direct effect on protein excretion by the kidney (40). Polyuria and decreased urine osmolality, frequently reported in aging rats, were prevented by food restriction. This was not due to reduced daily food intake, because a 30% food restriction for 1 wk in senescent animals did not alter their urine osmolality. The maintained kidney concentrating ability in restricted animals may be related either to an increase in vasopressin secretion in 30-mo-old treated rats or to prevention

Figure 6. Ultrastructural localization of AGE by immunogold labeling in the tubular basement membrane of 10-mo-old (A) and 30-mo-old (B) female WAG/Rij rats fed ad libitum and 30-mo-old animals food-restricted by 30% (C). Negative control in the absence of AGE antibody (D). TBM, tubular basement membrane; Epi, epithelial cells. Magnification: ×10,000.
of the vasopressin resistance reported in the aging kidney (45). Vasopressin measurements and determination of V2 receptor and aquaporin expression would be necessary to establish the mechanisms by which chronic diet restriction maintains water homeostasis.

In conclusion, the present data indicate that chronic food restriction in lean rats prevents the accumulation of AGE and inhibits structural and functional alterations of the renal and cardiovascular systems, yet does not affect survival of the animals between 10 and 30 mo.

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