Podocyte Hypertrophy, “Adaptation,” and “Decompensation” Associated with Glomerular Enlargement and Glomerulosclerosis in the Aging Rat: Prevention by Calorie Restriction

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Whether podocyte depletion could cause the glomerulosclerosis of aging in Fischer 344 rats at ages 2, 6, 17, and 24 mo was evaluated. Ad libitum–fed rats developed proteinuria and glomerulosclerosis by 24 mo, whereas calorie-restricted rats did not. No evidence of age-associated progressive linear loss of podocytes from glomeruli was found. Rather, ad libitum–fed rats developed glomerular enlargement over time. To accommodate the increased glomerular volume, podocytes principally underwent hypertrophy, whereas other glomerular cells underwent hyperplasia. Stages of hypertrophy through which podocytes pass en route to podocyte loss and glomerulosclerosis were identified: Stage 1, normal podocyte; stage 2, nonstressed podocyte hypertrophy; stage 3, “adaptive” podocyte hypertrophy manifest by changes in synthesis of structural components e.g., desmin but maintenance of normal function; stage 4, “decompensated” podocyte hypertrophy relative to total glomerular volume manifest by reduced production of key machinery necessary for normal podocyte function, e.g., Wilms’ tumor 1 protein [WT1], transcription factor pod1, nephrin, glomerular epithelial protein 1, podocalyxin, vascular endothelial growth factor, and α5 type IV collagen and associated with widened foot processes and decreased filter efficiency (proteinuria); and stage 5, podocyte numbers decrease in association with focal segmental glomerulosclerosis. In contrast, in calorie-restricted rats, glomerular enlargement was minor, significant podocyte hypertrophy did not occur, podocyte machinery was unchanged, there was no proteinuria, and glomerulosclerosis did not develop. Glomerular enlargement therefore was associated with podocyte hypertrophy rather than hyperplasia. Hypertrophy above a certain threshold was associated with podocyte stress and then failure, culminating in reduced podocyte numbers in sclerotic glomeruli. This process could be prevented by calorie restriction.


The podocyte, a key cell involved in glomerulosclerosis, is a highly differentiated neuron-like epithelial cell with limited capacity for cell division and replacement (1–4). Podocytes function to support and maintain the glomerular basement membrane (GBM) filtration mechanism (3,4). Genetic mutations resulting in a glomerulosclerotic phenotype occur in proteins that are expressed by the podocyte, thereby providing strong support for a link between podocyte dysfunction and glomerulosclerosis (3–7). Increasing evidence from experimental models suggests that podocyte injury and loss from the glomerulus may be key components of the process driving glomerulosclerosis (1,3,8–12). Analysis of biopsies from human types 1 and 2 diabetic glomerulosclerosis and IgA nephropathy show that podocyte number is reduced in proportion to the severity of injury and degree of proteinuria and that podocyte number is a predictor of progression (13–18). Loss of podocyte markers from glomeruli is associated with glomerulosclerosis in human biopsy samples from patients with focal segmental glomerulosclerosis (19,20), and increased appearance of podocytes and podocyte constituents in urine is associated with glomerulosclerosis and more rapid deterioration of renal function in focal segmental glomerulosclerosis, lupus nephropathy, IgA nephropathy, and diabetes (21,22). Together these data support the “podocyte depletion hypothesis” for glomerulosclerosis that has as its central tenet the concept that a failure of podocytes to cover the available GBM filtration surface area results in denuded areas of GBM, which in turn triggers matrix accumulation and glomerulosclerosis. A key question to be addressed is whether the acceleration of ESRD prevalence in older age cohorts somehow also could be related to podocyte injury and loss. Floege et al. (23) provided data to support the concept that the glomerulosclerosis of aging is “a podocyte disease,” although the mechanism by which this might occur was not elucidated. We have addressed this question using a rat model (Fischer 344) that does not develop diabetes or hypertension, so as to avoid these confounding variables, but does develop glomerulosclerosis with age when fed an ad libitum diet (24).
The structural changes that occur during glomerular development and aging are well established (25–27). Glomerular size, GBM thickness, and mesangial matrix increase with age in rats that are housed under laboratory conditions, where they receive an ad libitum diet and have limited exercise. We also know that calorie restriction has been reported to prevent glomerulosclerosis associated with aging and to ameliorate the sclerotic lesions in the partial nephrectomy model in the rat (28–30). In this report, we dissect the biology of the glomerulus over time in the Fischer 344 rat model to determine how high-calorie intake might cause age-associated glomerulosclerosis.

Materials and Methods

Rat Model
Fischer 344 rats were purchased from the National Institute on Aging (NIA) at 2, 5 to 7, 15 to 17, and 24 mo of age either as calorie-restricted or in the ad libitum–fed state (24). These rats were kept under identical conditions until after weaning, at which time they were assigned to calorie-restricted or ad libitum diets and maintained in identical cages. The specific food for calorie-restricted rats (supplemented to provide the same mineral and vitamin content as the ad libitum diet) was also purchased from the NIA so as to be able to continue to feed this diet (60% of the ad libitum diet calories) after transfer of animals.

Fixation of Kidneys for Morphometric Analysis
Rats (n = 5 per time point) were anesthetized by ketamine/xylazine injection. Before the heart stopped, the inferior aorta was cannulated, the aorta above the renal arteries was clamped, and perfusion with PBS at 4°C was started at 120 mmHg for 2 min until the kidneys were blanched. One kidney then was removed for further processing, frozen sectioning, or glomerular purification for RNA or protein extraction in some experiments. The perfusate then was changed to paraformaldehyde/lysine/periodate (PLP) fixative at 4°C for 8 min. Kidneys then were removed and sliced into 3-mm thick slices. Slices were processed in one of the following ways: (1) placed in formalin before being processed for histology; (2) diced and processed for transmission electron microscopy; (3) or stored overnight at 4°C in PLP, equilibrated in 20% sucrose, snap-frozen in liquid nitrogen, and stored at −80°C.

Morphometric Analysis

Three-micrometer sections of PLP perfusion-fixed kidney slices embedded in paraffin were used. After treatment with an unmasking agent (Retrieve-All-1; Signet Labs, Dedham, MA) for 2 h at 90°C, sections were incubated with a murine anti-rat glomerular epithelial protein 1 (GLEPP1) mAb, then developed with a peroxidase system using the substrate diaminobenzidine (DAB; Vector Labs, Burlingame, CA). Sections then were counterstained with periodic acid-Schiff (PAS) to stain mesangial matrix and basement membrane pink. Adjacent sections were developed using a monoclonal anti-Wilms’ tumor (WT1) antibody (SC-7385; Santa Cruz Biotechnology, Santa Cruz, CA) using the peroxidase system as described above. Other sections were stained with hematoxylin and eosin for total cell counts. Sections were examined using a Leica DM inverted microscope (Bannockburn, IL), and SPOT camera system (Diagnostic Instruments Inc., Sterling Heights, MI) with morphometry was performed using the calibrated Metamorph Imaging System (Universal Imaging Corp., Downingtown, PA).

Percentage of Podocytes in Glomerular Tuft Cross-Sections.
These estimations were made on 3-μm thick sections stained with peroxidase anti-GLEPP1 and counterstained by PAS/hematoxylin. Thirty consecutive glomerular tuft profiles were analyzed by a blinded investigator, who counted the total number of nuclear profiles in the cross-section that lay within podocytes as indicated by peroxidase-GLEPP1 staining and divided this value by the total number of hematoxylin-stained nuclei in the glomerular tuft cross-section. No correction was made for differences in nuclear size between podocytes and other glomerular cells.

Podocyte Counting, Method 1.
For the initial podocyte counts presented in Results, we used selected glomerular cross-sections and counted both WT1- and GLEPP1-positive cell nuclei as described previously (10). For these measurements, 12 glomerular cross-sectional profiles were identified as passing through the equator of the glomerulus. Each glomerular section was photographed at ×200 magnification, and the image was stored digitally for analysis. Two different individuals performed each set of counts. There were no differences between counts made by the two methods. The results of the four counts for each section were averaged and used for further analysis.

Podocyte Counting, Method 2.
A second method for quantifying podocytes using two sections of different thickness was developed and validated (31). Briefly, thin (3 μm) and thick (9 μm) sections were peroxidase-stained for WT1 on the same microscope slide and viewed under polarized light to facilitate identification of peroxidase-stained podocyte nuclei as described previously. We analyzed 50 consecutive glomerular cross-sections from the outer cortical and juxtamedullary regions without selection. Podocyte numbers calculated using the thin/thick method then were averaged for each individual and used to calculate the values shown in Results.

Glomerular Volume Calculation.
Each of 200 consecutively evaluated glomerular tuft cross-sections in the outer cortex and juxtamedullary region of the tuft area was measured using the calibrated Metamorph Imaging System. The average glomerular volume was calculated as described previously (31).

Total Glomerular Cell Counts.
These were made by counting all hematoxylin-stained nuclei in consecutive hematoxylin-stained sections 3 μm thick. These values, corrected for the known volume of the section and the known average glomerular volume for each rat, were used to calculate the average total cell number per glomerulus.

Glomerular Podocyte Total Volume and Podocyte Cell Size Estimations.
Three-micrometer sections that were stained with peroxidase anti-GLEPP1 and counterstained with PAS/hematoxylin were used. For podocyte measurements, the percentage area of each glomerular tuft profile that was stained brown with immunoperoxidase/DAB using the GLEPP1 antibody was measured using the Metamorph Imaging System. This value then was multiplied by the calculated average glomerular tuft volume to give an estimation of the average volume of podocyte cell content per glomerulus (podocyte mass). This number then was divided by the measured number of podocytes per glomerulus for each animal to give an estimation of podocyte size.

PAS-Positive Glomerular Volume Estimation.
The 3-μm thick sections that were stained with peroxidase anti-GLEPP1 and counterstained with PAS/hematoxylin were used. The area of each glomerular profile that stained pink by PAS was measured using the Metamorph Imaging System. The total PAS-positive volume was estimated by multiplying the glomerular tuft volume by the average percentage of the glomerular profile that stained pink.

Glomerular Volume per Cell.
This was estimated by dividing the average glomerular tuft volume by the average glomerular cell count.

PAS-Positive Volume per Cell.
This was estimated by dividing the average PAS-positive volume by the average glomerular cell number for each animal.
Ultrastructural Morphometric Measurements

Rat kidneys were perfusion-fixed as described above. After removal of the kidney, 1-mm³ fragments were fixed in 4% glutaraldehyde/cacodylate buffer and then prepared for transmission electron microscopy using standard methods. Transmission electron micrographs [(×2800) were made from five glomeruli from each of four different kidneys for the 2-mo-old and 24-mo-old ad libitum-fed and calorie-restricted rats. The 20 photomicrographs for each set of animals were coded by a blinded individual. A Vernier caliper was used to measure (1) the GBM thickness at five points on each photomicrograph and (2) the foot process width for 10 consecutive typical foot (minor) processes along a selected length of GBM.

Glomerular Isolation

Kidneys that were perfused with cold PBS at 4°C were excised, and the cortex was removed and diced with a razor blade. The diced tissue was pushed through a 100-µm nylon sieve (Sefar, Briarliff Manor, NY), and the material that passed through the sieve was collected on a second 75-µm nylon sieve. Glomerular preparations were checked for purity by counting the number of glomerular tufts and tubular fragments. The purity of the glomerular preparations was assessed as the percentage of the fragments present in the preparation that were glomeruli. The data (mean ± SEM) obtained are as follows: 2 mo, 84 ± 3%; 6 mo ad libitum, 83 ± 1%; 17 mo ad libitum, 81 ± 2%; 24 mo ad libitum, 81 ± 2%; 6 mo calorie-restricted, 83 ± 2%; 17 mo calorie-restricted, 85 ± 3%; 24 mo calorie-restricted, 80 ± 3%. There were no statistical differences between these values. Because the tubular fragments were smaller than the isolated glomeruli, >90% of the extracts made from these materials were of glomerular origin. In interpreting these experiments, we note that the glomerular isolation procedure does not capture sclerotic glomeruli, which cannot be extracted by sieving (32). Therefore, in the experiments using isolated glomeruli, there is little or no contribution from sclerotic glomeruli.

Glomerular Protein Extraction, Western Blot, Dot Blot, and Densitometry

Protein extracts of glomeruli were made by suspending isolated glomeruli in PBS-containing inhibitors (Protease Inhibitor Cocktail; Roche Diagnostics Inc., Mannheim, Germany) at 10,000 glomeruli/ml, followed by homogenization in RIPA buffer and centrifugation. Extracts then were aliquotted and stored at –80°C until use. Western blots were performed on glomerular extracts using antibodies to rat nephrin (rabbit polyclonal supplied by Dr. Lawrence Holzman, University of Michigan) or desmin (rabbit polyclonal antibody, cat no. 68122; ICN Biomedicals Inc., Aurora, OH) to confirm that the antibodies recognized the appropriate bands. Dot blots were performed by dotting 3 µl of glomerular protein extract onto Transblot Transfer Medium (BioRad Laboratories, Hercules, CA) paper, blocking with dried milk (2% in TBS) overnight, incubating with antibodies to rat nephrin or desmin followed by secondary antibody, and development with Lumi-light chemiluminescent substrate (Roche Diagnostics). Densitometry was performed on exposed film using the NIH Image 1.61 System. Quantification was achieved by making serial dilutions of the highest concentration sample and then using a standard curve made by serial dilutions of that sample from which to calibrate other samples and expressing data as a percentage of the control. Data were expressed as arbitrary units.

RNA Preparation and Real-Time PCR Measurements

TRIzol reagent (Invitrogen, Carlsbad, CA) was used, according to the manufacturer’s protocols, for isolation of total RNA from tissue speci-
the remaining differences were averaged to obtain (unnormalized) probe set intensities. The standard 230A array was scaled to give an average of 1500 units, and the remaining A arrays were normalized to this standard using a piecewise linear function that made 99 evenly spaced quantiles agree with the quantiles of the standard. The standard 230B array was scaled to give an average of 474 units, which was chosen because this made the average of the ratios for 100 probe sets that are identical on the A and B arrays equal 1.0 for the standard A and B arrays. The remaining B arrays then were quantile-normalized to the standard B array as described for the A arrays. These normalized data then were quantile-normalized to the standard A array as described for the A arrays. The remaining B arrays then were quantile-normalized to the standard B array as described for the A arrays. These normalized data were log-transformed using log[max(x + 50,0) + 50]. The basis for the analysis is as described previously (33) and as detailed at the web address http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=15705192.

As noted above, glomerular preparations were 80% pure as judged by counting. As a further step to confirm that the glomerular RNA preparations were not contaminated in a biased way by either renal cortical or medullary RNA, we performed DNA microarray analysis on RNA preparations of whole renal cortex and medulla at each time point. Within the renal cortex and medullary DNA arrays, we identified marker genes that were highly expressed in either renal cortex or medulla but were expressed by at least a 10-fold lower amount in glomeruli and were unaffected by diet and age. We used kynurenine aminotransferase as a marker of proximal tubules (34). We used claudin 16, an epithelial tight junction protein present in the thick ascending limb of Henle but not in glomerulus (35), as a medullary marker. Because the signal level in cortex and medulla was at least 10-fold higher than in glomerulus, if cortical or medullary RNA had contaminated the glomerular RNA preparations at a level of 10%, then this would have caused at least a doubling of the glomerular kynurenine aminotransferase or claudin 16 signals. In fact, there was no indication that any glomerular preparations used for analysis were significantly contaminated by cortex or medulla (data not shown). Thus, the glomerular RNA preparations are at least 90% free of tubular contamination. Therefore, differences between groups identified on the basis of DNA microarray or real-time PCR data could not be attributed to contamination of glomerular preparations from renal cortex or medulla.

Statistical Analyses

All data presented in figures and tables are shown as the mean ± 1 SD unless otherwise stated in the text. ANOVA was used to compare data sets from different diets. T tests were used to compare groups from different diets at the same time point. Unless otherwise stated * = P < 0.05 and ** = P < 0.01. For DNA microarray analysis, probe sets were selected on the basis of the following criteria. At a given time point (6, 17, or 24 mo), all of the following conditions must be met: (1) minimum 1.5-fold change (up or down) between the average of ad libitum–fed rats and the average of the calorie-restricted rats, (2) no more than 2-fold change (up or down) between any individual rats within the ad libitum–fed or calorie-restricted groups, and, (3) average expression of at least one of the ad libitum–fed or calorie-restricted groups must exceed 315 units (on arrays scaled to a mean of 1500 units). The overall significance of the resulting probe sets was assessed using random permutations of the 24 experimental measurements from each probe set. Less than 0.5% of the randomized data sets gave as many probe sets that met the selection criteria as the actual data.

Results

Fischer rats were housed under laboratory conditions and fed either an ad libitum diet or a calorie-restricted diet (60% of the ad libitum diet calories) from 2 mo of age (after weaning). Measurements were made at 2 mo (“adolescent”), 6 mo (“young adult”), 17 mo (“middle age adult”), and 24 mo (“old adult”).

Figure 1A and Table 1 show that ad libitum–fed rats rapidly gained weight between 2 and 6 mo (2.4-fold) and continued to gain weight up until 17 mo of age (2.9-fold). However, between 17 and 24 mo of age, ad libitum–fed rats lost weight (P < 0.05). Calorie-restricted rats gained much less weight by 6 mo (only 1.3-fold) and by 24 mo had reached only 1.6-fold the 2-mo value. At 24 mo, the ad libitum–fed rats looked frail, sluggish, and old, whereas the calorie-restricted rats of the same age were sleek and active.

As the animals aged, the ad libitum–fed rats developed increased proteinuria (Figure 1B). This did not occur in the calorie-restricted rats. By 24 mo of age, the ad libitum–fed rats showed focal glomerulosclerosis in 9.1 ± 1.8% of glomeruli (Figure 1C and Table 1) compared with 0% glomerulosclerosis in calorie-restricted rats of the same age (P < 0.001). Neither group of rats showed glomerulosclerosis at any other time point. Thus, the dietary difference in the two groups of rats resulted in differences in growth rate of the whole animal and the development of glomerulosclerosis and proteinuria in ad libitum–fed rats over time.

Figure 2 shows representative photomicrographs of glomeruli from the time course under ad libitum–fed and calorie-restricted conditions. These photomicrographs are stained with immunoperoxidase/DAB (brown) for GLEPP1, a podocyte marker, thereby allowing the podocyte area to be measured. They are counterstained with PAS to allow quantification of matrix material and with hematoxylin to allow cell nuclei to be counted. Morphometric analysis of glomeruli was performed to map the structural changes that occurred during aging in ad libitum–fed and calorie-restricted rats. The rapid gain in weight by 6 mo of age seen in the ad libitum–fed rats was accompanied by glomerular volume increases of 2.1-fold by 6 mo of age, 2.6-fold by 17 mo of age, and 3.1-fold by 24 mo of age (Figure 1D and Table 1). This change was much less in the calorie-restricted rats (1.6-fold by 24 mo). Thus, the rapid increase in glomerular volume with age is a calorie-dependent phenomenon, not an inherent property of aging rats.

The total cell number per glomerulus was measured (Figure 1E and Table 1). In ad libitum–fed rats, this number markedly increased between 2 and 6 mo (1.7-fold) and continued to increase throughout life (2.3-fold by 17 mo and 2.6-fold by 24 mo). The total glomerular cell number did not increase in calorie-restricted rats (1-fold at 6 mo, 0.9-fold at 17 mo, and 1-fold at 24 mo). When expressed as glomerular volume per cell, there was no difference between ad libitum–fed and calorie-restricted rats (Figure 1F). Similarly, the PAS-positive glomerular volume increased rapidly in ad libitum–fed rats by 2.2-fold at 6 mo of age, 3.8-fold by 17 mo of age, and 5.8-fold by 24 mo of age, whereas the increase was only 2.1-fold by 24 mo of age in calorie-restricted rats (Figure 1G). When expressed as PAS-positive volume per cell, there was no difference between ad libitum–fed and calorie-restricted groups through 24 mo of age (Figure 1H). Thus, both the glomerular volume and the PAS-
positive volume when expressed per glomerular cell did not differ between ad libitum–fed and calorie-restricted rats over the life of the rat regardless of dietary conditions. As can be seen from Figure 2, the PAS-positive area on glomerular tuft cross-sections largely but not exclusively measures the mesangial compartment.

To measure podocyte number per glomerulus, we used sections stained for both WT1 (a podocyte nuclear marker) and GLEPP1 (a podocyte apical cell membrane marker). Nuclei that stained for WT1 and nuclei of GLEPP1-positive cells were counted in different experiments and expressed as the proportion of total number of cell nuclei in glomerular cross-sections. The proportion of glomerular cells that were podocytes (as defined above) decreased markedly in ad libitum–fed rats over time (Figure 1I). At 2 mo of age, 41.7 ± 2.4% of glomerular cells were podocytes. In the ad libitum–fed rats, the proportion of cells that were podocytes rapidly decreased by 6 mo and further decreased to only 16.8 ± 0.6% by 24 mo. In the calorie-restricted rats, this proportion decreased to 30.6 ± 4.3% by 24 mo. This result suggested that the proportional increase in glomerular cell number seen (Figure 1E) was not reflected by a proportional increase in podocyte number. Rather, the increase in cells must have been in nonpodocyte glomerular cells.

To assess the question of podocyte number in more detail, we performed podocyte counts using a previously described method (10). The number of podocytes per glomerulus (mean ± SEM) at 2 mo was 331 ± 11. The number of podocytes per glomerulus in ad libitum–fed rats at 6, 17, and 24 mo were, respectively, 408 ± 14, 426 ± 11, and 355 ± 8. The values for the calorie-restricted rats at 6, 17, and 24 mo were, respectively, 390 ± 12, 381 ± 8, and 382 ± 10. There was no statistical difference between these values. In ad libitum–fed rats at the 24-mo time point, there were fewer podocytes per glomerulus than at 17 mo, although this difference did not achieve statistical significance.

We were concerned that the method used was not optimal.
We therefore developed a new method for counting podocytes (31). Using this new method, we then recounted the podocytes from the same animals and obtained the results shown in Figure 1J and Table 1. The results were similar to those obtained by the previous method except that we had previously overestimated the total glomerular podocyte count for the reasons described (31). There were no statistical differences between the *ad libitum*–fed and calorie-restricted data sets. However, there was a 1.4-fold increase in podocyte number from 2 to 6 mo in *ad libitum*–fed rats (*P* < 0.05) as the glomerular volume increased and rats gained weight. This value remained at 1.4-fold by 17 mo and decreased to 1.2-fold at 24 mo. The decrease in podocyte number in *ad libitum*–fed rats between 17 and 24 mo did not reach statistical significance. We therefore established that although the podocyte number did increase 1.4-fold in the early growth phase between 2 and 6 mo in *ad libitum*–fed rats, this increase was much less than the increase in glomerular volume and total glomerular cell number. In calorie-restricted rats, there was only a 1.1-fold increase in podocyte number and only a 1.6-fold increase in glomerular volume by 24 mo. These results explain the difference in the percentage of glomerular cells that were podocytes in the previous experiment shown (Figure 11).

Although the glomerular volume continued to increase throughout life in the *ad libitum*–fed rats, the number of podocytes did not increase between 6 and 17 mo and actually decreased by 24 mo. We therefore would anticipate that each podocyte must have become responsible for more basement membrane territory. This relationship (glomerular volume per podocyte) increased markedly in *ad libitum*–fed rats over time (Figure 1K). In parallel with this process, the size of each podocyte increased in the *ad libitum*–fed rats to 1.6-fold by 6 mo, 1.8-fold by 17 mo, and 2.2-fold by 24 mo of age (Figure 1L and Table 1). In contrast, podocyte cell volume changed little in the calorie-restricted rats through 24 mo of age (1.1-fold by 6 mo, 1.0-fold by 17 mo, and 1.2-fold by 24 mo). We conclude from these experiments that podocyte volume increased (hypertrophy) in *ad libitum*–fed rats in conjunction with the increase in glomerular volume. This result would be compatible with the concept that as glomeruli increase in size, the podocytes accommodate by increasing in size, in contrast to other glomerular cells, which increase in number.

The total podocyte complement in the glomerulus at each time point is shown in Table 1 under the heading Pod Mass (the podocyte number multiplied by the podocyte size). From Table 1, podocyte mass had increased to 2.2-fold by 6 mo, a finding that paralleled the increase in glomerular volume (2.1-fold). By 17 mo, the increase in podocyte mass had increased to 2.5-fold, whereas the glomerular volume had increased 2.6-fold. However, by 24 mo in *ad libitum*–fed rats, the podocyte mass had not increased further (only 2.6-fold consisting of a further increase in size and a reduction in number), whereas the glomerular volume had continued to increase (to 3.1-fold). Thus, there seemed to be a relative shortfall in further proportional podocyte mass expansion by 24 mo in *ad libitum*–fed rats at the same time that proteinuria and glomerulosclerosis appeared in these animals.

To provide an additional evaluation of molecular events that take place in glomeruli during the aging and glomerulosclerotic process, we also performed DNA microarray analysis of isolated glomerular RNA preparations from both *ad libitum*–fed and calorie-restricted rats during aging. To identify genes of potential importance to the glomerulosclerotic process and to control for the age affect, we examined the differences in gene expression between *ad libitum*–fed and calorie-restricted rats at each time point (6, 17, and 24 mo). A total of 497 of approximately 30,000 probe sets (1.6%) were significantly different according to the selection criteria used at one or more of the three time points. Of these, 302 probe sets coded for known genes.

Several podocyte marker molecules were present in the selected probe sets. The most striking result was that in each case,
vascular endothelial growth factor (41), and (to a lesser extent) synaptopodin (42), were also significantly reduced in the ad libitum–fed 24-mo-old group. Podocin and CD2AP were not represented on the Affymetrix chip. In contrast, there was no trend for the mesangial marker Thy1 (43) or matrix molecules (collagen I α1 or 2 chains, collagen IV α1 or 2 chains, or fibronectin) to be reduced at the 24-mo time point in ad libitum–fed rats (Table 2). Some of these matrix markers were increased at the 17- and 24-mo time points, consistent with increased matrix synthesis relative to the total glomerular mRNA pool, as might be expected given the increase in PAS-positive material in glomeruli in the ad libitum–fed rats (Table 1).

To confirm the finding that podocyte mRNA were relatively decreased at the 24-mo time point in ad libitum–fed rats we performed quantitative real-time PCR for nephrin (Figure 3A). As was seen in the nephrin DNA microarray data, there was a small increase at 6 mo in nephrin mRNA that approached statistical significance. However, the major difference was at 24 mo, when the ad libitum–fed rat glomeruli contained significantly less mRNA for nephrin (59%) than did calorie-restricted rats. This was confirmed at the protein level by measuring nephrin protein in glomerular protein extracts from the ad libitum–fed rats. The data (expressed in units in relation to total glomerular protein as the mean ± 1 SD) were as follows: 2 mo 113 ± 17, 6 mo 129 ± 8, 17 mo 141 ± 29, and 24 mo 75 ± 18. There was a statistically significant 47% decrease in nephrin protein concentration relative to total glomerular protein at 24 mo in ad libitum–fed rats compared with the 17-mo time point (P < 0.05). This result is consistent with the morphometric and glomerular mRNA data presented above. Thus, three independent measurements (morphometry, mRNA, and protein) all show that there is a 20 to 50% relative decrease of podocyte content of the glomeruli by 24 mo in ad libitum–fed rats, at a time when proteinuria and glomerulosclerosis was occurring.

Floege et al. (23) previously demonstrated that desmin, an intermediate filament, was increased in podocytes in association with aging in a rat model. Desmin was one of the molecules that appeared on the DNA microarray lists as significantly different between ad libitum–fed and calorie-restricted glomeruli (Table 2). We therefore examined desmin expression over time in ad libitum–fed and calorie-restricted rats (Figure 4). Glomerular desmin mRNA was increased significantly in ad libitum–fed rats by 17 and 24 mo as assessed by real-time PCR measurements (Figure 4A). Desmin protein was also increased at these time points as assessed by protein quantification in glomerular extracts (Figure 4, B and C). This increase was readily apparent in a podocyte distribution by immunoperoxidase histochemistry in ad libitum–fed rats (Figure 4, D through J). Desmin mRNA levels remained low in calorie-restricted rats. These results suggests that even by 17 mo in ad libitum–fed rats, before proteinuria was present and glomerulosclerosis had appeared, the biology of the podocyte had changed in a significant way.

As a potentially useful index of podocyte stress, we expressed the glomerular mRNA signal for desmin divided by the mRNA signal for nephrin. Both signals were quantified by real-time PCR in the same glomerular RNA preparations. By
using the ratio of these two podocyte products, we theoretically should provide a window into podocyte function that is unaffected by glomerular size or other variables. The result shown in Figure 3B emphasizes the difference in podocyte biology between ad libitum–fed and calorie-restricted rats already evident by 17 mo, before development of proteinuria and glomerulosclerosis, but increasing through the 24-mo time point, when glomerulosclerosis was present in some glomeruli of ad libitum–fed rats. As noted in Materials and Methods, the isolated glomerular preparations do not contain sclerosed glomeruli because they are not captured by the sieving method. Therefore, Figure 3 shows information derived from nonsclerosed glomeruli even at the 24-mo time point. The data for calorie-restricted rats is remarkably stable throughout the 22-mo period of observation. This result emphasizes that podocytes of ad libitum–fed rats had changed their biology by 17 mo. We designated the term “podocyte stress” to this phenomenon brought out powerfully by measuring the ratio of desmin:nephrin (podocyte stress index).

Podocytes have a complex structure in which interdigitating actin-filled foot processes support a filtration surface (the GBM) to allow for efficient glomerular filtration to occur. If hypertrophic stress were occurring in podocytes and desmin metabolism were altered, then we might expect this to be reflected by detectable changes in podocyte foot process structure. We therefore performed morphometry on transmission electron micrographs of glomerular capillary loops at 2 mo and at 24 mo for both ad libitum–fed and calorie-restricted groups (Table 3). There was a remarkable increase in GBM thickness with age, as is well established. However, the difference in GBM thickness between the 24-mo ad libitum–fed and calorie-restricted groups did not quite reach statistical significance (P = 0.06). Thus, the increase in GBM thickness was predominantly an age-associated phenomenon and largely unrelated to diet. In contrast, foot process width was significantly increased in the ad libitum–fed group compared with the calorie-restricted and 2-mo-old groups (P < 0.01). There was no statistical difference in foot process width between the 2- and 24-mo calorie-restricted groups. Therefore, foot process widening was associated with changed desmin metabolism by 24 mo in ad libitum–fed rats.

The number of podocytes in partially sclerotic glomeruli of ad libitum–fed rats at 24 mo was measured. The area per podocyte nucleus in both the total glomerular tuft area (including the sclerotic area) and the nonsclerotic areas of sclerotic glomeruli was measured. We compared these data with values obtained from nonsclerotic glomeruli in the same histologic section (Table 4). We found that the total area per podocyte was increased, as expected, in sclerotic glomeruli because we and others have previously shown that podocytes are absent from sclerotic areas (19,20). However, we also found that the podocyte number

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**Table 2. DNA microarray data from isolated glomerular RNA preparations of 6-, 17-, and 24-month-old ad libitum–fed and calorie-restricted rats**

<table>
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<th>Affymetrix No.</th>
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<th>Gene</th>
<th>Function/Name</th>
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<th>17 Months</th>
<th>24 Months</th>
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<td>0.51^d</td>
<td>0.26</td>
<td>-0.99^c</td>
</tr>
<tr>
<td>1388782_at</td>
<td>Rn0.1671</td>
<td>Pod1</td>
<td>Transcription factor</td>
<td>0.59^d</td>
<td>0.39</td>
<td>-0.82^b</td>
</tr>
<tr>
<td>1369153_at</td>
<td>Rn0.48745</td>
<td>Nphs1</td>
<td>Nephrin, slit diaphragm protein</td>
<td>0.25^d</td>
<td>-0.05</td>
<td>-0.62^c</td>
</tr>
<tr>
<td>1368412_a_at</td>
<td>Rn0.10163</td>
<td>Ptpro</td>
<td>GLEPP1, apical PTPase</td>
<td>0.23</td>
<td>0.03</td>
<td>-0.77^c</td>
</tr>
<tr>
<td>1374705_at</td>
<td>Rn0.17094</td>
<td>CollIVa5</td>
<td>Collagen IV α5</td>
<td>0.06</td>
<td>-0.21</td>
<td>-0.69^c</td>
</tr>
<tr>
<td>1367600_at</td>
<td>Rn0.1657</td>
<td>Des</td>
<td>Desmin</td>
<td>0.14</td>
<td>0.4^d</td>
<td>0.75^b</td>
</tr>
<tr>
<td>1387933_1_at</td>
<td>Rn0.13805</td>
<td>Podcallyxin</td>
<td>Podocalyxin</td>
<td>0.1</td>
<td>-0.04</td>
<td>-1.10^b</td>
</tr>
<tr>
<td>1370081_at</td>
<td>Rn0.1923</td>
<td>VEGF</td>
<td>VEGF</td>
<td>0.34</td>
<td>0.1</td>
<td>-0.89^b</td>
</tr>
<tr>
<td>1387801_at</td>
<td>Rn0.42910</td>
<td>Synpo</td>
<td>Synaptopodin</td>
<td>-0.15</td>
<td>-0.23</td>
<td>-0.45^d</td>
</tr>
</tbody>
</table>

Other podocyte markers

<table>
<thead>
<tr>
<th>Affymetrix No.</th>
<th>Unigene No.</th>
<th>Gene</th>
<th>Function/Name</th>
<th>6 Months</th>
<th>17 Months</th>
<th>24 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1398905_at</td>
<td>Rn0.34356</td>
<td>Thy1</td>
<td>Thy1</td>
<td>0.2</td>
<td>0.21</td>
<td>0.01</td>
</tr>
<tr>
<td>1370234_at</td>
<td>Rn0.1604</td>
<td>Fn</td>
<td>Fibronectin</td>
<td>0.1</td>
<td>0.23^d</td>
<td>0.3</td>
</tr>
<tr>
<td>1370281_at</td>
<td>Rn0.4064</td>
<td>CollIVa1</td>
<td>Collagen IV α1 chain</td>
<td>0.51</td>
<td>0.57^d</td>
<td>-0.14</td>
</tr>
<tr>
<td>1388494_at</td>
<td>Rn0.2237</td>
<td>CollIVa2</td>
<td>Collagen IV α2 chain</td>
<td>0.44</td>
<td>0.43^d</td>
<td>0.37^b</td>
</tr>
<tr>
<td>1370155_at</td>
<td>Rn0.2953</td>
<td>Colla1</td>
<td>Collagen I α1 chain</td>
<td>0.29</td>
<td>0.21</td>
<td>0.38^d</td>
</tr>
<tr>
<td>1370864_at</td>
<td>Rn0.16629</td>
<td>Colla2</td>
<td>Collagen I α2 chain</td>
<td>-0.19</td>
<td>-0.06</td>
<td>0.89^b</td>
</tr>
</tbody>
</table>

**Notes:**

a | Data are the differences of average log₂ transformed data for ad libitum–fed minus calorie-restricted rat glomerular DNA microarray signal (see Materials and Methods). Two probe sets for WT1 were provided on the Affymetrix chips. Podocin and CD2AP were not present on the Affymetrix rat chips. VEGF, vascular endothelial growth factor.

^b P < 0.01.

^c P < 0.001.

^d P < 0.05.
Fischer 344 rats are available at predetermined ages, through the NIA, and are known to develop spontaneous glomerulosclerosis with age. They do not develop either hypertension or overt diabetes even under ad libitum–fed conditions (24). Thus, we are not concerned with superimposed overt diabetes or hypertension in considering these data, although, as would be expected, ad libitum–fed Fischer rats do have higher insulin levels than calorie-restricted rats (44). The calorie-restricted and ad libitum–fed rats are maintained under identical conditions until after weaning, at which time glomerular development is complete. Thus, there is no difference in glomerular development, structure, or number at the 2-mo start point for the experiments. We mapped the changes in glomerular structure at 2 mo (after weaning, when development is complete), at 6 mo (young adult), at 17 mo (mature adult), and at 24 mo (old adult, by which time 50% of Fischer rats that are kept on an ad

libitum–fed have died of complications of old age). Calorie-restricted rats live on average to 32 mo (24). We hypothesize that the ad libitum–fed Fischer rats are analogous to humans who grow rapidly, attain a large body size, and become overweight on a high-calorie, low-exercise regimen and who may have a genetic propensity to develop glomerulosclerosis. Furthermore, we hypothesize that the calorie-restricted rats may be analogous to humans who have the same propensity to develop glomerulosclerosis but do not become large and overweight because of a lower calorie diet and/or exercise.

Our results from morphometric analysis in the Fischer 344 rat were comparable to those reported for other rat strains (25,27). Steffes et al. (15) also reported similar data for normal human glomeruli, in which the total glomerular cell number was shown to be related directly to glomerular size with both mesangial and endothelial cells contributing. In contrast, there was no increase in podocyte number with glomerular size, and podocyte number did not change measurably with age. Thus, rat and human glomeruli undergo similar age-related changes with respect to glomerular enlargement, total cell number, and podocyte number. Prevention of glomerular enlargement and glomerulosclerosis by dietary restriction in rats has also been reported previously to ameliorate glomerulosclerosis associated with both age and partial nephrectomy (28–30). Floege et al. (23) observed increased podocyte desmin staining in association with glomerulosclerosis of older Milan rats. They concluded that the glomerulosclerosis of age in the Milan rat was a "podocyte disease." The importance of the relationship between glomerulomegaly and glomerulosclerosis is well established, as has been emphasized by Fogo et al. (45,46).

There is now substantial evidence from both human and experimental animal data to support the concept that podocyte depletion is directly related to and can cause glomerulosclerosis (1,8–20). Kriz et al. (1,4,9) described a pathologic process by which denuded GBM resulting from podocyte injury/loss triggers glomerulosclerosis. The mature podocyte has limited capacity for cell division, so once a critical number of podocytes are lost, it seems that the podocyte complement cannot be readily replaced (1–4). At the same time, development of glomerulosclerosis is associated with age, and glomerulosclerosis is the major cause of ESRD with a peak prevalence of treated ESRD in the elderly (26,47). Thus, the hypothesis that we initially tested was that age-associated glomerulosclerosis was due to a linear progressive loss of podocytes with age. We did not find a simple reduction in podocyte number with age. Rather, we found that glomeruli enlarged over time in a calorie-dependant manner. This glomerular enlargement was associated with both age and partial nephrectomy (23) observed increased podocyte desmin staining in association with glomerulosclerosis of older Milan rats. They concluded that the glomerulosclerosis of age in the Milan rat was a "podocyte disease." The importance of the relationship between glomerulomegaly and glomerulosclerosis is well established, as has been emphasized by Fogo et al. (45,46).

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Figure 3. Real-time PCR quantitative mRNA measurements. Glomerular RNA was assayed for nephrin mRNA (A). The ratio of desmin:nephrin mRNA ("podocyte stress index") is shown in B. The data for B are derived from the data shown in A above and from Figure 4. The data shown are the mean ± 1 SD; n = 4 per group. *P < 0.05; **P < 0.01.
From the analysis provided, it seems that the ability of the podocyte to compensate for glomerular enlargement by a combination of hyperplasia and hypertrophy is also limited. By 6 mo of age in ad libitum-fed rats, the 2.1-fold increase in glomerular volume was associated with a 2.2-fold increase in podocyte mass (a 1.4-fold increase in podocyte number × a 1.6-fold increase in podocyte size). Therefore, at the 6-mo time point, there were parallel increases in all glomerular components.

Table 3. Morphometric ultrastructural data from transmission electron micrographs in 2- and 24-month-old calorie-restricted and ad libitum-fed rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 Months</th>
<th>24-Month Calorie-Restricted</th>
<th>24-Month Ad Libitum-Fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBM thickness (µm)</td>
<td>0.18 ± 0.01b</td>
<td>0.39 ± 0.06</td>
<td>0.48 ± 0.05b</td>
</tr>
<tr>
<td>Foot process width (µm)</td>
<td>0.31 ± 0.02</td>
<td>0.32 ± 0.03b</td>
<td>0.40 ± 0.02b</td>
</tr>
</tbody>
</table>

*Statistical indicators are as follows: Those in the 2-month column relate to a comparison between 2-month and 24-month calorie-restricted; those in the 24-month calorie-restricted column relate to a comparison between the 24-month calorie-restricted and ad libitum-fed rats; those in the 24-month ad libitum-fed column relate to a comparison of the 24-month ad libitum-rats with the 2-month rats. GBM, glomerular basement membrane.

bP < 0.01.
There was no measurable podocyte stress (as defined by an increase in desmin synthesis) at 6 mo. In contrast, by 17 mo, a 2.6-fold increase in glomerular volume was associated with no further increase in podocyte number but a further increase in podocyte size to 1.8-fold, resulting in a 2.5-fold increase in podocyte mass. This further increase in podocyte size was clearly associated with a change in podocyte biology, reflected by increased desmin mRNA and accumulation of desmin protein. There was no increase in proteinuria and no glomerulosclerosis present by 17 mo in *ad libitum*–fed rats, presumably reflecting an adequate, if stressed, contribution from the podocyte. However, by 24 mo, there was a reduction in podocyte number to 1.2-fold and a further increase in podocyte size to 2.2-fold to give a podocyte mass that was essentially unchanged from the 17-mo time point. By this time, glomeruli had continued to increase in size to 3.1-fold above baseline. Thus, the glomerular podocyte mass had not kept up with the increases in glomerular volume by 24 mo of age in *ad libitum*–fed rats. This conclusion was validated by three independent measurements (morphometry, glomerular content of specialized podocyte mRNA, and nephrin protein content).

The components that make up the PAS-positive glomerular volume were not defined precisely in this analysis. They consist mostly of mesangial matrix but also include mesangial and endothelial cells in different proportions at different times. When expressed per glomerular cell, this variable continued to increase throughout life, and the ratio did not differ between *ad libitum*–fed and calorie-restricted rats. However, the *ad libitum*–fed rats accumulated many more cells, so their glomeruli actually contained 5.8-fold more matrix by the 24-mo time point.

The failure of proportional podocyte hyperplasia and hypertrophy by 24 mo in *ad libitum*–fed rats was accompanied by widening of foot processes and dysfunction of the glomerular filter, manifest by proteinuria. Moreover, in some glomeruli of 24-mo-old *ad libitum*–fed rats, podocyte number had decreased further in association with segmental glomerulosclerosis, possibly as an attempt to repair areas of denuded GBM by the mechanisms described by Kriz *et al.* (1,4). We conclude that

### Table 4. Podocyte content of sclerotic and nonsclerotic glomeruli from 24-month-old *ad libitum*–fed rats

<table>
<thead>
<tr>
<th>Glomerulus Type</th>
<th>Total Tuft Area per Podocyte Nucleus (μm²)</th>
<th>GLEPP1-Positive Area per Podocyte Nucleus (μm²)</th>
<th>Glomerular Tuft Area (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsclerotic</td>
<td>1285 ± 113</td>
<td>564 ± 100</td>
<td>17085 ± 1238</td>
</tr>
<tr>
<td>Partially sclerotic</td>
<td>2224 ± 331b</td>
<td>715 ± 99c</td>
<td>15618 ± 974</td>
</tr>
</tbody>
</table>

*P < 0.01, *P < 0.05.

---

Table 5. Stages of hypertrophic podocyte injury

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>Normal podocyte</td>
</tr>
<tr>
<td></td>
<td>normal glomerular and podocyte structure and function</td>
</tr>
<tr>
<td>Stage 2</td>
<td>Nonstressed podocyte hypertrophy</td>
</tr>
<tr>
<td></td>
<td>enlarged podocytes but not associated with changes in podocyte biology such</td>
</tr>
<tr>
<td></td>
<td>as increased desmin synthesis and associated with a proportional level of</td>
</tr>
<tr>
<td></td>
<td>specialized podocyte machinery in relation to glomerular size and</td>
</tr>
<tr>
<td></td>
<td>no increase in proteinuria</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Adaptive podocyte hypertrophy</td>
</tr>
<tr>
<td></td>
<td>podocyte hypertrophy associated with a change in podocyte biology such that</td>
</tr>
<tr>
<td></td>
<td>desmin synthesis is increased but podocyte function remains normal as</td>
</tr>
<tr>
<td></td>
<td>reflected by a proportional increase in specialized podocyte machinery and</td>
</tr>
<tr>
<td></td>
<td>lack of overt proteinuria</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Decompensated podocyte hypertrophy</td>
</tr>
<tr>
<td></td>
<td>podocyte hypertrophy associated with changed podocyte biology (increased</td>
</tr>
<tr>
<td></td>
<td>desmin synthesis) but now manifest by decreased specialized podocyte</td>
</tr>
<tr>
<td></td>
<td>machinery (e.g., nephrin) in relation to glomerular volume, increased foot</td>
</tr>
<tr>
<td></td>
<td>process width, and manifest functionally by decreased filter efficiency</td>
</tr>
<tr>
<td></td>
<td>(increased protein excretion)</td>
</tr>
<tr>
<td>Stage 5</td>
<td>Podocyte depletion</td>
</tr>
<tr>
<td></td>
<td>podocyte hypertrophy associated with changed podocyte biology, reduced</td>
</tr>
<tr>
<td></td>
<td>specialized podocyte machinery, and failure of podocytes to meet demand,</td>
</tr>
<tr>
<td></td>
<td>resulting in relative or absolute podocyte loss and consequent</td>
</tr>
<tr>
<td></td>
<td>glomerulosclerosis</td>
</tr>
</tbody>
</table>

---
because mature podocytes have limited capacity to divide, they are largely dependent on hypertrophy to increase the podocyte mass (podocyte size × number) in mature glomeruli. From these data, we hypothesize that there are recognizable stages of hypertrophy through which podocytes pass en route to glomerulosclerosis (Table 5). Similar stages of podocyte hypertrophy, “adaptation,” and “decompensation” could occur in association with glomerulomegaly caused by partial nephrectomy, nephronopenia, obesity, diabetes, and other metabolic and endocrine abnormalities (9,45,46,48,49).

One of the difficulties inherent in analysis of the podocyte contribution to injury is the problem of counting podocytes. We used three different approaches to assess podocyte number per glomerulus: (1) The percentage of podocytes per glomerular cross-section method, (2) the podocyte number per cross-section method, and (3) the thick/thin section method. All three approaches provided a similar relative result. However, this study reinforces the point that the primary issue of concern is probably not the podocyte number per se but the podocyte number in relation to the territory that each podocyte has to support (sometimes designated “podocyte density” or “glomerular volume per podocyte”) (10,13–18,45,46,48). The “podocyte stress index” (desmin:nephrin mRNA ratio) is a related value that takes into account whether the podocyte is stressed by alterations in glomerular volume in relation to podocyte number under any circumstances and may prove to have utility in circumventing the complex problem of morphometrically measuring this relationship.

Calorie restriction prevented glomerular enlargement, foot process widening, PAS-positive volume expansion, podocyte stress, podocyte loss, and glomerulosclerosis seen in the ad libitum–fed rats by the 24-mo time point. This result draws attention to the potential role of high caloric intake throughout life and consequent high insulin levels, which may influence glomerular size (50) and may set up the glomerulus for glomerulosclerosis. Obesity itself is associated with proteinuria and glomerulosclerosis (51) and has been reported to be associated with increased prevalence of ESRD (52). We currently face an epidemic of obesity in children and adults who eat high-calorie fast food and exercise little. Obesity and overweight are also associated with diabetes and hypertension, two major causes of ESRD in the United States (47). It may be that high-calorie intake resulting in larger body size, obesity, and a high growth factor milieu is an important factor driving development of glomerulosclerosis through glomerular enlargement associated with podocyte hypertrophy, stress, and failure by the mechanisms outlined above.

Acknowledgments

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References


See related editorial, “Role of Podocytes in Focal Sclerosis: Defining the Point of No Return,” on pages 2830–2832.