Adult-onset calorie restriction delays the accumulation of mitochondrial enzyme abnormalities in aging rat kidney tubular epithelial cells

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Submitted 4 August 2006; accepted in final form 28 February 2007

McKiernan SH, Tuen VC, Baldwin K, Wanagat J, Djamali A, Aiken JM. Adult-onset calorie restriction delays the accumulation of mitochondrial enzyme abnormalities in aging rat kidney tubular epithelial cells. Am J Physiol Renal Physiol 292: F1751–F1760, 2007. First published March 6, 2007; doi:10.1152/ajprenal.00307.2006.—Adult-onset calorie restriction (A-CR) is an experimental model of life extension and healthy aging less explored, compared with calorie restriction begun at early ages, but one more realistic for human application. We examined the effect of A-CR on the aging rat kidney with respect to common structural age-dependent changes and the accumulation of mitochondrial enzyme abnormalities in tubular epithelial cells. A 40% calorie restriction was initiated in middle-aged rats, before the onset of significant age-related changes in the Fischer × Brown Norway rat kidney. This dietary intervention effectively reduced glomerulo-interstitial fibrosis formation within 1 yr and vascular wall thickening rat kidney. This dietary intervention effectively reduced glomerulo-interstitial fibrosis formation within 1 yr and vascular wall thickening rat kidney.

Mitochondrial enzyme abnormalities in tubular epithelial cells. A 40% calorie restriction was initiated in middle-aged rats, before the onset of significant age-related changes in the Fischer × Brown Norway rat kidney. This dietary intervention effectively reduced glomerulosclerosis and tubular atrophy within 6 mo and changed the rate of interstitial fibrosis formation within 1 yr and vascular wall thickening and the expression cytochrome c oxidase (COX)-deficient tubular epithelial cells in 18 mo compared with age-matched ad libitum-fed rats. Our histological approach (histochimical staining for mitochondrial enzyme activity and laser capture microdissection) coupled with mitochondrial DNA (mtDNA) PCR analyses demonstrated that COX-deficient renal tubular epithelial cells accumulated mtDNA deletion mutations and that these cells contained unique, clonally expanded mtDNA deletion mutations. Renal tubular epithelial cells with mitochondrial abnormalities presented cellular characteristics indicative of physiological dysfunction.

dietary restriction; cytochrome c oxidase; mtDNA deletion mutations

CHRONIC KIDNEY DISEASE (CKD) is a disorder primarily of the elderly, the fastest growing segment of American society (8). The incidence rates of CKD for individuals over 75 years of age have doubled in the last 20 years, whereas the incidence rates for patients younger than 65 are stable. This may be due to improved treatment of diabetes and hypertension, the most common causes of CKD; however, a complete understanding of the broader mechanisms that contribute to intrinsic kidney aging is lacking. Studies that reveal the molecular and biochemical changes that accompany aging in the kidney may lead to interventions that prevent or delay the onset of CKD.

Age-dependent alterations in mitochondrial function and mitochondrial DNA (mtDNA) are believed to play a fundamental role in the aging process (16, 53). A common mtDNA deletion mutation, 4,834 bp long, has been shown to increase in frequency with age in the kidneys of Wistar rats (47). Chronic renal failure has been associated with increased genomic damage (45). The pathogenesis of DNA damage involves enhanced reactive oxygen species (ROS) formation, advanced glycation end products, and carbonyl stress (46). Mitochondrial dysfunction and mtDNA mutations, specifically, have been observed in nephrotic syndrome (15, 43), focal segmental glomerulosclerosis (52), in kidneys of diabetic rats (20), in Adiramycin-induced nephrosis (23), and as peripheral (skeletal muscle and hair follicles) biomarkers of end-stage renal disease (25, 26).

The mitochondrion is unique among cellular organelles in that it contains its own genome and that 10s–100s of copies are present in a single mitochondrion. The mitochondrial genome is a 16-kb double-stranded circular DNA molecule encoding 22 tRNA and 13 polypeptides of the electron transport system (ETS). Cytochrome c oxidase (COX; complex IV of the ETS) has three of its subunits encoded by the mtDNA. COX is the final complex of the electron transport system and catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen and is strategic for the safe reduction of oxygen. The COX activity of individual cells can be determined, cytochemically, on frozen histological slide sections. The absence of COX activity within individual cells on histological sections is indicative of mitochondrial dysfunction within those cells. Cells lacking COX (COX−) activity are referred to as “ETS abnormal.” Succinate dehydrogenase (SDH) is the second complex of the ETS, embedded in the mitochondrial membrane, and is entirely nuclear encoded. On histological sections of skeletal muscle, COX-deficient cells are often found to have greatly increased SDH activity indicating a compensatory up-regulation of mitochondrial biogenesis (SDH+/+). Concomitant with ETS abnormal cells (COX−/SDH+/+) are deletion mutations of the mitochondrial genome (6, 11, 50). In aged skeletal muscle fibers, these age-dependent mtDNA deletion mutations are typically very large, lacking nucleotides that encode subunits of ETS complexes-I, -II, -IV, and/or -V as well as transfer RNA genes. For an abnormal mitochondrial enzyme phenotype to be detected histologically, in skeletal muscle fibers, the mutant mtDNA must persist and accumulate to >90% of the total cellular mtDNA (4, 14).

Diet has long been recognized as a modulator of kidney health in both humans and experimental models (2, 35). Protein restriction (30, 37), salt restriction (39), and calorie restriction (17, 18) delay the development of age- and disease-related glomerular sclerosis. Calorie restriction (CR), initiated at early

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ages in mammalian model systems, extends and retards the progression of many age-associated molecular, physiological, and pathological processes (51). Age-dependent changes that occur in tissues with high oxidative demand, such as skeletal muscle (5, 32), heart (28), brain (33), and kidney (17, 24, 44), were significantly attenuated after long-term CR. Chronic progressive nephropathy, a common age-related renal disease in Sprague-Dawley and Fischer 344 rats, was effectively reduced by restricted caloric intake (12). Subsequent studies on the kidney using life-long CR show effective reductions in apoptosis (24), Fas expression (36), and mitogen-activated protein kinases (21).

The effect of a 40% calorie-restricted diet, begun at middle age (A-CR), is a paradigm less frequently employed but one more relevant with respect to human health (i.e., CR initiated at a young age is not amenable to human application given its effects on development). The potential mechanisms responsible for the anti-aging effects of a calorie-restricted diet are wide ranging but no single mechanism has been defined (reviewed in Refs. 31 and 51). It is widely postulated that CR’s benefits derive partly from decreased mitochondrial ROS production and subsequent reduction in oxidative damage (19). CR promotes mitochondrial biogenesis, bioenergetic efficiency (27), and prevents the age-associated decline in oxidative capacity (3). The mitochondrion is central to cellular homeostasis and has emerged, from numerous CR studies, as an essential element in aging and anti-aging hypotheses.

Kidney tubular epithelial cells are rich in mitochondria, are highly dependent on oxidative phosphorylation, and display age-dependent decrements in function. We hypothesized that adult-onset CR inhibits the generation of mtDNA mutations, ETS abnormalities, preserves mitochondrial function in renal tubular epithelial cells, and limits age-associated structural changes. Using the Fischer × Brown Norway F1 hybrid rat (FBN), a long-lived rat model of healthy aging, we examined the effects of A-CR initiated in middle age (18 mo) on tubular epithelial cell mitochondrial enzyme activity together with age-associated morphological changes in the aging kidney at 24, 30, and 36 mo of age. This study represents the first study to examine long-term A-CR at extremely old ages in the rat kidney.

MATERIALS AND METHODS

Animals and calorie restriction. Two groups of male Fischer 344 × Brown Norway hybrid rats were purchased from the NIA aging rodent colony at Harlan (Oregon, W1). The first group of rats (n = 38) was 17 mo of age. The rats were acclimated to their surroundings and average food intake was monitored (~16.5 g/day). At 17.5 mo of age, a 20% dietary restriction was imposed, and by 18 mo, A-CR rats were brought to a full 40% (~10 g/day) reduction in dietary intake. The restricted diet was AIN 93M Fortified (F05495, BioServ, Frenchtown, NJ), enriched with caseins, fat, vitamins, and minerals. The second group of rats (controls) was purchased at 6-mo intervals beginning at 18 mo through 36 mo of age (n = 6 per age group) and were fed ad libitum (AL) using Harlan Teklad Global Diet 20/14s (Madison, WI). Not only is the overall diet composition important in CR to prevent malnutrition, but the relative proportion of fat, protein, and carbohydrate can affect tissue-specific aging. Since soy-based or low-protein casein diets diminish nephropathy in the Fischer 344 rat strain (35), the diets used in this study were low protein (~14% by weight) to avoid a dietary contribution to the age-related nephropathy being studied. Animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals and the institutional animal protocol review board approved experimental procedures.

Rats were euthanized, weighed, and kidneys were dissected from AL rats at 18, 24, 30, and 36 mo (n = 6 at each time point) of age and A-CR rats at 24 (n = 6), 30 (n = 6), and 36 mo (n = 5) of age. Kidneys were weighed, bisected sagitally, embedded in Optimal Cutting Temperature medium (Tissue Tek O.C.T. Compound, Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. Enzyme analyses require frozen tissue since enzymes can be completely inactivated by exposure to conventional fixatives. While formalin-fixed, paraffin-embedded sections are ideal for morphology, frozen sections are required for analysis of mitochondrial enzyme activity and still allow identification of general trends in kidney pathology. Fifty consecutive, 10-μm-thick midsagittal sections were cut at ~20°C using a cryostat, placed on glass slides, and stored at ~80°C until use.

Mitochondrial enzyme activity. To identify abnormal mitochondrial enzyme activity in kidney tubular epithelial cells, frozen sections of whole kidney were histochemically stained for COX and SDH enzyme activities using standard protocols described by Seligman et al. (41) and Dubowitz (10). Abnormal tubular epithelial cells were identified by the absence of COX activity and hyperreactive for SDH (Fig. 1, A and B, respectively). Dual staining for COX and SDH (48) provides high contrast and specificity to distinguish ETS normal cells (muted brown/blue color) from cells deficient in COX activity (bright blue; Fig. 1C). Two dual-stained sections of one kidney from each animal at each age and diet were examined for ETS enzyme activities. The volume density of ETS abnormalities (VDETSab) on whole sections of kidney was determined using contiguous digital images of the cortex (~24 images per kidney section) at ×4 magnification. The areas of each image, as well as the areas of the ETS abnormal tubular epithelial cells within that image, were measured using ImagePro

![Fig. 1](http://ajprenal.physiology.org/10.22033.2)
software (Media Cybernetics, Silver Spring, MD). The \( V_{D\text{ETS}_{ab}} \) was expressed as (total area of ETS abnormalities observed per kidney section)/(the total area of cortex examined) \( \times 100 \) (29). ETS abnormal tubules \( (n = 32) \) from 30-mo-old AL-fed rat kidneys were followed along their length on \( 10 \) (\( n = 14 \)) and \( 15 \) (\( n = 18 \)) consecutive dual-stained slide sections to characterize the spatial pattern of ETS abnormal cells within the tubules.

**Laser-capture microdissection and mtDNA analyses.** ETS normal and ETS abnormal tubules were isolated from slide sections using laser-capture microdissection. Slide sections, adjacent to those on which ETS abnormal tubules were identified, were stained for SDH activity and dehydrated through an ethanol and xylene series. Individual ETS normal and abnormal tubules were identified and microdissected using PixCell II laser-capture microdissection (Arcturus, CA) with a laser spot size of 30 \( \mu \)m, a pulse power of 30 mW, and a pulse width of 50-ms settings. A single tubule was captured per cap (Fig. 2). Total DNA was extracted using 1 \( \mu \)l of digestion solution (2 mg/ml proteinase K, 0.5% sodium dodecyl sulfate, and 10 mM EDTA) placed on the captured tissue and incubated at 37°C for 30 min. After digestion, 10 \( \mu \)l of water were added to the original 1 \( \mu \)l of digestion solution and the sample was collected and stored at -20°C. One microliter of the DNA solution underwent primary PCR amplification using primers specific to the rat mitochondrial genome using primers F4032 (5'-AAAAAGCCCAACATCACTGAAAGCA-3') and R1432 (5'-AGCTGATAAGCTTTCACC-3') for 30 cycles at 94°C for 30 s, 60°C for 30 s, and 70°C for 11 m. This reaction produced sufficient amplicons as to be visualized on an agarose gel. The amplicons from the ETS abnormal tubules were less than the expected 14-kb wild-type DNA. These DNA products were further amplified in preparation for gel extraction and sequencing during a second nested PCR reaction using the primers F7141 (5'-ACACAACAAGCCAGTACATGAA-3') and R14140 (5'-TTTCGGATGTTTGCAAAGCTAT-3') for 30 cycles at 94°C for 30 s, 56°C for 30 s, and 70°C for 5 min amplifying a 7,000-bp fragment of the wild-type mtDNA. PCR products were gel extracted using a QIAquick gel extraction kit (Qiagen, Valencia, CA) and sequenced using ABI PRISM BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA) at the University of Wisconsin-Madison, DNA Sequencing Center.

**Semiquantitative scoring of age-associated changes in the kidney.** Two slide sections of one kidney from each animal were stained with Gomori’s trichrome to assess interstitial fibrosis and vascular wall thickening (42). A minimum of twenty cortical fields were examined for each kidney section. A score of zero (0) indicated no tubular atrophy of cortical tubules, no glomerular basement wall thickening with no capillary irregularities, no interstitial fibrosis of the cortical area, or no vascular wall thickening/luminal area narrowing. A score of one (1), two (2), or three (3) indicated ≤25% (mild), 26–50% (moderate), or greater than 50% (severe) involvement, respectively, of the cortex or specific structure (45). Two independent observers evaluated pairs of slides from each kidney.

**Statistical analysis.** Average scores for each kidney, at each age and diet, for each index measured, were used for statistical analysis. Data were analyzed using regression analysis to determine whether there was an interaction between age and diet, while ANOVA was utilized to determine differences between ages and diets \( (P < 0.05) \). All statistical analyses were performed using the general linear model procedures of SAS (40).

**RESULTS**

**Anthropometric data.** The body weight of AL-fed rats significantly increased between 18 and 24 mo of age; weight was maintained through 30 mo, followed by an 18% decline at 36 mo (Fig. 3A). In A-CR rats, body weight did not change during the first 6 mo of dietary restriction but by 30 mo of age (12 mo of restriction), A-CR rats had lost 20% of their body mass with no further decline in body mass observed between 30 and 36 mo of age. Calorie-restricted rats weighed, on average, 38% less than the AL-fed rats. Regression analysis indicated a significant interaction between age and diet on rat body weight \( (P = 0.0217) \).

The mean kidney weight of the 18-mo AL rats was 1.22 ± 0.10 g. Between 18 and 24 mo, the mean kidney weight of the AL rats increased significantly to 1.54 ± 0.16 g and then remained unchanged through 30 and 36 mo. Kidney weights in A-CR rats \( (1.24 ± 0.12 \text{ g at 24 mo, } 1.23 ± 0.05 \text{ g at 30 mo, and } 1.17 ± 0.11 \text{ g at 36 mo}) \) were significantly less than age-matched AL kidneys (Fig. 3B) and maintained the baseline weight from 18 through 36 mo of age. Regression analysis indicated a significant interaction between age and diet on kidney weight indicating that A-CR prevented the age-associated weight increase seen in the AL rats \( (P = 0.0015) \).

**Mitochondrial ETS enzyme abnormalities.** Mitochondrial enzyme abnormalities were rarely identified in 18-mo kidneys (Fig. 4A) yet were observed in all kidney sections from AL and

![Fig. 2. Laser-capture microdissection of ETS abnormal renal tubular epithelial cells (36-mo-old ad libitum-fed rat) from a histological section. A: ETS abnormal tubule stained with SDH. B: slide section with remnants of captured tubule after laser-capture microdissection. C: ETS abnormal tubule isolated for mtDNA mutation analysis.](http://ajprenal.physiology.org/DownloadedFrom)
A-CR rats ≥24 mo of age. In midsagittal sections, COX-deficient cells (Fig. 4B) were concentrated in the cortex and populated discreet portions of individual tubules. Regions of the tubule were either overtaken by ETS abnormal cells (Fig. 4, C and F) or the ETS abnormal cells were interspersed among ETS normal cells (Fig. 4, D and E). Many single ETS abnormal tubular epithelial cells were also observed (Fig. 4, G and H). Thirty-two ETS abnormal tubules were followed through 15 serial sections (i.e., encompassing 150 μm from the first section) to determine the extent of the abnormal epithelium within the tubule. The pattern of involvement varied within individual affected tubules. ETS abnormal cells persisted throughout the 150 μm examined in three tubules. Fifteen of the abnormal tubules turned out of the field of view and their length could not be accurately determined. Three ETS abnormal tubules were cut along their longitudinal axis and each was present on at least 10 consecutive sections (i.e., these tubules were ≥100 μm in cross-section). Eleven tubules had segmental distributions of ETS abnormal cells flanked by ETS normal cells (Fig. 5, A and B); seven of which were very short (10–30 μm) and four with ETS abnormal regions extending 60–140 μm, indicating these abnormalities were centered within the tubule and did not extend to the glomerulus.

The mean number of renal tubules deficient in COX enzyme activity increased in AL-fed rats with age, from 0.41 ± 0.80 ETS abnormal tubules per 10-μm-thick kidney section in the 18-mo-old rat to 44 ± 13 per section in 24-mo-olds, 56 ± 19 per section in 30-mo-old and 80 ± 21 per section in 36-mo-old rats. A-CR rats also had an age-dependent increase in ETS abnormal tubules (24 mo = 32 ± 13, 30 mo = 43 ± 14, and 36 mo = 65 ± 19). To account for the change in kidney size with age, the volume density of ETS abnormal cells (VDETSab) in kidney sections were calculated (29). The VDETSab in AL-fed rats significantly increased with age from 0.0015 ± 0.0034% at 18 mo to 0.6892 ± 0.2475% at 36 mo of age (Fig. 6). The A-CR rat kidneys had a significantly lower volume density of ETS abnormal cells (VDETSab = 0.4067 ± 0.1367%) compared with AL kidneys at 36 mo; however, no differences were observed between A-CR and AL rats at 24 and 30 mo of age. Regression analysis indicated a significant interaction between age and diet (P < 0.0001) indicating that

![Fig. 3](http://ajprenal.physiology.org/) A: body weights of FBN rats with age on ad libitum (AL) and calorie-restricted (A-CR) diets. B: FBN hybrid rat kidney weights with age and diet, n = 6 per age and diet. Bars with different letters indicate significant differences, P < 0.0001.

![Fig. 4](http://ajprenal.physiology.org/) A: 18-mo histochemical section with normal staining for ETS enzyme activity. B: 36 mo with 3 COX-deficient tubules. C: 36-mo rat kidney ETS abnormal tubule. D: hyperplasia of tubular epithelial cells within an ETS abnormal tubule showing disorganization of the epithelial cell monolayer and nuclear depolarization. E: heterogeneous population of ETS normal and ETS abnormal epithelial cells within a tubule (note the cellular hypertrophy of cells within the abnormal tubule compared with normal tubular epithelial cells). F: proximal tubule completely overtaken by COX-deficient epithelial cells. G and H: single ETS abnormal tubular epithelial cells. Histological sections were obtained from 36-mo-old ad libitum-fed rats. Bars indicate 50 μm.
the rate of ETS abnormal cell accumulation in kidney tubular epithelium was different between AL- and A-CR-fed rats. As shown in Fig. 4, C and D, renal tubule epithelial cells with abnormal COX activity often appeared hypertrophic compared with ETS normal cells and the orderly single cell epithelial layer became hyperplastic. The cellular organization of these cells was disrupted with nuclei centrally located suggesting a loss of polarization.

Laser-capture microdissection and mtDNA PCR analyses. To determine whether mtDNA mutations were concomitant with the ETS enzymatic abnormalities, individual tubules were microdissected from histological slides. Three ETS normal tubules (N) and four ETS abnormal tubules, three of these being sampled at two different locations along the tubule (1a, 1b, 2a, 2b, 3a, 3b and 4), were laser capture microdissected from the 10-μm-thick histological sections. Samples were PCR

Fig. 5. Serial sections of ETS abnormal tubules dual stained for COX and SDH facilitated the analysis of tubules along their length. A): renal tubules with ETS abnormal epithelial cells. B): tubule with ETS abnormal cells present on 10 of 14 sections flanked by ETS normal cells.
amplified using primers specific to and bracketing the major arc of the mitochondrial genome. The expected wild-type (7 kb) size product was observed from the ETS normal tubules (Fig. 7 sample N) while the major amplification products from the ETS abnormal tubule were smaller than wild-type. A single, smaller than wild-type amplification product was observed in each ETS abnormal tubule (Fig. 7). The size of the deletion mutations differed between tubules; however, when the same abnormal tubule was sampled at two different regions, the same mtDNA amplification product was obtained. Samples 2a and 2b (Fig. 7) were heteroplasmic for wild-type and mutant DNA. The amplification products were directly sequenced and deletion breakpoint position determined. In all cases, the deletion mutations were the result of a loss of a single region of the genome. The same deletion event was present in samples 1a and 1b (3,135-bp deletion product with breakpoints at nucleotides 9358 and 12493) and in samples 2a and 2b (1,687-bp deletion product with breakpoints at nucleotides 11218 and 12905). Sample 3 (3a) identified a 3,137-bp deletion mutation between nucleotide numbers 9792 and 12905. Sample N, an ETS normal tubule, had an intact PCR product of 6999 bp. Sample 4 had a 4,499-bp deletion product with breakpoints at nucleotides 7652 and 12151.

Histomorphometric analyses of age-dependent kidney injury. Kidney structural integrity was analyzed by semiquantitative scoring of tubular, interstitial, glomerular, and vascular changes to determine the impact of A-CR on age-dependent kidney remodeling. Representative H&E- and Gomori-stained sections from 18-mo-old, 36-mo-old AL-fed, and 36-mo-old A-CR-fed rat kidneys are presented in Fig. 8. Glomerulosclerosis significantly increased with age in both the AL- and A-CR-fed rats (Fig. 9A); however, these lesions were significantly greater in the AL-fed rats at corresponding time points (ANOVA, $P < 0.0001$). There was a significant interaction between age and diet (regression, $P = 0.0007$). Renal tubular atrophy increased with age in the AL rats with mean severity scores of $0.602 \pm 0.575$ for 18-mo-old animals to $1.875 \pm 0.857$ at 36 mo of age (Fig. 9B). Tubular atrophy scores were significantly lower in the A-CR rats compared with the AL rats at all ages (ANOVA, $P = 0.002$), with a significant interaction between age and diet (regression, $P = 0.0262$). Interstitial fibrosis was mild ($<1.5$ score) for all ages and diets; however, there was an age-dependent increase in extracellular matrix in both the AL- and A-CR-fed rats ($P < 0.001$; Fig. 9C). At 24 mo, A-CR-fed rats exhibited interstitial fibrosis scores similar to AL rats but were lower than AL-fed rats at 30 and 36 mo. Regression analysis identified a significant interaction between age and diet indicating that A-CR slowed the rate of interstitial fibrosis ($P = 0.0017$). Vascular wall thickening for the 18-, 24-, and 30-mo AL and A-CR animals was mild and did not significantly change within this time frame. At 36 mo of age, vascular wall thickening in the AL rats increased to moderate and was significantly different from the earlier ages (Fig. 9D). In contrast, the 36-mo A-CR rats had mild wall thickening with a severity score of 1.3, significantly lower than the vascular wall thickening observed in the 36-mo AL rats ($P < 0.0001$), no interaction between age and diet was observed ($P = 0.0807$).

**DISCUSSION**

Adult-onset CR is an experimental model of life extension and healthy aging less explored, but one more realistic for human application. While CR begun in middle age may not have the same life extension benefits of CR begun at an early age in rodent models (reviewed in Ref. 31), long-term application may reduce or at least delay age-dependent deterioration. In this study, we observed a significant impact of A-CR on the progression of age-related changes in the kidney. A-CR was very effective in reducing glomerulosclerosis and tubular atrophy within 6 mo of onset and continued to slow these age-related changes through very old ages compared with
AL-fed rats. Significant changes in interstitial fibrosis, vascular wall thickening, and mitochondrial ETS enzyme activities between AL- and A-CR-fed rats took longer to emerge (≥12 mo of CR). These data indicate that A-CR has a positive effect on the rate of aging in the rat kidney and that CR must be maintained for 6 mo and longer to achieve those benefits.

Since renal tubular epithelial cells are rich in mitochondria and highly dependent on oxidative phosphorylation, we hypothesized that these cells would accumulate mtDNA mutations and ETS enzyme abnormalities, much like the age-dependent accumulation of aberrant mitochondria observed in muscle and brain, and that A-CR would reduce or delay their accumulation. Renal tubule epithelial cells deficient in COX activity were present, and similar in number at 24 mo of age, in both AL-fed and A-CR rats (6 mo postinitiation of a reduced calorie diet). Significant age-dependent increases in the volume density of ETS abnormal tubules in AL-fed rats occurred between 24 and 30 mo as well as between 30 and 36 mo of age. A-CR reduced both the number of ETS abnormal tubules at 36 mo of age and the rate at which they occurred compared with AL-fed rats.

We determined that ETS abnormal renal tubular epithelial cells contain mtDNA bearing deletion mutations. The etiology of mtDNA mutations is unknown; however, it has been postulated that ROS generated by the electron transport system during normal oxidative phosphorylation, over time, damages mitochondrial lipids, proteins, and nucleic acids, leading to functionally impaired respiratory chain subunits causing ETS dysfunction and a further increase in ROS production (1, 9, 13). Even though the mutation event is stochastic in nature, once mtDNA with a deletion mutation becomes established within a mitochondrion, it can overtake wild-type mtDNA. In aged skeletal muscle, this population of mutant mtDNA, with time, reaches a threshold level (≥90% in muscle fibers) (4, 14) and phenotypic expression of abnormal mitochondrial enzymes can be observed on histological sections. The same mtDNA deletion mutation is always observed at different points along an affected muscle fiber (4, 6, 14). A similar phenomenon

Fig. 8. Representative photomicrographs of 10-μm-thick frozen sections of rat kidney stained with H&E and Gomori trichrome. A and B: from 18-mo-old AL-fed rats. C and D: from 36-mo-old AL-fed rats. E and F: from 36-mo-old A-CR-fed rats. Scale bars = 100 μm.
occurs in renal epithelial cells, as the same deletion mutation was observed at different locations within the same ETS abnormal tubule. These data indicate that the deletion mutation was clonal among cells in an affected tubule, persisting in cells during replication.

Since mitochondrial deletion mutations occur randomly and their accumulation to threshold levels within a cell is time dependent, we suggest that events that trigger the formation and accumulation of mtDNA deletion mutations occur on a continuum. Mutations established within a mitochondrion before the initiation of CR would likely continue to expand within the affected cell and among cells within a tubule. After a year of A-CR, however, we see a change in the steady-state levels of cells expressing ETS abnormalities compared with AL-fed rat kidneys, indicating that the events that initiate deletion mutations and/or their accumulation have been attenuated (but not completely eliminated) by reduced calorie intake. In rat skeletal muscle, life-long CR limited the number of ETS abnormalities in very aged rats but had no effect on the progression of the ETS abnormality once established (5).

Renal epithelial cells replicate at a very slow rate and our data showing the same mtDNA deletion event in different abnormal cells within the same tubule suggest that the mitochondria harboring the deletion mutation are being partitioned to daughter cells. In response to physiological signals or pathological processes, the growth rate and growth characteristic of renal tubular epithelium are perturbed and result in hyperplasticity and/or hypertrophy (34). Many tubules containing ETS abnormal epithelial cells were hyperplastic (Fig. 4, C and D), much larger in size (Fig. 4E) and the cellular organization of these cells was disrupted with nuclei centrally located, suggesting a loss of polarization. The histological concordance between the mitochondrial enzymatic abnormal phenotype, mtDNA deletion mutations, cell hypertrophy, cell proliferation, and nuclei displacement suggests a detrimental physiological impact that would likely interfere with normal epithelial cell function.

Renal tubule cells that exhibit mitochondrial enzyme dysfunction may be a result of nephron failure secondary to glomerular failure. Alternatively, the ETS abnormal phenotype in epithelial cells may precede rather than being a coincidence of glomerular failure. ETS abnormal cells within a tubule were often flanked by ETS normal regions, indicating that events leading to the ETS abnormality did not involve the entire tubule and, thus appear to be independent of the glomerulus. The nature of mtDNA (10–100s of copies within a mitochondrion and 100–1,000s of mitochondria per cell), the time dependence to threshold for mutant mtDNA phenotypic expression, and the fact that the mtDNA deletion mutations observed in ETS abnormal tubule cells are clonal indicate that the epithelial cells bearing mtDNA deletion mutations are replicating. Whether a cell containing high levels of mutant mtDNA expressing an abnormal phenotype can actively divide or whether low levels of mutant mtDNA are passed onto daughter cells followed by an accumulation of the mtDNA mutation and subsequent ETS abnormality expression are important questions to answer.

Initiation of calorie restriction at 18 mo, postadolescent development, and before the onset of significant age-related changes in the FBN rat kidney effectively reduced glomerulosclerosis and tubular atrophy within 6 mo, changed the rate of interstitial fibrosis within 1 yr, and vascular wall thickening and the abundance of COX-deficient tubular epithelial cells in 18 mo compared with AL-fed rats. Our histological approach demonstrated that COX-deficient renal tubular epithelial cells involved mtDNA deletion mutations and that these cells con-
tain unique, clonally expanded mtDNA deletion mutations. Renal tubular epithelial cells with mitochondrial abnormalities presented cellular characteristics indicative of physiological dysfunction.

ACKNOWLEDGMENTS

The authors thank Dr. D. McKenzie for critical review of the manuscript.

GRANTS

This work was supported by National Institutes of Health Grants AG-11604, AG-17543, and DK-067981.

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