Calorie Restriction Modulates Renal Expression of Sterol Regulatory Element Binding Proteins, Lipid Accumulation, and Age-Related Renal Disease

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Sterol regulatory element binding proteins (SREBP) are major regulators of fatty acid and cholesterol synthesis. This study found that age-related renal matrix deposition and proteinuria were associated with increased renal expression of SREBP-1 and SREBP-2 and increased renal accumulation of triglyceride and cholesterol. Because calorie restriction (CR) modulates age-related renal disease, it then was determined whether the effects of CR are mediated partially by modulation of renal lipid metabolism. Compared with ad libitum (AL)-fed 24-month-old (24m) F344BN rats, CR resulted in significant decreases in extracellular matrix accumulation (periodic acid-Schiff staining and immunofluorescence of type IV collagen and fibronectin) and proteinuria. A significant decrease was also observed in the renal expression of growth factors (connective tissue growth factor and vascular endothelial growth factor) and matrix metalloproteinase inhibitor (plasminogen activator inhibitor-1). These structural and functional changes were associated with significant decreases in renal nuclear SREBP-1 (5.2 in 24m AL versus 3.3 densitometry units in 24m CR; \( P < 0.01 \)) and SREBP-2 (7.1 in 24m AL versus 4.1 densitometry units in 24m CR; \( P < 0.01 \)) protein abundance and renal triglyceride and cholesterol contents. It is interesting that serum leptin level was significantly increased as a function of aging, and CR resulted in significant reduction in serum leptin level. Because it was shown previously that increased renal expression of SREBP-1a per se caused renal lipid accumulation, glomerulosclerosis, and proteinuria, the results suggest that CR modulates age-related renal disease in part by modulation of renal SREBP expression and renal lipid accumulation.


n humans and in laboratory animals, the normal aging process is associated with progressive structural and functional alterations in the kidney, including glomerulosclerosis, tubulointerstitial fibrosis, decreases in renal blood flow and GFR, and changes in tubular functions that lead to defects in urinary concentration, dilution, acidification, and phosphate transport (1–3). The elderly are at least five times more prone to develop ESRD than young adults (3). Several factors, including angiotensin II, advanced glycosylation end products (AGE), oxidative stress, and nitric oxide, may contribute to age-related renal diseases (4–7). It is interesting that similar mechanisms have also been implicated in the pathogenesis of diabetic nephropathy. Modulation of these processes may help prevent or at least attenuate the age-related changes in renal structure and function.

It has long been known that calorie restriction (CR) slows the aging process and prevents age-related diseases reported in most species (8). Over the decades, many investigators have shown that this protection can lead to a reduction of incidence and severity in renal injury in aging animal models (9,10). Several mechanisms have been suggested for the beneficial effects of CR. These include decreased accumulation of AGE, decrease in oxidative damage as a result of a slowing of metabolism, increase in protein turnover, increased insulin sensitivity, and more efficient glucose utilization (11–14). However, the precise molecular mechanism of the specific effects of CR on age-related renal disease remains to be elucidated.

The sterol regulatory element binding proteins (SREBP) have been shown to be master regulators of both fatty acid and cholesterol metabolism. Three SREBP isoforms have been identified and characterized: SREBP-1a, SREBP-1c, and SREBP-2. SREBP are synthesized as precursors bound to the endoplasmic reticulum. After a two-step cleavage process, the NH2-terminal segment of SREBP is released from the membrane and translocated to the nucleus, where it binds to enhancer regions of target genes to activate transcription. SREBP-1 preferentially activates genes that are involved in fatty acid synthesis, whereas SREBP-2 preferentially activates genes that are involved in cholesterol biosynthesis (15,16). Whereas the role of SREBP in lipogenesis has been examined intensively in liver and adipose tissues (17,18), less is known of its role in renal diseases.

In a recent study, we found that in a type 1 diabetes rat
model, in addition to glomerulosclerosis, there is increased renal accumulation of lipids, mediated by increased expression of SREBP-1. In SREBP-1a transgenic mice, in the absence of any changes in serum glucose or serum lipids, there is increased accumulation of triglyceride and cholesterol in the kidney, associated with increased expression of TGF-β, vascular endothelial growth factor (VEGF), and the extracellular matrix (ECM) proteins type IV collagen and fibronectin, resulting in glomerular hypertrophy, glomerulosclerosis, and proteinuria (19). These renal alterations are very similar to the renal pathology in diabetic and aging animals (1–3). Therefore, increased expression of SREBP-1 resulting in renal lipid accumulation may play an important role in the pathogenesis of diabetic and age-related kidney diseases. We performed this study to determine (1) whether age-related renal disease is associated with increased accumulation of lipids in the kidney, (2) whether the increase in renal lipid accumulation is mediated by increased renal expression of SREBP, and (3) whether CR modulates age-related renal disease at least in part by modulating SREBP expression and renal lipid metabolism.

Materials and Methods

**Animal Models**

Four-month-old (4m), 12m, and 24m male F344 × Brown-Norway F1-hybrid (F344BN) rats were obtained from the National Institute on Aging aging colony (Bethesda, MD). There were 20 rats in each age group; 10 were fed ad libitum (AL), and 10 were restricted to 40% of AL. Both CR and AL are individually housed. CR is initiated at 14 wk of age at 10% restriction, increased to 25% restriction at 15 wk and to 40% restriction at 16 wk, where it is maintained throughout the life of the animal. They were acclimated at our animal care facility for 3 wk before study and were maintained on a 12-h light/dark cycle. The studies were approved by the Institutional Board of the Denver Veterans Affairs Medical System and the University of Colorado Health Sciences Center. All experimental procedures involving the production and use of laboratory rats complied with National Institutes of Health guidelines.

After the collection of urine, six rats in each group were anesthetized by intraperitoneal injection of pentobarbital. Blood was obtained via aortic puncture at time of killing. The kidneys then were rapidly removed and homogenized, and aliquots were set up for (1) nuclei isolation, (2) lipid extraction and measurement of lipid composition, (3) Western blot to examine growth factors. In addition, four rats in each group underwent in vivo perfusion fixation of the kidneys, and the kidneys then were processed for histologic staining and immunofluorescence microscopy.

**Blood and Urine Chemistries**

Glucose was measured using the glucose C2 kit (Wako Chemicals USA, Inc., Richmond, VA). Total cholesterol was measured using the cholesterol CII kit (Wako Chemicals USA). Triglycerides were measured by the L-Type TG H kit (Wako Chemicals USA). Leptin was determined by competitive ELISA via the rat leptin kit (Crystalchem Inc., Downers Grove, IL). Urine albumin concentration was determined by competitive ELISA via the Albuwel M kit (Exocell, Philadelphia, PA). Urine creatinine concentration was determined by the Creatinine Companion kit (Exocell). Results are expressed as the urine albumin to creatinine ratio (mg/mg).

**Homogenization and Nuclei Isolation**

Kidneys were homogenized at 4°C in homogenization buffer (20 mM Tris·Cl [pH 7.4], 75 mM NaCl, 2 mM EGTA, 2 mM EDTA, 1 mM Na2VO4, and 1 mM dithiothreitol), supplemented with protease inhibitor cocktail that consisted of 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St. Louis, MO).

Nuclear extracts were prepared according to the method of Morooka et al. (20) with modifications. An aliquot of homogenate was centrifuged at 3300 × g for 15 min at 4°C. The supernatants were discarded, and the packed nuclear volume was estimated. The nuclei were resuspended in 0.5 packed nuclear volume of low-salt buffer (20 mM HEPES [pH 7.9], 25% glycerol, 1.5 mM MgCl2, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSE, and 0.5 mM DTT); 0.5 packed nuclear volume of high-salt buffer (20 mM HEPES [pH 7.9], 25% glycerol, 1.5 mM MgCl2, 0.8 M KCl, 0.2 mM EDTA, 0.2 mM PMSE, and 0.5 mM DTT) then was added. After 30 min of continuous gentle mixing, the nuclei were pelleted by centrifugation (30 min at 25,000 × g). The supernatants were saved, and the protein concentration was determined by the method of Lowry (21).

**Protein Electrophoresis and Western Blot**

Nuclear extracts were subjected to SDS-PAGE (10% wt/vol), as described by Laemmli (22), and then transferred to nitrocellulose membranes. Membranes were blocked in 5% dried milk in TBST (0.2% Tween 20 in 1× TBS), incubated with anti-SREBP-1 and anti–SREBP-2 (1:1000; BD Biosciences, Pharmingen, San Jose, CA) followed by horse-radish peroxidase–labeled anti-mouse IgG (1:5000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), washed four times with 1× TBS, and then developed using the chemiluminescence detection system (Pierce Biotechnology, Rockford, IL). The signals were quantified in a Phosphor Imager (Bio-Rad Laboratories, Hercules, CA).

Cortical homogenates were similarly subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes then were probed with antibodies for hydroxymethylglutaryl (HMG)-CoA reductase (1:500; Upstate, Lake Placid, NY), plasminogen activator inhibitor-1 (PAI-1, 1:1000), VEGF (1:1000), and connective tissue growth factor (CTGF; 1:1000; Santa Cruz Biotechnology).

**Lipid Extraction and Analysis**

Total lipid was extracted from kidney cortex using the method of Bligh and Dyer (23). Total cholesterol and triglycerides were determined as described above.

**Perfusion Fixation of Rat Kidney**

Rats were anesthetized and perfused at a pressure of 180 mmHg through the abdominal aorta with a fixative buffer that consisted of 3% paraformaldehyde and 0.05% picric acid in a 6:4 mixture of cacodylate buffer (pH 7.4; adjusted to 300 mOsm with sucrose) and 10% hydroxyethyl starch. After 5 min of fixation, the rats were perfused for an additional 5 min with the cacodylate buffer.

**Periodic Acid-Schiff Staining and Immunofluorescence Microscopy**

Paraffin sections were stained for hematoxylin and eosin and for periodic acid-Schiff (PAS) for histologic examination of glomerular and tubulointerstitial matrix deposition. The stained kidney sections were imaged with an Olympus microscope and analyzed in a blinded manner by two independent renal pathologists (C.P. and S.L.). For glomerulosclerosis, at least 100 glomeruli per section, three or four sections per animal were checked. Glomerulosclerosis was expressed as a percentage of sclerotic glomeruli based on total glomeruli. Sclerotic glomeruli
were defined by increased mesangial matrix based on PAS-positive area. For the extent of tubular atrophy and interstitial fibrosis, the evaluation was based on the following four grades: Minimal (<5%), mild (6 to 34%), moderate (34 to 66%), and severe (>66%). For immunofluorescence, frozen sections were preincubated for 10 min at room temperature with 3% milk powder in PBS that contained 0.05% Triton X-100. They then were covered overnight at 4°C with the type IV collagen or fibronectin antibody (Chemicon, Temecula, CA) diluted 1:200 in the preincubation solution. The sections were rinsed three times with PBS before incubation for 1 h at 4°C with the secondary antibody, goat anti-rabbit IgG conjugated to Alexa 488 (Molecular Probes, Inc., Eugene, OR). After rinsing with PBS, the sections were mounted using DAKO-Glycergel (Dakopatts, Glostrup, Denmark) plus 2.5% 1,4-diazabicyclo-[2.2.2]-octane (Sigma) as a fading retardant. The kidney sections then were imaged with a Laser Scanning Confocal microscope (Zeiss LSM 510).

**Statistical Analyses**

Results are presented as the means ± SE for at least three independent experiments. Data were analyzed by ANOVA and Dunnett or Student-Newman-Keuls tests for multiple comparisons. Statistical significance was accepted at the $P < 0.05$ level.

**Results**

**Body Weight, Serum Glucose, Triglyceride, Cholesterol, and Leptin Levels**

Table 1 demonstrates the changes in body weight and serum chemistries in AL and CR rats as a function of age. There was a significant age-related increase in body weight in AL rats. However, this change with age was not observed in CR rats. In addition, at each age, CR rats had significantly lower body weight than AL counterparts. Blood glucose kept relatively

![Figure 1](image-url)
constant across age within each diet group, and no significant
effect of CR was observed. Serum triglyceride and cholesterol
levels were significantly higher in 24m AL group relative to the
4m AL group, whereas levels were lower in the 12m and 24m
CR groups compared with age-matched AL groups but were
not statistically significant as a result of large individual vari-
ation. Serum leptin level was significantly higher in the 24m AL
group compared with both the 4m and 12m AL groups. CR diet
dramatically reduced the serum leptin level relative to that in
AL rats, and this reduction achieved statistical significance in
both the 12m and 24m groups. These results indicate age-
related increases in body weight, serum triglyceride, choles-
terol, and leptin levels in AL rats and significant CR effects on
body weight and serum leptin level.

Renal Histopathology and Proteinuria

To evaluate the effects of diet and age on renal histopa-
thology, we performed the PAS staining on renal sections
from each study group. Increased PAS staining indicates
accumulation of ECM proteins. Compared with 4m and 12m
AL rats, significant increase of segmental glomerulosclerosis
(Figure 1, A and B) was observed in 24m AL rats, and CR diet
markedly attenuated it. In addition, we found mild tubular
atrophy (Table 2) and interstitial fibrosis (Table 3) in 24m AL
rats, and these changes were prevented by CR diet.

### Table 2. Tubular atrophy in the kidney of AL- and CR-
treated rats of different ages

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<th>Group</th>
<th>Animal</th>
<th>Minimal &lt;5%</th>
<th>Mild 6 to 34%</th>
<th>Moderate 34 to 66%</th>
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### Table 3. Interstitial fibrosis in the kidney of AL- and
CR-treated rats of different ages

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<th>Group</th>
<th>Animal</th>
<th>Minimal &lt;5%</th>
<th>Mild 6 to 34%</th>
<th>Moderate 34 to 66%</th>
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Immunofluorescence microscopy indeed confirmed the in-
creased expression of two ECM proteins, type IV collagen
(Figure 2) and fibronectin (Figure 3), in glomeruli and tubulo-
tubular-interstitial space in AL rats with age. CR decreased the ac-
cumulation of ECM proteins in all age groups clearly. The immu-
nofluorescence data for ECM proteins therefore is in agreement
with the PAS staining data.

The glomerular histopathologic changes observed in AL rats
were associated with a three- to six-fold increase in the ratio of
urinary albumin to creatinine in the 24m group relative to the 4m
and 12m groups (Figure 4). In CR rats, there was a significantly
lower level of ratio of urinary albumin to creatinine in the 24m
group compared with AL rats at the same age. Therefore, the
proteinuria, which is an indicator of glomerular injury, was also
prevented by CR.

Renal Expression of CTGF, PAI-1, and VEGF

Growth factors such as CTGF and VEGF and matrix metal-
loproteinase inhibitors such as PAI-1 are involved in the regu-
lation of synthesis and degradation of ECM. Thus, the accumu-
lation of ECM proteins and mesangial expansion in
glomerulosclerosis are mediated by increased expression of
those factors. As anticipated, renal cortex from AL-fed rats
exhibited a significant age-dependent increase in the protein
expression level of CTGF (Figure 5A), PAI-1 (Figure 5B), and VEGF (Figure 5C), whereas in rats that were maintained on the 24m CR diet, there were significantly lower values when compared with AL rats of the same age.

Renal Triglyceride and Cholesterol Content

To study the effects of aging and CR on renal lipid composition, we analyzed renal content of triglyceride (Figure 6A) and cholesterol (Figure 6B). In the AL group, there was an age-dependent increase in triglyceride (12m or 24m versus 4m; \( P < 0.01 \)) and cholesterol (24m versus 4m, \( P < 0.01 \); 24m versus 12m; \( P < 0.05 \)) content. In CR rats, renal triglyceride content was reduced to 76% in the 12m group and 78% in the 24m group, respectively, of age-matched AL controls. Renal cholesterol content showed statistically significant 23% reduction in 24m CR rats compared with 24m AL rats. These results demonstrated renal lipid accumulation in aging AL rats and prevention of lipid accumulation in aging CR rats.

SREBP-1 and SREBP-2 Expression in Kidney

The above results indicate accumulation of lipids in the kidney of aging models. We then investigated possible alterations in the expression of the transcription factors that are important for lipid metabolism, SREBP, in the kidney of rats that were maintained on both CR and AL diets. We examined the active/mature nuclear form of SREBP-1 and SREBP-2 by Western blot after nuclei isolation. There was a significant age-related increase in the expression of SREBP-1 (Figure 7A) and SREBP-2...
The expression levels were dramatically lower in CR rats (SREBP-1 in the 24m group; SREBP-2 in the 12m and 24m groups) than those in age-matched AL groups. Our data indicate that the observed increases in renal triglyceride and cholesterol contents in aging AL rats were associated with increased renal expression of SREBP, which was attenuated by CR diet.

HMG-CoA Reductase Expression in Kidney

HMG-CoA reductase is an important SREBP-2 transcription target enzyme for cholesterol synthesis. Consistent with the age-related increase in SREBP-2 expression in AL rats and the role of CR in the regulation of SREBP-2, there was a significant age-dependent increase in the expression of HMG-CoA reductase, and the CR diet prevented the increased expression in both the 12m and 24m groups compared with AL rats at the same age (Figure 8).

Discussion

CR Prevents Age-Related Renal Morphologic Changes and Increase in Proteinuria

Previous studies have shown that CR extends the mean and maximal life span of animals and significantly reduces age-associated diseases (13,14). F344BN rats developed by the National Institute on Aging live considerably longer and have less organ pathologies at any given age versus inbred strains (24). To our knowledge, except for some impairments of renal function in aged F344BN rats, there are no data regarding the effects of age and CR on renal histopathologic alterations in this strain of rat (25). Our studies demonstrate increased incidence of age-related glomerular and tubulointerstitial lesions judged by quantitative analysis of PAS staining and immunofluorescence for fibronectin and type IV collagen in kidney. We also found an age-related increase in urinary albumin excretion, which is an indicator of glomerular injury. These changes were prevented or markedly attenuated by CR, in agreement with earlier studies in other animal models (10,11,13,14).

CR Prevents Age-Related Increase in Lipid Accumulation and Increased Expression of SREBP

A novel finding of our study was that we observed significant age-related increases in renal triglyceride and cholesterol
alterations in renal SREBP-1 and SREBP-2 expression. We found that in the nuclear extracts from the kidney, there were significant age-related increases in the cleaved active nuclear form of SREBP-1 and SREBP-2, which suggests that the age-related increases in triglyceride and cholesterol content are mediated by increased activity of the transcriptional factors SREBP-1 and SREBP-2. In agreement, HMG-CoA reductase protein abundance, a major target enzyme of SREBP-2, was increased as a function of aging. This is the first demonstration of increased SREBP expression and activity in the aging kidneys. In liver from aging Fischer-344 rats, there is upregulation of mRNA level of SREBP-1c (26), whereas in adipose tissue from aging monkeys, there is downregulation of mRNA level of SREBP-1 (27). These differences may be due to tissue-specific effect of aging on SREBP expression.

The regulation of SREBP expression in the kidney has not been well described. However, studies in other systems have generated a number of possible candidates that are involved in SREBP regulation. Aging is associated with insulin resistance and compensatory hyperinsulinemia (28). Studies have suggested that insulin upregulates SREBP-1c mRNA level in other tissues, such as skeletal muscle (29), adipose tissue (30), and liver (31). Aging rats also develop leptin resistance and compensatory hyperleptinemia (32,33). Hyperleptinemia induced by adenovirus gene transfer lowers hepatic SREBP-1c (34). Leptin deficiency has been implicated as a potential mediator of increased SREBP-1c in adipose tissue (35). In lep−/− mice, an increase in liver SREBP-1c has been reported (36). However, these mice tend to be hyperinsulinemic as well, making it difficult to ascribe the increase in SREBP solely to the leptin resistance per se. In contrast, our data showed that the age-related increase in serum leptin was associated with the upregulation of SREBP expression. Therefore, tissue-specific effects of hyperleptinemia and/or hyperinsulinemia may be responsible for the age-related increase in SREBP.

The mechanisms by which CR attenuates age-related renal morphologic changes and proteinuria remain to be determined. We reported previously that in SREBP-1α transgenic mice overexpressing SREBP-1 in kidney, in the absence of any increases in serum glucose, triglyceride, or cholesterol, there were increased renal triglyceride accumulation and upregulation of TGF-β and VEGF, resulting in accumulation of fibronectin and type IV collagen, glomerulosclerosis, and proteinuria (19).

Therefore, our main purpose in this study was to determine whether the effects of CR are mediated at least in part by the modulation of renal lipid metabolism. We found that CR partially prevented the age-related increases in nuclear SREBP-1 and SREBP-2 expression, as well as triglyceride and cholesterol accumulation in the kidney. We also examined the expression of HMG-CoA reductase, which is one of the important target enzymes of SREBP-2 and a key enzyme involved in cholesterol synthesis. CR-induced downregulation of age-related increase in the protein abundance of HMG-CoA reductase is consistent with the effect of CR on the decrease of nuclear SREBP-2 protein abundance and cholesterol accumulation. The correlation between improvements in renal structure and function and decrease in SREBP expression suggests that SREBP and lipids...
Figure 9. Summary of our results showing the effects of CR on age-related renal disease partially by modulation of renal SREBP-1 and SREBP-2 expression and renal triglyceride and cholesterol content.

Role of Altered Lipid Metabolism in Renal Disease

There is increasing evidence to show the role of lipid metabolism in renal disease. It has been reported that inhibition of cholesterol synthesis by HMG-CoA reductase inhibitors (statins) and triglyceride synthesis by peroxisome proliferator-activated receptor-α agonists (fibrates) protect against diabetic and nondiabetic renal disease (37,38). A recent meta-analysis of several small-scale interventional studies in diabetic and nondiabetic human subjects with glomerulosclerosis and proteinuria indicated that long-term treatment with statins and/or fibrates significantly prevents the decline in GFR (39).

The mechanisms by which lipids modulate renal disease are not entirely known. In this study, we found an age-related increase in the expression of CTGF, PAI-1, and VEGF in renal cortex, which was partially prevented by CR. These changes were consistent with the effects of aging and CR on the expression of nuclear SREBP-1 and SREBP-2. In the SREBP-1a transgenic mouse, we have found increased renal expression of TGF-β and VEGF as well as increased expression of type IV collagen and fibronectin, well-established mediators of glomerulosclerosis and proteinuria (19). In addition, cell culture studies have shown that in mesangial cells, LDL or VLDL induces upregulation of TGF-β and PAI-1 and accumulation of ECM proteins (40–42). These data indicated a direct role for lipids in activating the mediators of renal disease.

Our finding of decreased fatty acid and cholesterol deposition in the kidneys of CR animals is in agreement with several other studies that have examined tissue lipid deposition in the setting of CR. Gondret et al. (43) demonstrated that in rabbits, CR led to a decrease in lipid content in skeletal muscle compared with the muscle of AL-fed animals. Studies in rat kidneys have shown that the brush border membranes of CR animals had decreased content of several phospholipids (phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin) and cholesterol (44). In addition to modulating renal lipid metabolism, other mechanisms may be involved in CR effects on age-related renal disease, such as modulating BP, which definitely plays an important role in the progression of renal disease (45–48).

In conclusion, our studies indicate that age-related renal disease is associated with increased accumulation of lipids in the kidney. This increase in renal lipid accumulation is mediated by increased renal expression of SREBP. Furthermore, our data suggest that CR attenuates age-related renal disease at least in part by modulating renal SREBP expression and lipid accumulation (Figure 9).

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