Antioxidant Ceruloplasmin Is Expressed by Glomerular Parietal Epithelial Cells and Secreted into Urine in Association with Glomerular Aging and High-Calorie Diet

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Biologic aging is accelerated by high-calorie intake, increased free radical production, and oxidation of key biomolecules. Fischer 344 rats that are maintained on an ad libitum diet develop oxidant injury and age-associated glomerulosclerosis by 24 mo. Calorie restriction prevents both oxidant injury and glomerulosclerosis. Ceruloplasmin (Cp) is a copper-containing ferroxidase that functions as an antioxidant in part by oxidizing toxic ferrous iron to nontoxic ferric iron. Glomerular Cp mRNA and protein expression were measured in ad libitum–fed and calorie-restricted rats at ages 2, 6, 17, and 24 mo. In ad libitum–fed rats, Cp mRNA expression increased six-fold \( (P < 0.01) \) and protein expression increased five-fold \( (P = 0.01) \) between 2 and 24 mo of age. In calorie-restricted rats, Cp mRNA expression increased three-fold \( (P < 0.01) \) and protein expression increased 1.6-fold (NS) between 2 and 24 mo of age. Both the cell-associated alternately spliced variant and secreted variants of Cp were expressed. Immunofluorescent analysis showed that Cp was expressed by the parietal epithelial cells that line the inner aspect of Bowman’s capsule in the glomerulus. Cp also was present in urine, particularly of old ad libitum–fed rats with high tissue Cp expression. Cp expression by Bowman’s capsule epithelial cells therefore occurred in direct proportion to known levels of oxidant activity (older age and high-calorie diet) and is secreted into the urine. It is suggested that Cp expression at this site may be part of the repertoire of the glomerular parietal epithelial cell to protect the glomerular podocytes and the downstream nephron from toxic effects of filtered molecules, including ferrous iron.

Ceruloplasmin Increases with Age in Parietal Epithelial Cells

**Materials and Methods**

**Rat Model**

Fischer 344 rats were purchased from the 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 (23–26). 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 Immunohistochemical 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 fixative at 4°C for 8 min. Kidneys then were removed, cut into 3-mm slices, and placed in formalin before being processed for histology.

**Immunofluorescence**

Four-micrometer-thick paraformaldehyde/lysine/periodate fixation, paraffin-embedded sections were deparaffinized, hydrated, and treated with Retrieve-All 1 target unmasking fluid (Signet Labs, Dedham, MA) for 2 h at 90°C. Sections were permeabilized with 1% SDS and blocked with 10% goat and 10% rat serum in PBS. Immunofluorescent staining was performed using a mouse mAb against rat Cp (Clontech, San Jose, CA). A FITC-conjugated goat anti-mouse secondary antibody was used (Jackson ImmunoResearch, West Grove, PA). Sections were examined using a Leica DM inverted microscope (Bannockburn, IL) and SPOT camera system (Diagnostic Instruments Inc., Sterling Heights, MI).

**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, Briarcliff Manor, NY), and the material that passed through the sieve was collected on a second 25-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) were as follows: 2 mo 84 ± 3%, 6 mo *ad libitum* 83 ± 1%, 17 mo *ad libitum* 82 ± 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. All glomerular preparations contained approximately 50% of glomeruli that contained Bowman’s capsule. Because the tubular fragments were smaller than the isolated glomeruli, >90% of the extracts that were 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 (29). Therefore, in the experiments that used isolated glomeruli, there is little or no contribution from sclerotic glomeruli.

**RNA Purification**

TRIzol reagent (Invitrogen, Carlsbad, CA) was used, according to the manufacturer’s protocols, for isolation of total RNA from isolated glomeruli. Total RNA was purified further using the Qiagen RNeasy cleanup kit (Qiagen, Valencia, CA). The A260/A280 ratio was at least 1.8. The quality of RNA also was assessed by agarose gel electrophoresis to assess intactness of major RNA bands. All preparations showed intact well-defined RNA bands at 18S and 28S.

**DNA Microarray Analysis**

Samples were processed (*n* = 4 for each group including the common 2-mo-old group, and then at 6, 17, and 24 mo for the *ad libitum*–fed and calorie-restricted groups) by standard protocols using the rat RAE230A and RAE230B chips (Affymetrix, Santa Clara, CA). These microarrays produce gene expression levels on 31,142 known genes and expressed sequence tags (both microarrays combined). Preparation of cRNA, hybridization, and scanning of the arrays were performed according to manufacturer's protocols and as described previously (23).

**Reverse Transcription–PCR Analysis and cDNA Sequencing**

Quantitative real-time reverse transcription–PCR (RT-PCR) was performed using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA). Total RNA (100 ng) was used as a template. The Cp sense primer was selected from exon 18, 5'-acctacagtgtttacactaaatc-3', and the secreted variant antisense primer from exon 19 in the 3' untranslated region (3' UTR), 5'-cttattgtgtctttacctac-3'. RT-PCR for the membrane-tethered variant used the same sense primer as the secreted version and the antisense primer from exon 19 in the 3' UTR, 5'-ggttcgttggaggaactct-3'. RT-PCR reactions were made according to package insert directions. Amplification was done in a Rotor-Gene 3000 real-time thermal cycler (Corbett Research, Sydney, Australia). Reactions were incubated at 50°C for 10 min followed by 95°C for 5 min to achieve first-strand synthesis. PCR was cycled for 40 iterations, 95°C for 10 s, 55°C for 30 s, and 72°C for 60 s. Reaction was completed at 72°C for 10 min. PCR product was purified using MinElute PCR Purification Kit (Qiagen) according to package directions and sequenced at the University of Michigan DNA Sequencing Core.

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

Protein extracts of glomeruli were made by suspending isolated glomeruli in PBS with added inhibitors (Protease Inhibitor Cocktail; Roche Diagnostics Inc., Mannheim, Germany) at 10,000 glomeruli/ml, followed by homogenizing with RIPA buffer and centrifugation. Extracts then were aliquotted and stored at −80°C until use. Western blots were performed on glomerular extracts (100 μg of protein loaded onto each lane) using a mouse mAb to rat Cp (Clontech) followed by...
secondary antibody and development with Lumi-light chemiluminescent substrate (Roche Diagnostics). Equal loading was confirmed using a housekeeping protein (actin) evaluated by Ponceau stain of the same gel. Gels were developed on x-ray film (BioMax MR; Eastman Kodak Co., Rochester, NY) and quantified by densitometry using the National Institutes of Health Image1.61 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.

Urine Cp Protein Analysis
Urine protein was concentrated from 500 µl of rat urine by precipitation using an equal volume of 30% trichloroacetic acid on ice. The protein pellets were washed in acetone and resuspended in 100 µl of PBS. Aliquots then were analyzed by SDS-PAGE and Western blot using the anti-Cp antibody as outlined above.

Statistical Analyses
All data are presented as the mean ± 1 SD unless otherwise stated in the text. 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 two-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 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
Cp mRNA Steady-State Expression by the Glomerulus
We identified Cp as significantly increased with age in glomerular mRNA preparations from both ad libitum-fed and calorie-restricted rats by DNA microarray. We therefore used quantitative RT-PCR to measure Cp mRNA expression in the glomerulus throughout the lifespan of the Fischer rat. Figure 1A shows data for the 2-, 6-, 17-, and 24-mo time points for ad libitum-fed and calorie-restricted rats. Between 2 and 24 mo, there was a six-fold increase in ad libitum-fed rats (P = 9 × 10⁻⁷), and a three-fold increase (P = 4 × 10⁻⁷) for calorie-restricted rats. The difference in expression between ad libitum-fed and calorie-restricted rats was NS at the 6-mo time point but was significant for both the 17- and 24-mo time points (P < 0.05). We conclude that Cp mRNA increased in glomerular RNA preparations with age for both groups but more rapidly in the ad libitum-fed group.

Alternate Splice Cp Variants Expressed by the Glomerulus
Cp exists in both secreted and alternatively spliced GPI-linked membrane-bound forms. To determine which form is present in the glomerulus, we performed RT-PCR to amplify the glomerular Cp mRNA using splice-specific primers. Figure 1A (inset) shows that bands for both splice variants are present in glomerular RNA. Nucleotide sequencing of the PCR products confirmed that both the secreted and the alternatively spliced cell-associated forms were expressed. Therefore, we conclude that both versions of Cp are made in the glomerulus.

Cp Protein Expression by the Glomerulus
Figure 1B shows data for Cp protein expression from quantitative Western blots using a mouse mAb that recognizes rat Cp (see inset). Overall, there was a five-fold increase in protein expression on Western blot between 2 and 24 mo in the ad libitum-fed Fischer rats (P = 0.01). The calorie-restricted rats...
showed only a 1.6-fold increase, which was not statistically significant. The difference between ad libitum–fed and calorie-restricted rats was significant at 17 and 24 mo (P < 0.01). Therefore, the glomerular Cp protein amount was closely correlated with the mRNA data, compatible with the conclusion that Cp expression is regulated by steady-state mRNA levels.

**Localization in Parietal Epithelial Cells of the Glomerulus**

Figure 2 shows immunofluorescence that was developed using the mouse anti-Cp mAb that gives a single band on Western blotting (Figure 1B, inset). Cp was detected in occasional parietal epithelial cells at 2 mo in the Fischer rat kidney. At 24 mo, there was a robust signal in the parietal epithelial cells of Bowman’s capsule and a detectable signal in the tubulointerstitial compartment, particularly in ad libitum–fed rats. There was minimal signal within the glomerular tuft at any time point. We conclude that the source of the Cp in glomerular preparations is the parietal epithelial cell.

**Urinary Excretion of Cp**

Figure 3 shows that urine contains Cp at all ages examined but that Cp is present at higher concentrations in urine from older ad libitum–fed rats compared with younger rats and calorie-restricted rats. In all urine samples, the major band of Cp was at 140 kD, which co-migrates with the main secreted form of Cp present in plasma. However, there was another, lower molecular weight band at 125 kD. This lower molecular weight form was present in relatively higher amounts in urine, particularly in the urine of the 24-mo-old ad libitum–fed rat, but also was seen in small amounts in plasma. This low molecular weight Cp may represent a proteolytically modified or a less glycosylated form of Cp but probably is not the alternately spliced form that has a higher molecular weight than the secreted form of Cp (10). Therefore, we can say that secreted Cp is present in normal rat urine and that its concentration in urine increases with age and increases especially in older rats that are fed an ad libitum diet in association with high Cp expression in the kidney.

**Discussion**

In this report we analyzed Cp expression in the Fischer 344 rat as it relates both to aging and to diet. Cp is an important antioxidant, and oxidant injury is thought to be important in the kidney. However, Cp expression has not previously been reported in the kidney, probably because it is present at low levels in young kidneys in contrast to old kidneys. For these experiments, rats were fed either an ad libitum diet, which accelerates the aging process (and is associated with enhanced oxidant activity and development of glomerulosclerosis), or a calorie-restricted diet, which delays the aging process (and is associated with reduced oxidant injury and prevention of glomerulosclerosis) (23–28). Cp expression increased linearly with age in glomeruli under both ad libitum–fed and calorie-restricted conditions. The amount of Cp expressed (both as mRNA and as protein) in the glomerulus was significantly higher under ad libitum–fed conditions, showing that diet directly affects the antioxidant profile. We found that Cp was particularly synthesized in the parietal epithelial cells that line the glomerular Cp protein amount was closely correlated with the mRNA data, compatible with the conclusion that Cp expression is regulated by steady-state mRNA levels.

![Figure 2. Cp distribution in glomeruli and interstitium. Cp was identified by immunofluorescence at 2 mo (A) and at 24 mo in calorie-restricted (B) and ad libitum–fed (C) rats (see arrowheads). Note that at 2 mo, occasional positive fluorescence is seen in the parietal epithelial cells along Bowman’s capsule. At 24 mo in calorie-restricted rats, positive fluorescence is present in most parietal epithelial cells that line Bowman’s capsule as well as weakly in the tubulointerstitial compartment (**). By 24 mo in ad libitum–fed rats, staining of parietal epithelial cells is particularly strong, but there is also widespread fluorescence in tubular epithelial cells (**). Weak staining also is present in the glomerular tuft. We conclude that Cp accumulates with age, particularly in the parietal epithelial cells that line Bowman’s capsule but also in tubular cells and in the interstitial compartment.

![Figure 3. Urinary Cp. Cp was identified in urine by Western blot. The samples shown were from trichloroacetic acid (TCA)-precipitated urine from a 24-mo-old ad libitum–fed rat (25 µl in lane A; 12.5 µl in lane B), a 24-mo-old calorie-restricted rat (100 µl of urine in lane C), a 2-mo-old rat (100 µl of urine in lane D), and from rat plasma (2 µl of plasma in lane E). To confirm that the method used to precipitate the samples with TCA did not result in loss of Cp, lane F shows non–TCA-precipitated Cp from 2 µl of plasma. The secreted form of Cp that is present in plasma was seen as a single major band with an apparent molecular weight of 140 kD (lanes E and F). This major secreted form of Cp also was the major form of Cp that was present in urine of both 2– (lane D) and 24-mo-old ad libitum–fed and calorie-restricted rats (lanes B and C). However, particularly in the urine of ad libitum–fed 24-mo-old rat, there was an additional major band at 125 kD that also was seen at low amounts in plasma and urine of 24-mo-old calorie-restricted rat. This may represent a proteolytic product of Cp or be less glycosylated. We conclude that Cp is normally present in urine of rats and that the amount of Cp in urine is greatly increased in old rats, particularly in old ad libitum–fed rats.****
the inner aspect of Bowman’s capsule. Both splice variants of Cp were found in glomerulus, suggesting that some Cp would be secreted into the glomerular filtrate. We therefore looked for and found Cp in urine of both old and young rats. The amount of Cp that was present in urine correlated directly with the amount of Cp that was found in kidney, suggesting that the kidney was a likely source of urinary Cp. Urine of older rats, particularly those that were ad libitum fed, contained higher amounts of Cp. Therefore, there is a proportional relationship between the amount of Cp present in glomeruli and urine and the known levels of tissue oxidant activity during aging, which are increased further on the high-calorie diet.

Cp is an acute-phase reactant whose expression is regulated in part by an IL-6 response element (5). IL-6 levels increase with age and are closely linked to accelerated aging and frailty (30). Therefore, IL-6 may play a role in the age-related Cp expression identified in this report. Insulin also has been shown to regulate Cp expression (31). Fischer rats that are fed an ad libitum diet have higher insulin levels than those that are fed a calorie-restricted diet (32). Therefore, our finding that the ad libitum–fed rats had higher Cp levels than did calorie-restricted rats would be compatible with IL-6 and/or insulin-induced Cp expression in the glomerulus, although other factors, such as epigenetic factors, also could result in this age-dependant increase in Cp expression. We also note that this is an example of dietary content having a direct impact on glomerular gene expression.

Cp functions as an important antioxidant mechanism. It serves as a ferroxidase that converts toxic ferrous iron to nontoxic ferric iron, which thereby becomes available for binding to and transport by transferrin (1). At the same time, Cp serves as an antioxidant by removing the free ferrous iron, which acts as a major producer of oxidants (superoxide and hydroxyl radical) (33). Cp also serves as a general antioxidant by catalyzing the destruction of oxygen radicals (2,3,34) and can bind to and inhibit neutrophil myeloperoxidase oxidant activity (4). Oxidative damage is thought to be of major importance in tissue aging, including lipid peroxidation and damage to DNA and proteins (3,14,15). Increased levels of lipid peroxidation products as well as oxidant-induced DNA damage have been shown to be associated with aging and with accelerated aging states such as diabetes and chronic kidney disease (14–22). Decreased levels of available antioxidants also have been associated with frailty and chronic disease states (21).

The finding that Cp is expressed at high levels by parietal epithelial cells of Bowman’s capsule suggests that Cp might function specifically at this site to detoxify molecules as they pass through the glomerular filter. This concept was supported by our showing that the glomerulus contained the secreted form of Cp mRNA and by finding easily identifiable levels of Cp in urine. Therefore, the separation of high molecular weight proteins and blood cells from low molecular weight molecules by the glomerular filter thereby could remove protective antioxidant proteins and cellular mechanisms. This would expose the podocytes in Bowman’s space and the downstream nephron to toxic oxidant products in the protein- and cell-free filtrate (35). For example, De Tata et al. (36) reported that aldehydes that are generated by lipid peroxidation are present in urine and that levels of aldehyde excretion are affected by both age and diet. Cp that is present on the surface of and secreted by parietal epithelial cells could detoxify filtered ferrous iron and other oxidants and thereby reduce oxidant injury in Bowman’s space and in the downstream nephron.

Conclusion

We speculate that we have uncovered part of a novel function of the parietal epithelial cell as a protective mechanism situated immediately downstream of the glomerular filter. Cp may be one part of a more complex repertoire of this cell, which functions to protect the glomerular podocytes in Bowman’s space and the downstream nephron from oxidant injury. The finding that this antioxidant mechanism was clearly increased with age and was enhanced by a process that is known to accelerate oxidant injury and the aging process (a high-calorie diet) raises the question of whether this could be one part of a mechanism that protects the aging kidney. On the basis of these results, evaluation of other antioxidant processes in older kidneys also is warranted, because conclusions that are drawn on the basis of younger kidneys may not be applicable to old kidneys. This report shows that, at least with respect to Cp, an old kidney is not the same as a young kidney. This is important when one considers that ESRD is a disease of older age. Reduced capacity of the kidney to produce Cp such as may occur in chronic renal diseases also could render the kidney less able to detoxify the large amounts of iron that commonly are administered to these patients for treatment of anemia.

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