Impaired glomerular and tubular antioxidative defense mechanisms in nephrotic syndrome

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Impaired glomerular and tubular antioxidative defense mechanisms in nephrotic syndrome. Am J Physiol Renal Physiol 299: F898–F904, 2010. First published August 4, 2010; doi:10.1152/ajprenal.00124.2010.—The molecular mechanisms behind acquired nephrotic syndrome (NS) are still largely unknown. One possible explanation for the development of proteinuria is oxidative damage to the glomerular cells. Our hypothesis was that the oxidative defense is weakened in NS, and we focused on measurements of the antioxidative-antioxidative stress in the glomerular and tubular parts of the nephron. Gene expression was analyzed in renal biopsies from patients with NS. In addition, to compare the acute and chronic phases of the disease, we studied puromycin-treated rats. In the biopsy material, the expression of enzymes involved in the antioxidative defense was higher in the tubulointerstitial compartment than in the glomerular cells. Real-time PCR analysis revealed a decreased glomerular expression in nephrotic kidneys for the antioxidant enzymes catalase and glutathione peroxidase-3, and -4. The tubular gene expression was downregulated for catalase, glutathione peroxidase-3, and thioredoxin reductase-1 and -2. The altered gene expression was accompanied by increased lipid peroxidation in urine. In rats, serum concentrations of ascorbyl-free radicals, measured with electron spin resonance, were elevated in the acute phase of the disease, suggesting increased oxidative stress in the circulation. In addition, we saw an increase in the plasma antioxidant capacity combined with a decreased oxidation of proteins in sera from nephrotic rats, but not from humans. In conclusion, there is a marked downregulation of several antioxidative enzymes in nephrotic kidneys, especially in glomerular structures. Our data suggest that oxidative damage to glomerular cells may contribute significantly to the course and prognosis of nephrotic syndrome.

puromycin aminonucleoside; oxidative stress

NEPHROTIC SYNDROME (NS) is one of the most common renal disorders in humans. In recent years, the mechanisms behind the hereditary kidney diseases have been elucidated with the discovery of nephrin (20, 37), podocin (5), α-4-actinin (15, 30), laminin (25), and more. However, the pathogenesis of the large number of acquired NS is still unclear. Thus there are several possible causes behind the leakage of albumin into urine. Generally, proteinuria will occur if there is a defect in any of the major components of the glomerular filtration barrier, i.e., podocytes, the basement membrane, or the endothelium with its glycocalyx. In a recent study, the importance of the receptor for advanced glycation end products (RAGE) and its glycocalyx. In a recent study, the importance of the receptor for advanced glycation end products (RAGE) was clearly demonstrated in adriamycin-treated mice (16). Indeed, signs of increased oxidative stress are seen in many different pathological conditions, including renal disorders.

Oxidative stress occurs due to an excess of reactive oxygen species (ROS), an impaired antioxidant system, or a combination of both these factors (36). Exposure of cells to ROS can damage most major cellular constituents and processes, including DNA and protein synthesis, and may trigger cell death by apoptosis or necrosis.

Increased levels of oxidative stress markers have been described in sera and urine from patients in dialysis (38), in nephrotic children (7), as well as in animal models of NS (18, 33). By treating nephrotic rats with various antioxidants (such as SOD, catalase, or α-tocopherol), the proteinuria was, at least partly, reversed (2, 11, 32). Still, there are some controversies regarding the positive effects of catalase and/or SOD treatment, due to their short half-life in the circulation, low membrane permeability, and method of administration (3, 28).

Effects of exogenous antioxidants, as outlined above, indicate that the endogenous antioxidant defense system is somehow overwhelmed in NS. In this light, it is of considerable interest to investigate whether this system is operating on full effect, upregulated in response to increased oxidative stress, or whether expression of antioxidant genes is somehow suppressed by some factor(s) related to the disease, weakening the defense and leading to oxidative damage, possibly without any increase in the stress level per se.

However, when studying gene expression in glomerular disease, one is immediately confronted with the problem that the glomeruli constitute just a minute part of the renal cortex, the majority consisting of tubular tissue. Given the fact that glomerular and tubular cells differ vastly in morphology as well as metabolism, it is obvious that expression of various genes, both under normal and pathological circumstances, could differ significantly between these two fractions. Indeed, such differences has been noted in normal rat kidneys (17). Still, there are no previous studies on antioxidant gene expression in human kidneys.

In this study, we have separated renal biopsies from nephrotic patients and healthy kidney donors into glomerular and tubulointerstitial fractions. We then compared the expression levels of several specific antioxidant enzymes (AOE) in these fractions. By separating glomerular and tubulointerstitial material, we could evaluate more specifically how renal disease affects antioxidant activity in different parts of the nephron.

To compare the oxidative aspects of the chronic human disease to a well-established animal model, we have also included data from experiments with rats in which NS was acutely induced by treatment with puromycin aminonucleoside (PAN). Our hypothesis was that the oxidative defense would be diminished in NS in both cases.
MATERIALS AND METHODS

Renal Biopsies From Patients with NS

The study was conducted according to the Declaration of Helsinki, and patients received written and oral information before giving their consent to an extra renal biopsy to be taken during their otherwise routine procedure. The studies were approved by the regional ethical board. Biopsies from patients diagnosed with NS (n = 8) were used for gene expression analysis (Table 1). Biopsies from healthy age- and gender-matched transplant kidneys were used as controls (n = 10). The tissue was placed in RNAlater (Qiagen, Roche) and stored at −20°C until preparation. Single glomeruli were dissected from the rest of the cortical material, leaving a fraction containing mostly tubular structures. RNA was prepared from both fractions by using a Qiagen Micro-kit (Roche), and the quality and concentration were determined with an Agilent 2100 bioanalyzer (Nano-chip).

The renal progression rate was determined from estimated creatinine clearance, repeated 3–12 times over a period of 1–5 yr (3.4 ± 0.5 yr).

Experimental Animals

Experiments were performed using female Sprague-Dawley rats (Harlan), weighing ~200 g (n = 7). The animals had free access to food and water, and the local ethical board approved the experiments.

PAN (Sigma, St. Louis, MO) was administered to the rats in a single injection (150 mg/kg ip); the control group received an equal dose of physiological saline. Five days after PAN administration, glomerular function was quantified as previously described (4). The kidneys and blood were collected for further analysis.

Infusion and Tracers

The tracer solution had the following composition: 13 mM NaHCO3, 132.5 mM NaCl, 83 mM glucose (Merck, Darmstadt, Germany), and 51Cr-EDTA (0.37 MBq/l, Amersham Pharmacia Biotech, Buckinghamshire, UK). All solutions were made with distilled water and protected from light.

Isolation of RNA From Rat Glomeruli

After the functional measurements, the kidneys from each rat were removed, minced, and forced through a 106-μm sieve. The glomeruli were transferred to a tube and washed by sequential centrifugation steps. RNA isolation was performed by using the Qiagen mini-kit. The concentration and quality of the RNA samples were determined by using the Agilent 2100 bioanalyzer (Nano-chip).

Data Analysis

Plasma and urine samples were analyzed for 51Cr-EDTA using a gamma-counter (Packard Instrument, Meriden, CT).

Albumin was quantified by size exclusion chromatography by UV detection at 226 nm.

Calculations

Glomerular filtration rate. Rat glomerular filtration rate (GFR) was determined from the urine/plasma glomerular filtration rate (GFR) concentration ratio of 51Cr-EDTA times urine flow.

Fractional clearance for albumin. By dividing the U/P concentration for albumin by the U/P for 51Cr-EDTA, we could calculate the fractional clearance for albumin in rats.

Calculated clearance for creatinine. Patient creatinine clearance was calculated by using the Cockcroft-Gault equation.

Real-Time PCR

Reverse transcription was performed using a standard protocol. The mRNA level of target genes was quantified by real-time PCR on the ABI Prism 7900HT Sequence Detection system [Taquin, Applied Biosystems (ABI), Foster City, CA], as previously described (4). All of the primer-probe pairs were ordered from ABI. We used “Low density array micro fluidic cards” to analyze the genes, allowing us to use small sample volumes. One sample [15–30 ng (human) or 50 ng (rat) cDNA in 100 μl Taqman universal PCR mastermix] was distributed into small compartments of 2 μl each by centrifugation. The samples were then run in triplicate (human) or quadruple (rat) for 16–23 different genes (including endogenous control genes) in one run. The samples were denatured and then subjected to 40 cycles of 2-step PCR (15 s at 95°C, 1 min at 60°C). The comparative ΔΔCT method of relative quantification was used to calculate the differences in gene expression between the groups. 18S and GAPDH (predesigned assay reagent applied by ABI) were used as endogenous controls. mRNA samples without reverse transcription served as negative controls.

Ascorbyl Free Radical Analysis

Electron spin resonance (ESR) was used to measure the amount of ascorbyl free radicals (AFR) in plasma from patients with NS (n = 27, 15 men and 12 women, age 22–73 yr) and from healthy blood donors (n = 22, gender and age matched). In addition, blood samples from the PAN-treated rats were analyzed and compared with control animals. Plasma (50 μl) was drawn into a hematocrit tube (KEBO-Lab, Stockholm, Sweden). The AFR signal intensity was then measured by an X-band spectrometer Bruker ECS 106 (Bruker Biospin, Rheinsteryt, Germany). The instrument settings were microwave power 10 mW, center field 3,480.30 G, sweep width 5.0 G, modulation amplitude 1.0 G, modulation frequency 100.0 kHz, sweep time 5.24 s, time constant 163.84 ms, conversion time 5.12 ms, and number of scans 20. The AFR gives a characteristic two-peak signal, and the combined amplitudes of the peaks were measured to estimate the AFR concentration in the sample. The AFR concentration was deter-
minded by using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Sigma-Aldrich) as a standard.

Antioxidant Capacity

The plasma total antioxidant capacity was assessed with the TEAC method [for 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) equivalent antioxidant capacity] and compared between groups (NS vs. controls), essentially as described previously (13). This method is based on the ability of plasma antioxidant to reduce, and thus decolorize, the stable radical cation ABTS+. Briefly, the green-colored ABTS+ (λmax 734 nm) was produced by mixing equal volumes of 14 mM 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Sigma-Aldrich) and 4.9 mM potassium peroxodisulfate. The reduction rate of ABTS+ is directly dependent on the antioxidant capacity of the added plasma and was calculated by monitoring the decreasing absorbance at 734 nm. A known amount of the antioxidant Trolox (Sigma-Aldrich) was used as a standard, and the results were expressed in Trolox units, one unit representing the reduction rate produced by 1 μM Trolox.

Measurement of Protein Carbonyls

Oxidation of proteins was determined by measuring protein carbonyl content in sera with a spectrophotometric carbonyl assay (31). This method is based on the reaction between protein carbonyl groups and 2,4-dinitrophenylhydrazine (Sigma), which results in formation of a hydrazone with a strong yellow color. The concentration of hydrazone, which is directly correlated to the concentration of carbonyl groups in the sample, was calculated by measuring the absorbance at 360 nm.

Measurement of Lipid Peroxidation

Urine samples from patients with NS and healthy controls were analyzed for lipid peroxidation, a major indicator for oxidative stress. The thiobarbituric acid-reactive substances method by Yagi et al. (41) uses the binding between malondialdehyde and thiobarbituric acid. The resulting product is measured by fluorometry at 553 nm with 515-nm excitation.

Statistics

Results are presented as means ± SE, and differences were tested using one-way ANOVA.

RESULTS

Patient Baseline Data

Baseline data for the patients included in the study are shown in Table 1. The disease progression, calculated over 1–5 yr and expressed as GFR reduction rate, was 2.8 ± 1.2 ml·min⁻¹·yr⁻¹ (n = 8). Repeated measurements of total urinary albumin (collected for 24 h) over the same period revealed a decreased (n = 5) or unaltered (n = 2) excretion for all but one of the patients.

Gene Expression Analysis in Human Biopsies

The mRNA isolated from the human biopsies was of high quality (Fig. 1). Expression of several genes, coding for enzymes involved in the antioxidative defense, were altered in glomerular (n = 6) and tubulointerstitial (n = 8) fractions of biopsies from patients with NS compared with corresponding fractions from healthy controls (n = 10, Table 2 and Fig. 2). Expression of glutathione peroxidase (Gpx)-4 was decreased in glomerular structures only (by 40% in humans). Catalase and Gpx-3 expression was reduced in both fractions, but the effects were more pronounced in glomeruli (by 42 and 56%, respectively, compared with 27 and 29% in the tubulointerstitial part). Thioredoxin reductase (Txnr)-1 and -2 were decreased in the tubulointerstitial structures only (by 48 and 18%, respectively).
expression level ($\Delta C_T = C_T \text{target gene} - C_T \text{housekeeping gene}$) of the enzymes differed markedly between the two control fractions, with a significantly lower expression in glomeruli compared with the tubular structures (Table 3).

**GFR and Fractional Clearance for Albumin in Rats**

Treatment of rats with PAN for 5 days reduced the GFR from 0.99 ± 0.03 (control) to 0.19 ± 0.01 ml/(min × g kidney).

The fractional clearance for albumin in the control group was 0.0008 ± 0.0004. A 10-fold increase in albumin clearance was induced by the administration of PAN ($n = 5$ in each group) as partly presented (4). Electron micrographs confirmed a malformation of the glomerular barrier 6 days after PAN administration (Fig. 3).

**Gene Expression Analysis in Rat Glomeruli**

PAN treatment downregulated the antioxidative enzymes SOD-3 (by 46%), Gpx-3 (by 62%), and catalase (by 37%). Two enzymes, Gpx-1 and hemeoxygenase-1, were upregulated by the PAN treatment, whereas SOD-1 and Gpx-4 seemed to be unaffected by the stimulation (Table 2). Measurement of the gene expression over time was performed for some of the Gpx and SOD genes ($n = 1–7$), and we could see that the expression for Gpx-3 continued to decrease even further at days 6 and 7 (Fig. 3).

**AFR Measurements**

Rats. A significant increase in the ESR signal from ascorbyl radicals in the nephrotic sera was seen compared with control ($P \leq 0.01$) (Fig. 4).

Humans. Ascorbyl radical levels in sera from patients with NS were not significantly different from those of healthy controls.

**Antioxidant Capacity**

Rats. Antioxidant capacity was significantly higher in sera from PAN-treated rats (3.18 ± 0.26 mM Trolox equivalents) compared with control animals (2.47 ± 0.12 mM, $P \leq 0.05$).

Humans. No significant differences were detected between NS (4.31 ± 0.14 mM, $n = 27$) and control (3.95 ± 0.21 mM, $n = 22$).

**Table 3. Gene expression level ($\Delta C_T$ values) in glomerular and tubulointerstitial fractions**

<table>
<thead>
<tr>
<th>Glomerular Fraction</th>
<th>$\Delta C_T$</th>
<th>Tubular Fraction $\Delta C_T$</th>
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<tbody>
<tr>
<td>Catalase</td>
<td>14.4</td>
<td>12.9</td>
</tr>
<tr>
<td>Gpx-1</td>
<td>14.7</td>
<td>15.2</td>
</tr>
<tr>
<td>Gpx-3</td>
<td>10.2</td>
<td>7.4</td>
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<td>Gpx-4</td>
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<tr>
<td>SOD-1</td>
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<td>SOD-3</td>
<td>16.7</td>
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<tr>
<td>Txnrd-1</td>
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<td>14</td>
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<tr>
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Comparison of $\Delta C_T$ values (CT target gene - CT housekeeping gene) in glomerular and tubulointerstitial fractions from healthy controls is shown. We have correlated the expression for the gene of interest to the amount of loaded material to the LDA cards (internal control). The housekeeping gene (18S) was detected at the average CT value 14.1 for the glomerular fraction (average amount of cDNA = 15 ng) and 13.7 for the tubulointerstitial fraction (average amount of cDNA = 30 ng).
Oxidative Protein Damage

Rats. Protein oxidation, measured as protein carbonyl groups, was significantly decreased in sera from PAN-treated rats (11.8 ± 0.6 nmol/ml) compared with control (18 ± 1.4 nmol/ml, P ≤ 0.001, n = 8).

Humans. When comparing sera from nephrotic patients (15.23 ± 1.5 nmol/ml, n = 12) with sera from healthy controls (15.43 ± 1.2 nmol/ml, n = 29), we could not detect any difference.

Lipid Peroxidation

Thiobarbituric acid-reactive substances were significantly increased in urine from nephrotic patients (13.3 ± 0.9 nmol/ml, n = 14, P ≤ 0.001) compared with healthy controls (6.6 ± 0.7 nmol/ml, n = 29), compatible with an increase in oxidative stress in the urinary system.

DISCUSSION

In this study, we have used unique renal biopsy material from nephrotic patients as well as healthy kidney donors. By directly transferring the renal biopsy to Rinalater, the material is well preserved pending further dissection into a glomerular and a tubulointerstitial fraction. Separation of the specimens into glomerular and tubulointerstitial fractions allows local analysis of gene expression in various parts of the nephron without amplification.

One of our main findings is the difference in expression of antioxidant enzymes between the tubular and glomerular fractions in kidneys from both healthy and nephrotic subjects. Most of the AOE were expressed at a markedly higher level in tubular cells compared with glomerular structures. Our results confirm and extend the findings of Gwinner et al. (17), who showed that there is a difference in the expression levels of both SOD and catalase between glomerular and tubular structures in healthy rats. It has been suggested that the higher tubular level of AOE is due to the greater number of mitochondria in the highly metabolically active proximal tubular cells (8).

The rat model of PAN-induced NS is known to increase oxidative stress in the glomerular structures (18, 39). However, the situation in the nephrons of nephrotic patients is poorly investigated. We saw a decrease of several AOE in both structures in nephrotic patients, indicating an impairment of the system that normally protects the cells against oxidative stress. Also, the change was more dramatic in the glomerular cells for most of the affected genes. This is in line with the idea that NS is a glomerular disease, but with an involvement of the tubular structures as well.

The mRNA expression of Gpx-3 and -4 was significantly reduced in glomeruli, indicating a reduced capacity of detoxifying H₂O₂, as well as other peroxides, in the kidney. Gpx reduces various peroxides, including hydrogen peroxide, to water by oxidizing glutathione. Previous reports describe a reduction in plasma and/or erythrocyte Gpx activity in adults and children with NS (6, 12, 42, 43). Our data extend these...
findings since we measured the expression locally in the nephron, which is the most prominent source of circulating plasma Gpx measured in blood (40, 42).

Both Gpx-3 (24) and Txnrd-1 (34) have been reported to be prominently expressed in the proximal tubules of rodent kidneys. Only a few papers describe a faint immunolocalization to glomeruli for both Gpx (9) and Txnrd-1 (27). In our assay, we found that, in addition to the tubular expression, these enzymes are clearly expressed in isolated human glomeruli. In addition, recent data suggest that Gpx-3 is produced by podocytes and may have a connection to podocin (29).

To link the altered gene expression to functional data, we performed three different serum analyses: ESR measurement of the ascorbyl radical, which is a general measure of the utilization of vitamin C as a radical scavenger; assessment of plasma antioxidant capacity, which indicates the ability of plasma to resist oxidation; and measurements of protein carbonyls, reflecting the extent of oxidative protein damage.

The increase in ascorbyl radicals seen in sera from nephrotic rats correlated with their increase in proteinuria. In a comparison of sera from nephrotic patients and healthy blood donors, however, none of the three analyses yielded any differences. This indicates that there is no general increase in oxidative burden associated with chronic human NS. However, urine analyses revealed an increased level of lipid peroxidation locally in the kidney of nephrotic patients. This confirms our reported decrease in gene expression for several AOEIs in the glomerular and tubular structures. An increase in thiobarbituric acid-reactive substances has previously been reported in isolated glomeruli from PAN-treated rats (26). The observed downregulation of Gpx in human and rat glomeruli should reduce plasma antioxidant capacity. However, other factors could elevate this parameter, e.g., the massive upregulation of heme oxygenase-1 (HO-1) seen in rat glomeruli. HO-1 is an indicator of oxidative stress and catalyzes the oxidation of heme into iron, carbon monoxide, and biliverdin-bilirubin (a potent antioxidant) (23). An increase in HO-1 would reduce lipid peroxidation and formation of oxygen free radicals, and it has previously been reported that HO-1 is upregulated as a protective and antiapoptotic response to different renal disorders (10, 22).

There are several reasons for the discrepancy between the rat and human forms of NS. First, PAN induces an acute NS, whereas the patients may have passed the acute phase of their disease and thereby lost some early oxidative stress markers. Second, the increase in ascorbyl radicals seen in rats, by both us and Nakakura and colleagues (26), could be a reaction of the chemical induction by PAN rather than a cause of the nephrotic state. PAN is an adenosine analog and has been suggested to induce oxygen free radical generation via the xanthine oxidase system (11). Third, the patient material is more heterogenic according to age, gender, and medication than in the bred animals. However, when comparing the altered glomerular gene expression between rats and humans, we could see that in most cases the two species responded in similar ways, with the exception that Gpx-1 was upregulated in rats.

We have previously reported that the synthesis of negatively charged proteoglycans is disrupted during PAN-induced NS, causing reduced charge selectivity (4). In addition, there are reports describing a protective role for the glycosaminoglycans (GAGs; anionic sugar chains attached to the proteoglycans) against free radical damage. By binding transition metals, which are known catalysts of oxidative stress, the GAGs in the extracellular matrix will inhibit peroxidation and thereby prevent the induction of oxidative damage (1). Other studies have shown that superoxide-mediated oxidant injury may lead to degradation of the glomerular basement membrane (35) and a decreased synthesis of proteoglycans by the glomerular cells (19). Extracellular SOD (SOD-3) is known to bind tightly to heparin sulfate GAGs and thereby prevent oxidative damage to many extracellular matrix components (14, 21). When combining our present and previous results, we demonstrated that two important components of the glomerular defense against oxidative stress are impaired in NS. Decreased expressions of the enzymes that are involved in free radical detoxification, and degradation of the protective endothelial pericellular coat, containing first and foremost proteoglycans and GAGs, are two possible mechanisms behind the induction of proteinuria.

In summary, our data suggest that NS is associated with weakening of a vital part of the endogenous antioxidative defense. Thus levels of oxidative stress that would otherwise be regarded as normal could now be sufficient to cause damage to glomerular structures and contribute to the main symptoms of the NS, i.e., protein leakage and impaired renal clearance. The issue of whether such increased vulnerability is a main cause of the loss of renal function, or merely exacerbates it to some degree, remains to be solved.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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