Oxidant stress in hyperlipidemia-induced renal damage

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SEVERAL HEMODYNAMIC AND METABOLIC factors have been shown to contribute to the progression of renal disease in animal models and in humans (23). Hyperlipidemia, specifically hypercholesterolemia, can induce or exacerbate glomerular injury in mammals (14, 52). Data from animal experiments and clinical studies suggest that lipids and lipoproteins affect not only glomeruli but also the tubulointerstitium (15).

The lipid-induced tubulointerstitial damage is of special pathophysiological importance as Risdon et al. (45) and Mackensen-Haen et al. (30) have shown that tubules and interstitium are major determinants of renal excretory function.

Interstitial fibrosis and tubular atrophy have been documented in both female and male hypercholesterolemic rats without primary glomerular disease (15, 10). Renal mRNA of the profibrogenic cytokine, transforming growth factor-β1 (TGF-β1), and the chemokine, monocyte chemoattractant protein-1 (MCP-1), were found to be increased in these experiments. The pathogenetic mechanisms, however, leading to an increase in inflammatory mediators in lipid-induced tubulointerstitial fibrosis and the relationship between glomerular and tubulointerstitial damage have not been examined in detail.

In cell culture experiments, oxygen radicals were identified as messengers for expression of the chemokine MCP-1 and monocyte colony-stimulating factor (CSF-1) (46).

It has been stressed recently that oxidants and oxidative modifications do indeed play a major role in permanent tissue damage (4). The detrimental effects of reactive oxygen species (ROS) have also been documented in renal parenchyma, mesangial cells in culture, and on matrix components (2, 3, 44, 47).

In human inflammatory (e.g., interstitial nephritis) and degenerative (e.g., nephrosclerosis) renal disease, we have identified proteins modified by reactive oxygen compounds, e.g., hypochlorous acid/hypochlorite (HOCl/OCl−) in the tubulointerstitium (33). Human tubular epithelia in culture can oxidatively modify native lipoproteins (40).

In hyperlipoproteinemia, cell membranes and the extracellular matrix can change their lipid composition and be more prone to radical generation (21). Hypercholesterolemia has been reported to increase superoxide anion production in endothelial cells (39). Oxidative...
modification of lipids is able to lead to a self-perpetuating cycle of oxygen radical generation and modification of proteins (11).

It was postulated that lipid-induced tubulointerstitial disease might occur via oxygen radical generation. As the ratio of oxidant and antioxidant enzyme activities determines the oxidant status of renal cells and tissues (17), a failure of a compensatory rise in antioxidant enzyme activities or an increase in oxidant enzyme activity may both contribute to renal oxidant stress.

Thus, two main aims were pursued in this experimental study. 1) The generation of ROS and the activities of oxidant and antioxidant enzymes in glomeruli and tubulointerstitium were determined during the development of glomerular and tubulointerstitial damage in uninephrectomized rats with diet-induced hypercholesterolemia. 2) As the renal effects of hyperlipidemia have not been analyzed and may change in immune complex glomerulonephritis, we also studied the effect of hyperlipidemia on glomeruli and tubulointerstitium with regard to oxidant stress and antioxidant defense in uninephrectomized rats with mesangioproliferative glomerulonephritis. The present findings demonstrate that hyperlipidemia increased oxidant stress. A rise in the ratio of oxidant to antioxidant enzyme activities in generation of ROS and oxidatively modified compounds was observed. Simultaneously, the number of myofibroblasts in the tubulointerstitium was increased. Significant chronic tubulointerstitial damage was observed after 5 mo of hyperlipidemia.

METHODS

Animal Model

Male Wistar rats (160 g body wt; Charles River, Sulzfeld, Germany) were unilaterally nephrectomized to accelerate renal damage. Two weeks after nephrectomy, Thy-1 nephritis was induced by an intravenous injection of rabbit anti-rat thymocyte antiserum (ATS; 5 mg kg/100 g body wt). The antiserum was obtained from R. A. K. Stahl (University of Hamburg) and produced as described (48). The mesangioalytic activity of the ATS was evaluated by light microscopy in two animals chosen at random in every group, 4 days after ATS injection. Pronounced focal mesangioysis and mesangial hypercellularity were regarded as necessary to include animals into the experimental groups. One week after the first antibody injection, rats received a second intravenous ATS injection at the same dose to achieve a progressive glomerulonephritis (36). After the second injection of the antiserum, animals were fed a high-fat (40%)/cholesterol (5%) diet. The fat diet contained cholic acid (0.35%) to enhance the enteral absorption of lipids and cholesterol. The diet has been described in detail (13).

Unilaterally nephrectomized rats without ATS injection and with regular pellet chow or fat/cholesterol diet served as controls.

Daily calorie intake was maintained identical between animals of the different diet groups by measurement of food intake and daily portioning of diet. Animals were allowed free access to tap water. Functional and morphological studies were carried out at 70 and 150 days after primary ATS administration.

Experimental Groups

Unilaterally nephrectomized animals were divided into the following four groups: group 1, rats on regular pellet chow (70 days, n = 10; 150 days, n = 7); group 2, rats on cholesterol/fat-enriched diet (70 days, n = 10; 150 days, n = 7); group 3, rats with Thy-1 nephritis on regular pellet chow (70 days, n = 6; 150 days, n = 18); group 4, rats with Thy-1 nephritis on cholesterol/fat diet (70 days, n = 8; 150 days, n = 13).

Measurements

Arterial pressure. Systolic arterial pressure was measured by tail plethysmography under light ether anesthesia (12).

Urinary protein excretion and serum chemistry parameters. Animals were housed in metabolic cages for 24 h with free access to drinking water. Urine was collected under liquid paraffin to prevent evaporation. Protein content was measured according to Lowry et al. (29). Serum levels of creatinine, urea, cholesterol, and triglycerides were measured by autoanalyzer technique.

Analysis of plasma lipoproteins. At the end of the study (150 days), plasma from the animals of every group was pooled for separation of various lipoproteins by sequential ultracentrifugation (Beckman Ultracentrifuge L8–55, rotor 75Ti at 10°C). Chylomicrons were separated at specific gravity (d) > 1.006 g/ml by centrifugation at 12 × 10^4 g for 30 min; the infranate was brought to d = 1.01 g/ml and centrifuged at 12 × 10^4 g for 24 h. After collecting the floating lipoproteins, the very low-density lipoprotein (VLDL) and intermediate density lipoprotein (IDL) fraction were brought to d = 1.006 g/ml and centrifuged at 12 × 10^4 g for 24 h to separate VLDL. The infranate was adjusted to d = 1.060 g/ml and centrifuged for 24 h. After collecting the floating lipoproteins (low-density lipoprotein, LDL), the infranate was adjusted to d = 1.21 g/ml by addition of solid KBr and centrifuged for 48 h to isolate high-density lipoprotein (HDL). All lipoprotein fractions were washed and concentrated by recentrifugation at their respective floating densities. Lipoproteins were dialyzed against 5 mM Tris buffer, pH 7.4, containing 150 mM NaCl and 0.25 mM EDTA and analyzed for lipid and protein composition. The purity of lipoproteins was checked by electrophoresis (Lipidophor; Immuno, Heidelberg, Germany).

Morphological studies. At the end of the respective experiments, organs (e.g., heart, liver, kidney, spleen) were removed under deep ether anesthesia. Organs were quickly blotted free of blood, weighed, and then processed for histology, immunohistochemistry, and in situ hybridization.

Organs were cut into 1-mm slices and immersion-fixed in phosphate-buffered formaldehyde (4%, pH 7.35) at 4°C for 24 h and embedded in paraffin. Additional slices of kidney were fixed in methacarn solution (60% methanol, 30% chloroform, and 10% acetic acid), fixed for 8 h and embedded in paraffin. For immunohistochemistry, slices were frozen in liquid nitrogen and consequently stored at −80°C until used.

Light microscopy. Light microscopy was done on 3-µm sections of formaldehyde-fixed paraffin-embedded tissue stained by periodic acid-Schiff or Goldner-Elastica-trichrome.

Immunohistochemistry. The monoclonal antibody ED1 (Serotec/Camon, Wiesbaden, Germany) was used on methacarn-fixed sections (3 µm) to demonstrate monocytes/macrophages. The mouse monoclonal antibody detecting the intermediate filament desmin was applied to frozen sections fixed in acetone for 5 min. The specificity of this antibody has been described in detail (8). An alkaline phosphatase/anti-alkaline phosphate system was used for immunohistochemical demonstration of the intermediate filament desmin.
phosphatase detection system was applied (Dako, Hamburg, Germany). Controls omitting the first or second antibody and taking nonimmune mouse IgG instead of the first antibody were negative.

**In situ hybridization.** Single-stranded RNA probes were generated by in vitro transcription of a cDNA derived from rat TGF-β1 (955-bp fragment of the major coding region of rat TGF-β1 precursor) described by Qian et al. (42) in 1990. In vitro transcription was carried out using a Trans-Probe kit (PharMacia, Freiburg, Germany) and digoxigenin-labeled UTP (Boehringer, Mannheim, Germany). The vector [pBlueScript KS (+); Stratagene, Heidelberg, Germany] was cut with BamHI and transcribed with T3 RNA polymerase to yield antisense probe; to yield sense probe, the plasmid was cut with EcoRI followed by transcription with T7 RNA polymerase. After deparaffinization, kidney sections were digested with 20 µg/ml proteinase K (Sigma) in PBS for 16 min. Sections were postfixed for 5 min in 4% formaldehyde and acetylated (0.25% acetic anhydride in 0.1% triethanolamine, 10 min). The following hybridization buffer was used: 5× standard saline citrate (SSC), 50% formamide, 50 µg/ml tRNA, 50 µg/ml heparin, 0.1% SDS.

After hybridization at 56°C for 16 h, slides were washed once in 4× SSC and 2× SSC for 10 min at 37°C, followed by a washing step in 0.5× SSC for 30 min at 60°C. Slides were then digested with RNase A (20 µg/ml; Sigma, Deisenhofen, Germany) and washed again with 0.5× SSC at 37°C for 30 min and 0.1× SSC at 22°C for 15 min. Antidigoxigenin antibody incubation and alkaline phosphatase reaction was done according to guidelines by the manufacturer (Boehringer), using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as color reagents.

**Morphometry (Ref. 49).**

Glomerular sclerosis. Glomerular section areas without lesion were judged as grade 0; with up to 25% obliterated capillary tuft and adhesions to Bowman’s capsule as sclerosis grade 1; with 25–50% as grade 2; 50–75% as grade 3; and >75% as grade 4.

Mesangial matrix. Mesangial matrix was examined similarly: grade 0, no matrix increase; grade 1, slight broadening of the mesangium; grade 2, moderate mesangial matrix increase; and grade 3, pronounced mesangial matrix increase.

Mesangial cells were taken as such when cell nuclei were clearly embedded within the intercapillary matrix; evaluation was done on 3-µm thin sections.

Mesangial cell density. Mesangial cell density was judged as normal (less than 3 cells/mesangial field = grade 0), slightly elevated (3–4 cells/mesangial field = grade 1), moderate (5–7 cells/mesangial field = grade 2), or pronounced (more than 7 cells/mesangial field = grade 3).

Fifty glomeruli per kidney were examined using a ×40 objective by passing from a randomly chosen subcapsular area to the juxtapositional and vice versa with 2 glomerular diameters between measurement lines. The indexes of glomerular sclerosis, of mesangial matrix, and of mesangial cell density were calculated as the sum of the respective grades, after lesions with grade 1 were multiplied by a factor 1, grade 2 by 2, grade 3 by 3, grade 4 by 4. A mean was determined for every group. Glomerular ED1 antigen-positive cells were counted in 50 glomerular cross sections and given as the mean per glomerular section. Total interstitial cells and interstitial ED1 antigen-positive cells and TGF-β mRNA-labeled cells were counted in 20 cortical high-powered fields (HPF, ×400) of cortex and outer stripe of outer medulla per kidney and have been recorded as mean per HPF. For TGF-β1-positive cells, the only cells counted were those showing a clear-cut blue perinuclear signal.

The percentage of tubulointerstitial area, affected by infiltration of mononuclear cells and tubular damage (e.g., tubular atrophy with flattened epithelium and broadened basement membranes, tubular dilatation, cast formation, interstitial fibrosis) was measured in a fashion comparable to the glomerular morphometric measurements, in 10 areas of cortex and outer stripe of outer medulla using a ×20 objective and a computer-aided image analysis system (Cue 3 Colour image analyzer, Galai version 4.6p; Olympus Haemek, Israel).

**Oxygen Radicals and Oxidant and Antioxidant Enzyme Activities.**

Isolation of glomeruli and cortical tubulointerstitium. At the end of experiments, the animals were killed by cervical dislocation. Blood samples were drawn by cardiac puncture. The abdomen was opened by a midline incision; the aorta was dissected and cannulated with a polyethylene catheter. Kidneys were flushed with 20 ml of ice-cold Krebs-Henseleit saline (29 mM NaHCO₃, 1.2 mM Na₂PO₄, 5 mM KCl, 2 mM CaCl₂, and 1.09 mM NaCl, pH 7.4) until they were completely free of blood. Kidneys were removed. Glomeruli and cortical tubulointerstitial samples (TIS) were prepared by the sieving method: briefly, the medulla was removed, and the remaining renal parenchyma was minced and subsequently passed through sieves of 300, 150, and 75 µm mesh width. Tubulointerstitial material was taken from the 150-µm sieve, and glomeruli were from the 75-µm sieve. Glomerular preparations had a purity of greater than 90% as examined by light microscopy. The tubulointerstitial fraction contained proximal and distal tubules and approximately 25% of interstitial tissue with only few ED1 cells. Samples were collected for measurement of oxygen radical generation and activities of oxidant enzymes and antioxidant enzymes.

Measurement of oxygen radicals. Glomeruli and TIS were resuspended in “Dulbecco’s Minimal Essential Medium for Chemiluminescence” (Boehringer) and stored on ice until measurements.

Oxygen radicals were measured at 37°C using a Biolumat LB 9505 C liquid scintillation counter (Berthold, Wildbad, Germany) with lucigenin and luminol at a final concentration of 0.26 and 0.75 mM, respectively. The validity of lucigenin for detecting superoxide anion has recently been ascertained (18, 28); luminol chemiluminescence was taken as a general detector of ROS. After recording background activity for 5 min in 400 µl, the samples (100 µl) were added (final volume = 500 µl). Basal oxygen-radical generation was recorded for 40 min, then glomeruli or tubules were stimulated with phorbol myristate acetate (PMA, 5 µM) to determine the capacity of radical generation. Protein concentration in the reaction vials was determined according to Lowry et al. (29). Results were expressed as counts per minute per microgram protein. Measurements were shown to be linear in the range of 50–250 µg protein/vial.

**Measurement of oxidant enzyme activities.** Samples for determination of oxidant enzyme activities were stored in Krebs-Ringer solution (KRS: 136 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 10 mM HEPES, and 5 mM glucose, pH 7.4) at −70°C until measurement. Samples were homogenized on ice using a Teflon Potter homogenizer.

In general, oxidant enzyme activities were determined by lucigenin enhanced chemiluminescence (ECL) in a final volume of 500 µl. Lucigenin solutions were prepared freshly every day. The reaction vials containing glomeruli or TIS
homogenates were prewarmed at 37°C for several minutes, and background chemiluminescence was recorded over 5 min. NADH/NADPH oxidase activity was measured in 50 mM KPO₄ buffer (pH 7.0) containing 1 mM EGTA, 100 mM sucrose, and 0.26 mM lucigenin (16). The reaction was started by addition of 50 µl NADPH or NADH stock solution (1 mM) dissolved in 1% NaHCO₃ resulting in a final substrate concentration of 0.1 mM. Measurements were done at signal maximum. Oxidase activities were calculated as average cpm minus background cpm and related to the protein concentration of the sample.

Xanthine oxidoreductase activity was measured in 100 mM Tris buffer containing 1 mM EDTA (pH 9.0, 44 µl) and lucigenin at a concentration of 0.26 mM (35).

Samples containing 50 µg protein were added in 50 µl KRS at 37°C, and the reaction was started by addition of 10 µl of a 2.5 mM xanthine stock solution (final concentration: 50 µM). Subsequent steps were identical to that of NAD(P)H oxidase measurements. Specificity of the measurement was tested by addition of oxyquinol at a final concentration of 100 µM, which resulted in complete inhibition of the signal.

Measurement of antioxidant enzyme activities.

Superoxide dismutase (SOD) activity was determined by the cytochrome c assay according to Crapo et al. (7) in 1978. Total SOD activity was measured in the presence of 10 µM potassium cyanide to avoid interference by cytochrome c oxidase. Mn-SOD activity was determined separately after inhibition of CuZn-SOD by 1 mM potassium cyanide. Enzyme activities in the samples were calculated from a standard curve (17). One unit of SOD was defined as the amount of SOD activity necessary to inhibit the reduction of cytochrome c by 50%.

Polycrylamide Gel Electrophoresis and Western Blotting Technique

Detection of xanthine oxidoreductase. Frozen kidneys were homogenized in a solution of 25 mM Tris and 1 mM EDTA with 0.1% SDS, 20 µM phenylmethylsulfoxide, and pepstatin, leupeptin, and aprotinin (2% each), and Triton X-100, pH 6.8. The protein content of the supernatant, after centrifugation at 13,000 rpm for 10 min, was determined. Samples (50 µg protein) were separated on a 1-mm thick SDS/8% polyacrylamide gel and blotted to nitrocellulose. The polyclonal rabbit antibody against rat xanthine oxidase was a kind gift from T. Nishino (Japan) (38). Bound primary antibody was detected with an anti-rabbit IgG antibody from donkey coupled with horseradish peroxidase.

Detection of HOCl-modified proteins. Homogenized kidneys were dissolved in 1 mL of O’Ferral’s buffer (3.04 mol/l

### Table 1. Serum cholesterol, creatinine, urea, and systolic arterial pressure in experimental groups with and without high-fat diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Cholesterol, mg/dl</th>
<th>Serum Creatinine, mg/dl</th>
<th>Serum Urea, mg/dl</th>
<th>SAP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 day RD</td>
<td>110 ± 10 (9)</td>
<td>0.73 ± 0.04 (9)</td>
<td>56 ± 3 (9)</td>
<td>94 ± 3 (10)</td>
</tr>
<tr>
<td>70 day FD</td>
<td>132 ± 6* (9)</td>
<td>0.74 ± 0.02 (10)</td>
<td>44 ± 4* (10)</td>
<td>101 ± 2 (10)</td>
</tr>
<tr>
<td>150 day RD</td>
<td>71 ± 5 (7)</td>
<td>0.70 ± 0.02 (7)</td>
<td>45 ± 4 (7)</td>
<td>86 ± 5 (7)</td>
</tr>
<tr>
<td>150 day FD</td>
<td>174 ± 12* (7)</td>
<td>0.78 ± 0.03 (7)</td>
<td>43 ± 4 (7)</td>
<td>98 ± 2 (7)</td>
</tr>
<tr>
<td>Thy-1 nephritis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 day RD</td>
<td>115 ± 12 (6)</td>
<td>0.71 ± 0.03 (6)</td>
<td>68 ± 6 (6)</td>
<td>105 ± 7 (6)</td>
</tr>
<tr>
<td>70 day FD</td>
<td>256 ± 75* (5)</td>
<td>1.01 ± 0.33 (5)</td>
<td>129 ± 81 (5)</td>
<td>111 ± 4 (7)</td>
</tr>
<tr>
<td>150 day RD</td>
<td>139 ± 13* (18)</td>
<td>0.99 ± 0.27 (18)</td>
<td>62 ± 6 (10)</td>
<td>109 ± 5* (10)</td>
</tr>
<tr>
<td>150 day FD</td>
<td>244 ± 31* (13)</td>
<td>0.95 ± 0.18 (13)</td>
<td>71 ± 22 (9)</td>
<td>113 ± 7 (9)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n values are in parentheses. SAP, systolic arterial pressure. RD, regular diet; FD, fat diet; for details see METHODS. *Significant difference between the diets at the same time of controls or Thy-1 nephritis. †Significant difference between Thy-1 nephritis and control at the same time and diet.

### Table 2. Lipid-protein composition of plasma lipoproteins in experimental groups with and without fat diet after 150 days

<table>
<thead>
<tr>
<th>Group</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 day RD</td>
<td>24.4</td>
<td>25.3</td>
<td>37.3</td>
</tr>
<tr>
<td>70 day FD</td>
<td>6.9</td>
<td>36.3</td>
<td>20.3</td>
</tr>
<tr>
<td>150 day RD</td>
<td>56.3</td>
<td>21.4</td>
<td>22.0</td>
</tr>
<tr>
<td>150 day FD</td>
<td>12.5</td>
<td>17.0</td>
<td>20.3</td>
</tr>
<tr>
<td>Thy-1 nephritis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 day RD</td>
<td>19.5</td>
<td>27.9</td>
<td>29.7</td>
</tr>
<tr>
<td>70 day FD</td>
<td>12.5</td>
<td>32.7</td>
<td>21.0</td>
</tr>
<tr>
<td>150 day RD</td>
<td>48.8</td>
<td>18.9</td>
<td>25.4</td>
</tr>
<tr>
<td>150 day FD</td>
<td>19.2</td>
<td>20.4</td>
<td>23.9</td>
</tr>
</tbody>
</table>

Values are in percent. VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
The Wallis test followed by the Mann-Whitney U rank sum test.

Statistical Analysis were supplied by Sigma (Munich, Germany) and Dako.

Glomerular morphology in experimental groups with and without fat diet (Table 3).

- Table 3. Glomerular morphology in experimental groups with and without fat diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mesangial Matrix, index</th>
<th>Mesangial Cell Density, index</th>
<th>ED1+ Cells, cells/glomerulus</th>
<th>Glomerulosclerosis, index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>6 ± 1.0 (10)</td>
<td>7 ± 1.3 (10)</td>
<td>1.4 ± 0.3 (10)</td>
<td>0 ± 0.1 (10)</td>
</tr>
<tr>
<td>70 day RD</td>
<td>24 ± 9.0* (9)</td>
<td>36 ± 12.7* (9)</td>
<td>4.5 ± 0.8* (10)</td>
<td>2 ± 1.1 (9)</td>
</tr>
<tr>
<td>150 day RD</td>
<td>14 ± 1.4 (7)</td>
<td>13 ± 1.2 (7)</td>
<td>2.2 ± 0.9 (7)</td>
<td>0 ± 0.1 (7)</td>
</tr>
<tr>
<td>150 day FD</td>
<td>66 ± 9.8* (7)</td>
<td>47 ± 8.2* (7)</td>
<td>7.8 ± 2.1* (7)</td>
<td>8 ± 3.2* (7)</td>
</tr>
<tr>
<td>Thy-1 nephritis</td>
<td>70 day RD</td>
<td>113 ± 16.9* (6)</td>
<td>2.8 ± 0.2* (6)</td>
<td>19 ± 9.2* (6)</td>
</tr>
<tr>
<td></td>
<td>70 day FD</td>
<td>132 ± 24.1* (8)</td>
<td>5.5 ± 1.1* (8)</td>
<td>65 ± 36.4* (8)</td>
</tr>
<tr>
<td></td>
<td>150 day RD</td>
<td>152 ± 23.9* (12)</td>
<td>9.0 ± 12.5* (12)</td>
<td>30 ± 8.6* (11)</td>
</tr>
<tr>
<td></td>
<td>150 day FD</td>
<td>154 ± 16.7* (12)</td>
<td>8.1 ± 1.6* (9)</td>
<td>47 ± 16.8* (9)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n values are in parentheses. For details see Methods. *Significant difference between the diets at the same time of controls or Thy-1 nephritis. †Significant difference between Thy-1 nephritis and control at the same time and diet.

RESULTS

As shown in Table 1, animals fed a high-fat/cholesterol diet had higher plasma cholesterol levels (up to 2.4-fold) than those animals without fat diet.

After 150 days, analysis of protein, cholesterol, triglycerides, and phospholipids in VLDL, LDL, and HDL demonstrated a rise of cholesterol content in VLDL and LDL during the high-fat/cholesterol diet (Table 2). The lipoprotein particles were depleted of their triglyceride content. Animals consuming a high-fat diet had fatty livers with a significantly higher liver-to-body weight ratio than in animals fed normal pellets. There were no histological signs of hepatic inflammation and necrosis.

The mean weight of kidneys, serum creatinine, and urea of animals with Thy-1 nephritis tended to be higher than in controls; systolic arterial pressure did not vary appreciably between groups (Table 1). Urinary protein excretion was higher in rats with Thy-1 nephritis compared with controls and was increased during high-fat/cholesterol diet (Fig. 1).

Renal Morphology

Hyperlipidemia led to a statistically significant rise in glomerular monocytes/macrophages, in mesangial cell density and matrix, and in segmental glomerular scarring in rats without Thy-1 nephritis (Table 3).

Independent of the diet, rats with Thy-1 nephritis showed a significant increase in these mesangial parameters and in the glomerulosclerosis index (Table 3). The number of ED1-positive cells in glomeruli and the incidence of glomerulosclerosis tended to be higher in hyperlipidemic Thy-1 nephritics than in normolipidemic Thy-1 nephritic rats, but due to the variability of extent of glomerular sclerosis in hyperlipidemic nephritic rats, statistical significance could not be attained.

In groups without glomerulonephritis, relevant chronic tubulointerstitial damage was only noticed in rats with a cholesterol/fat diet (Fig. 2). In contrast, tubulointerstitial lesions were far more advanced in Thy-1 nephrosis at 70 and 150 days. Hyperlipidemic Thy-1 nephritis rats demonstrated a significantly higher extent of tubulointerstitial damage than normolipidemic Thy-1 nephritics. This clear-cut difference was
in contrast to the overlapping glomerular damage indexes in Thy-1 nephritic rats with normo- and hyperlipidemia. The development of chronic lesions of the tubulointerstitium was associated with an influx of mononuclear cells and ED1-positive monocytes/macrophages. Interstitial monocytes/macrophages constituted up to 50% of interstitial mononuclear cells. Even though the number of ED1-positive cells was generally higher in hyperlipidemic rats, the cell count did not tightly correlate with tubulointerstitial damage. Concurrent with tubulointerstitial damage, the number of desmin-positive cells rose in the interstitium. These desmin-positive cells were regarded as myofibroblasts (Fig. 3, a–d). Myofibroblasts were most numerous in kidneys with severe tubulointerstitial damage, e.g., Thy-1 nephritic rats with hyperlipidemia.

mRNA of the Fibrogenic Cytokine: TGF-β1

TGF-β1 could be shown in cells of glomeruli with mesangial cell and matrix increase and in interstitial cells as soon as the number of monocytes and myofibroblasts was increased. TGF-β1 mRNA was not detected...
in tubular epithelia. In the interstitium, the number of TGF-β1 mRNA-expressing cells was consistently higher in rats with hyperlipidemia than in normolipidemic rats and highest in nephritic rats with a high-fat/cholesterol diet (Fig. 4, A and B).

Reactive Oxygen Species and Oxidant/Antioxidant Enzymes

We attempted to correlate these data of greater chronic tubulointerstitial damage in hyperlipidemia to the renal oxidant status in two renal compartments: glomeruli and cortical tubulointerstitium.

Luminol ECL was taken as a measure for generation of ROS (Fig. 5, A and B). High-fat/cholesterol diet generally increased mean basal ROS generation in glomeruli and tubulointerstitium (Fig. 5). Lucigenin ECL gave similar results (data not shown). PMA-stimulated radical generation confirmed the results of basal oxygen species generation. In tubulointerstitial isolates, a high-fat/cholesterol diet was associated with a significant elevation of PMA-stimulated lucigenin ECL (control, regular diet 66 ± 11 vs. fat diet 189 ± 38 (P < 0.05); Thy-1 nephritis, regular diet 128 ± 31 vs. fat diet 542 ± 199 cpm/µg protein (P < 0.05)).

In the cortical tubulointerstitial fraction of normolipidemic Thy-1 nephritic rats, the severity of chronic damage was not accompanied by a consistent rise in ROS. Glomerular damage was quite pronounced in normolipidemic Thy-1 nephritis after 70 and 150 days, but luminol and lucigenin ECL did not increase appreciably in comparison to normolipidemic rats without glomerulonephritis (Fig. 5, A and B).

Since the effects of oxygen radicals are largely determined by the ratio of oxidant to antioxidant enzyme activities, enzyme activities of NADH oxidase, NADPH oxidase, xanthine oxidase, myeloperoxidase (MPO), SOD, GSH-PX, and catalase were quantitated.

Oxidant Enzyme Activities

Enzyme activities of NADH oxidase, NADPH oxidase, and xanthine oxidoreductase were measured in glomeruli and cortical tubulointerstitial fractions. MPO activity was assessed indirectly by detection of HOCl-modified proteins in cortical tissue homogenates and in urine.

Hyperlipidemia did not significantly influence activities of NADH and NADPH oxidases. Both NADH and NADPH oxidase activities decreased with age in control rats; this effect was not observed in rats with mesangioproliferative glomerulonephritis. Since activities of NADH oxidase and NADPH oxidase activities were similar, only data on NADPH oxidase activities in tubulointerstitium are shown in Fig. 6A.

A high-fat/cholesterol diet significantly increased xanthine oxidoreductase activity in glomeruli (data not shown) and tubulointerstitium in rats with and without mesangioproliferative glomerulonephritis (Fig. 6B). In renal tissue homogenates, xanthine oxidoreductase protein was also expressed more strongly in hyperlipidemia (up to 3-fold) than in normolipidemia (Fig. 7).

HOCl-Modified Proteins

After 150 days in normolipidemic nephritic rats with moderate tubulointerstitial damage, no HOCl-modified proteins were detected in urine and renal tissue homogenates by Western blot with specific monoclonal antibody (Figs. 8 and 9); in contrast, in hyperlipidemic Thy-1 nephritis with conspicuous chronic tubulointerstitial damage, HOCl-modified proteins were detected in urine and renal tissue after 150 days (Figs. 8 and 9).

Antioxidant Enzyme Activities

Total SOD, its isoform Mn-SOD, GSH-PX, and catalase activities were measured in glomeruli and cortical tubulointerstitial fractions. Antioxidant enzyme activities were severalfold (2- to 8-fold) higher in the tubulointerstitium than in glomeruli. In glomeruli of control rats, antioxidant enzyme activities decreased with age (data not shown). In the cortical tubulointerstitial fractions of Thy-1 nephritic rats, all four measured antioxidant enzymes fell in activity by 25–65% (Table 4). In conjunction with the increased protein and


activity of xanthine oxidoreductase, a rise in oxidant stress was thus demonstrated in the tubulointerstitium of hyperlipidemic Thy-1 nephritis.

**DISCUSSION**

It has been convincingly demonstrated that rats with chronic hyperlipidemia and hypercholesterolemia develop glomerulosclerosis in kidneys without immune complex disease (14, 34, 45, 53). The present study confirms these data. Hyperlipidemia can also be associated with chronic tubulointerstitial damage (10, 15). Several lines of evidence indicate that oxidant stress is a pathogenetic factor in lipid-induced nephropathy. By immunohistology, oxidatively modified lipoproteins were demonstrated in focal segmental glomerulosclerosis in rat and humans (26, 27, 31). However, the steps leading to oxidative modification of proteins and lipoproteins have not been elucidated.

The present study demonstrates that oxygen radical generation was increased in unstimulated and PMA-stimulated glomeruli of hypercholesterolemic, uninephrectomized male Wistar rats. This increase was evident as soon as the number of glomerular ED1-positive monocytes/macrophages and the incidence of glomerulosclerosis increased. In contrast to the dominant role of NADH oxidase and NADPH oxidase activity in the generation of oxidatively modified noncollagenous domains of collagen IV in the glomerular basement membranes of rats with Heymann nephritis (37), no change in these oxidant enzyme systems in glomeruli or tubulointerstitium could be detected.

Xanthine oxidase, infused into rabbit kidneys, can initiate dramatic glomerular damage, e.g., endothelial damage, microaneurysms, mesangiolysis (50). In hypercholesterolemic rabbits, a twofold elevation of plasma xanthine oxidase activity has been reported (56). It was also shown in that study that xanthine oxidase caused the enhanced superoxide production of aortic vessel rings. These data are analogous to our data that suggest that increased glomerular oxygen radical production was mainly caused by xanthine oxidoreductase. Xanthine dehydrogenase, with high xanthine/NAD⁺ activity, can be converted into xanthine oxidase with a high capacity of O₂⁻ generation either by reversible thiol oxidation or by irreversible proteolytic cleavage (38). Western blot experiments on whole renal tissue homogenates demonstrated an increase in protein of xanthine oxidoreductase and no additional peptide fragments in hyperlipidemia, thus excluding the possibility of conversion to xanthine oxidase by proteolysis.

Expression of xanthine oxidoreductase may be regulated by various cytokines (6, 41, 51). Furthermore, hyperlipidemia also favors the expression of cytokines that may modulate xanthine oxidase activity.

In uninephrectomized normolipidemic and hyperlipidemic rats without glomerulonephritis, glomerular antioxidant enzyme activities decreased with age, a finding corroborated by a recent study on the role of free radicals in the pathogenesis of lipid-induced glomerulosclerosis in Sprague-Dawley rats (19).

The increased ROS generation in glomeruli of hyperlipidemic rats without glomerulonephritis observed in
In the present study, however, seems primarily to result from elevated xanthine oxidase activity. Data on any direct effect of hypercholesterolemia on rat kidneys with glomerular immune complex disease have not yet been reported.

After 70 and 150 days of normolipidemic Thy-1 nephritis, a pronounced rise in glomerular cell number and an increase in mesangial matrix and in glomerulosclerosis was observed. Glomerular oxygen radical generation was not significantly altered. It may be speculated that in normolipidemic mesangioproliferative glomerulonephritis the immune complex stimulus was potent and used pathogenetic mechanisms other than oxygen radicals for glomerular scarring to occur. Oxidant stress was evident in hyperlipidemic rats with Thy-1 nephritis. Mean glomerulosclerosis was higher in nephritic and hyperlipidemic rats, but this did not attain statistical significance due to a large variability of single animal values.

In contrast, hyperlipidemia significantly aggravated tubulointerstitial damage in uninephrectomized rats with glomerulonephritis. A disturbed balance between oxidant and antioxidant enzyme activities may have been involved. The generation of ROS and the activity of xanthine oxidase in the cortical tubulointerstitium rose significantly in hyperlipidemia.

Although tubulointerstitial damage worsened from day 70 to day 150, high oxygen radical generation and xanthine oxidase activity either slightly decreased or did not change. This lack of synchrony between chronic irreversible damage and oxidant biochemical parameters may not contradict a pathogenetic interaction. It may be surmised that an altered oxidant/antioxidant balance prob-
It is unlikely that antioxidant enzymes decreased secondarily due to severe chronic tubular damage, because the reduction of enzyme activity already occurred at 70 days when pronounced tubular damage had not yet taken place. As our measurements did not include heme oxygenase-1, which has been shown to increase in glomerular inflammation, the decrease in tubular antioxidant defense may only have been partial in nephritic rats (54).

Nevertheless, it has to be stressed that the present data do not provide unfailing evidence for a causal relation between lipid-induced oxygen radical generation and chronic tubulointerstitial injury. Specifically, the temporal dissociation between an increase in radical species and tubulointerstitial injury in Thy-1 nephritis may also be interpreted as a lack of relation between these parameters.

Oxidatively modified proteins may contribute to the progression of irreversible renal damage. HOCl is generated in vivo by the MPO/H2O2/halide system, and HOCl-modified (lipo) proteins have been identified in human kidney tissues (33).

HOCl-modified (lipo) proteins are chemotactic for mononuclear cells in vitro and increase secretion of interleukin-8 in these cells (57). Other oxidative modifications exerted similar activities on glomerular cells, in culture (20, 25).

Indeed, the presence of HOCl-modified proteins in renal cortical tissue and urine of hyperlipidemic nephritic rats underlined the pathogenetic event of oxidant stress in hyperlipidemic nephritic rats. Whether the presence of HOCl-modified proteins is directly correlated with an increase in tissue lipids, i.e., fatty acids, remains to be elucidated. Free fatty acids increased HOCl synthesis in activated mononuclear cells (43).

In hyperlipidemic rats, proteinuria was significantly higher than in rats with normolipidemia.

Proteinuria has been correlated to progressive tubulointerstitial disease in human and experimental renal diseases (23). A high protein/albumin load and increased endocytosis apparently could lead to growth factor/cytokine expression such as M-CSF, MCP-1, and TGF-β1 in tubular epithelia; also, exposure of proximal tubular cells at their apical border to serum proteins, ably precedes severe damage and that a more or less constant level of oxidant stress can maintain ongoing damage.

In addition, antioxidant enzyme activity in the cortical tubulointerstitium tended to decrease in nephritic rats.

| Table 4. Antioxidant enzyme activities in tubulointerstitium in experimental groups with and without fat diet |
|---|---|---|---|---|
| Group | Total SOD, mU/µg protein | MnSOD, mU/µg protein | Glutathione Peroxidase, pmol NADPH·min⁻¹·mg⁻¹ | Catalase, µmol H2O2·min⁻¹·mg⁻¹ |
| Controls | | | | |
| 70 day RD | 46.4 ± 5.0 (10) | 15.5 ± 1.7 (10) | 489 ± 48 (10) | 183 ± 12.2 (10) |
| 70 day FD | 47.9 ± 9.3 (10) | 20.3 ± 4.5 (10) | 838 ± 145* (10) | 192 ± 27.6 (10) |
| 150 day RD | 29.8 ± 1.8 (7) | 12.5 ± 1.2 (7) | 749 ± 46 (7) | 176 ± 8.4 (7) |
| 150 day FD | 34.6 ± 5.9 (7) | 14.6 ± 1.9 (7) | 1002 ± 78* (7) | 241 ± 31.4* (7) |
| Thy-1 nephritis | | | | |
| 70 day RD | 25.1 ± 2.8† (6) | 11.6 ± 1.1 (6) | 400 ± 41 (6) | 144 ± 7.1† (6) |
| 70 day FD | 24.7 ± 6.7† (8) | 10.4 ± 2.5 (8) | 350 ± 87† (8) | 126 ± 30.5 (8) |
| 150 day RD | 22.4 ± 4.8 (10) | 8.7 ± 1.5 (10) | 398 ± 71† (10) | 68 ± 18.9† (10) |
| 150 day FD | 18.7 ± 2.7† (9) | 6.7 ± 0.9† (9) | 412 ± 55† (10) | 87 ± 14.2† (9) |

Values are means ± SE; n values are in parentheses. For details, see METHODS. SOD, superoxide dismutase. * Significant difference between the diets at the same time of controls or Thy-1 nephritis. † Significant difference between Thy-1 nephritis and control at the same time and diet.
albumin, or transferrin stimulated release of endothelin, platelet-derived growth factor, and fibronectin (9, 58). Tubular catabolism of albumin could lead to release of an inflammatory lipid (22).

The pronounced aggravation of tubulointerstitial damage by hyperlipidemia, especially noticeable in nephritic rats, was accompanied by a dramatic increase in the number of myofibroblasts. Kliem et al. (24) have also pointed to desmin-positive myofibroblasts as markers of tubulointerstitial damage.

We propose that these cells are involved in the pathogenetic sequence leading to tubular atrophy and progression of tubulointerstitial damage by hyperlipidemia, especially noticeable in nephritic rats, was accompanied by a dramatic increase in the number of myofibroblasts. Kliem et al. (24) have also pointed to desmin-positive myofibroblasts as markers of tubulointerstitial damage.

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In summary, the present study has demonstrated that in uninephrectomized male rats hyperlipidemia increased glomerular and tubulointerstitial infiltration by mononuclear cells and aggravated glomerulosclerosis and particularly chronic tubulointerstitial injury. Oxidant stress seemed to have significantly contributed to these chronic degenerative processes by enhancing glomerular generation of ROS caused mainly by enhanced xanthine oxidase activity.

In Thy-1 mesangiproliferative glomerulonephritis, hyperlipidemia did not lead to a significantly higher incidence of immune complex-mediated glomerulosclerosis but did induce significantly greater chronic tubulointerstitial damage; the latter was associated with a shift of oxidant/antioxidant enzyme activity ratio. Xanthine oxidoreductase activity was raised, and antioxidant enzyme activities decreased. In parallel, urinary excretion of oxidatively modified proteins was elevated.

The ability of hyperlipidemia to modulate renal disease and damage different renal compartments seems to be dependent on the primary renal disease and increased oxidant stress.

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