Remodeling of Resistance Arteries in Renal Failure: Effect of Endothelin Receptor Blockade

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Abstract. Remodeling of vessels is a known feature of renal failure, but it is unclear whether this represents an appropriate or inappropriate response to the known changes in blood flow, shear stress, and wall tension. To investigate remodeling in response to variations in blood flow, first-order mesenteric arteries were exposed to high- and low-flow conditions via the ligation of second-order branches, according to the technique described by Pourageaud and De Mey. The resulting changes in vessel geometric features, relative proportions of intima and media, submicroscopic structure, and immunostaining for proliferating cell nuclear antigen (PCNA), endothelin-1 (ET-1), and ETA receptors were assessed in first-order mesenteric arteries under low-flow and high-flow conditions. Subtotally nephrectomized (SNX) animals were compared with sham-operated rats. Animals either were left untreated or were treated with the ETA receptor antagonist (ET-RA) LU-135252, because of suggestions in the literature that ET is involved in vascular remodeling in uremia. A highly significant increase in intimal thickness was noted in low-flow arteries (4.21 ± 1.39 μm) of SNX animals, compared with normal-flow arteries (2.06 ± 0.61 μm), but this increase was not observed in sham-operated rats (1.38 ± 0.77 in low-flow arteries versus 2.40 ± 0.35 μm in normal-flow arteries). The increase in intimal thickness in low-flow arteries was abrogated by ET-RA. The medial thickness was increased in untreated SNX animals (19.5 ± 3.61 μm), compared with sham-operated rats, and this increase was also prevented by ET-RA. The medial thickness was not affected by low flow in either sham-operated or SNX animals. In parallel, the number of PCNA-positive intimal cells was higher in low-flow, but not high-flow, arteries of SNX rats, compared with sham-operated rats. No significant change was observed in sham-operated animals. In the media, the number of PCNA-positive cells was higher in untreated SNX animals than in sham-operated rats. The number was even more markedly increased in high-flow, but not low-flow, vessels. This increase was abrogated by ET-RA. It is concluded that, in uremic animals, the response of the intima to low flow and the response of the media to high flow are exaggerated. Both responses are apparently mediated by ET.

Among renal patients, functional (1) and imaging (2) studies have documented vascular remodeling of central arteries, i.e., increased wall thickness and reduced elasticity. The abnormal cushioning function of central arteries increases cardiac workload and impairs diastolic coronary perfusion (3). It may thus importantly contribute to the high cardiovascular mortality rate among renal patients (4). Much less is known regarding the important consequences, i.e., wall thickening and luminal narrowing. Endothelin-1 (ET-1) plays a pivotal role in vascular remodeling (5–7). Both experimental (8,9) and clinical (10) studies suggest that this is also true in renal failure, at least for elastic arteries.

Uncontrolled observations in the hearts of uremic patients with coronary artery disease suggested more pronounced poststenotic thickening of the intima and media in uremic patients, compared with nonrenal patients (Prof. Dr. G. Mall, Darmstadt, Germany, personal communication). We considered the hypothesis that the remodeling of resistance arteries in response to modifications in blood flow is abnormal in uremia. We also hypothesized that ET is involved in this process.

To address this issue, we adopted the technique described by Pourageaud and De Mey (11), to modify flow rates in first-order mesenteric arteries and to evaluate vascular remodeling under low-flow and high-flow conditions. Vessel geometric and histologic features were compared in sham-operated and subtotally nephrectomized (SNX) rats, using light and electron microscopy.

Materials and Methods
Surgery and Study Design
Twelve-week-old, male, Sprague-Dawley rats (150 to 200 g; Ivanovas Co., Kisslegg, Germany) were maintained in single cages,
under conditions of constant temperature and humidity. The animals had free access to Altromin 1324 pellets (18% protein, 0.2% sodium). After 1 wk of adaptation, the animals were subjected to either a two-step surgical SNX or sham operation. In brief, animals were anesthetized with 0.02 ml of xylazine (Rompun, 2%; Bayer Co., Leverkusen, Germany) and 0.2 ml of ketamine (Ketanest, 10%; WDT, Garbsen, Germany). In the first operation, the right kidney was decapsulated (sham operation), with or without subsequent nephrectomy. The weight of the removed right kidney was carefully determined. After 1 wk, surgical subtotal resection of the cortex of the left kidney was performed. The resected tissue was weighed, and the exact amount of cortex corresponding to two-thirds of the weight of the right kidney was removed. Sham operations were performed as described above.

Sham-operated and SNX animals were randomly allocated to the following groups: group 1, sham operation; group 2, sham operation plus ET receptor antagonist (ET-RA); group 3, sham operation plus mesenteric artery ligation; group 4, sham operation plus mesenteric artery ligation plus ET-RA; group 5, SNX; group 6, SNX plus ET-RA; group 7, SNX plus mesenteric artery ligation; group 8, SNX plus mesenteric artery ligation plus ET-RA. The numbers of animals in each group are presented in Table 1. From day 3 after the second operation until the end of the study, ET-RA-treated animals received the selective ET_{A}-receptor antagonist LU-135252 (Knoll AG, Ludwigshafen, Germany) in tap water, designed to deliver a daily dose of 50 mg/kg body wt.

Six days after the second intervention (i.e., SNX), surgery was performed to modify the mesenteric blood flow, using the technique described by Pourageaud and De Mey (11). The cecum and the adjoining portion of the ileum, with mesentery and vessels, were exteriorized and placed on sterile surgical gauze; the tissues were kept warm and wet in physiologic salt solution. A heating platform maintained the body temperature at 37.5°C. By using a dissection microscope, a segment of a first-order mesenteric artery was gently prepared. The second-order arteries from every other first-order mesenteric artery side branch were ligated with 5-0 nonabsorbable surgical thread (Figure 1).

The rats subsequently had access to rat chow and tap water. BP was measured biweekly in conscious rats, using tail plethysmography. The study was terminated 4 wk after mesenteric surgery.

**Surgical Treatment of the Femoral Artery**

The mesenteric arteries did not yield sufficient material for molecular biologic studies. Therefore, an ancillary experiment was performed with sham-operated (see above) or SNX (see above) animals.

**Table 1. Animal data***

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Animals</th>
<th>Body Weight (g)</th>
<th>Plasma Urea Concentration (mg/dl)</th>
<th>Plasma Creatinine Concentration (mg/dl)</th>
<th>Hemoglobin Level (g/dl)</th>
<th>Systolic BP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham operation</td>
<td>11</td>
<td>337 ± 16.3</td>
<td>31.8 ± 2.5</td>
<td>0.36 ± 0.03</td>
<td>13.4 ± 1.12</td>
<td>100 ± 17</td>
</tr>
<tr>
<td>2. Sham operation + ET-RA</td>
<td>10</td>
<td>347 ± 15.7</td>
<td>40.4 ± 12.4</td>
<td>0.43 ± 0.06</td>
<td>11.4 ± 2.14</td>
<td>100 ± 17</td>
</tr>
<tr>
<td>3. Sham operation + mesenteric artery ligation</td>
<td>10</td>
<td>334 ± 12.3</td>
<td>36.2 ± 7.0</td>
<td>0.36 ± 0.04</td>
<td>14.3 ± 1.00</td>
<td>108 ± 11</td>
</tr>
<tr>
<td>4. Sham operation + mesenteric artery ligation + ET-RA</td>
<td>12</td>
<td>337 ± 10.6</td>
<td>35.4 ± 5.9</td>
<td>0.38 ± 0.03</td>
<td>13.4 ± 1.27</td>
<td>103 ± 19</td>
</tr>
<tr>
<td>5. SNX</td>
<td>15</td>
<td>341 ± 12.6</td>
<td>69.4 ± 13.1\textsuperscript{b}</td>
<td>0.64 ± 0.09\textsuperscript{h}</td>
<td>13.0 ± 0.80</td>
<td>131 ± 12\textsuperscript{b}</td>
</tr>
<tr>
<td>6. SNX + ET-RA</td>
<td>14</td>
<td>338 ± 7.8</td>
<td>69.7 ± 10.9\textsuperscript{b}</td>
<td>0.68 ± 0.07\textsuperscript{h}</td>
<td>13.4 ± 1.39</td>
<td>135 ± 14\textsuperscript{b}</td>
</tr>
<tr>
<td>7. SNX + mesenteric artery ligation</td>
<td>13</td>
<td>334 ± 10.3</td>
<td>70.7 ± 11.7\textsuperscript{b}</td>
<td>0.63 ± 0.07\textsuperscript{h}</td>
<td>14.0 ± 1.44</td>
<td>134 ± 14\textsuperscript{b}</td>
</tr>
<tr>
<td>8. SNX + mesenteric artery ligation + ET-RA</td>
<td>15</td>
<td>337 ± 9.5</td>
<td>85.0 ± 10.7\textsuperscript{b}</td>
<td>0.70 ± 0.07\textsuperscript{h}</td>
<td>13.6 ± 1.83</td>
<td>132 ± 10\textsuperscript{b}</td>
</tr>
</tbody>
</table>

ANOVA 100 NS P < 0.05 P < 0.05 NS P < 0.05

\* ET-RA, endothelin receptor antagonist; SNX, subtotal nephrectomy.
\*\textsuperscript{b} P < 0.05 versus groups 1, 2, 3, and 4.
male Sprague-Dawley rats \((n = 10 \text{/ group})\). Six days after the second operation of the SNX procedure, animals were anesthetized as described above and the left femoral artery was exteriorized and carefully prepared under a microscope. The artery was ligated with 5-0 nonabsorbable surgical thread. The right femoral artery remained untouched.

**Tissue Preparation**

Animals were anesthetized, the aorta was catheterized, and blood samples were obtained. For 58 animals, the organs were subsequently fixed with 4% glutaraldehyde solution at a controlled pressure of 100 mmHg (Table 2). For 42 animals, the organs were prepared for immunohistochemical analyses by perfusion with physiologic saline solution (Table 3). For morphologic measurements, the aorta and individual first-order mesenteric arteries were prepared and embedded in Epon-Araldite (Ciba-Geigg, Basel, Switzerland). Semithin sections of the vessels (0.5 \(\mu m\)) were cut and stained with basic fuchsin and methylene blue. For immunohistochemical analyses, the sections were fixed in paraformaldehyde, embedded in paraffin, and sectioned (5 \(\mu m\)).

The additional study was terminated 10 d after ligation of the left femoral artery, by perfusion-fixation with physiologic saline solution (see above). A 0.5-cm poststenotic segment of the artery (approximately 40 mg) was carefully excised and immediately frozen at \(-70^\circ\) for RNA extraction.

**Quantitative Morphologic Assessments**

The lumen diameter, intimal thickness, medial thickness, wall thickness, and wall/lumen ratio were measured in semithin sections, using a light microscope (Zeiss GmbH, Oberkochen, Germany) and a semiautomatic image analysis system (Videoplan; Kontron Co., Eching, Germany). First-order mesenteric arteries were evaluated at a magnification of \(\times 200\), and aortae were evaluated at a magnification of \(\times 25\). With the use of a cursor, the contours of the lumen, intima, and media of the vessels were marked. Areas, perimeters, and maximal and minimal diameters were calculated. The thicknesses of the intima and media were calculated as the means of the measurements of two opposite walls at the level of the minimal diameter, because this is where measurements are least affected by the sectioning angle. On paraffin sections used for proliferating cell nuclear antigen (PCNA) immunohistochemical analyses (see below), intimal and medial areas were also measured using the semiautomatic image analysis system (Videoplan; Kontron), as described above.

**Electron Microscopy**

Ultrathin sections (0.08 \(\mu m\)) were prepared, stained with uranyl acetate and lead citrate, as described elsewhere (12), and qualitatively evaluated using a Zeiss EM 10 microscope (Zeiss GmbH), at various magnifications.

**Immunohistochemical Analyses (PCNA, ET-1, and ET\(_{A}\) Receptors)**

For immunohistochemical staining, 5-\(\mu m\)-thick paraffin sections were used. Staining for PCNA was performed using an anti-PCNA antibody (DSC-Diagnostik Systeme, Hamburg, Germany), at a dilution of 1:150, as described in detail elsewhere (13). Staining for ET-1 was performed using an unconjugated, human ET-1-specific antibody (Biotrend GmbH, Cologne, Germany); for immunohistochemical de-

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**Table 2. Quantitative morphologic assessments of first-order mesenteric arteries**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Animals</th>
<th>Lumen Diameter ((\mu m))</th>
<th>Intimal Thickness ((\mu m))</th>
<th>Medial Thickness ((\mu m))</th>
<th>Wall/Lumen Ratio ((\mu m/\mu m \times 10^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham operation</td>
<td>5</td>
<td>210 ± 63.9</td>
<td>2.40 ± 0.35</td>
<td>14.0 ± 2.26</td>
<td>84.0 ± 21.0</td>
</tr>
<tr>
<td>2. Sham operation + ET-RA</td>
<td>5</td>
<td>208 ± 42.7</td>
<td>1.28 ± 0.35</td>
<td>12.9 ± 1.24</td>
<td>70.7 ± 14.7</td>
</tr>
<tr>
<td>3. Sham operation + mesenteric artery ligation</td>
<td>5</td>
<td>189 ± 23.0</td>
<td>1.38 ± 0.77</td>
<td>13.9 ± 5.68</td>
<td>82.5 ± 34.0</td>
</tr>
<tr>
<td>low flow(^a) (a)</td>
<td>5</td>
<td>231 ± 68.8</td>
<td>1.78 ± 0.34</td>
<td>15.1 ± 5.79</td>
<td>75.4 ± 26.8</td>
</tr>
<tr>
<td>high flow (b)</td>
<td>5</td>
<td>227 ± 28.1</td>
<td>2.62 ± 1.55</td>
<td>14.2 ± 2.89</td>
<td>74.0 ± 12.3</td>
</tr>
<tr>
<td>high flow (b)</td>
<td>7</td>
<td>286 ± 62.1</td>
<td>1.64 ± 0.81</td>
<td>16.4 ± 3.37</td>
<td>64.3 ± 10.9</td>
</tr>
<tr>
<td>5. SNX</td>
<td>9</td>
<td>196 ± 62.2</td>
<td>2.06 ± 0.61</td>
<td>16.2 ± 4.68</td>
<td>110 ± 74.2</td>
</tr>
<tr>
<td>6. SNX + ET-RA</td>
<td>9</td>
<td>207 ± 37.2</td>
<td>1.80 ± 0.48</td>
<td>15.5 ± 2.89</td>
<td>87.5 ± 26.7</td>
</tr>
<tr>
<td>7. SNX + mesenteric artery ligation</td>
<td>8</td>
<td>185 ± 58.9</td>
<td>4.21 ± 1.39(^b)</td>
<td>13.8 ± 3.67</td>
<td>102 ± 21.7</td>
</tr>
<tr>
<td>low flow (a)</td>
<td>8</td>
<td>266 ± 80.3(^c)</td>
<td>2.09 ± 1.00(^d)</td>
<td>19.5 ± 3.61(^e)</td>
<td>86.1 ± 22.2</td>
</tr>
<tr>
<td>high flow (b)</td>
<td>10</td>
<td>233 ± 53.2</td>
<td>1.20 ± 0.71</td>
<td>16.4 ± 2.71</td>
<td>79.7 ± 25.0</td>
</tr>
<tr>
<td>high flow (b)</td>
<td>289 ± 43.0</td>
<td>0.92 ± 0.83</td>
<td>15.5 ± 1.94</td>
<td>58.7 ± 15.1</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Low flow, first-order mesenteric branches with ligation of the daughter second-order branches (see Figure 1).

\(^b\) \(P < 0.05\) versus all other groups.

\(^c\) \(P < 0.05\) versus groups 5 and 7a.

\(^d\) \(P < 0.05\) versus group 8b.

\(^e\) \(P < 0.05\) versus groups 7a and 8b.

\(^f\) By Kruskal-Wallis test.
**Reverse Transcription-PCR**

reduce run-to-run variations in staining intensity. Experiments were performed by omitting the primary antibody. Staining with hematoxylin and examined using light microscopy. The antibody served as a chromogen. Subsequently, sections were counterstained buffered saline. Fast red substrate (Dako GmbH, Hamburg, Germany) incubation step was followed by two thorough rinses with Tris-gated streptavidin (Biogenex) for 30 min at room temperature. Each then incubated with supersensitive-label, alkaline phosphatase-conjugated streptavidin (Biogenex, San Ramon, CA) was applied for 30 min. The sections were then rinsed in Tris-buffered saline (pH 7.6) and subjected to the incubation at 42°C for 45 min, the enzyme was denatured for 5 min at 95°C. For each animal, reverse transcription was repeated four times. The resulting cDNA was pooled and redivided for PCR, with each aliquot containing 0.25 μg of reverse-transcribed RNA. The cDNA was amplified in 100 μl of a solution containing PCR buffer (Life Technologies-BRL, Grand Island, NY), and 200 U of Superscript reverse transcriptase (Life Technologies-BRL). After incubation at 42°C for 45 min, the enzyme was denatured for 5 min at 95°C. For each animal, reverse transcription was repeated four times. The resulting cDNA was pooled and redivided for PCR, with each aliquot containing 0.25 μg of reverse-transcribed RNA. The cDNA was amplified in 100 μl of a solution containing PCR buffer (same final concentrations as above), 8 pM levels of each primer, and 2.5 U of Taq polymerase (Life Technologies-BRL). The reaction was performed in a Perkin Elmer/Cetus thermal cycler (for conditions, see reference 14). Gel electrophoresis was used to verify the predicted sizes for the amplification products.

**Competitive PCR.** Quantification of ET-1 levels was performed by using deletion mutants of the ET-1 cDNA as internal standards. Amplification of the mutants resulted in a PCR product of 295 bp for ET-1. For each animal, four competitive PCR were performed.

**Analysis of PCR Fragments.** The relative amounts of ET-1 mRNA in each sample were quantified by using a gel documentation system (Intas Co., Göttingen, Germany); the intensities of the bands were densitometrically measured (National Institutes of Health Image 1.44 program), as described in detail elsewhere (14). For each reaction, the relationship of the intensity of the endogenous cDNA band to its respective mutant cDNA band was calculated. For each animal, the mean of four reactions was calculated.

**Statistical Analyses**

Data are presented as mean ± SD. After testing for distribution normality and variance homogeneity, variance was investigated using either ANOVA (if data were normally distributed) or the Kruskal-Wallis test (if distribution normality could not be proven), with the

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**Table 3. Cell proliferation evaluated in PCNA immunohistochemical analysesa**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Animals</th>
<th>PCNA-Positive Cells (no./mm² area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham operation</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2. Sham operation + ET-RA</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3. Sham operation + mesenteric artery ligation low flow (a)</td>
<td>5</td>
<td>484 ± 423 477 ± 383</td>
</tr>
<tr>
<td>4. Sham operation + mesenteric artery ligation + ET-RA low flow (a)</td>
<td>5</td>
<td>466 ± 518 564 ± 361</td>
</tr>
<tr>
<td>high flow (b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. SNX</td>
<td>6</td>
<td>353 ± 532 244 ± 196 517 ± 130</td>
</tr>
<tr>
<td>high flow (b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. SNX + ET-RA</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>low flow (a)</td>
<td>5</td>
<td>206 ± 274 27 ± 53 146 ± 83</td>
</tr>
<tr>
<td>high flow (b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. SNX + mesenteric artery ligation low flow (a)</td>
<td>5</td>
<td>1692 ± 702c 1098 ± 834d</td>
</tr>
<tr>
<td>high flow (b)</td>
<td>5</td>
<td>549 ± 672 683 ± 182</td>
</tr>
<tr>
<td>8. SNX + mesenteric artery ligation + ET-RA low flow (a)</td>
<td>5</td>
<td>608 ± 351</td>
</tr>
<tr>
<td>high flow (b)</td>
<td>5</td>
<td>617 ± 305</td>
</tr>
</tbody>
</table>

Kruskal-Wallis test: P < 0.0001  P < 0.0005

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a PCNA, proliferating cell nuclear antigen.
b P < 0.05 versus groups 1, 2, 3, and 4.
c P < 0.05 versus groups 1, 2, 3, 4, 6, 7b, and 8.
d P < 0.05 versus groups 1, 2, 3, 4, 6, and 8.

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Tection of ETₐ receptors, a sheep antibody (Biotrend) was used. To reduce nonspecific background staining, the sections were incubated for 20 min at 37°C with tissue-unmasking factor. The samples were then rinsed in Tris-buffered saline (pH 7.6) and subjected to the avidin-biotin system. In brief, the slides were incubated with the primary antibody, at a dilution of 1:50, for 60 min at room temperature. The secondary antibody (biotinylated goat anti-rabbit Ig; Biogenex, San Ramon, CA) was applied for 30 min. The sections were then incubated with supersensitive-label, alkaline phosphatase-conjugated streptavidin (Biogenex) for 30 min at room temperature. Each incubation step was followed by two thorough rinses with Tris-buffered saline. Fast red substrate (Dako GmbH, Hamburg, Germany) served as a chromogen. Subsequently, sections were counterstained with hematoxylin and examined using light microscopy. The antibody used had been tested for specificity in rat samples. Negative control experiments were performed by omitting the primary antibody. Staining of control and SNX sections was performed in the same run, to reduce run-to-run variations in staining intensity.

**Reverse Transcription-PCR**

**Sample Preparation and Primers.** Because measurement of ET-1 gene expression in mesenteric arteries could not be performed by using reverse transcription-PCR, because of the small amount of extractable mRNA, an ancillary experiment was performed (see above). Briefly, RNA extraction and reverse transcription-PCR were performed as described in detail elsewhere (14). For ET-1, the sense primer was 5'-TGGCTTTCCAAGGAGCTCC-3' and the antisense primer was 5'-GCTTTGGCAGAATTCCAGC-3', resulting in a PCR fragment of 339 bp.

**Reverse Transcription and PCR.** Total RNA (1 μg) was dissolved in 40 μl of a reaction mixture containing 100 pmol of random hexamers (Boehringer Mannheim GmbH, Mannheim, Germany), PCR buffer (Life Technologies-BRL, Mannheim, Germany), and 200 U of Superscript reverse transcriptase (Life Technologies-BRL). After incubation at 42°C for 45 min, the enzyme was denatured for 5 min at 95°C. For each animal, reverse transcription was repeated four times. The resulting cDNA was pooled and redivided for PCR, with each aliquot containing 0.25 μg of reverse-transcribed RNA. The cDNA was amplified in 100 μl of a solution containing PCR buffer (same final concentrations as above), 8 pM levels of each primer, and 2.5 U of Taq polymerase (Life Technologies-BRL). The reaction was performed in a Perkin Elmer/Cetus thermal cycler (for conditions, see reference 14). Gel electrophoresis was used to verify the predicted sizes for the amplification products.
SPSS program. If ANOVA or the Kruskal-Wallis test indicated significance, a post hoc test (least-significant difference test) was performed to analyze differences between the groups. The null hypothesis was rejected at $P < 0.05$.

**Results**

**Animal Data**

Deliberately, only a modest amount of remnant hypertrophied renal tissue was resected, to prevent advanced uremia. Consequently, body weights and hemoglobin concentrations at the end of the experiment were not different between sham-operated and SNX rats (Table 1). Plasma urea and creatinine concentrations were significantly higher in SNX rats, as was systolic BP. Treatment with ET-RA had no significant effect on plasma creatinine or urea concentrations or systolic BP.

**Quantitative Morphologic Assessments of First-Order Mesenteric Arteries**

**Lumen Diameter.** Lumen diameters were not significantly different for untreated SNX rats, compared with sham-operated animals. In SNX but not sham-operated animals, the lumen area of low-flow, first-order, mesenteric arteries was less than that of high-flow arteries (Table 2).

**Intimal Thickness.** Under low-flow conditions, the intimal thickness was significantly greater in SNX animals, compared with sham-operated animals (Figure 2A); this increase in SNX animals was abrogated by ET-RA. Although the intimal thickness was not significantly higher in high-flow vessels from SNX animals, compared with sham-operated animals, ET-RA treatment produced lower values (Table 2). The intimal area of low-flow mesenteric arteries was also greater than that of high-flow vessels in the paraffin-embedded samples used for PCNA staining, which were obtained from SNX animals without ET-RA treatment ($26.2 \pm 11.0 \times 10^{-4}$ mm$^2$ in low-flow arteries versus $12.8 \pm 6.98 \times 10^{-4}$ mm$^2$ in high-flow arteries, $P < 0.05$, compared with $8.75 \pm 7.99 \times 10^{-4}$ mm$^2$ in sham-operated low-flow arteries and $17.5 \pm 20.4 \times 10^{-4}$ mm$^2$ in sham-operated high-flow arteries). Intimal thickening in low-flow arteries of SNX animals was prevented by ET-RA treatment (intimal area, $11.7 \pm 6.52 \times 10^{-4}$ mm$^2$ in SNX plus ET-RA low-flow arteries and $9.72 \pm 8.83 \times 10^{-4}$ mm$^2$ in sham-operated plus ET-RA low-flow arteries). The contrasting intimal thicknesses in low-flow and high-flow mesenteric arteries of individual SNX animals are illustrated in Figure 2A.

**Medial Thickness.** There was no significant difference in medial thickness between untreated sham-operated and SNX animals. The medial thickness in SNX animals was significantly greater in high-flow arteries, compared with low-flow arteries (Table 2 and Figure 2B). The increased medial thickness in high-flow arteries was not demonstrable in animals treated with ET-RA. Typical light-microscopic findings are presented in Figure 3. The observation of more pronounced vascular thickening in SNX animals was confirmed by the finding that, in the paraffin-embedded mesenteric arteries used for PCNA staining, the vessel area was also significantly greater in SNX arteries ($0.035 \pm 0.007$ mm$^2$) than in sham-operated arteries ($0.016 \pm 0.005$ mm$^2$, $P < 0.05$).

**Ultrastructural Studies**

Using qualitative electron-microscopic assessment of randomly selected sections from two or three animals per group, thickening of the lamina elastica interna and activation of endothelial cells were observed after SNX. In addition, in SNX animals with mesenteric artery ligation, marked intimal thickening attributable to an increase in smooth muscle cells and fibrous tissue was observed. These changes were almost completely absent after treatment of SNX animals with ET-RA (Figure 4).

**Cell Proliferation, as Evaluated by PCNA Immunostaining**

For sham-operated animals, no statistically significant differences in the numbers of PCNA-positive cells per cross-sectional area (i.e., cell density) were noted for the different intervention groups, compared with untreated animals without modification of mesenteric flow (Table 3). There was a highly significant difference in the number of PCNA-positive medial cells per entire vessel cross-section in sham-operated high-flow
arteries, compared with low-flow arteries (15.4 ± 5.85 versus 7.0 ± 7.48 cells/cross-section) (Figure 5B). Cell densities were not significantly different, however; more positive cells were observed in the vessel cross-sections because there were larger vessels, i.e., greater medial area (Table 3).

There was a significantly greater number of PCNA-positive cells per intimal area for untreated SNX animals, compared with sham-operated animals (Table 3). There was a tendency toward higher PCNA-positive cell numbers in the media of SNX animals, but this was not statistically significant. The absolute (Figure 5A) and relative (Table 3) numbers of PCNA-positive cells per intima in low-flow arteries of SNX animals were significantly higher than those in high-flow arteries, and this difference was completely abrogated by ET-RA (Table 3). The number of PCNA-positive cells per medial area in SNX animals was particularly high in low-flow arteries, and this effect was also abrogated by ET-RA (Table 3).

**Immunohistochemical Analyses of ET-1 and ET-RA Protein Expression**

ET-1 expression was markedly greater in endothelial cells and vascular smooth muscle cells (VSMC) from untreated SNX animals (Figure 6C), compared with sham-operated control animals (Figure 6B). In SNX animals, the staining was even more intense when the mesenteric artery was ligated (low flow) (Figure 6D). After treatment with ET-RA, ET-1 protein expression was markedly reduced (Figure 6E). In the ancillary study of ligated femoral arteries, ET-1 mRNA levels were similar in the unligated femoral arteries of SNX and sham-operated animals (0.56 ± 0.01 ratio versus 0.66 ± 0.53 ratio) but were significantly (P < 0.05) higher in the ligated arteries (surrogates for low-flow arteries) of SNX animals (0.42 ± 0.12 ratio), compared with sham-operated animals (0.24 ± 0.09 ratio).

Endothelial ET<sub>A</sub> receptor expression at the protein level was comparable in mesenteric arteries of untreated sham-operated animals (Figure 7B) and untreated SNX animals (Figure 7C). Expression was enhanced in endothelial cells of SNX animals when the mesenteric artery was ligated (low flow) (Figure 7D). Expression was lower after treatment with ET-RA (Figure 7E).

**Quantitative Morphologic Assessments of Aortae**

There was no significant difference in lumen diameter between the sham-operated and SNX groups (Table 4). In con-
Discussion

The main result of this study was the demonstration of inappropriately marked intimal cell proliferation and intimal thickening of resistance vessels, i.e., first-order mesenteric arteries, in response to low-flow conditions in rats with renal failure. The responses of the intima and the media to high-flow conditions were not significantly altered, compared with those in sham-operated control animals. In untreated SNX animals, both intimal and medial thicknesses were increased. ET receptor blockade with an orally bioavailable ET-RA significantly reduced both intimal and medial thicknesses in SNX animals. Receptor blockade also prevented the abnormal responses of the intima and the media to low-flow and high-flow conditions, respectively. Taken together, these results suggest an abnormal response of endothelial cells to low-flow conditions. An important role of ET-1- and ETA receptor-mediated events in arterial remodeling in renal failure is suggested by the findings that the intimal response to low-flow conditions and the medial response to high-flow conditions were abrogated by ET-RA.

A number of methodologic points were taken into consideration when the study was being designed. A moderate degree of renal failure was deliberately induced, to avoid some confounding factors associated with advanced uremia, i.e., severe hypertension and hypervolemia, severe anemia, metabolic acidosis, and hyperparathyroidism. There was some elevation of BP in this study, and we did not include a group with antihy-
pertensive intervention. In a previous study (16), however, we could demonstrate that the mesenteric wall changes observed in response to uremia were not affected by antihypertensive intervention.

We are also aware of the limitations of PCNA as an index of cell proliferation, because PCNA reflects cell activation without obligatory commitment to subsequent mitosis. In this context, it should be emphasized that thickening of the intima and media, as observed in this study, must not necessarily be interpreted as being the result of cell proliferation alone, because cell hypertrophy and apoptosis have not been investigated.

On the basis of the investigations performed by Mulvany (17,18), the mesenteric artery is widely accepted as a well characterized model of resistance arteries. The structural and functional adaptations to altered hemodynamics have been investigated in great detail (11,19–21). The extent to which findings in the mesenteric arteries can be extrapolated to other arteries that are potentially of greater clinical importance, e.g., arteries and arterioles of the coronary circulation, is currently unknown. We are encouraged, however, by the finding that a response to ET receptor blockade similar to that observed in this study was noted in cardiac arterioles in a previous study in our laboratory (8).

The flow-induced arterial remodeling process has been investigated in great detail by Pourageaud and De Mey (11) and Tulis et al. (19). According to Pourageaud and De Mey (11), the density of VSMC is markedly higher in small muscular arteries than in large arteries. Remodeling of the wall of small muscular arteries thus affects VSMC more than the extracellular matrix and its constituents, such as collagen and elastin. Therefore, we examined primarily the cellular components, although we acknowledge that the extracellular matrix is certainly also important.

The selection of an ET receptor-specific antagonist requires some comment. d’Uscio et al. (22) studied salt-loaded Dahl rats and documented that the ET receptor-specific blocker LU-135252 prevented eutrophic remodeling and endothelial cell dysfunction. Furthermore, Li et al. (23) studied the rat model of deoxycorticosterone acetate (DOCA)/salt-induced hypertension and demonstrated that hypertrophic remodeling in various vascular territories was reduced by ET receptor-selective antagonists but also by nonselective ET receptor antagonists. The stimulatory effect of ET-1 on DNA synthesis by VSMC was observed to be attributable to transactivation of the epidermal growth factor receptor (24). ET-1 not only stimulates proliferation of VSMC (24) but also suppresses endothelial cell apoptosis (25). The relative roles of these processes require further study. In this context, it is important to note that Kayashima et al. (26) observed ET-1 immunoreactivity in the proximal part of the mesenteric artery (which we studied) but not in the distal arterioles. ET-1 abundance paralleled the presence of Weibel-Palade bodies. Therefore, our findings regarding the reversibility of abnormal vascular remodeling responses in uremia may not be generalizable to all arterial vessels.

A number of observations argue for a particular role of ET in vascular remodeling in renal failure. Our previous observations demonstrated that wall thickening in cardiac arterioles of SNX animals was nearly obliterated when animals were treated with ET receptor blockers (8), including LU-135252. In this context, it is of interest that ET-dependent vascular changes are particularly observed under conditions of salt loading. This was noted in DOCA/salt-hypertensive rats (22,23) and in stroke-prone spontaneously hypertensive rats (27). On a priori grounds, it might be assumed that in renal failure, i.e., a state of salt loading, sodium would also be involved. ET-1 is apparently also associated with abnormalities of central arteries, because Demuth et al. (10) observed a correlation between central artery function and circulating ET-1 concentrations among uremic patients. Admittedly, circulating ET-1 levels may not accurately reflect the role of ET at the tissue level.

For all of these reasons, we studied pharmacologic blockade of the ET system, but we emphasize that the dramatic effects of ET RA by no means exclude the participation of other systems, e.g., growth factors, cytokines, nitric oxide (NO), and the local renin-angiotensin system. What our observations do suggest, however, is that ET-1 plays a particularly important role; it may represent the final common pathway in several pathogenic sequences.

The exaggerated intimal proliferation that is observed in renal failure under low-flow conditions indicates endothelial cell dysfunction. Disturbed endothelial cell function in uremia has been documented by the demonstration of abnormal endo-
The marked increases in the wall thicknesses of mesenteric arteries from untreated SNX animals (C), compared with sham-operated animals (A), and of SNX low-flow arteries (D), compared with high-flow arteries (C), should be noted. ET-1 protein expression was markedly enhanced in endothelial and vascular smooth muscle cells in untreated SNX animals (C), compared with sham-operated animals (B). This increase was not present in SNX animals after treatment with ET-RA (E).

Figure 6. ET-1 immunohistochemical analysis of first-order mesenteric vessels. (A) Negative control (sham-operated animal). (B) Sham operation. (C) Untreated SNX. (D) SNX plus mesenteric artery ligation, low flow. (E) SNX plus mesenteric artery ligation (low flow) plus ET-RA. Lu, lumen; i, intima; m, media. Magnification, ×600. The marked increases in the wall thicknesses of mesenteric arteries from untreated SNX animals (C), compared with sham-operated animals (A), and of SNX low-flow arteries (D), compared with high-flow arteries (C), should be noted. ET-1 protein expression was markedly enhanced in endothelial and vascular smooth muscle cells in untreated SNX animals (C), compared with sham-operated animals (B). This increase was not present in SNX animals after treatment with ET-RA (E).
compared with nonuremic control subjects. Several observations suggest that, in addition to the restriction of coronary artery flow by plaques, further abnormalities of the coronary circulation occur in uremia. Koga et al. (34) noted diminished vasodilation in response to acetylcholine in the coronary arteries of uremic patients. We demonstrated that the capillary network is rarified in the hearts of uremic patients (35). This was paralleled by thickening of intramyocardial arterioles (14,36). These observations indicate that, in addition to stenosing plaques, further functional and structural abnormalities are present in the coronary circulation of uremic patients. Exaggerated intimal proliferation under conditions of low flow, e.g., distal to coronary artery stenoses, may be a factor that further compromises myocardial blood flow.

Acknowledgments

Drs. Miltenberger-Miltenyi and Koch were supported by the Deutsche Forschungsgemeinschaft (Graduiertenkolleg Nieren- und Kreislaufregulation). Dr. Miltenberger-Miltenyi worked in Heidelberg as an exchange student of Semmelweis University (Budapest, Hungary). We thank Dr. Klaus Münter (Knoll AG, Ludwigshafen, Germany) for kindly providing LU-135252. The study was supported by a grant from the Deutsche Forschungsgesellschaft (Am/93-2-3, SFB 423). Dr. Chris Baylis served as Guest Editor for this manuscript and supervised the review and final disposition of this manuscript.

References


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**Table 4. Quantitative morphologic assessment of the aorta**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Animals</th>
<th>Lumen Diameter (mm)</th>
<th>Wall Thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham operation</td>
<td>5</td>
<td>1.82 ± 0.06</td>
<td>82.4 ± 6.64</td>
</tr>
<tr>
<td>2. Sham operation + ET-RA</td>
<td>5</td>
<td>1.76 ± 0.14</td>
<td>89.3 ± 8.45</td>
</tr>
<tr>
<td>3. Sham operation + mesenteric artery ligation</td>
<td>5</td>
<td>1.75 ± 0.08</td>
<td>91.1 ± 5.41</td>
</tr>
<tr>
<td>4. Sham operation + mesenteric artery ligation + ET-RA</td>
<td>7</td>
<td>1.76 ± 0.09</td>
<td>88.9 ± 8.06</td>
</tr>
<tr>
<td>5. SNX</td>
<td>9</td>
<td>1.73 ± 0.11</td>
<td>99.4 ± 12.0a</td>
</tr>
<tr>
<td>6. SNX + ET-RA</td>
<td>9</td>
<td>1.74 ± 0.08</td>
<td>96.4 ± 12.9a</td>
</tr>
<tr>
<td>7. SNX + mesenteric artery ligation</td>
<td>8</td>
<td>1.77 ± 0.05</td>
<td>104.0 ± 6.84a</td>
</tr>
<tr>
<td>8. SNX + mesenteric artery ligation + ET-RA</td>
<td>10</td>
<td>1.76 ± 0.09</td>
<td>96.8 ± 8.39a</td>
</tr>
<tr>
<td>ANOVA</td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

*a P < 0.05 versus groups 1, 2, 3, and 4.*

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Figure 7. ET_A receptor immunostaining of first-order mesenteric arteries. (A) Negative control (sham-operated animal). (B) Sham operation. (C) Untreated SNX. (D) SNX plus mesenteric artery ligation, low flow. (E) SNX plus mesenteric artery ligation (low flow) plus ET-RA. Lu, lumen; i, intima; m, media. Magnification, ×600. The elevated endothelial expression of ET_A receptor protein in SNX plus mesenteric artery ligation (low flow) arteries (D), compared with untreated SNX arteries (C), should be noted. This increase in protein expression was prevented by treatment with ET-RA (E). In addition, wall thickening of the mesenteric artery was observed in low-flow arteries (D), compared with untreated SNX animals with arteries with high flow (C).


