Chronic Uremia Induces Permeability Changes, Increased Nitric Oxide Synthase Expression, and Structural Modifications in the Peritoneum

SOPHIE COMBET,* ‡ MARIE-LAURE FERRIER,* MIEKE VAN LANDSCHOOT,§ MARIA STOENOIU,* PIERRE MOULIN, † TOSHIO MIYATA,¶ NORBERT LAMEIRE, § and OLIVIER DEVUYST*
Departments of *Nephrology and † Pathology, Université Catholique de Louvain Medical School, Brussels, Belgium; ‡ Department of Cell Biology, CEA, Saclay, France; § Department of Nephrology, Rijksuniversiteit Gent, Gent, Belgium; and ¶ Institute of Medical Science and Department of Internal Medicine, Tokai University School of Medicine, Kanagawa, Japan.

Abstract. Advanced glycation end products (AGE), growth factors, and nitric oxide contribute to alterations of the peritoneum during peritoneal dialysis (PD). These mediators are also involved in chronic uremia, a condition associated with increased permeability of serosal membranes. It is unknown whether chronic uremia per se modifies the peritoneum before PD initiation. A rat model of subtotal nephrectomy was used to measure peritoneal permeability after 3, 6, and 9 wk, in parallel with peritoneal nitric oxide synthase (NOS) isoform expression and activity and structural changes. Uremic rats were characterized by a higher peritoneal permeability for small solutes and an increased NOS activity due to the up-regulation of endothelial and neuronal NOS. The permeability changes and increased NOS activities correlated with the degree of renal failure. Focal areas of vascular proliferation and fibrosis were detected in uremic rats, in relation with a transient up-regulation of vascular endothelial growth factor and basic fibroblast growth factor, as well as vascular deposits of the AGE carboxymethyllysine and pentosidine. Correction of anemia with erythropoietin did not prevent the permeability or structural changes in uremic rats. Thus, in this rat model, uremia induces permeability and structural changes in the peritoneum, in parallel with AGE deposits and up-regulation of specific NOS isoforms and growth factors. These data suggest an independent contribution of uremia in the peritoneal changes during PD and offer a paradigm to better understand the modifications of serosal membranes in uremia.

The unique structure of the peritoneal membrane (PM) allowed the development of peritoneal dialysis (PD), a therapeutic modality that now accounts for 15% of the total number of patients worldwide who are undergoing dialysis (1). The long-term use of PD is limited by progressive alterations in the PM. These alterations include fibrosis and vascular proliferation, the latter being responsible for an increased effective peritoneal surface area (EPSA), an increased permeability for small solutes and glucose, and, thereby, an impaired ability to sustain ultrafiltration (2). The relevance of the problem has been confirmed by recent studies demonstrating that high PM permeability is associated with increased mortality and morbidity in patients undergoing PD (3). Studies in both animal models and patients undergoing PD have shown that these structural changes are mainly related to the exposure of the PM to high glucose concentrations, with eventual accumulation of advanced glycation end products (AGE), release of growth factors, and increased production of nitric oxide (NO) (4–6). The latter is mediated by regulation of specific NO synthase (NOS) isoforms within the PM (6).

Thus far, the potential contribution of uremia per se to the changes of PM structure and function has not been considered. Among a constellation of symptoms known for decades, uremia is associated with an increase in the permeability of serosal membranes with eventual pleural or pericardial effusion (7). Several molecular mechanisms associated with uremia might have potential effects on the PM, irrespective of exposure to glucose-based dialysate. NO is an obvious candidate, because it regulates vascular tone and reactivity and is involved in vascular proliferation (8). However, the issue of the L-arginine–NO pathway in uremia appears to be complex. Evidence for decreased excretion of NO metabolites (9) and reduced whole-body NO production (10) have suggested low basal NO production in uremia. In contrast, other studies have shown increased NO production in the systemic circulation of uremic rats (11) and patients (12). The availability of L-arginine, the NOS substrate, in uremia has also been debated (9,12), although renal synthesis of endogenous arginine appears to be maintained in uremic rats (13) and patients with end-stage
renal disease (12). Interestingly, an up-regulation of NOS isoforms in extrarenal tissues has been documented in uremic rats (14). Uremic patients are also characterized by high levels of circulating AGE (15) and vascular endothelial growth factor (VEGF) (16). Several growth factors, including the basic fibroblast growth factor (FGF2) and VEGF, have been implicated in structural changes induced by experimental uremia in the remnant kidney (17) or in the heart (18).

In this study, we tested the hypothesis that chronic uremia induces functional and structural changes in the PM of rats that have undergone subtotal nephrectomy. This procedure provides a classical model of chronic uremia that has been extensively used for descriptive and interventional studies focused thus far on the remnant kidney or other target organs such as the heart (18) or the bone (19). Our investigations of peritoneal permeability parameters were correlated with determinations of NOS expression and enzymatic activities, as well as with expression of AGE and growth factors in the peritoneum. The role of anemia in the genesis of the modifications was further investigated in uremic rats treated with erythropoietin (EPO).

Materials and Methods

Rat Model of Chronic Uremia

Adult (8 wk) male Sprague-Dawley rats (Iffa Credo, Brussels, Belgium) weighing 300 ± 3 g were randomly assigned to uremic and sham-operated groups. Uremia was induced by a standard procedure of tissue removal (5/6 nephrectomy followed 1 wk later by contralateral nephrectomy), as described previously (20). The amount of renal tissue removed (approximately 85%) was quantified by weighing the tissue, and less than 10% variation was observed between series (20). Sham-operated (control) rats were subjected to flank incisions separated by 1 wk. All rats had access to a standard rat laboratory diet (Pavan, Oud-Turnhout, Belgium) containing 21% protein (1.3% L-arginine) and tap water ad libitum until the evening before they were killed. A first series of uremic and control rats were studied 3, 6, and 9 wk after the last surgery/incision. Subsequently, a second series of 8-wk-old male Sprague-Dawley rats underwent the same procedure of subtotal nephrectomy to investigate the effects of a subcutaneous injection of EPO (100 IU/kg, Boehringer, Mannheim, Germany) or normal saline twice a week from the time of nephrectomy and until 6 wk thereafter. The cumulative mortality, including the nephrectomy/sham procedure, was 6% (1/17) in controls versus 34% (18/53) in uremic rats. About 60% of the deaths (11/18) in the uremic group occurred within 10 d, i.e., during or immediately after the two-step surgery. This mortality is expected for this degree of tissue removal in large rats that are prone to pre- or postoperative bleeding (20,21). Accordingly, the other rats (7/18) died during the 9-wk follow-up period, corresponding to the chronic uremic state. Four rats died within the first week, one during the fifth week, and two during the eighth week of follow-up. Light microscopy examination of the peritoneum of rats that died within the first week of uremia did not show signs of acute peritonitis or any specific change.

PD and Biologic Parameters: Tissue Sampling

At the time they were killed, rats were anesthetized with subcutaneous Nembutal and placed on a heated pad to perform a 2-h exchange PD, as described previously (22). The rats were weighed, and their BP was measured by means of a transducer (Pressure Set Uniflow type 43-600F, Bentley Laboratories Europe, Uden, The Netherlands) implanted in the carotid artery and connected to a Linear-corde polygraph (Ankersmit, Groot-Bijgaarden, Belgium). The BP values were obtained after 30-min equilibration, i.e., before starting the dwell. Blood and dialysate samples were obtained before (t0) and at 30 min (t30), 60 min (t60), and 120 min (t120) of dwell time. At the end of the dwell, animals were killed by exsanguination, and the dialysate was collected. Urea, creatinine, hematocrit, glucose, sodium, potassium, total protein, and osmolality were assayed by standard methods (22). Plasma levels of prealbumin were measured in 6-wk control and uremic rats (four randomly selected samples in each group) by nephelometry (Behring Nephelometer Analyzer II, Dade Behring, Marburg, Germany) and amino acids by ion-exchange chromatography (Biochrom 20 Analyzer, Pharmacia Biotech, Rosendaal, The Netherlands). White blood cells were counted in a Bürker chamber, and dialysate cultures were routinely obtained (22). Identical samples from the visceral and parietal peritoneum were processed for fixation in 4% paraformaldehyde or protein extraction as previously detailed (22,23). All experiments were conducted in accord with local prescriptions and the NIH Guide for the Care and Use of Laboratory Animals.

Antibodies

The NOS isoforms were detected with monoclonal antibodies raised against human endothelial NOS (eNOS) and neuronal NOS (nNOS) and mouse inducible NOS (Transduction Laboratories, Lexington, Kentucky) (23). Other antibodies included affinity-purified rabbit antibodies against FGF2 and a monoclonal antibody against VEGF (both from Santa Cruz Biotechnology, Santa Cruz, California); a goat anti–type III collagen (Southern Biotechnology Associates, Birmingham, Alabama); a monoclonal antibody against human α-smooth muscle actin (Dako, Glostrup, Denmark); affinity-purified rabbit antibodies against carboxymethyllysine (CML) and pentosidine (24), and a monoclonal antibody against CML (25); and a monoclonal antibody against β-actin (Sigma, St. Louis, Missouri). Positive immunoblot controls included lysates from bovine aortic endothelial cells (eNOS), mouse macrophages (iNOS), rat pituitary gland (nNOS), and recombinant FGF2 and VEGF (Santa Cruz Biotechnology).

Western Blot Analyses

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting were performed as described previously (22). Efficiency of transfer to nitrocellulose was routinely tested by Ponceau red (Sigma) staining and β-actin immunoreactivity. The membranes were blocked for 30 min at room temperature and then incubated with the primary antibody at 4°C for 16 to 18 h. The membranes were then washed and incubated for 1 h at room temperature with the appropriate peroxidase-labeled secondary antibodies (Dako) before visualization with enhanced chemiluminescence (Amersham, Little Chalfont, UK). Densitometry analyses were performed with a studioStar Scanner (Agfa-Gevaert, Mortsel, Belgium) by use of the NIH-Image V1–57 software. The relative optical densities (in %, relative to the age-matched control) were obtained in duplicate.

Immunohistochemistry

Immunostaining was performed on 6-μm sections from peritoneum samples embedded in paraffin (22,23). After blocking in 0.3% H2O2 and incubation with 10% normal serum, sections were incubated successively for 45 min each with the primary antibody, biotinylated IgG (Vector Laboratories, Burlingame, California), and avidin-biotin peroxidase (Vector). Immunolabeling was visualized by use of ami-
Table 1. Clinical and biologic parameters of sham-operated (control) and uremic rats. Two series of 8-wk-old male Sprague-Dawley rats were used for evaluating the effects of uremia (A) and, subsequently, the role of anemia (B)\(^a\)

<table>
<thead>
<tr>
<th>Series</th>
<th>Groups</th>
<th>Time (wk)</th>
<th>(n)</th>
<th>Body Weight (g)</th>
<th>BP (mm Hg)</th>
<th>Plasma Urea (mg/dl)</th>
<th>Plasma Creatinine (mg/dl)</th>
<th>Hematocrit (%)</th>
<th>Dialysate WBC (cells/mm(^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>3</td>
<td>6</td>
<td>375 ± 18</td>
<td>123 ± 13</td>
<td>22 ± 1</td>
<td>0.23 ± 0.02</td>
<td>49 ± 1</td>
<td>362 ± 69</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>6</td>
<td>4</td>
<td>404 ± 8</td>
<td>130 ± 5</td>
<td>24 ± 1</td>
<td>0.26 ± 0.05</td>
<td>48 ± 2</td>
<td>498 ± 139</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9</td>
<td>6</td>
<td>496 ± 11(^d)</td>
<td>125 ± 7</td>
<td>27 ± 1(^d)</td>
<td>0.26 ± 0.03</td>
<td>48 ± 1</td>
<td>735 ± 59(^d)</td>
</tr>
<tr>
<td></td>
<td>Uremic</td>
<td>3</td>
<td>7</td>
<td>297 ± 17(^b)</td>
<td>142 ± 4</td>
<td>144 ± 26(^b)</td>
<td>1.7 ± 0.2(^b)</td>
<td>33 ± 3(^b)</td>
<td>416 ± 62</td>
</tr>
<tr>
<td></td>
<td>Uremic</td>
<td>6</td>
<td>6</td>
<td>321 ± 23(^b)</td>
<td>129 ± 7</td>
<td>183 ± 56(^b)</td>
<td>1.9 ± 0.3(^b)</td>
<td>36 ± 2(^b)</td>
<td>342 ± 92</td>
</tr>
<tr>
<td></td>
<td>Uremic</td>
<td>9</td>
<td>7</td>
<td>418 ± 13(^b,c)</td>
<td>123 ± 7</td>
<td>83 ± 9(^b)</td>
<td>1.0 ± 0.1(^b,c)</td>
<td>44 ± 1(^b,c)</td>
<td>726 ± 82(^c)</td>
</tr>
<tr>
<td>B</td>
<td>Uremic EPO−</td>
<td>6</td>
<td>7</td>
<td>370 ± 13</td>
<td>109 ± 8</td>
<td>123 ± 23(^b)</td>
<td>1.5 ± 0.3(^b)</td>
<td>36 ± 2(^b)</td>
<td>546 ± 101</td>
</tr>
<tr>
<td></td>
<td>Uremic EPO+</td>
<td>6</td>
<td>7</td>
<td>370 ± 13</td>
<td>113 ± 9</td>
<td>126 ± 26(^b)</td>
<td>1.4 ± 0.4(^b)</td>
<td>48 ± 1(^c)</td>
<td>599 ± 85</td>
</tr>
</tbody>
</table>

\(^a\) EPO, erythropoietin; WBC, white blood cells; EPO (+) or control (−) injections were given to the second series of 6wk-uremic rats. 
\(^b\) \(^P\) < 0.05 versus control at corresponding time; \(^c\) \(^P\) < 0.001 versus 6-wk uremic; \(^d\) \(^P\) < 0.005 versus 3-wk control.
permeability for urea and glucose, decreased sodium sieving) were observed in uremic rats at 3 and 9 wk. Analysis of permeability parameters in all groups showed a significant correlation between the increased permeability for small solutes and the degree of renal failure (Figure 2A). Significant correlations were also obtained with the other permeability parameters (including the dialysate-over-plasma ratio at 120 min) and when plasma urea levels were used instead of plasma creatinine (data not shown).

**NOS Enzymatic Activities**

In comparison with controls, total NOS activity in the visceral peritoneum of uremic rats was significantly increased at 3 and 6 wk and returned to control levels at 9 wk (Table 2). Determination of Ca\(^{2+}\)-independent NOS activity yielded similar and low values in all groups. As a consequence, the differences in total NOS activity observed between uremic and control rats at 3 and 6 wk were solely due to Ca\(^{2+}\)-dependent NOS activity (Table 2). The level of total NOS activity within the peritoneum was significantly correlated with plasma creatinine levels (Figure 2B). Significant correlations were also observed with Ca\(^{2+}\)-dependent NOS and when plasma urea levels were used instead of creatinine (data not shown).

**Expression of NOS Isoforms**

Immunoblot analyses were performed to identify which NOS isoform(s) was (were) involved in the increased Ca\(^{2+}\)-dependent NOS activity measured in uremic peritoneum. Representative blots shown on Figure 3A demonstrated that the expression of both nNOS (155 kD) and eNOS (140 kD) in the peritoneum was increased in uremic versus control rats. Denitometry analysis (Figure 3B) confirmed a significant upregulation of nNOS in uremic rats at 3 wk (132% increase; \(P < 0.006\)) and 6 wk (70% increase; \(P < 0.05\)), with a return to control levels at 9 wk. In contrast, eNOS expression was significantly higher at 6 wk (60% increase; \(P < 0.01\)) and 9 wk (52% increase; \(P = 0.04\)). No signal was detected when the blots were probed with an anti-iNOS (data not shown), as expected, given that none of the rats suffered from peritonitis and Ca\(^{2+}\)-independent NOS activity was very low (23).

![Figure 1](image-url)

**Table 2.** Nitric oxide synthase (NOS) enzymatic activities in the peritoneum of sham-operated (control) and uremic rats

<table>
<thead>
<tr>
<th>Series</th>
<th>Groups</th>
<th>N</th>
<th>NOS Activity (pmol citrulline/mg protein per min)</th>
<th>Ca(^{2+})-Independent</th>
<th>Ca(^{2+})-Dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3-wk control</td>
<td>4</td>
<td>0.11 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>3-wk uremic</td>
<td>4</td>
<td>0.21 ± 0.02(^a)</td>
<td>0.03 ± 0.01</td>
<td>0.18 ± 0.01(^a)</td>
</tr>
<tr>
<td></td>
<td>6-wk control</td>
<td>3</td>
<td>0.17 ± 0.02(^b)</td>
<td>0.03 ± 0.01</td>
<td>0.15 ± 0.03(^b)</td>
</tr>
<tr>
<td></td>
<td>6-wk uremic</td>
<td>3</td>
<td>0.24 ± 0.03(^a)</td>
<td>0.02 ± 0.01</td>
<td>0.22 ± 0.03(^a)</td>
</tr>
<tr>
<td></td>
<td>9-wk control</td>
<td>4</td>
<td>0.15 ± 0.02</td>
<td>0.01 ± 0.05</td>
<td>0.14 ± 0.02(^b)</td>
</tr>
<tr>
<td></td>
<td>9-wk uremic</td>
<td>4</td>
<td>0.16 ± 0.03(^c)</td>
<td>0.02 ± 0.01</td>
<td>0.15 ± 0.03(^c)</td>
</tr>
<tr>
<td>B</td>
<td>6-wk uremic EPO−</td>
<td>4</td>
<td>0.20 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>6-wk uremic EPO+</td>
<td>4</td>
<td>0.20 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
</tbody>
</table>

\(^a\) \(P < 0.02\) versus control at corresponding time; \(^b\) \(P < 0.005\) versus 3-wk control; \(^c\) \(P < 0.02\) versus 6-wk uremic.
Structural Changes of the Peritoneum

Immunostaining for NOS isoforms was performed in 6-wk rats (Figure 4, A through I). As previously described (6,22), a faint signal for eNOS was located in the endothelium that lined peritoneal blood vessels of control rats (Figure 4A). The signal intensity, as well as the density of stained capillaries, were increased in uremic rats (Figure 4B), and focal areas of capillary proliferation were observed in the latter (Figure 4C). A very faint staining for nNOS could be detected in the nerve sections found in the peritoneum of control rats (Figure 4, D and E). The signal was more intense in uremic rats in large nerve sections and also within the media of peritoneal arteries (Figure 4F). The absence of cross-reactivity between antibodies against nNOS and eNOS was demonstrated on serial sections (Figure 4, F through H). The specificity of the signal was confirmed by the absence of staining when incubation was performed with control mouse IgG (Figure 4I). Examination of trichrome- and reticulin-stained sections from 6-wk rats showed focal areas of increased collagen matrix in submesothelial and perivascular tissue of uremic versus control rats (Figure 4, compare J with K and L, and compare N with O and P). The focal areas were located both in the isolated visceral peritoneum (Figure 4, J and K) and above the omentum (Figure 4, N and O). Staining on serial sections demonstrated strong immunoreactivity for type III collagen in these areas (Figure 4, M and Q), whereas staining for α-smooth muscle actin was consistently negative. These structural changes were detected in 3- and 9-wk uremic rats, but they were more marked at 6 wk. Computer-assisted morphometry in random sections of the visceral peritoneum confirmed a significant increase in the density of capillaries stained for eNOS (6.6 ± 1.2 versus 2.7 ± 0.5 stained capillaries/field, P = 0.03) in 6-wk uremic versus control rats.

To substantiate the link among uremia, carbonyl stress, and AGE deposition in the peritoneum (6,15), we performed immunostaining for CML and pentosidine in representative sections of the peritoneum (Figure 5). A weak staining for CML and pentosidine was detected in the peritoneum of 3-wk (Figure 5A) and 6-wk (Figure 5D) control rats; the staining intensity was significantly increased in uremic rats at 3 (Figure 5, B and C) and 6 wk (Figure 5, E through G), mainly located in the arterial walls. Although the labeling for CML was consistently more intense than pentosidine, staining on serial sections allowed us to demonstrate that both AGE accumulated on the same structures (Figure 5, compare B with C and F with G). The AGE immunostaining was specific, as was demonstrated by complete inhibition of staining in the presence of an excess of CML- or pentosidine-conjugated bovine serum albumin (data not shown).

Expression of Growth Factors

Immunoblot analysis confirmed that expression of type III collagen (150 kD) was increased in the peritoneum of uremic rats, mostly at 6 wk (Figure 6A). Compared with controls, expression of both dimeric VEGF and FGF2 (45 and 17 kD, respectively, identified by comigration with full-length recombinant human factors) markedly increased in 3-wk uremic rats (171% and 549% increase, respectively) (Figure 6, B and C). The VEGF expression returned to control levels in 6- and 9-wk uremic rats. By contrast, expression of FGF2 was sustained in 6-wk uremic rats (Figure 6C), essentially located within vascular and capillary walls within the peritoneum (Figure 4R).

Effects of EPO Treatment in Uremic Rats

The potential effect of anemia on the changes observed in uremic peritoneum was evaluated by treating a second series of uremic rats for 6 wk with EPO or vehicle (Table 1). These 6-wk uremic rats showed slight (but NS) differences for body weight, BP, and plasma urea, and creatinine levels when compared with 6-wk uremic rats investigated in the first series, but their hematocrit was exactly similar (36 ± 2%). Apart from the...
subcutaneous injection of EPO/vehicle twice a week, all the 6-wk uremic rats were identical in terms of age, race, gender, and experimental procedure. The differences might thus reflect a certain degree of variability in the response to surgery, anesthesia, or preparation for the dwell between two batches of rats. Treatment with EPO managed to fully correct anemia in the 6-wk uremic rats, whereas BP, weight, and other biologic (Table 1) parameters remained unchanged. Also, treatment with EPO had no effect on permeability parameters: in comparison with control rats, EPO-treated uremic rats were still characterized by an increased permeability for urea (Figure 7A) and increased removal of glucose from the dialysate (Figure 7B). Similarly, correction of anemia with EPO was not reflected by significant changes in NOS activities (Table 2) or in the expression levels of nNOS, eNOS, VEGF, and FGF2 (Figure 7C).

**Discussion**

In this article, we demonstrate that chronic renal failure induced by subtotal nephrectomy in rats induces significant changes in peritoneal permeability before exposure to glucose-containing solutions. The changes parallel an increased NOS activity due to the up-regulation of specific NOS isoforms. The peritoneum of uremic rats is also characterized by structural modifications that include angiogenesis, fibrosis, and a major increase in AGE deposits. The increased permeability and NOS activities correlate with the degree of uremia. The changes are independent of renal failure–induced anemia as demonstrated in EPO-treated uremic rats.

The functional characteristics of the PM were evaluated with the clinical peritoneal equilibration test (20,22). The PM of uremic rats is characterized by an increased permeability for small solutes such as urea and creatinine, a faster reabsorption of glucose from the dialysate, and a loss of free water permeability (attested by a lower sodium sieving in the presence of an effective osmotic gradient) (Figure 1). These permeability features, which are explained by an increased EPSA, are similar to those observed in acute peritonitis (22) and long-term PD (6). Acute peritonitis, a potential complication of any animal model, was ruled out in our uremic rats by negative cultures, absence of infiltrate on pathologic examination, and NOS studies (*vide infra*).
Figure 4. Expression of NOS isoforms and structural modifications in the peritoneum of uremic rats. (A through C) Staining for eNOS in the visceral peritoneum of 6-wk control (A) and uremic (B and C) rats. Staining for eNOS is located in the endothelium lining blood vessels and capillaries, whereas the mesothelium (m) is negative (A). The signal intensity, as well as the density of capillaries stained for eNOS are increased in uremic rats (B), with distinct areas of capillary proliferation (C). The mesothelium (m) remains unstained. (D and E) Serial sections
The relation between the increased peritoneal permeability and the degree of renal failure thus includes the peritoneum among serosal membranes modified by uremia.

Studies performed in rat models and patients undergoing PD have shown that an enhanced NO release within the peritoneum, mediated by specific NOS isoforms, is a critical factor for increased EPSA (22,26). This study further illustrates the potential link between peritoneal permeability and NOS activity; both parameters are directly correlated with the degree of uremia in the rats (Figure 2). The demonstration of increased

of the visceral peritoneum of 6-wk control rats stained for nNOS (D) or with hemalun-eosine (E). nNOS is detected in nerve sections (*) within the peritoneum. (F through H) Serial sections of the visceral peritoneum of 6-wk uremic rats stained for nNOS (F), trichrome blue (G), and eNOS (H). The signal for nNOS (located in nerve sections, *) is more intense than in control rats, in particular within the media of large peritoneal arteries (F and G). The absence of cross-reactivity between nNOS (nerve staining, *) and eNOS (endothelium staining, arrowheads) is demonstrated by comparing the serial sections. (I) The specificity of the immunostaining is shown by the lack of signal when sections of 6-wk uremic rats are incubated with control mouse IgG. (J and K) Trichrome blue staining of the visceral peritoneum of 6-wk control (J) and uremic (K) rats. Focal areas of fibrosis, with thickening of the visceral peritoneum, are identified in uremic rats. (L and M) Serial sections with (K), stained with reticulin (L) or type III collagen (M). The colocalization of these markers confirm the focal area of fibrosis within the visceral peritoneum of 6-wk uremic rats. (N and O) Fibroblast growth factor (FGF2) location in the vascular and capillary walls located in the visceral peritoneum of a 6-wk uremic rat. No signal is observed in the mesothelium (m). Magnifications: ×335 for A and B; ×225 for C; ×200 for D and E; ×315 for F through H; ×250 for I and N through Q; ×125 for J through M; ×270 for R.

Figure 5. Immunostaining for carboxymethyllysine and pentosidine in the peritoneum of control and uremic rats. (A through C) Peritoneal arteries of 3-wk control (A) and uremic (B and C) rats stained with polyclonal antibodies against carboxymethyllysine (CML) (A and B) or pentosidine (C). The weak staining for CML detected in the arterial walls of controls is strongly enhanced in uremic rats. Staining on serial sections demonstrates the colocalization of CML and pentosidine. (D through G) Peritoneal arteries of 6-wk control (D) and uremic (E through G) rats stained with polyclonal antibodies against pentosidine (D through F) and a monoclonal antibody against CML (G). The staining for pentosidine is significantly increased in 6-wk uremic rats. Staining on serial sections demonstrates that pentosidine (F) and CML (G) accumulated in the arterial walls. Note that some nerve sections are also stained. Magnifications: ×250 for A through C; ×275 for D and E; ×300 for F and G.
NOS activities in the uremic peritoneum relies on the specific and sensitive L-citrulline assay, confirmed by expression studies for NOS isoforms (23). At variance with acute peritonitis, the increased NOS activity observed in uremic rats is only due to Ca\(^{2+}\)-dependent NOS (Table 2), associated with the up-regulation of both eNOS and nNOS (Figure 3). Just as in long-term PD, increased eNOS expression probably reflects vascular proliferation and increased endothelial area (6). The nNOS expression also increases in the peritoneum of uremic rats, in contrast with its stability in acute peritonitis (22). The intricate regulation of nNOS is implicated in many processes, including neuronal development and plasticity (27). One hypothesis is that nNOS up-regulation in the peritoneum somehow reflects uremia-induced peripheral neuropathy. A similar increase in both eNOS and nNOS has been reported in the penis and pelvic ganglia of rats with subtotal nephrectomy.
A specific role of the uremic state in NOS regulation is further suggested by the positive correlation between the level of NOS activity within the peritoneum and the degree of uremia (Figure 2).

The state of the L-arginine–NO pathway in uremia remains debated, probably because (1) different methods are used to investigate the complex NO metabolism and (2) the release of NO by a given organ might be different from its systemic production. Studies have shown decreased excretion of NO oxidation products (9) and a reduction of basal whole-body NO production (10) in patients with chronic renal disease. In contrast, increased NO production has been documented in patients with end-stage renal disease (12) and also in the circulation of rats made uremic by subtotal nephrectomy (11).

Whether NOS substrate deficiency occurs in uremia is another open question. Our data confirm previous reports of the maintenance of (sub)normal plasma levels of L-arginine in uremia (9,12,28,29). This implies a compensatory mechanism, either increased synthesis of L-arginine in the remaining nephrons (12,13) or increased extrarenal release (29). The repeated observations of increased plasma levels of citrulline in uremia (9,12) might thus reflect an adaptation mechanism or, alternatively, originate from reduced renal clearance and arginine synthesis (28). At any rate, the plasma arginine levels observed here are well above the $K_m$ of NOS (32 to 35 μmol/L), although this does not exclude a deficit in intracellular availability (28).

Different structural modifications—vascular proliferation and fibrosis—are observed in focal areas of the uremic peritoneum (Figure 4). The structural changes were quantified by image analysis and confirmed by immunoblot analyses for eNOS (Figure 3) and type III collagen (Figure 6). The changes were more conspicuous when uremia peaked at 6 wk. The functional consequences of each structural change are different (30). Submesothelial and perivascular fibrosis increases the distance between the dialysate and the endothelium and, thus, decreases peritoneal permeability. In contrast, vascular proliferation increases EPSA and, thus, permeability for small solutes. The increased peritoneal permeability documented in uremic rats suggests that, quantitatively, vascular proliferation is functionally more important than fibrosis in this model. However, because fibrotic changes predominate in the peritoneum (O. Devuyst and N. Lameire, unpublished observations) and the myocardium (31) of uremic rats that survived over 6 mo, the nature of structural changes might change over time.

This study identifies several mediators potentially involved in the structural changes of the uremic peritoneum. The overexpression of angiogenic factors such as VEGF and FGF2 at the third wk of uremia (Figure 6) suggests indeed that they are instrumental in the vascular proliferation—and the fibrotic changes—observed at 6 wk. Previous studies in human and rat have suggested a link between VEGF expression and vascular proliferation in the peritoneum (5,6). NO is necessary for the biologic activity of VEGF (32), and enhanced expression of both mediators is thought to play a determining role in the neovascularization observed in the peritoneum of patients undergoing long-term PD (6). On the other hand, FGF2 is impli-

---

**Figure 7.** Peritoneal permeability and expression of NOS and growth factors in uremic rats: influence of treatment with erythropoietin (EPO). (A and B) The D/P ratio of urea (A) and the removal of glucose from the dialysate (D/D$_{D_0}$) (B) were determined in 6-wk control (○) and uremic rats treated with EPO (▲) or vehicle (▼) during a 2-h exchange with 15 ml of 7% glucose. All variables were obtained for six rats in each group. *$P < 0.05$ between control and EPO-treated uremic rats. (C) Representative immunoblots for neuronal nNOS (155 kD), eNOS (140 kD), dimeric VEGF (45 kD), and FGF2 (17 kD) in the visceral peritoneum of 6-wk uremic rats treated with vehicle (EPO−) or EPO (EPO+). Thirty μg of samples were run on SDS-PAGE (7.5% gel for NOS; 14% gel for VEGF and FGF2). The expression levels are similar in both groups.
cated in processes including angiogenesis, smooth muscle cell proliferation, wound healing, and tissue repair (33). It induces endothelial cell proliferation, migration, and angiogenesis, possibly under the control of VEGF (34). In addition, FGF2 is a potent mitogen for various cell types, including fibroblasts (35), and its expression correlates with renal fibrogenesis (36). A similar increase in FGF2 expression has been detected in the peritoneum of uremic patients at time of PD catheter insertion (M. L. Ferrier and O. Devuyst, unpublished data).

Immunostaining with specific antibodies (24,25) demonstrates a striking increase in CML and pentosidine deposits in the peritoneum of uremic rats (Figure 5). Both in vitro and in vivo studies have shown that chronic uremia is associated with high levels of circulating AGE, as a result of the accumulation of reactive carbonyl compounds (15). Glucose-derived carbonyls promote the liberation of VEGF in cultured peritoneal cells (37), and AGE colocalizes with VEGF in the peritoneum (6,37). Taken together with these observations, our data suggest that AGE deposition in the peritoneum might contribute, via enhanced VEGF liberation, to the structural changes observed in this uremic rat model.

The putative role of anemia in VEGF/eNOS up-regulation and vascular proliferation in uremic rats was specifically addressed. At 6 wk, the mean hematocrit level of uremic rats had fallen by 25% (Table 1). In vivo, both hypoxia and anemia are potent inducers of VEGF expression (38), and increased NO levels are associated with anemia (39). Furthermore, chronic hypoxia induces permeability changes in endothelial cells (40), as well as remodeling of the vasculature and surrounding tissues (including smooth muscle proliferation and fibrosis) (41). Nevertheless, full correction of anemia with EPO failed to reverse the changes in PM permeability, NOS activity, and growth factors expression in uremic rats (Figure 7). Uremic anemia as such was thus ruled out in the genesis of the observed modifications.

Thus, our data demonstrate that significant changes of permeability and structure of the PM occur in this uremic rat model. These modifications might be secondary to the adaptive response (e.g., renal compensatory growth) to renal ablation (42). However, the correlations between the degree of uremia and changes in permeability or NOS activity, the similar effect of uremia on NOS expression in target organs, and the involvement of a series of mediators—NO, VEGF, and AGE—known to operate in uremia (9–18) support an independent contribution of uremia in the PM changes that occur during PD. These findings, which confirm the earlier observation by Rubin et al. (43) that peritoneal transport might be increased in uremic patients when compared with nonuremic patients, are also important in terms of biocompatibility because most of the studies evaluating dialysates are performed in normal rats.

Finally, the combination of functional and expression studies used here offers a paradigm to better understand the interplay between various factors in the modification of serosal membranes induced by uremia. Thus, the retention of carbonyl compounds leads to a significant accumulation of AGE in the peritoneum, as early as 3 wk after induction of uremia. This is paralleled by a peak in VEGF and FGF2 expression within the PM, returning to normal within 6 (VEGF) or 9 wk (FGF2).

There is a progressive increase in total NOS activity—in relation with the up-regulation of NOS isoforms—from 3 to 6 wk of uremia, with a fall at 9 wk. Vascular proliferation and functional alterations of the PM peak at 6 wk. It is also important to point out that the changes are variable through evolution. Although variability in renal function and expression of growth factors over time is characteristic in this rat model (21,42), our findings open perspectives because of potential regression of peritoneal alterations in parallel with renal functional improvement.

Acknowledgments

These studies were supported in part by the Belgian agencies FNRS and FRS/FM (crédit 9.4540.96 and convention 3.4566.97) and the ARC 00/05–260 (to O.D.) and Grants from Baxter (to S.C.) and the Société de Néphrologie (to M.S.). We thank Prof. P. Courtoy, Dr. E. Goffin, Dr. W. van Biesen, and Prof. Ch. van Ypersele de Strihou for suggestions and critiques. The expert technical assistance of Y. Cnops, T. Dheuvaert, S. Ruttens, and L. Wenderickx is highly appreciated. S.C. and M.L.F. contributed equally to this study.

These results were presented in part at the 33rd Annual Meeting of the American Society of Nephrology, October 11 to 16, 2000, Toronto, Canada, and have been published in abstract form (J Am Soc Nephrol 11: 615A, 2000).

References