Tyrosine Nitration in Plasma Proteins From Patients Undergoing Hemodialysis

Zoi Mitrogianni, MD, Alexandra Barbouti, PhD, Dimitrios Galaris, PhD, and Kostas C. Siamopoulos, MD

**Background:** A growing body of evidence suggesting that oxidative stress might be one of the most important complications occurring during hemodialysis (HD) has accumulated. However, although the role of reactive oxygen species has been investigated extensively, little is known about the involvement of reactive nitrogen species. In the present investigation, levels of protein modifications in the form of tyrosine nitration in patients undergoing long-term HD therapy were evaluated.

**Methods:** Tyrosine nitration of plasma proteins was detected by means of Western blotting using a specific nitrotyrosine-recognizing monoclonal antibody, and band intensity was evaluated by using image analysis software. Immunoprecipitation of plasma proteins by antinitrotyrosine-agarose–conjugated antibodies, followed by Western blotting, was used in an attempt to identify the nitrated proteins.

**Results:** Although several proteins with nitrated tyrosine residues were observed in plasma of healthy individuals, increased nitration levels were observed in some specific proteins in all patients tested (n = 25) compared with controls (n = 6). At least 6 apparent bands appeared to be more nitrated than their counterparts in plasma from controls. Ceruloplasmin was identified as 1 of the proteins with significantly increased nitration in patients.

**Conclusion:** Results of the present investigation show that specific plasma proteins of HD patients are post-translationally modified by nitration of their tyrosine residues. The nature of these proteins, as well as the exact molecular mechanisms and consequences of these modifications, warrant additional investigation.


© 2004 by the National Kidney Foundation, Inc.

INDEX WORDS: Tyrosine nitration; hemodialysis (HD); oxidative stress (OS).
HD. Moreover, an attempt was made to identify some of the proteins with increased tyrosine nitration.

METHODS

Patient Population and Blood Collection

Patients on long-term HD therapy were recruited from the HD unit of our university hospital. Basic characteristics of these patients are listed in Table 1. Twenty-five HD patients compared with 6 adult healthy controls were used for experiments estimating plasma nitrated proteins. Causes of end-stage renal disease (ESRD) were diabetic nephropathy in 2 patients, polycystic kidney disease in 2 patients, obstructive nephropathy in 2 patients, and glomerulonephritis in 12 patients (2 patients, membranous; 3 patients, focal segmental; and 7 patients, chronic glomerulonephritis), whereas 7 patients had ESRD of unknown cause. Six of 25 patients, age- and sex-matched to the 6 healthy controls, were studied in an attempt to identify the nitrated proteins. Causes of ESRD in these patients were diabetic nephropathy in 2 patients, glomerulonephritis in 3 patients (2 patients, focal segmental; 1 patient, chronic glomerulonephritis), and unknown in 1 patient. Patients with acute or chronic inflammation caused by any virulent agent, those administered vitamin supplementation, and smokers were excluded from the study.

All patients were in a stable condition for at least 6 months and were undergoing HD 3 times a week for 240 minutes per dialysis session. For all studied patients, standard bicarbonate-based dialysate was used with low-molecular-weight heparin as an anticoagulant, whereas membrane type was not used as 1 of the criteria in selecting patients. Regarding the first group (patients who participated in the estimation of nitrated proteins), in 16 of 25 patients, biocompatible membranes (polysulfone, polyacrylonitrile) were used, whereas for the second group (patients who participated in the identification of nitrated proteins), these membranes were used in 5 of 6 patients. For the rest of the patients, hemophane was used. Blood was drawn just before dialysis, after access cannulation, and at the end of the session from the arterial dialyzer tubing into EDTA tubes. Samples were centrifuged at 1,700 g for 15 minutes to obtain plasma (stored at −80°C until used; 1-month storage time). Informed consent was obtained from all subjects, and the protocol was approved by the local hospital committee.

Detection of Nitrotyrosine Formation in Plasma Proteins

Total plasma protein concentrations were determined by means of the Bradford method, using bovine serum albumin as a standard. Plasma proteins (30 μg) were separated on duplicate 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. One of these 2 gels then was transferred to nitrocellulose membranes by electroblotting. After blocking with 5% nonfat dry milk, membranes were exposed to a mouse monoclonal antitryptosine antibody (Upstate Biotechnology, Lake Placid, NY), followed by horseradish peroxidase–conjugated secondary antibody. Bands were visualized with chemiluminescent chemicals (Amersham Biosciences, Buckinghamshire, England) and captured on films (Kodak Company, Rochester, NY). The second blot was stained with Coomassie Brilliant Blue G 250 (Merck, Darmstadt, Germany) for proteins.
Identification of Nitrated Proteins

For immunoprecipitation of nitrated plasma proteins, each plasma protein sample (500 μg) was diluted in 500 μL of fresh buffer, pH 7.4, constituted of 20 mmol/L of Tris base (Merck), 150 mmol/L of sodium chloride, 10% glycerol, 1% Triton X-100 (Merck), 4 mmol/L of ethylenebis(oxyethylenenitrilo) tetra-acetic acid, 1 mmol/L of phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin. Samples were incubated at 4°C with shaking overnight with 10 μL anti-nitrotyrosine (mouse monoclonal immunoglobulin G–agarose–conjugated antibody [Upstate Biotechnology]). The supernatant was removed after a 2-minute centrifugation at 10,000 rpm, and beads were washed 3 times with 500 μL of fresh buffer. After the final wash, the buffer was replaced with 60 μL of sample-loading buffer 2 times containing SDS and DTT-dithiothreitol, and samples were boiled for 5 minutes. Immunoprecipitated proteins were separated on a 10% SDS-PAGE and transferred to nitrocellulose paper. After blocking with 5% nonfat dry milk, membranes were exposed to polyclonal antibodies against ceruloplasmin (goat from Sigma, St. Louis, MO), transferrin (goat from Sigma), or albumin (rabbit from Abcam, Cambridge, UK), followed by the specific horseradish peroxidase–conjugated secondary antibody. Bands were visualized with chemiluminescent chemicals and captured on films.

Evaluation of Intensities of Nitrated Bands

Intensities of bands corresponding to tyrosine nitrated proteins were evaluated by using the Kodak Digital Science 1D Image Analysis Software. Each Western blot included both patient and healthy control samples so that patient samples were compared with healthy volunteers developed under the same conditions in all experiments. For each experiment, band density was determined from the blot that produced clearly visible bands.

Statistical Analysis

Patient and control nitrated protein intensity readings were logarithmically transformed and compared by using analysis of variance (ANOVA) and Kruskal-Wallis test, when applicable. For identification of nitrated proteins, paired comparisons (sign test) of optical density readings in “patient-versus-healthy-control” samples that were on the same film were performed. All P are 2 tailed.

RESULTS

Figure 2A shows a representative blot in which tyrosine nitration of plasma proteins from 5 patients (lines 1, 2, 3, 5, and 7) and 3 healthy controls (lines 4, 6, and 8) were analyzed. Several distinct proteins with molecular weights (MWs) of 30 to 300 kD were found to be nitrated, even in healthy controls. However, optical density readings of the corresponding bands apparently were greater in all patients tested, indicating that an intense modification process took place in the case of HD patients (Table 2).

Conversely Coomassie blue staining of corresponding gels did not show differences in amounts of individual proteins (Fig 2B). It is apparent that in both healthy individuals and HD patients, only some distinct plasma proteins were nitrated, indicating a degree of specificity for the nitration process. Specificity of the antinitrotyrosine antibody used was shown by the absence of staining when the antibody was preincubated with free nitrotyrosine for 1 hour at room temperature before use (results not shown). Moreover, treatment of nitrated plasma proteins for 60 minutes with 20 mmol/L of dithionite resulted in almost disappearance of the detected staining (Fig 3).

By using an Image Analysis software system (Kodak), at least 6 distinct nitrated bands (denoted by numerals 1 to 6, from high to low MWs) were identified. Molecular masses of the identified proteins, estimated by Western blotting, were approximately 156, 110, 79, 67, 49, and 29 kD from 1 to 6, respectively (Fig 2A).
However, in some cases, although the system identified 1 band, it was obvious that this band consisted of 2 or more overlapping proteins. Statistical analysis of intensity readings elaborates a significant difference between patients and healthy controls, evident with both methods used (ANOVA and Kruskal-Wallis). This difference is not replicated in band 6 (Table 2).

Some bands that were most intensively stained were shown to represent (by their corresponding MWs) albumin, transferrin, and ceruloplasmin. To test the validity of these findings, nitrated plasma proteins were first immunoprecipitated by using antinitrotyrosine-agarose–conjugated antibodies (Upstate Biotechnology). An attempt to identify the precipitated proteins was made by Western blotting and the use of specific antibodies. As shown in Fig 4 (left panel), similar amounts of ceruloplasmin, albumin, and transferrin were detected by Western blotting directly on plasma proteins. However, when nitrated plasma proteins were first immunoprecipitated and then analyzed by Western blotting (Fig 4, right panel), ceruloplasmin was detected in greater amounts in patients than healthy controls ($P = 0.031$). Nitrated albumin also was detected, but the band density was not significantly increased in patients ($P = 1.00$). No transferrin was detected among immunoprecipitated proteins. Attempts to identify the rest of the nitrated plasma proteins are presently under way in our laboratory.

No detectable differences in band intensities were observed in experiments comparing predialysis and postdialysis plasma proteins for tyrosine nitration. Moreover, type of dialysis membrane used did not affect band intensities. Also, no strong differences were found among patients with different causes of primary disease.

**DISCUSSION**

Increased OS, induced by biocompatibility phenomena occurring during extracorporeal circulation or the influence of uremia itself in long-term HD patients, as repeatedly shown,4-14 may have serious consequences on these individuals.

Formation of RNS, especially peroxynitrite and nitrogen dioxide, has been recognized as an important component of OS. It is suggested that these species contribute to the pathogenesis of many diseases, such as acute and chronic inflammatory processes, sepsis, neurodegenerative diseases, and ischemia-reperfusion, among others.15-22,30-36 In particular, peroxynitrite is a highly reactive and short-lived species that promotes oxidative cell and tissue damage. How-

### Table 2. Statistical Analysis of Nitrated Protein Results

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Optical Density</th>
<th>$P$ (ANOVA)</th>
<th>$P$ (Kruskal-Wallis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>671 (225-3,185)</td>
<td>12,505 (2,880-32,037)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>2</td>
<td>1,800 (141-4,315)</td>
<td>11,507 (3,187-32,518)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>3</td>
<td>6,226 (3,213-17,851)</td>
<td>41,521 (7,252-77,133)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>4</td>
<td>5,351 (2,425-21,604)</td>
<td>30,143 (15,535-42,782)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>5</td>
<td>27,554 (17,161-42,616)</td>
<td>42,709 (38,945-73,230)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>6</td>
<td>2,076 (970-4,896)</td>
<td>3,066 (2,036-6,835)</td>
<td>0.087</td>
</tr>
</tbody>
</table>

NOTE. Values expressed as median (interquartile range).
ever, direct detection of peroxynitrite in vivo is not possible because of the elusive nature of this molecule. Moreover, detection of footprints of its existence are complicated further by: (1) the multiple reactions in which it is involved, and (2) difficulty discriminating between peroxynitrite and other nitric oxide–derived oxidants. Additionally, yields of peroxynitrite formation in biological systems are usually low because of competing reactions of nitric oxide. Especially in blood, peroxynitrite formation is inhibited because of the presence of high amounts of hemoglobin that outcompete superoxide anion for interaction with nitric oxide. However, when formed in appreciable amounts, peroxynitrite promotes nitration (incorporation of a nitro \(\text{NO}_2\) [nitrogen dioxide] group), mainly in aromatic residues of proteins. Most notably, protein tyrosine residues constitute key targets for peroxynitrite-mediated nitration, and the presence of 3-nitrotyrosine in proteins represents a usual modification introduced by peroxynitrite.\(^{15,20}\)

In the present work, we detected increased tyrosine nitration in specific plasma proteins of patients undergoing HD. Although other mechanisms, such as: (1) the hydrogen peroxide/nitrite (\(\text{H}_2\text{O}_2/\text{NO}_2^–\))–hemeperoxidase system, (2) direct reaction of NO\(_2\) (formed from the aerobic oxidation of nitric oxide) with tyrosine residues, and (3) the oxidation of unstable nitrosotyrosines,\(^{37-39}\) also may be responsible, peroxynitrite remains the main suspected agent for inducing protein tyrosine nitration. Detection of nitrated proteins, even in plasma of healthy controls, possibly indicates a low flux of RNS that may cause molecular damage, even under normal conditions.

![Fig 4. Identification of proteins with increased tyrosine nitration in plasma of HD patients. Plasma proteins from patients undergoing HD or healthy controls (C) were analyzed by means of Western blotting, either (left) directly or (right) after immunoprecipitation by using agarose-conjugated antinitrotyrosine antibodies. Ceruloplasmin, albumin, and transferrin were detected by using polyclonal antibodies specific for each of these proteins.](image-url)
shown previously that nitration of ceruloplasmin at its tyrosine residues resulted in a substantial loss (~50%) of its ferroxidase activity. The impaired ability of ceruloplasmin to oxidize iron may have serious consequences for patients regarding the capacity of the reduced iron to participate in Fenton-type reactions, with peroxides generating the extremely toxic hydroxyl- and alcoxyl-radicals.

Regarding band intensities, no significant differences were found among patients with different primary diseases. Therefore, this finding could be attributed to either the chronic process of renal replacement or the uremic syndrome itself. However, according to our recent preliminary data for patients with chronic kidney disease stages 3 to 5, no statistically significant difference in plasma nitrated protein level was detected compared with healthy individuals. In conclusion, the HD procedure seems to be mainly responsible for tyrosine nitration in plasma proteins in HD patients.

ACKNOWLEDGMENT

The authors thank Dr Thomas Trikalinos for help with statistical analysis of the results.

REFERENCES


34. Szabo C: The pathophysiological role of peroxynitrite in shock, inflammation, and ischemia-reperfusion injury. Shock 6:79-88, 1996


