Activation of 5-Lipoxygenase and Related Cell Membrane Lipoperoxidation in Hemodialysis Patients

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Abstract. Lipid peroxidation was shown at the membrane level in peripheral blood cells of patients hemodialyzed on cuprophan dialyzers, and was mainly attributable to the generation of conjugated hydroperoxides in the lipid bilayer. The oxidative index (i.e., the A234/205 ratio) of membrane lipids was 3.2-fold higher in hemodialysis patients than in healthy control subjects, and also the level of leukotriene B4 was significantly increased (up to 1.7-fold over control). Both membrane peroxidation and release of leukotriene B4 were linked to upregulation of 5-lipoxygenase activity (up to 2.4-fold over control) and expression at the protein level (up to 1.9-fold). Vitamin E, the most important lipophilic antioxidant, prevented both membrane peroxidation and release of leukotriene B4 by inhibiting 5-lipoxygenase activity without affecting enzyme expression. Similar results were observed in patients hemodialyzed on polymethylmetacrylate membranes, but in this case the activation of 5-lipoxygenase was less pronounced. The use of a purified 5-lipoxygenase demonstrated that vitamin E was a reversible inhibitor of enzyme activity (IC50 = 35 ± 4 μM), further characterized as noncompetitive (Ki = 30 ± 3 μM). Taken together, the results reported here shed some light on the mechanism responsible for the oxidative damage in hemodialysis. Moreover, the beneficial effect of vitamin E described here may have relevance for the therapy of patients with kidney disease.

Hemodialysis (HD) treatment of uremic patients may have several adverse long-term side effects, such as pulmonary complications, possibly due to induction of a highly peroxidative state (1). HD has been demonstrated to decrease serum antioxidant activity (2), and in parallel HD patients showed an increased need for supplementation of the main hydrophilic antioxidant vitamin C (3). Supplementation of vitamin E, the main lipophilic antioxidant of the body, has been reported to reduce in uremic patients the lipoperoxidative damage of human peripheral blood mononuclear cells (4), platelets (5), and lymphocytes (6). Moreover, dietary administration of vitamin E has been shown to significantly reduce glomerulosclerosis in rats (7). Unlike blood cells, serum polyunsaturated fatty acids (8) and lipoproteins (9) were not significantly oxidized upon HD, suggesting that oxidative damage in uremic patients occurs mainly at the cellular level. However, the mechanism of lipoperoxidation, measured until now as the amount of the terminal product malondialdehyde, remains elusive. In fact, it is not clear which lipoperoxides are being produced, where they might be localized, and which enzyme(s) are responsible for their generation. Remarkably, exposure of blood to artificial kidney membranes (especially those derived from cellulose) causes activation of the alternative pathway of the complement system, leading to the release of the active products C3a, C5a, and C5b-9. Also, other biologically active compounds are released upon blood exposure to dialyzers, such as eicosanoids (10) and cytokines (11). In particular, the biosynthesis of leukotriene B4 (LTB4), the main product of 5-lipoxygenase (E.C. 1.13.11.34), was shown to rapidly increase in patients with end-stage renal failure undergoing chronic HD (12). More recently, it has been reported that the interaction between blood and HD membranes upregulates 5-lipoxygenase gene expression at the mRNA level (13). Interestingly, lipoxygenase activity has been implicated in membrane lipid peroxidation of various human cells in culture (14,15) due to its ability to form hydroperoxides in the polyunsaturated fatty acids of membranes (16). In this context, the effect of HD on lipoxygenase-mediated membrane lipid peroxidation and 5-lipoxygenase activity and protein level was investigated in peripheral blood mononuclear cells (PBMC). The effect of vitamin E on lipid peroxidation and 5-lipoxygenase expression was also studied, extending the analysis to the in vitro interaction of the antioxidant with purified 5-lipoxygenase. The results reported here shed some light on the mechanism responsible for the oxidative damage in HD. Moreover, the beneficial effect of vitamin E described in the article may have relevance for the therapy of patients with kidney disease.

Received October 15, 1998. Accepted March 27, 1999.
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1046-6673/1009-1991
Journal of the American Society of Nephrology
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Materials and Methods

Patients

After obtaining informed consent, 13 chronic uremic male patients (age: 54 ± 5 yr, mean ± SD), subjected to maintenance HD for a period of 60 ± 5 mo, and 13 age-matched healthy control subjects (52 ± 5 yr) were enrolled in this study. All HD patients received a standard bicarbonate dialysis schedule of 4 h, three times weekly, using hollow-fiber cuprophan membranes (eight patients) or polyethylene/paramethylacrylate (PMMA) Filtryzers B2 (five patients). The surface area of both dialyzers was 1.3 m², and patient treatment with either membrane lasted for 6 mo. The cause of end-stage renal disease was chronic glomerulonephritis in eight patients and nephroangiosclerosis in five patients. Healthy control subjects showed normal parameters of renal function. Both HD patients and control subjects were on a free diet with a normal and constant intake of essential fatty acids, and none of them took any drug with established or potential oxidizing effect. Cuprophan membranes were from Spiraflo NT 1311 Bellco (Mirandola, Italy), whereas PMMA Filtryzers B2 1.3 were purchased from Toray Industries (Tokyo, Japan).

Isolation of PBMC

Blood samples (10 ml per donor) were drawn from the antecubital vein in healthy subjects and from the arterial side of the arterovenous fistula before dialysis in HD patients. Then blood was collected into heparinized sterile test tubes and processed within 2 h. HD patients were then treated with vitamin E (EVION 300 mg, Bracco, Milan, Italy) in daily doses of 300 mg (intramuscularly) for 15 consecutive days (4,6). Posttreatment blood sampling was then performed as described above. Isolation and purification of PBMC were performed by centrifugation on a concentration gradient (d = 1077), as reported (4).

Materials

All chemicals were of the purest analytical grade. Arachidonic (eicosatetraenoic) acid, ATP, phenylmethylsulfonyl fluoride, vitamin E (α-tocopherol) used in the inhibition experiments and soybean trypsin inhibitor were from Sigma Chemical Co. (St. Louis, MO). Anti-human 5-lipoxygenase rabbit polyclonal antibodies were a kind gift from Dr. A. W. Ford-Hutchinson (Merck Frosst Center for Therapeutuic Research, Canada), and goat anti-rabbit polyclonal antibodies conjugated with alkaline phosphatase were purchased from Bio-Rad (Richmond, CA). Authentic LTB₄ was from Cayman Chemical Co. (Ann Arbor, MI).

Analysis of Membrane Lipid Spectra

Membrane lipids were isolated from PBMC (10 × 10⁶/test) as reported (17). Briefly, cell homogenates were prepared in 200 µl of phosphate-buffered saline with an UltraTurrax T25, then 800 µl of ice-cold methanol/chloroform (2:1, vol/vol) was added with vortexing. This mixture was allowed to stand at room temperature for 30 min, then 240 µl of chloroform and 240 µl of water were added with vortexing. After 10 min at room temperature, the mixture was centrifuged at 3000 × g for 5 min, the upper aqueous layer was removed by suction, and the lower organic phase was dried by spinning the samples in a DNA MINI Speedvac (Heto-Holten, Allerød, Denmark), at 100 mbar and 30°C for 30 min. The residue was dissolved into 100 µl of methanol and subjected to spectrophotometric analysis. Absorption spectra were recorded in the wavelength range 200 to 350 nm, to measure the oxidative index, i.e., the A₂₃₄/A₂₀₅ ratio (16). Difference spectra were obtained by subtracting the absorption spectrum of healthy control subjects from that of HD patients. Spectra were recorded at room temperature in an ultraviolet-VIS spectrometer Lambda 18 (Perkin Elmer, Norwalk, CT).

5-Lipoxygenase Activity and Expression

The activity of 5-lipoxygenase (E.C. 1.13.11.34) was measured by incubating cell extracts, prepared from 5 × 10⁶ PBMC as described (18), for 10 min at 37°C in the presence of 1 mM ATP, 2 mM CaCl₂, and 40 µM arachidonic acid (19). Reaction products were separated by reverse phase-HPLC (RP-HPLC) on a Perkin Elmer Nelson model 1022 Plus Chromatograph, equipped with a Perkin Elmer series 200 LC Pump, a LC295 ultraviolet-VIS detector, and a 7125 BIO injector with a 20-µl loop. The system was interfaced with a Compaq Prolinea 5100 computer, using a Perkin Elmer Turbochrom software for system control and data processing. Separations were carried out on a C18 (5 µm, 30 × 3 mm inner diameter) column (SGE, Austin, TX) at a flow rate of 1.2 ml/min, using methanol/water/trifluoroacetic acid (70/30/0.007, by vol) as mobile phase. Chromatograms were recorded at 234 nm. 5-Lipoxygenase activity was expressed as pmol 5-hydroperoxyeicosatetraenoic acid (5-HPETE) formed per min per mg protein (15). Protein concentration of cell extracts was determined as reported (20).

Enzyme-linked immunosorbent assay was performed as reported (15) by coating each well overnight with cell extracts (25 µg/well) and reacting with rabbit anti-5-lipoxygenase polyclonal antibodies (diluted 1:200) as first antibody. Goat anti-rabbit IgG conjugated with alkaline phosphatase were used as second antibody (diluted 1:2000), and color development of the alkaline phosphatase reaction was followed at 405 nm, using p-nitrophenyl phosphate as substrate.

Analysis of LTB₄

Arachidonate metabolite LTB₄ was extracted from PBMC (5 × 10⁶/test) by solid-phase octadecyl-SPE columns (Baker, Deventer, The Netherlands), using ethyl acetate as eluent, and was analyzed by RP-HPLC as described above for the products of 5-lipoxygenase activity. The eluate was monitored at 270 nm, assessing peak identity by comparison with an authentic standard (15). Quantitative determinations were performed by integrating peak areas.

Effect of Vitamin E on Purified 5-Lipoxygenase

5-Lipoxygenase was purified to homogeneity from barley (Hordeum vulgare) by immunoaffinity chromatography, as reported (21). Enzyme activity was measured spectrophotometrically by recording the formation of conjugated hydroperoxides at 234 nm (22). A stock solution of linoleic acid (30 µM), dissolved in 1% Tween 20 in water, was diluted to the appropriate final concentration in 0.1 M sodium phosphate buffer, pH 6.8, according to Lomnitski et al. (23). The effect of vitamin E on linoleic acid dioxygenation by 5-lipoxygenase was analyzed by Lineweaver-Burk double reciprocal plots, taking into account the effect of vehicle alone (23). Reversibility of inhibition was assessed by assaying lipooxygenase activity after dialysis at 4°C of enzyme/inhibitor mixtures (15 nM/100 µM, preincubated at 4°C for 5 min in a final volume of 1 ml) against 100 ml 0.1 M sodium phosphate buffer, pH 6.8 (22).

Statistical Analyses

The data reported here are the mean ± SD of three independent determinations, each in triplicate. Statistical analysis was performed by t test, elaborating experimental data by means of the InStat program (GraphPad Software, San Diego, CA).
Results

Membrane Lipid Peroxidation in HD Patients

In HD patients treated with cuprophan hollow-fiber dialyzers, an increase in the absorbance spectrum between 200 and 350 nm of PBMC membranes was shown (Figure 1A), a hallmark of lipid peroxidation (16). The increase in absorbance was most remarkable around 240 nm (Figure 1B), where the absorption is mainly attributable to conjugated hydroperoxides (15) and was paralleled by a 3.2-fold increase of the oxidative index compared to the healthy control (Table 1). Therefore, HD-induced membrane peroxidation could be attributed to the generation of hydroperoxides in the lipid bilayer, a process that can be catalyzed by lipoxygenase activity (14–16). Consistent with this observation, the amount of the 5-lipoxygenase product LTB4 increased in HD patients 1.7-fold over the control (Table 1), and 5-lipoxygenase activity and protein levels increased 2.4-fold and 1.9-fold, respectively (Table 1). Remarkably, treatment of uremic patients with PMMA hollow-fiber dialyzers yielded changes in membrane lipoperoxidation and 5-lipoxygenase activity and expression similar to those observed with cuprophan, although they were less pronounced (Table 2). In particular, the oxidative index, the level of LTB4, the 5-lipoxygenase activity, and expression in patients hemodialyzed on PMMA membranes were, respectively, 65, 78, 78, and 81% of those measured in patients treated with cuprophan dialyzers (compare pre-vitamin E data in Tables 1 and 2). These differences were statistically significant ($P < 0.05$ in all cases).

Effects of Vitamin E Supplementation to HD Patients

In previous studies, we showed that daily doses of 300 mg (intramuscularly) of vitamin E for 15 consecutive days exert beneficial effects in uremic patients undergoing HD (4–6). Therefore, parenteral supplementation was chosen to further the study of the effect of vitamin E. Here, it is shown that vitamin E administration caused a significant reduction in both the oxidative index (from 3.2- to 1.6-fold compared with healthy controls) and the LTB4 content (from 1.7- to 1.2-fold) in HD subjects, as shown in Table 1. These effects could be attributed to the reduction of 5-lipoxygenase activity, which indeed decreased from 2.4- to 1.2-fold compared with control subjects (Table 1). Unlike the activity, 5-lipoxygenase expression at the protein level was not significantly affected by treatment with $\alpha$-tocopherol (Table 1), suggesting that the antioxidant acted as an inhibitor of the enzyme. Analogously, vitamin E supplementation was able to inhibit membrane peroxidation and 5-lipoxygenase activity, but not the expression, in patients treated with PMMA dialyzers (Table 2).

In Vitro Interaction between Vitamin E and 5-Lipoxygenase

To further support the hypothesis of a direct interaction in vivo between vitamin E and 5-lipoxygenase, in vitro experiments were performed using 5-lipoxygenase purified from barley (Hordeum vulgare). This plant enzyme is widely used as a model of the mammalian counterpart, which is not available in sufficient amounts. Moreover, the overall similarity between the two enzymes makes it possible to extrapolate to the mammalian lipoxygenase the kinetic experiments with the plant enzyme (21). Vitamin E was found to inhibit purified 5-lipoxygenase, with an IC$_{50}$ of 35 ± 4 $\mu$M (Figure 2A). Enzyme inhibition by vitamin E was fully reversible, with 5-lipoxygenase recovering its full activity 10 min after dialysis of enzyme/inhibitor mixtures (Figure 2B). Lineweaver-Burk analysis of the 5-lipoxygenase-catalyzed reaction, in the presence or absence of vitamin E, showed that the latter was a noncompetitive inhibitor of 5-lipoxygenase, with an inhibition constant, $K_i$, of 30 ± 3 $\mu$M. In keeping with these observations, addition of 35 $\mu$M vitamin E to PBMC extracts from healthy donors reduced in vitro the 5-lipoxygenase activity, from 1500 ± 150 pmol/min per mg protein of the untreated control to 900 ± 90 pmol/min per mg protein.
The various parameters were determined in peripheral blood mononuclear cells isolated from healthy volunteers and hemodialyzed patients, before and after treatment with vitamin E. Values in parentheses represent percentages of the control, arbitrarily set to 100.

Table 1. Oxidative index, leukotriene B₄, and 5-lipoxygenase activity and expression in patients hemodialyzed on cuprophan membranes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Subjects</th>
<th>Hemodialyzed Patients Pre-Vitamin E</th>
<th>Post-Vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative index (A₂₃₄/₂₀₅)</td>
<td>0.062 ± 0.007 (100%)</td>
<td>0.200 ± 0.020 (322%)</td>
<td>0.102 ± 0.010 (164%)</td>
</tr>
<tr>
<td>Leukotriene B₄ (pmol/mg protein)</td>
<td>45 ± 5 (100%)</td>
<td>77 ± 8 (171%)</td>
<td>56 ± 6 (124%)</td>
</tr>
<tr>
<td>5-Lipoxygenase activity (pmol/min per mg protein)</td>
<td>1500 ± 150 (100%)</td>
<td>3600 ± 380 (240%)</td>
<td>1800 ± 180 (120%)</td>
</tr>
<tr>
<td>5-Lipoxygenase expression (A₄₀₅ units)</td>
<td>0.430 ± 0.050 (100%)</td>
<td>0.825 ± 0.085 (192%)</td>
<td>0.795 ± 0.080 (185%)</td>
</tr>
</tbody>
</table>

The various parameters were determined in peripheral blood mononuclear cells isolated from healthy volunteers and hemodialyzed patients, before and after treatment with vitamin E. Values in parentheses represent percentages of the control, arbitrarily set to 100.

Table 2. Oxidative index, leukotriene B₄, and 5-lipoxygenase activity and expression in patients hemodialyzed on polymethylmetacrylate membranes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Subjects</th>
<th>Hemodialyzed Patients Pre-Vitamin E</th>
<th>Post-Vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative index (A₂₃₄/₂₀₅)</td>
<td>0.062 ± 0.007 (100%)</td>
<td>0.130 ± 0.015 (210%)</td>
<td>0.074 ± 0.008 (119%)</td>
</tr>
<tr>
<td>Leukotriene B₄ (pmol/mg protein)</td>
<td>45 ± 5 (100%)</td>
<td>60 ± 6 (133%)</td>
<td>47 ± 5 (104%)</td>
</tr>
<tr>
<td>5-Lipoxygenase activity (pmol/min per mg protein)</td>
<td>1500 ± 150 (100%)</td>
<td>2800 ± 300 (187%)</td>
<td>1650 ± 170 (110%)</td>
</tr>
<tr>
<td>5-Lipoxygenase expression (A₄₀₅ units)</td>
<td>0.430 ± 0.050 (100%)</td>
<td>0.670 ± 0.070 (156%)</td>
<td>0.625 ± 0.006 (145%)</td>
</tr>
</tbody>
</table>

Discussion

Recent studies have revealed that uremia is an oxidative stress-related disease (1,2), although the precise nature of oxidants involved and the target or mechanism of oxidative damage have not been elucidated (1–9). Results reported here show for the first time that in HD patients lipid peroxidation is occurring at the membrane level in peripheral blood mononuclear cells (Figure 1), and is mainly due to the generation of conjugated hydroperoxides in the lipid bilayer (Table 1). Up-regulation of 5-lipoxygenase activity (Table 1) plays a key role in such a membrane peroxidation, because it generates the conjugated hydroperoxides in the lipid bilayer. Also, the synthesis and the release of bioactive molecules such as LTB₄, observed here (Table 1) and in previous studies (10–12), are shown to correlate with the increase in 5-lipoxygenase activity. Therefore, this enzyme seems critical for the oxidative damage associated with uremia. The concept of a critical role for 5-lipoxygenase is corroborated by the evidence that enzyme expression is upregulated at the translational level in HD subjects (Table 1). These data extend recent observations on the increase of 5-lipoxygenase mRNA in HD (13). Remarkably, 5-lipoxygenase activation and related membrane lipoperoxidation were significant in patients hemodialyzed on bio-compatible PMMA hollow-fiber dialyzers, although they were significantly less pronounced than those observed in patients treated with bioincompatible cuprophan membranes (compare Tables 1 and 2). This suggests that the oxidative stress was a consequence of uremia, possibly amplified by the bioincompatible dialyzer. Parenteral supplementation of vitamin E to uremic patients undergoing HD has been shown by our group (4–6) and others (7) to exert beneficial effects. In this context, such beneficial effects of vitamin E in the therapy of uremic patients might be attributed at least in part to the direct inhibition of 5-lipoxygenase activity, which was indeed shown for the first time (Figure 2). Such an inhibition was dose-dependent, reversible, and noncompetitive, suggesting that a chronic administration of α-tocopherol should be effective in the control of lipid peroxidation-related disorders in HD patients.
Remarkably, trolox (a water-soluble analogue of α-tocopherol) has been reported to inhibit competitively lipoxygenase activity, with $K_i = 18 \mu M$ (22). These findings may also have clinical relevance, suggesting that lipoxygenase-oriented therapies might be helpful in the course of dialytic treatment. In keeping with this concept, growing evidence indicates that leukocyte activation in HD patients (24,25) takes part in the pathogenesis of a number of dialysis-related complications, from atherosclerosis (9) to coronary heart disease (26–28).

Finally, it is noteworthy that results reported here give biochemical background to the newly discovered ability of nitric oxide to mimic HD damage (29). Indeed, prolonged exposure of human cells to nitric oxide enhances lipoxygenase activity (15), thus promoting the release of bioactive eicosanoids from the arachidonate cascade (30).

In conclusion, the results reported here describe a potentially important mechanism responsible for the oxidative damage in HD. They also strongly suggest that the beneficial effect of vitamin E may have relevance for therapy of patients with kidney disease, yet not therapy for the renal disease itself.

Acknowledgments
This investigation was supported in part by Ministero Università e Ricerca Scientifica Tecnologica-Progetto Ricerca Interesse Nazionale (1997). The authors thank Dr. M. Bari (University of Rome Tor Vergata) for her skillful assistance with the RP-HPLC analysis.

References

**Figure 2.** Inhibition of purified 5-lipoxygenase by vitamin E. (A) Concentration dependence of 5-lipoxygenase-catalyzed dioxygenation of 90 μM linoleic acid by vitamin E. (B) Residual activity of 5-lipoxygenase (15 nM) after 5 min incubation at 4°C with vitamin E (100 μM) and dialysis for different periods of time. Values were expressed as percentage of the activity in the absence of vitamin E, arbitrarily set to 100 (100% = 1.94 ± 0.20 nM/min).


