Favorable Effect of Hemodialysis on Decreased Serum Antioxidant Activity in Hemodialysis Patients Demonstrated by Electron Spin Resonance

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Abstract. There is considerable evidence that uremic patients are in a highly peroxidative state. The purpose of this study was to investigate the serum antioxidant activity that may regulate, or represent, the redox state in vivo. Serum from pre- and posthemodialysis patients and from healthy control subjects was added to a system generating the hydroxyl radical, and then the signal intensities of reactive oxygen species were measured by electron spin resonance and spin-trapping technique. The electron spin resonance signals of the reaction mixture containing prehemodialysis sera were significantly stronger than those of the reaction mixture containing healthy sera (P < 0.001, n = 19), and there was no significant difference in the signals between the reaction mixture containing posthemodialysis and healthy sera. These findings demonstrated that serum antioxidant activity in hemodialysis patients is significantly decreased, and this pathological condition is improved by hemodialysis treatment. (J Am Soc Nephrol 8: 1157-1163, 1997)

Healthy organisms combat oxygen toxicity with a variety of defense mechanisms. In pathologic states, these protective mechanisms appear to be attenuated, leading to a number of important sequelae, such as inflammation, fibrosis, hemolysis, arteriosclerosis, cancer, and the concomitants of aging (1-6). We have reported previously that methylguanidine, a potent uremic toxin, is a peroxidative product of creatinine (7-10), a substance once thought to be biologically inert. This strongly supports the evidence that uremia is a state of oxidative stress (11-16).

Recent progress in electronics makes it possible, using electron spin resonance (ESR) and a spin-trapping technique, to determine and quantify the species of reactive oxygen involved in a reaction (17,18). Biological applications of ESR include detecting the production of the hydroxyl radical during NADPH-dependent microsomal lipid peroxidation (19), measuring the increased activity of serum superoxide dismutase in patients with chronic glomerulonephritis or chronic renal failure (20), and showing the accumulation of oxidizing components in uremic plasma (21). In this study, we investigated serum antioxidant activity by measuring serum-scavenging activity against the hydroxyl radical, a very reactive and short-lived free radical, using ESR. This was accomplished by adding serum from patients before and after hemodialysis (HD) treatment to a system generating the hydroxyl radical, which reacts with spin-trapping reagent and produces a relatively long-lived product (spin adduct) that can be detected by ESR and by comparison with healthy control subjects. These ESR values were correlated with various biochemical parameters to examine the factors that affect the peroxidative state of serum.

Materials and Methods

Patients

After giving informed consent, 19 stable adult patients (14 men, 5 women) aged 44.8 ± 14.8 yr (mean ± SD) who had been undergoing maintenance HD 545.6 ± 422.8 times and 23 healthy control subjects (20 men, 3 women) with normal renal function aged 44.7 ± 8.5 yr were enrolled in this study. All of the patients were routinely dialyzed for 3 to 4 h three times weekly using polysulfone membranes (Fresenius, Kawasumi Chemical Co., Tokyo, Japan) with no dialyzer reuse. The dialysate used was bicarbonate-based Kinderly AF-2 (Fuso Pharmaceutical Co., Osaka, Japan). The cause of end-stage renal disease was chronic glomerulonephritis in 11 cases, diabetic nephropathy in 3 cases, nephrosclerosis in 2 cases, polycystic kidney disease in 2 cases, and myeloma kidney disease in 1 case.

Blood samples were drawn from the antecubital veins of healthy subjects and from the arterial side of the arteriovenous fistula of HD patients before and after HD. Sera were separated by centrifugation at 1100 × g for 15 min at 4°C and kept refrigerated at −20°C until used.

Clinical Parameters

Serum total protein, albumin, urea nitrogen, creatinine, uric acid, total and direct bilirubin, glutamic-oxaloacetic transaminase, glutamic
pyruvic transaminase, lactate dehydrogenase, alkaline phosphatase, total and HDL cholesterol, triglyceride, glucose, total calcium, inorganic phosphorus, Na+, K+, and Cl− levels were determined by an autoanalyzer (Hitachi 736-15, Ibaraki, Japan), and tocopherol levels were measured by HPLC analysis (22). Serum iron level was measured colorimetrically, and serum ferritin level was measured by an enzyme immunoassay. Red and white blood cells, hemoglobin level, hematocrit level, and platelets were determined by an autoanalyzer (Coulter STKS, Hialeah, FL).

**ESR Measurement of the Serum-Scavenging Activity against the Hydroxyl Radical**

The serum-scavenging activity against the hydroxyl radical, derived from Fenton's reaction (23), was directly measured by the inhibition rate of ESR signals after the addition of sera to a system generating the hydroxyl radical. The ESR spectrum allows the identification and quantification of the original reactive radical. The reaction mixture consisted of 1 ml of distilled water containing 10% serum, 0.5 mM FeCl2, 5 mM hydrogen peroxide, and 100 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin-trapping reagent in final concentrations. The ESR spectra at 1 min after the initiation of the reaction by the addition of hydrogen peroxide were recorded by ESR machine JES-FE2XG (Nippon Densi, Tokyo, Japan), at room temperature (25°C). The spectrum of the reaction without serum was considered as a control, and the signal intensities were expressed as a percentage of the control.

**Measurement of Serum Catalase Activity**

The rate of serum degradation of hydrogen peroxide was measured spectrophotometrically by following the decrease in ultraviolet absorption at 250 nm (24). The reaction mixture consisted of 1 ml of 50 mM phosphate buffer (pH 7.0 at 25°C) containing 10 mM hydrogen peroxide and 25 μl/ml serum. The breakdown of hydrogen peroxide was calculated from the absorption before and after a 10-min incubation at 25°C. One unit of catalase activity was defined as the amount of enzyme that consumed 1 μmol of hydrogen peroxide per minute in this condition.

**Statistical Analyses**

Statistical analyses were performed using commercially available personal computer software, Stat-View 4.11 (Abacus Concepts, Inc., Berkeley, CA). Data were presented as mean ± SD and analyzed by t test and ANOVA. Difference was considered significant at P < 0.01.

**Results**

**Direct Proof of Serum-Scavenging Effect on the Hydroxyl Radical by ESR**

The signal intensities of the hydroxyl radical decreased as the concentration of sera from healthy control subjects in the incubation mixture rose, as shown in Figure 1. On the basis of these results, 100 μl of serum was added in 1 ml of the reaction mixture to determine the serum-reducing activity. The typical ESR spectra are shown in Figure 2. As shown in this figure, the signal intensity of the reaction mixture containing pre-HD serum (Figure 2B) is stronger than that containing serum from a healthy control individual (Figure 2A). The intensity recovered to the healthy value after HD treatment (Figure 2C). Figure 3 shows the individual results of 19 pairs of pre- and post-HD sera. These results demonstrate that the scavenging activity against the hydroxyl radical of pre-HD sera increased after HD treatment in almost every patient. Figure 4 clearly demonstrates that the ESR signals of the reaction mixture containing pre-HD sera are significantly stronger than those containing healthy sera (unpaired t test, P < 0.001, n = 19 and 23) or those containing post-HD sera (paired t test, P < 0.001, n = 19), and there is no significant difference in the signal intensities between the reaction mixture containing post-HD sera and healthy sera. These results are confirmed by ANOVA.

**Correlation among ESR Signal Intensity and Various Parameters**

The correlations among ESR signal intensity and various biological parameters were investigated to clarify the factors affecting serum antioxidant activity. No parameters changed in the same manner as ESR signal other than blood urea nitrogen, creatinine, and uric acid, all of which are easily removed by HD. The blood cell count and hemoglobin level in HD patients were significantly lower than in control subjects because of the disease condition. There was no significant difference in cata-
Effect of HD on Serum Antioxidant Activity

Figure 2. Typical spectra of ESR signals. The reaction mixture consisted of 1 ml of distilled water containing 10% serum, 0.5 mM FeCl₂, 5 mM hydrogen peroxide, and 100 mM DMPO in the following experiments using ESR. The signal intensity of the reaction mixture containing pre-HD serum (B) is stronger than that containing serum from a healthy control individual (A), and the intensity recovered to the healthy value after HD treatment (C).

In the correlation analysis of the total number of subjects, i.e., 19 pre-HD and 23 healthy sera, ESR signal intensity negatively correlated with total cholesterol level \( (r = -0.539, P < 0.001, n = 42; \text{Figure 5A}) \), total bilirubin level \( (r = -0.513, P < 0.001, n = 42; \text{Figure 5B}) \), hemoglobin level \( (r = -0.617, P < 0.001, n = 42) \), and red blood cell count \( (r = -0.651, P < 0.001, n = 42) \) and positively correlated with creatinine \( (r = 0.642, P < 0.001, n = 42) \) and urea nitrogen \( (r = 0.664, P < 0.001, n = 42) \) levels, but did not correlate with other parameters measured such as serum albumin levels. However, the significant correlations between ESR signal intensity and the parameters other than total cholesterol and bilirubin level are questionable in spite of the high correlation.
coefficients, because both of these parameters and ESR signal intensity are separated clearly between healthy and pre-HD patients as mentioned above. In this situation, the statistics have no significance. To examine the contribution of these parameters to ESR signal intensity, the changes of ESR signal caused by HD (ESR signal of the reaction mixture containing pre-HD sera minus that containing post-HD sera) were correlated with the changes of these parameters (pre-HD value minus post-HD value) in 19 HD patients. Because no change in any parameter correlated significantly with the changes of ESR signal, we concluded that only the correlations between ESR signal intensity and total bilirubin or total cholesterol are clinically relevant.

Discussion

Recent studies have revealed that uremia is a peroxidative state (4–6). There are several markers that indicate peroxidative condition, such as lipid peroxides (12,13,15), conjugated diene (16), antioxidant enzymes (14), and methylguanidine (8,10). However, all of these markers do not demonstrate the existence of reactive oxygen species directly. On this point ESR offers an advantage, and using this technique, we proved decreased serum-scavenging activity against the hydroxyl radical in pre-HD patients and restoration of activity after HD.

The reduced scavenging activity against the hydroxyl radical in the serum may be due to decreased serum activity for the degradation of the sources of the hydroxyl radical, i.e., hydrogen peroxide and free iron. However, because serum iron-binding capacity is approximately 0.05 mM and the serum concentration in this experiment is 10%, the iron-binding capacity in the reaction mixture is 0.005 mM. This is only 1% of the iron present in the reaction mixture. On the basis of this calculation, we concluded that serum iron-binding capacity does not affect the concentration of free iron in the reaction mixture. In addition, there was no significant difference in the
Table 2. Comparison of serum tocopherol levels among healthy, pre-HD, and post-HD groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Tocopherol (μg/ml; mean ± SD)</th>
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<tbody>
<tr>
<td>Healthy (n = 23)</td>
<td>11.43 ± 3.85</td>
</tr>
<tr>
<td>Pre-HD (n = 19)</td>
<td>8.97 ± 2.09</td>
</tr>
<tr>
<td>Post-HD (n = 19)</td>
<td>9.77 ± 2.46</td>
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</tbody>
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* HD, hemodialysis. Serum levels of α-, β-, and γ-tocopherols were measured by HPLC analysis. There was no significant difference among the three groups.

The activity of serum catalase among healthy, pre-HD, and post-HD subjects. The second possibility is reduced serum-scavenging action against the hydroxyl radical, although there is no significant difference of the serum concentration of tocopherols, which are naturally occurring scavengers, among healthy, pre-HD, and post-HD subjects and no correlation between the concentration of tocopherols and ESR signal levels. However, the intensities of the ESR signal in the reaction mixture decreased as the serum concentration of total cholesterol or bilirubin rose (Figure 5, A and B), although there was no significant difference in the serum concentration of these substances among the three groups. This strongly suggests that the significant correlations are not only statistical, but that total cholesterol and bilirubin contribute to serum antioxidant activity. In addition, we concluded that the statistically significant correlations between ESR signal intensity and creatinine, urea nitrogen, hemoglobin level, or red blood cell count have no relevance. In our reaction mixture, the hydroxyl radical is detected by ESR after binding with DMPO. Therefore, a scavenger in the serum must compete with DMPO for binding the hydroxyl radical to reduce the ESR signals. The concentration of DMPO in the reaction mixture is 100 mM, which is relatively high, and there are no substances of comparable concentration in the serum. This suggests that serum-scavenging activity may be derived from a combination of several molecules, including cholesterol, bilirubin, and other substances that were not measured in this study. In the patients undergoing HD, these protective mechanisms against reactive oxygen species may be attenuated in quality or quantity by the effects of toxic, but dialyzable, substances, which accumulate in the disease condition.

Our findings do not conflict with the reported fact that the activity of serum superoxide dismutase in patients with chronic glomerulonephritis or chronic renal failure is significantly higher than that in healthy control subjects (20), because different species of reactive oxygen are involved. A recent report described the accumulation of an endogenous, stable oxidizing agent in uremia that was dialyzable (21). The oxidant was...
detected by its capacity to oxidize the spin-trapping reagent using ESR. That study strongly supports the peroxidative condition of uremic patients. In contrast, in our study we examined serum-scavenging activity against the hydroxyl radical and show that there are dialyzable uremic compounds that decrease antioxidant activity. In addition, it is well known that hepatic microsomes catalyze an NADPH-dependent peroxidation of endogenous lipids on microsomal membranes (25), and the inhibition rate of this reaction by the addition of serum to the reaction mixture also demonstrates serum antioxidant activity. In an unreported study, we found that the serum inhibitory effect on microsomal lipid peroxidation is significantly weaker in pre-HD sera than in post-HD sera and that there is no significant difference between post-HD and healthy sera using this microsomal system. This trend is similar to the results of this study.

We have clearly demonstrated that serum-scavenging activity against the hydroxyl radical in HD patients is significantly decreased and that this pathological condition is improved by HD treatment. However, this study does not account for all of the factors that contribute to the regulation of serum antioxidant activity. Serum-scavenging activity against the hydroxyl radical may be derived from a collaboration of several substances that may have antioxidant activity, such as bilirubin, cholesterol, and other substances that were not measured in this study. Improvement of serum antioxidant activity by HD could be derived from the beneficial effect of high-flux membranes, similar to the beneficial effect on plasma lipoprotein profiles reported previously (26). However, we can make no definitive conclusion on this point because of a lack of data regarding low-flux membranes.

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References


lyzed patients before and during erythropoietin treatment. Nephron 65: 288–293, 1993
7. Nagase S, Aoyagi K, Narita M, Tojo S: Biosynthesis of methy-
