Ascorbate Supplement Reduces Oxidative Stress in Dyslipidemic Patients Undergoing Apheresis

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Objective—The effect of ascorbate treatment on apheresis-induced oxidative stress in uremic and dyslipidemic patients was evaluated.

Methods and Results—We developed a chemiluminescence-emission spectrum and high-performance liquid chromatography analysis to assess the effect of ascorbate supplement on plasma reactive oxygen species (ROS) scavenging activity and oxidized lipid/protein production in hyperlipidemic and uremic patients undergoing apheresis. Apheresis was efficient in reduction of atherogenic lipoproteins, complement, fibrinogen, soluble intercellular adhesion molecule-1, and oxidative parameters including phosphatidylcholine hydroperoxide (PCOOH), malonaldehyde, methylguanidine, and diotyrosine. Apheresis itself, however, activated leukocytes to increase ROS activity and reduced the plasma ROS scavenging activity. Ascorbate administration selectively diminished apheresis-enhanced H2O2 and inflammatory mediators such as tumor necrosis factor alpha (TNF-α) and monocyte chemoattractant protein-1. Chronically dyslipidemic and uremic patients undergoing biweekly apheresis plus ascorbate treatment had lower levels of C-reactive protein and PCOOH than did those without ascorbate treatment during a 6-month follow-up study period.

Conclusions—We demonstrate that apheresis with ascorbate treatment provides a therapeutic potential in reducing atherosclerotic risk via inhibition of H2O2-induced oxidative stress in patients with uremia or dyslipidemia. (Arterioscler Thromb Vasc Biol. 2004;24:1111-1117.)

Key Words: apheresis ■ reactive oxygen species ■ atherosclerosis ■ dialysis ■ vitamin C

Patients with end-stage renal disease or hyperlipidemia have complex dyslipidemia consisting of quantitative and qualitative abnormalities in serum lipoproteins.1 The highly atherogenic low-density lipoprotein (LDL) accumulates preferentially in hyperlipidemic patients who have nephropathy or are using hemodialysis treatment, and it exerts profound effects on the vasomotor response of blood vessels to various stimuli that closely mimic the vascular dysfunction associated with hypercholesterolemia and atherosclerosis in humans. These alterations in lipoprotein composition not only passively accompany chronic renal disease but also promote its progression and the development of atherosclerosis. Lipid-lowering therapy may have a beneficial role in normalizing vascular function and greatly decreasing the frequency of clinical events associated with atherosclerosis, combined with the ability of antioxidants to alleviate vasomotor disturbances in hypercholesterolemia and to slow the progression of atherosclerosis.²

Hemodialysis or apheresis of the extracorporeal system is often used for removal of excessive toxins, metabolic products, and blood components (eg, LDL) from patients with uremia or hyperlipidemia. The treatment is efficient in preventing fatal and nonfatal cardiovascular events in patients with these conditions.1-5 Nevertheless, the extracorporeal treatment per se is associated with increased production of reactive oxygen species (ROS) by granulocytes (polymorphonuclear neutrophils [PMNs])⁴ and a reduction in the antioxidant defense.⁵ The increased oxidative stress can cause oxidation of biological macromolecules, including proteins and lipids.⁵,⁷

The 2 major ROS generated from activated PMNs via the myeloperoxidase (MPO) system are hydrogen peroxide (H2O2) and hypochlorite (HOCl),⁸ which can produce lipid peroxidation products, malonaldehyde (MDA), and phosphatidylcholine hydroperoxide (PCOOH)⁹,¹⁰ and protein oxidation products, diotyrosine and methylguanidine, as indirect indicators of ROS and/or free radical activity.¹¹,¹² Among these oxidized products, oxidized LDL (oxLDL) can increase the adhesion of monocytes to the endothelium and transformation of macrophages into foam cells and impair endothe-
limum-dependent vasorelaxation.13–15 These changes could lead to the development of atherosclerosis and coronary artery disease.16,17

Ascorbate is a well-known ROS scavenger that can effectively prevent the initiation of lipid peroxidation and the formation of lipid peroxides.18 To minimize the oxidative stress of apheresis, we have considered administering ascorbate to the patients during the apheresis course. Our study showed that ascorbate administration selectively restored plasma ROS scavenging activity for H2O2 and resulted in lower plasma levels of oxidized lipids/proteins as compared with those in patients without ascorbate treatment. Apheresis with simultaneous ascorbate administration might be of clinical importance for preventing atherosclerotic disease in patients with hyperlipidemia or those undergoing long-term hemodialysis.

**Methods**

**Human Subjects**

Forty-nine hyperlipidemic patients (19 women and 30 men; mean age, 49.2 ± 11 years) and 39 uremic patients (16 women and 23 men; mean age, 52.2 ± 11 years) were included in the study. The uremic patients have been continuously on treatment with hemodialysis with an antecubital vein before and at the end of apheresis by centrifugation. The mean creatinine level was 1.0 ± 0.6 mg/dL, and 0.9 ± 0.1 mg/dL for hyperlipidemic patients, uremic patients, and healthy volunteers, respectively. Among these 49 hyperlipidemic patients, 9 were administered simvastatin (a hydroxymethylglutaryl-coenzyme A reductase inhibitor) 10 mg and 4 received probucol 1000 mg per day. Uremic patients were not treated with statins or fibrates. No angiotensin-converting enzyme inhibitors or angiotensin II type-I receptor blockers were prescribed in all the patients. Informed consent was obtained from all patients and volunteers. The permission for the clinical trial was approved by the Human Ethics Committee of the National Taiwan University Hospital.

**Apheresis and Ascorbate Administration**

Apheresis was performed by KM-8800 (Kuraray Co, Ltd, Osaka, Japan) with a double-filtration plasmapheresis procedure (Plasmacure PS-O6 and Evaflux 4A, Kuraray Medical Inc, Okayama, Japan). Total blood volumes of 10.5 ± 0.3 L and 9.8 ± 0.3 L were processed in the 2-hour plasmapheresis procedure for uremic and hyperlipidemic patients. Ascorbate (Tai-Yu Pharmaceutical, Taipei, Taiwan), prepared as 2.5 g in 150 mL of 5% albumin solution, was administered during the apheresis session.

**Blood Samples and Biochemical Analysis**

Plasma was separated from 10 mL of blood drawn from the antecubital vein before and at the end of apheresis by centrifugation at 1500g for 5 minutes at 4°C. Plasma total cholesterol (CHOE), triglycerides (TG), and high-density lipoprotein (HDL), LDL, and very low-density lipoprotein (VLDL) levels were measured.19 The measurement of lipoprotein(a) [Lp(a)] was performed with a commercial LPA kit (463560; Beckman Coulter-Array System, Denmark).

In addition, quantitative determination of fibrinogen levels in plasma was made by FIBRI-PREST AUTOMATE (Diagnostica Stago, Asnieres-Sur-Seine, France). Plasma complement 3 (C3) and component 4 (C4) were measured by use of C3 and C4 commercial kits (Beckman-Coulter Inc, Fullerton, Calif), respectively. Quantification of TNF-α and soluble intercellular adhesion molecule-1 (sICAM-1) and monocyte chemotactrant protein-1 (MCP-1) was performed by use of ELISA kits (R&D Systems Inc, Minneapolis, Minn).

**Measurement of Specific Plasma Antioxidant Activity**

A test mixture of 0.03% H2O2 or 0.012% NaOCl and a chemiluminescence (CL)-emitting substance [i.e., luminol (5-amino-2,3-dihydro-1,4-phenazinedione), Sigma, St. Louis, Mo] can emit CL signals that can be measured with a multichannel CL spectrum analyzer (CLA-SP2; Tohoku Electronic Ind. Co, Sendai, Japan).9 The recorded CL signals from the test mixtures containing plasma were referred to as plasma “reference H2O2 counts” (pRH2O2) or plasma “reference HOCl counts” (pRHOC). A higher pRH2O2 or pRHOC indicated higher ROS activity or lower antioxidative activity (lower ROS scavenging ability), or both, in plasma.

In addition, the total antioxidant status (TAS) in 20 μL of plasma was measured with a TAS kit (Randox, San Francisco, Calif) according to the manufacturer’s instructions. Ascorbate content in deproteinized plasma was estimated by colorimetry using the L-Ascorbic Acid kits (Boehringer Mannheim, Mannheim, Germany).

**Measurement of Oxidized Amino Acid/Protein Products and Lipid Products**

Two protein oxidation products, di tyrosine and methylguanidine, were measured. In the presence of H2O2, tyrosyl radicals generated by MPO can cross-link to give a fluorescent adduct, di tyrosine, which can be determined with a fluorometer (Hitachi F-2500, Tokyo, Japan). The methylguanidine with fluorescent activity, as an indirect measure of hydroxyl radical activity, was measured as described previously.12

Two lipid primary and secondary peroxidation products, PCOOH and MDA, were determined. The amounts of PCOOH were measured in duplicate by CL high-performance liquid chromatography (CL-HPLC) (Tohoku Electronic Ind. Co). MDA levels were assayed as previously described.20

**Measurement of ROS Activity in PMNs as the Primary Source of ROS in Plasma**

Intracellular ROS activity of PMNs was analyzed with a FACScalibur flow cytometer (Becton Dickinson, San Jose, Calif) after being stained with 2’,7’-dichlorofluorescin diacetate (DCF-DA) (5 mmol/L; Sigma).21 To determine the PMNs as the source of ROS in plasma, we isolated PMNs from patients with or without ascorbate treatment at various time points during apheresis. After centrifugation, PMNs (purity >95%) were washed and resuspended in RPMI 1640 medium containing 10% fetal serum at a density of 5 × 106 cells/mL at 37°C. The PMN suspension was immediately added to the luminol solution as described, and CL signals were measured. The effect of intravenous ascorbate, oral simvastatin, or probucol treatment on PMN CL signals was also evaluated. To determine the type of ROS derived from PMNs, we added superoxide dismutase (SOD) (an O2− scavenger, 30 U), catalase (a H2O2 scavenger), or epigallocatechin-3-gallate (EGCG, HOCl, and H2O2 scavenger, 100 μg) to the PMNs (2 hours after apheresis)/RPMI suspension. We compared the CL signals with or without treatment.

To test whether ROS may be released into the culture medium, we isolated PMNs from patients 2 hours after apheresis and incubated the PMN in RPMI suspension containing 10% fetal calf serum for various times. The RPMI medium after removal of PMNs was subjected to the CL assay.

**C-Reactive Protein Assay**

Apheresis was administered biweekly for ≥6 months. Serums were collected for measurement of C-reactive protein (CRP) level.23 The serum CRP concentrations were determined with an autoanalyzer (Tectron U-240 Plus, Tokyo, Japan). The lower limit of detection of CRP was 0.3 mg/L. The study is intended to compare the effect of ascorbate treatment on a possible reduction of the known marker, CRP, associated with atherogenesis.
Antioxidant Activity
Ascorbate, but Not Vitamin E, Exerted Potent Antioxidant Activity

Figure 1A shows a typical CL emission from H$_2$O$_2$ in plasma (Figure 1A). Ascorbate added to the test mixture revealed strong H$_2$O$_2$ scavenger activity in a dose-dependent manner, as shown by a nearly 70% to 80% reduction in RH$_2$O$_2$ (with ascorbate at $>10^{-4}$ M; Figure 1B). Vitamin E had weaker H$_2$O$_2$ scavenger activity as compared with that for ascorbate. Both vitamins C and E did not seem to be effective scavengers for RHOCl (not shown).

Ascorbate Reduced Apheresis-Enhanced Plasma RH$_2$O$_2$ Counts

We tested the antioxidant effects of plasma obtained from healthy controls and patients. The pRH$_2$O$_2$ values obtained from uremic and hyperlipidemic patients were higher than that for healthy controls. The pRH$_2$O$_2$ was further increased (~60% to 70%) in postapheresis plasma in patients with uremia or hyperlipidemia, indicating oxidative stress accompanied by apheresis. With ascorbate, the pRH$_2$O$_2$ was reduced by 80% to 93% as compared with that without ascorbate in the uremic and hyperlipidemic patients (Figure 2A and 2B). The degrees of reduction in pRH$_2$O$_2$ by ascorbate were similar in the hyperlipidemic patients treated with simvastatin (90%±3%, n=9), probucol (92%±3%, n=4), and in patients without drug treatment (90%±2%, n=36).

The TAS level was significantly decreased in the uremic or hyperlipidemic plasma when compared with normal plasma. Apheresis reduced the plasma TAS in uremic and hyperlipidemic plasma, and the reduction could be effectively reversed by ascorbate administration (Figure 2E).

The pre-apheresis plasma ascorbate was 72±7, 29±3, and 59±7 μmol/L in the controls, uremic, and hyperlipidemic patients, respectively. The plasma ascorbate was reduced by ~40% after apheresis. Apheresis plus ascorbate treatment elevated the plasma ascorbate concentration to 89±7 and 95±7 μmol/L in uremic and hyperlipidemic patients, respectively.
Ascorbate Only Partly Reduced Apheresis-Enhanced RHOCl Counts

The HOCl CL has a wide emission wavelength region of 350 to 670 nm with an Emax of 520 nm. The RHOCl in the PBS was 10,200±680 counts. When control plasma was added, the pRHOCl was reduced to 836±74 counts (Figure 2C). The pRHOCl values obtained from uremic and hyperlipidemic patients were higher than that for healthy controls. The pRHOCl values were increased by ~40% after apheresis in uremic and hyperlipidemic groups. Unlike the situation for \( \text{H}_2\text{O}_2 \), the increased pRHOCl associated with apheresis was not significantly reduced (<10%) by ascorbate administration (Figure 2C and 2D).

Ascorbate Reduced Apheresis-Induced ROS Formation in PMNs

The percentage of intracellular ROS production of DCF-positive PMNs was 24.6±7.8% for apheresis patients with ascorbate treatment, compared with increased DCF-positive PMNs (46.5±6.0%) for apheresis patients without ascorbate treatment. The percentage of DCF-positive PMNs obtained from patients before apheresis was 5% to 8%. The results indicated that the ROS in the dialysis membrane-activated PMNs increased and that ascorbate could act as a ROS scavenger within activated PMNs.

In hyperlipidemic (n=13) and uremic patients (n=15), apheresis increased luminol ROS activity from the activated PMNs. Ascorbate treatment could effectively suppress the enhanced PMN ROS formation (Figure 3A). The augmented CL counts of activated PMNs were greatly inhibited by the \( \text{H}_2\text{O}_2 \) scavenger of catalase (67±9%) and partially decreased by SOD (16±3%) and EGCG (17±3%), respectively (Figure 3B). We also tested the ROS activity from the RPMI medium after removal of postapheresis PMNs. An increase in RPMI CL counts was detected, indicating that the increased ROS from apheresis-activated PMNs may be released into RPMI medium (or plasma) (Figure 3C). Ascorbate treatment decreased the enhanced ROS in medium.

We also evaluated whether the 2 drugs (simvastatin and probucol) may display significant in vivo antioxidant effect mimic with that of vitamin C. The postapheresis PMN CL counts were only slightly reduced in patients with simvastatin (n=5) or probucol (n=4) treatment, as compared with patients without treatment (Figure 3A).

Effects of Apheresis-Induced Oxidative Stress on Biochemical Parameters

The pre-apheresis levels of CHOE, TG, LDL, VLDL, Lp(a), C3, C4, and fibrinogen were higher in the uremic and hyperlipidemic patients than those in the normal plasma. After apheresis, these parameters were reduced significantly by 40% to 60%, indicating a reduction of proteins/lipoproteins of various sizes during apheresis (Table). It was noteworthy that there was a significant reduction in the LDL/VLDL fraction after apheresis, ie, by 50% to 60% in uremic patients and 60% to 74% in hyperlipidemic patients. A single apheresis course alone also efficiently reduced sICAM-1 (by 60%), but not MCP-1 and TNF-\( \alpha \), suggesting increased production of the latter 2 proteins during the 2-hour apheresis course. Ascorbate treatment effectively suppressed MCP-1 and TNF-\( \alpha \) production (Table).

Effect of Apheresis on Oxidized Lipid and Protein Products

The levels of lipid-peroxidation products and protein/amino acid oxidation products in healthy controls, and in uremic and hyperlipidemic patients before and after apheresis, are displayed in Figure 4. In uremic and hyperlipidemic patients, baseline levels of PCOOH, MDA, methylguanidine, and diotyrosine were significantly higher than those in healthy
Effects of apheresis and ascorbate supplement on plasma oxidized parameters. PCOOH (A), MDA (B), methylguanidine (MG) (C), and diotyrosine (DT) (D) in plasma were obtained from normal individuals and from uremic and hyperlipidemic patients. Apheresis efficiently reduced oxidized products, especially in PCOOH. Ascorbate supplementation resulted in further reduction of these oxidized parameters in hyperlipidemic and uremic subjects. *P<0.05 versus the pre-apheresis value of normal plasma; †P<0.05 versus the pre-apheresis value of the respective group; and Δ P<0.05 versus the value for patients without ascorbate administration in each respective group.

Figure 4. Effects of apheresis and ascorbate supplement on oxidized parameters. PCOOH (A), MDA (B), methylguanidine (MG) (C), and diotyrosine (DT) (D) in plasma were obtained from normal individuals and from uremic and hyperlipidemic patients. Apheresis efficiently reduced oxidized products, especially in PCOOH. Ascorbate supplementation resulted in further reduction of these oxidized parameters in hyperlipidemic and uremic subjects. *P<0.05 versus the pre-apheresis value of normal plasma; †P<0.05 versus the pre-apheresis value of the respective group; and Δ P<0.05 versus the value for patients without ascorbate administration in each respective group.

Long-Term Effect of Apheresis with Ascorbate on CRP Level
We compared the CRP levels in 2 groups of uremic patients: 10 patients with biweekly apheresis treatment and 10 patients with biweekly apheresis plus ascorbate treatment. The 2 groups had a similar age distribution, clinical conditions, and blood chemistry including CRP levels before the study. Biweekly apheresis plus ascorbate treatment significantly reduced CRP levels after 6 months of therapy (from 1.33±0.36 mg/dL to 0.28±0.09 mg/dL, P<0.05). The CRP levels in patients receiving biweekly apheresis alone were under control, but at a higher level (1.18±0.38 mg/dL), as compared with that in patients receiving ascorbate treatment (0.28±0.09 mg/dL).

Figure 5. Correlation between pRH2O2 and pRHOCl and plasma total antioxidant status (TAS). In healthy controls and in uremic and hyperlipidemic patients, RH2O2 and RHOCl were negatively correlated with TAS (A, C). A positive correlation between RH2O2 and RHOCl counts with plasma PCOOH was detected in the 98 human subjects (B, D).
Discussion
Apheresis efficiently removed atherogenic substances from patients with hypercholesterolemia6 or those undergoing long-term hemodialysis. Apheresis also ameliorated blood rheology and the endothelial function of coronary arteries,25,26 and improved the prognosis of atherogenic patients. This may explain the beneficial effect of LDL apheresis in preventing fatal and nonfatal cardiovascular events from occurring in patients with hyperlipidemia and uremia.2–4 However, apheresis itself appears to cause an acute and transient reduction in endogenous antioxidant ability, as shown by the decrease in TAS and increased production of ROS by PMNs after apheresis. As a result, there was an increase in patients’ plasma RH2O2 and RHOCI after apheresis. Ascorbate administration during or after apheresis increased TAS and selectively diminished RH2O2, but not RHOCI. More significantly, the level of oxidative products of lipid and protein (including 2 inflammatory mediators, MCP-1 and TNF-α) can be reduced further by ascorbate supplement, despite the oxidative stress accompanying apheresis. MCP-1 is highly expressed in macrophage-rich areas of atherosclerotic lesions and plays an important role in the pathogenesis of atherosclerosis.25,27

The increased ROS found in patients with end-stage renal disease undergoing chronic hemodialysis or in patients with apheresis could originate from complement-, platelet-, and even dialysis membrane-activated PMNs.28 The self-perpetuating formation of H2O2 and HOCl from PMNs may potentially oxidize protein and LDL, change the lipid composition of cell membranes and the extracellular matrix,29–30 and consequently lead to vascular dysfunction and atherosclerotic injury. Cells (such as endothelial cells) are more prone to oxidative damage when exposed to H2O2 as compared with HOCl.31 However, when combined with H2O2, HOCl increased H2O2-mediated oxidative damage and compromised the repair process. HOCl, but not H2O2, has been shown to play a critical role in LDL thiol oxidation by PMNs in vitro.32

An important implication of the oxidative modification hypothesis of atherosclerosis is that increased antioxidants present in the extracellular fluid (plasma) or increased uptake of antioxidants in endothelial cells and PMNs may inhibit atherogenesis by protection of LDL against oxidative modification. Therefore, antioxidant treatment that can reduce apheresis-induced ROS, particularly H2O2, should be beneficial for minimizing oxidative damage to leukocytes and endothelial cells. Aside from crystalluria and rare stones that may occur in patients with renal dysfunction, ascorbate is without important adverse effects at doses 2 to 10 g/d.33 Ascorbate was selected not only for its strong scavenging H2O2 activity within plasma and PMNs but also for its effect in improving endothelial function and vasodilatation and preventing the formation of oxLDL-induced leukocyte–platelet aggregates in the blood stream.34 Ascorbate can protect vascular smooth muscle cells against apoptosis induced by oxLDL/lipid hydroperoxides (eg, PCOOH).35 Ascorbate is more effective than vitamin E in scavenging plasma H2O2 activity, and vitamins C and E appeared to be less effective for protection of HOCl-mediated oxidative stress. Ascorbate protects HOCl-mediated LDL oxidation for only a short period.32 Simvastatin and probucol are mild antioxidants that did not appear to have a significant effect on reduction of acute apheresis-induced oxidative stress in the hyperlipidemic patients. However, lipid-lowering therapy using the combination of LDL apheresis and lipid-lowering drugs may have a long-term potential role to ameliorate atherosclerotic injury in hyperlipidemic patients.36

Three lipid peroxidation products, MDA, PCOOH, and oxidized phosphatidylcholine (oxPC), in plasma are generally correlated well with the degree of LDL oxidation.10,22,37 Among these molecules, oxPC is the key molecule in oxLDL, is capable of inducing monocyte adhesion to endothelial cells and PMN migration, and is directly involved in the early development of atherosclerosis.37 PCOOH levels have a positive correlation with RH2O2 and RHOCI activity, whereas secondary oxidized products like MDA do not, indicating a critical role of PCOOH in lipid peroxidation. Simultaneous treatment with ascorbate further effectively attenuated PCOOH and other oxidative products in uremic and hyperlipidemic patients.

Inflammation plays a pivotal role in atherogenesis. In uremic and hyperlipidemic patients, morbidity may result from repetitive induction of the acute phase response and chronic inflammation.38 Apheresis is associated with damage to PMNs, increased production of ROS, and oxidative stress that leads to inflammation.4,39 In our study, levels of MCP-1 and TNF-α remained unchanged despite a significant reduction of most proteins after apheresis, suggesting increased production of the 2 inflammatory mediators during the 2-hour apheresis course. However, apheresis plus ascorbate administration effectively suppressed MCP-1 and TNF-α production. The patients receiving ascorbate had lower CRP levels than did patients without ascorbate treatment. Previous studies38,39 had shown that the CRP level is closely correlated with oxidative stress (including that evoked by leukocytes) and plays an important role in the pathogenesis of atherosclerosis. Lower ascorbate concentrations in association with higher CRP levels and severity of peripheral arterial diseases are found in intermittent claudicant patients.40

In summary, ascorbic-acid treatment that can reduce ROS, particularly H2O2, should be beneficial for minimizing oxidative damage to leukocytes and endothelial cells. This finding is of particular interest inasmuch as patients with renal disease receiving dialysis are susceptible to a deficit in ascorbate caused by its loss during dialysis and a restricted dietary intake of ascorbate. Increased antioxidant activity and decreased oxidative stress after long-term apheresis plus ascorbate treatment is expected as a long-term outcome that has therapeutic potential in improving ROS-induced atherogenic injury in uremic and hyperlipidemic patients. Ascorbate administration together with lipid-soluble antioxidants might be of clinical importance for preventing atherosclerotic disease in patients with uremia or hyperlipidemia who are undergoing hemodialysis.

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