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Oxidative Stress and Antioxidant Defenses in Renal Patients Receiving Regular Haemodialysis

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Patients with chronic renal failure, and particularly those receiving regular haemodialysis, have a high incidence of premature cardiovascular disease. Oxidative stress, which causes lipid peroxidation, may contribute to increase the risk of atherosclerosis. The results of the present study indicate that lipid peroxidation products (malonaldehyde and 4-hydroxyalkenals) are significantly increased in plasma of renal patients before dialysis and, although reduced, remained above the normal range after this treatment. Moreover, production of free radicals and reactive oxygen metabolites was increased in chronic renal failure patients, especially after dialysis. On the other hand, the antioxidant defenses of those patients were higher than those of normal subjects, as judged from the plasma levels of specific antioxidant molecules and from the plasma antioxidant capacity.

We also found that triglycerides were significantly higher in renal patients, both before and after dialysis, than in the control group.

These results suggest that patients on chronic haemodialysis are particularly prone to oxidative stress and that dialysis itself may worsen this condition. Rather than to a weakening of antioxidant defenses, the susceptibility of chronic renal failure patients to oxidative stress might be ascribed to an increased free radical and reactive oxygen metabolite production and to increased levels of oxidizable substrates, notably triglycerides with their unsaturated fatty acids.

Key words: Oxidative stress; Haemodialysis.

Introduction

Many pathological processes, including atherogenesis, carcinogenesis and ageing, have been associated with the damage induced by free radicals or reactive oxygen metabolites (ROMs) (1). In biological systems, the superoxide radical, hydrogen peroxide and the hydroxyl radical are the main representatives of these species, the last being the most reactive and potentially dangerous because it frequently attacks biological molecules by abstracting hydrogen (2).

The effects of ROMs and of other free radicals, mostly the induction of oxidative processes, notably lipoperoxidation, are opposed by a number of antioxidant defenses which act by removing the reactive species and limiting cell and tissue damage (3,4). Increased production of ROMs or free radicals, as well as an impairment of antioxidant defenses, may result in the condition known as "oxidative stress". This leads to a variety of biochemical lesions which could play a part in the development of pathological processes. In the case of atherosclerosis it is thought that oxidation of polyunsaturated fatty acids in plasma lipids, notably in low density lipoproteins (LDL) (5, 6), leads to the release of short-chain aldehydes such as malonaldehyde, which can modify key lysine residues on apolipoprotein B. The resulting modified LDL can be taken up by tissue macrophages in the arterial wall, leading to the formation of foam cells, a key early step in the initiation of the atherosclerotic plaque (7).

Other mechanisms by which oxidative stress may favour atherosclerosis are: high density lipoprotein (HDL) oxidation, resulting in an impairment of their function in reverse cholesterol transport (8); endothelial dysfunction, affecting vasoregulation, platelet and monocyte adhesion, vascular smooth muscle cell growth, and coagulation.

Several reports indicate a high incidence of premature cardiovascular disease in patients with chronic renal failure (9). This has been ascribed to various factors, such as an increased prevalence of hypertension, diabetes, and dyslipidaemia (10); however these conditions do not seem sufficient by themselves to account for the higher risk of cardiovascular disease observed in patients with renal disease compared with the general population. On the other hand, several strands of evidence suggest that an accelerated lipid peroxidation may occur in plasma of patients with chronic renal failure (CRF), notably in those on maintenance haemodialysis (11, 12). Thus, oxidative stress may favour atherogenesis in these patients.

The present paper reports a study that we conducted on a group of subjects receiving regular haemodialysis, in order to verify the effect of this treatment on the development of oxidative stress and to identify the main mechanisms involved. Plasma susceptibility to oxidation and total antioxidant capacity were also investigated in our subjects to ascertain the efficiency of protective mechanisms involved in limiting free radical damage.

Materials and Methods

Twenty patients (mean age 62.6 years) receiving regular haemodialysis for severe renal failure and twenty healthy individuals matched for age and sex were recruited. Smokers, diabetics, and patients with chronic inflammatory conditions or hepatic or respiratory disease were excluded.

All patients had been on regular dialysis for an average of 142 months and were dialyzed three times a week, each time for 4 hours receiving heparin as anticoagulant.

Venous blood samples were taken after an overnight fast immediately before and after haemodialysis. When plasma samples were not used immediately they were stored at -70 °C until assay. All the subjects included in the present study gave their informed consent.

All chemicals were purchased from Sigma Chemicals, (Milano, Italy). The Bioxytech LPO-586 kit from OXIS International S.A. (Portland, OR, USA), the d-ROMs Test, Diacron (Grosseto, Italy) and the Total Antioxidant Status (TAS) Test, Randox, (Gumlin, UK), were used.

Measurement of malonaldehyde and 4-hydroxyalkenals

Estimation of lipid peroxidation products, malonaldehyde and 4-hydroxyalkenals, in plasma of renal patients and control subjects was performed by colourimetric assay (Bioxytech LPO-586 kit from OXIS International S.A.). For this assay blood was collected into EDTA and centrifuged at 3000 x *g* for 15 min, within 30 min after venesection.

Plasma (200µl) was added to 650µl of reagent containing one volume of 100% methanol and three volumes of the chromogenic reagent (N-methyl-2-phenylindole in acetonitrile). After mixing, 150µl of methanesulfonic acid were added. The mixture was then incubated for 40 min at 45 °C, cooled on ice and centrifuged at 10000 x *g* for 5 min. The clear supernatant was used for spectrophotometric assay at 586 nm. Values were calculated on the basis of a previously determined standard curve which ranged from 0 to 20 µmol/l.

Assessment of plasma total antioxidant capacity and determination of susceptibility to oxidative stress

Total antioxidant capacity (TAC) was performed using an assay (TAS, Randox) based on the capacity of plasma antioxidants to suppress the blue-green colour of the radical cation $ABTS^{\cdot+}$. This is produced by the reaction of metmyoglobin on 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) in the presence of H_2O_2 (13). Antioxidants contained in the sample inhibit the production of colour to a degree which is proportional to their concentration. TAC was expressed as mmol/l on the basis of a standard solution of 6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid (Trolox). The sample used was heparinized plasma (20µl), which could be stored frozen up to 14 days before the assay. The test was performed at 37 °C and at a wavelength of 600 nm.

Plasma susceptibility to oxidative stress was assessed by the procedure of Cestaro *et al.* (14) with slight modifications. One millilitre of an aqueous solution of cupric sulphate (2.5 mmol final concentration) was added to 1 ml of heparinized plasma. The mixture was then incubated at 37 °C for 3 min. The kinetics of plasma oxidation were determined at 37 °C by monitoring the development of fluorescence at 430 nm, setting the excitation wavelength at 360 nm on a Shimadzu RF-5000 spectrofluorophotometer (Kyoto, Japan). The fluorimetrically determined kinetic profile of each plasma sample was divided into latency, propagation and steady state phases. The lag time, expressed in minutes, was calculated as the intercept of the linear regression line of the propagation phase with that of the lag phase. The lag time was used as a measure of plasma oxidation resistance. For these calculations the FigP Microsoft software was used.

Determination of ROMs

This was accomplished by an assay (d-ROMs test, Diacron) based on the ability of free radicals to react at 37 °C with a phenol-derived compound; this in turn is converted to a free radical producing a stable colour detected photometrically at 505 nm (15).

For this assay freshly drawn heparinized plasma (5µl) was incubated for 75 min at 37 °C and pH 4.8 with the chromogenic reagent. Under these conditions ferric ions are dissociated from transferrin, thus the free radicals which react may include both those already present in plasma and those produced from hydroperoxides (H_2O_2 and lipid hydroperoxides) through the Fenton reaction. Results are expressed in conventional units, 1 unit being equivalent to 23.5 µmol/l H_2O_2.

Biochemical assays

Plasma transferrin, iron, copper, triglycerides, total cholesterol, urea, uric acid, creatinine and bilirubin were determined by standard laboratory methods.

Plasma ascorbic acid concentration was determined by the modified HPLC procedure of Lazzarino *et al.* (16).

Statistical analysis

All data are expressed as mean values ± S.E.M.. Statistical analysis was performed using Student's *t* test. Significance was accepted at the $p < 0.05$ level.

Results

Plasma concentrations of lipid peroxidation late products, malonaldehyde and 4-hydroxyalkenals, measured in the renal patients before dialysis were significantly higher than the values observed in the control group. The levels of these compounds, although markedly reduced, still remained above the normal range after dialysis (Fig. 1).

Figure 2 presents the results of measurements of the plasma levels of ROMs in normal subjects and in CRF patients before and after dialysis. Our data, in agreement with other reports (17), indicate a significant enhancement of ROM production in renal patients compared to the control group. We also found that plasma ROM levels in CRF patients were significantly higher after dialysis than before this treatment, which suggests a role of dialysis itself in the generation of these species.

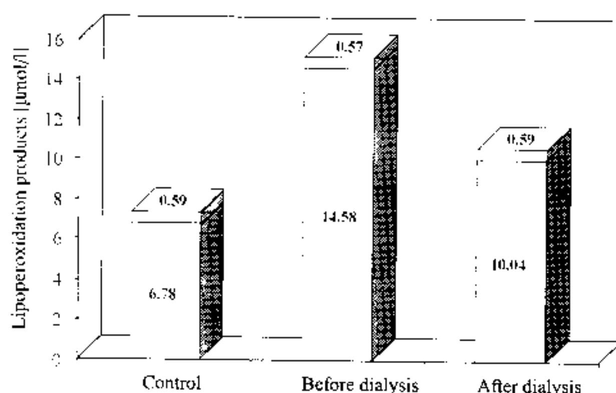

Fig. 1 Plasma levels of malonaldehyde and 4-hydroxyalkenal lipoperoxidation products in subjects before and after haemodialysis compared to normal controls. Columns represent mean values ± S.E.M. All differences were significant at the $p<0.05$ level.

Table 1 shows the biochemical parameters determined in the plasma of the subjects. Triglycerides were significantly higher in the renal patients, both before and after dialysis, than in the control group. As for the plasma levels of endogenous antioxidant substances, urate and creatinine concentrations in pre-dialysis patients were significantly increased with respect to control subjects. After haemodialysis there was a fall in urate, while creatinine concentrations decreased but still remained higher than control levels. Ascorbic acid concentrations, which showed a marked decrease in pre-dialysis patients with respect to normal subjects, fell to even lower levels after dialysis. We found a significant increase of bilirubin in pre-dialysis patients with respect to the control group; after dialysis plasma bilirubin concentrations remained higher than control values. Transition metal ions iron and copper, which are able to cause free radical formation, did not show significant modifications in CRF patients with respect to normal controls. Transferrin, which has an antioxidant action because of its ability to reduce free iron, did not alter either.

The results of measurements of TAC are shown in Figure 3. TAC was significantly higher in pre-dialysis patients than in the control group and it remained above normal values after dialysis, indicating a persistence of considerable amounts of antioxidant substances in plasma. The lag time before oxidation was used as an index of plasma resistance to oxidation. In the pre-dialysis patients the mean lag time was about

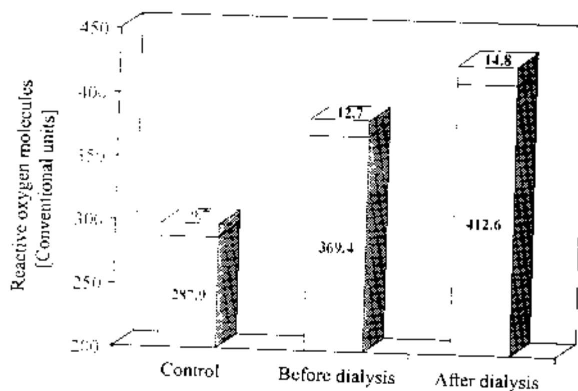

Fig. 2 Plasma reactive oxygen molecules (ROM) levels determined in subjects before and after haemodialysis compared to normal controls. ROM level was assessed by the d-ROMs assay. Values are expressed in conventional units, 1 unit being equivalent to 23.5 µmol/l H_2O_2. Columns represent mean values ± S.E.M. All differences were significant at the p<0.05 level.

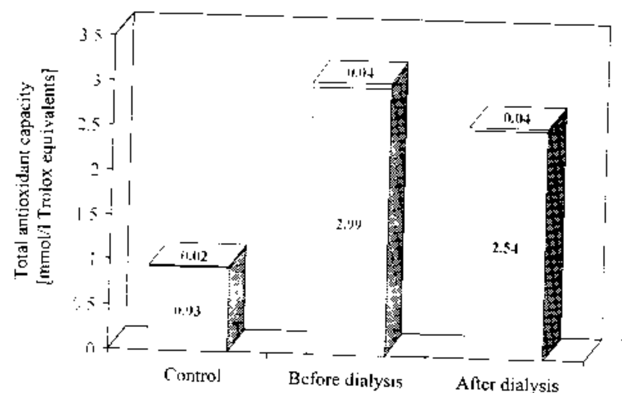

Fig. 3 Plasma total antioxidant capacity (TAC) in pre-dialysis and post-dialysis patients. TAC is expressed as mmol/l Trolox equivalents. Columns represent mean values ± S.E.M. All differences were significant at the p<0.05 level.

Tab. 1 Substances determined in the plasma of pre-dialysis and post-dialysis patients and in the control group.

	Pre-dialysis n = 20	Post-dialysis n = 20	Control n = 20
Transferrin (mg/l)	1771.9±92.4	1963.8±108.9	2002.1±125.5
Iron (µmol/l)	16.05±1.00	18.3±1.26	17.04±1.13
Copper (µmol/l)	18.68±0.71[b]	21.56±0.84	18.92±0.82
Triglycerides (mmol/l)	2.38±0.27[a]	2.01±0.21[a]	1.26±0.13
Cholesterol (mmol/l)	4.88±0.28	5.48±0.30	4.99±0.29
Urea (mmol/l)	24.66±2.06[a,b]	7.20±0.70	7.52±0.71
Uric acid (µmol/l)	428.80±2.08[a,b]	204.61±13.09[a]	274.8±14.87
Creatinine (µmol/l)	771.73±41.55[a,b]	303.21±16.80[a]	99.00±7.96
Bilirubin (µmol/l)	16.24±1.37[a,b]	15.05±0.68	12.31±0.51
Ascorbic acid (µmol/l)	118.2±4.9[a,b]	59.5±3.1	419.5±11.2

Values are means ± S.E.M. [a] significantly different at p<0.05 from the control group; [b] significantly different at p<0.05 from the post-dialysis group.

four fold greater than in the controls (Fig. 4) indicating the presence, in the plasma of these patients, of a higher degree of "protection" against oxidative stress. After dialysis the lag time, although lower, was still significantly higher than that of normal subjects.

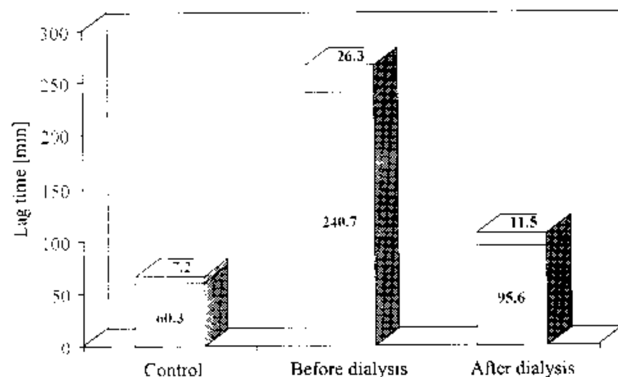

Fig. 4 Plasma susceptibility to copper-induced oxidation. All differences were statistically significant at the p<0.05 level.

Discussion

In keeping with previously published findings (18), the increased concentrations of malonaldehyde and 4-hydroxyalkenals clearly indicate an increased rate of lipid peroxidation in CRF patients compared to the control group. Lipid peroxidation represents one of the main effects of oxidative stress, which results from an imbalance between the production of free radicals (or other reactive species) and the efficiency of antioxidant defenses. The d-ROMs assay procedure which was used to measure ROM concentrations, determines both free radicals endogenously contained in the sample and hydroperoxides, notably H_2O_2 and lipid hydroperoxides. Since H_2O_2 is itself a ROM and lipid hydroperoxides are early products of lipid peroxidation, a process for which ROMs are chiefly responsible, this assay may be considered to indicate both directly and indirectly the formation rate of these reactive species. The observed increase in plasma ROM concentrations in our study groups leads to the conclusion that CRF patients exhibit an enhanced production of reactive oxygen species and that this process is triggered or, at least, exacerbated, by haemodialysis. Neutrophil leukocytes are probably responsible for this effect of dialysis, particularly since changes in concentrations of transition metal ions, which might represent another possible mechanism, were not observed in the present study. Also, haemodialysis could result in activation of complement which, in turn, could stimulate neutrophils to generate free radicals, notably superoxide, through the NADPH oxidase system (19). A similar mechanism has been invoked to explain the pulmonary dysfunction in early haemodialysis (20) and further support for the concept that dialysis itself plays a role in the generation of free radicals comes from studies where different plasma levels of lipid peroxidation products were observed in dialyzed subjects according to the type of membrane that was used (21).

Besides an enhanced generation of free radicals and other reactive oxygen species, another factor contributing to the accumulation of lipid peroxidation products in CRF patients may be their lipid levels. In agreement with other authors we found that both pre-dialysis and post-dialysis subjects showed plasma triglyceride concentrations significantly higher than normal controls; in fact it has been observed that patients with chronic kidney disease have an abnormal lipid profile characterized by hypertriglyceridaemia and low HDL cholesterol with a low HDL-cholesterol to total cholesterol ratio (23). As a consequence of their hypertriglyceridaemia our subjects probably have increased concentration of plasma unsaturated fatty acids which are substrates for peroxidative reactions. Furthermore, heparin treatment of haemodialyzed patients could result in an increase in plasma concentration of free fatty acids and it is possible that free unsaturated fatty acids are more susceptible to free radical attack than when they are esterified (17). Nevertheless, in our CRF patients, the protection against oxidative stress appeared not to be impaired. In fact, it was higher than in normal controls, a finding which may explain the apparent discrepancy between the increased levels of lipid peroxidation products and the reduced susceptibility of the plasma to copper-induced oxidation. As for the behaviour of individual antioxidant substances, the plasma concentrations of ascorbic acid that we measured in the present study showed a marked decrease in CRF patients compared to normal subjects. This is probably due to the patients' diet which permits only a very small ascorbate intake in order to restrict potassium intake. In addition, ascorbic acid is a small, water-soluble molecule which can be easily lost during dialysis. On the other hand, studies indicating that other important antioxidant compounds (α-tocopherol, retinol, β-carotene and thiol groups) are not significantly modified in plasma of patients with CRF (18, 23) suggest that the increased plasma TAC of our patients is mainly due to the increased levels of urate. This could be expected (23), given the key role of the kidney in the elimination of urate. Creatinine and bilirubin could contribute to the same effect and, given the marked reduction of urate after dialysis, are probably responsible for the fact that post-dialysis CRF patients exhibited a still higher plasma TAC than normal controls.

In conclusion, the results of the present study suggest that patients on chronic haemodialysis are particularly susceptible to oxidative stress and that dialysis itself may facilitate this. Rather than to a weakening of antioxidant defenses, the propensity of these patients to develop oxidative stress seems to be due to an increased generation of free radicals, together with other reactive oxygen species, and to increased levels of oxidable substrates, notably triglycerides with their unsaturated fatty acids.

In any case, it seems reasonable to suggest that therapeutic treatments aimed at strengthening the antioxidant defenses and normalizing lipid concentrations would be useful for protecting chronically haemodialyzed patients against oxidative stress.

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