Increased intracellular reactive oxygen species in patients with end-stage renal failure: Effect of hemodialysis

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**Background.** Reactive oxygen species (ROS) have been implicated in various forms of cellular injury. ROS may cause cell damage and are involved in the pathophysiology of several diseases, including atherosclerosis and chronic inflammation.

**Methods.** Disturbances of intracellular ROS levels were investigated in 28 patients with end-stage renal failure. The intracellular ROS levels were measured in lymphocytes before and after hemodialysis using biocompatible membranes and were compared with those from 11 patients with end-stage renal failure, not yet on renal replacement therapy, and 27 healthy control subjects. ROS levels were measured spectrophotometrically using the intracellular dye dichlorofluorescin diacetate.

**Results.** The spontaneous production of ROS was significantly higher in lymphocytes from patients with end-stage renal failure compared with healthy control subjects (P < 0.01). The addition of 100 nmol/L phorbol-myristate-acetate (PMA) produced a significant increase of ROS, both in lymphocytes from patients with end-stage renal failure and healthy control subjects. The PMA-induced ROS increase was significantly higher in lymphocytes from patients with end-stage renal failure compared with healthy control subjects (P < 0.01). In patients with end-stage renal failure, not yet on renal replacement therapy, the PMA-induced ROS was also significantly higher compared with healthy control subjects. The PMA-induced ROS increases were significantly inhibited by catalase, but not by superoxide dismutase or the superoxide dismutase mimetic, tempol. PMA-induced ROS was significantly reduced by tyrphostin A51 in lymphocytes from patients with end-stage renal failure and from healthy control subjects (each P < 0.01), indicating the involvement of a tyrosine kinase-dependent pathway. In patients with end-stage renal failure, the spontaneous and the PMA-induced production of ROS was not significantly different before and after hemodialysis.

**Conclusions.** Regular hemodialysis sessions using biocompatible membranes have no effect on the elevated intracellular ROS in patients with end-stage renal failure.

Reactive oxygen species (ROS) are byproducts of normal metabolic processes in cells. The major sources of these radicals are leakages from the electron transport chains of mitochondria and endoplasmic reticulum. At low concentrations, ROS act as physiological mediators of cellular responses, including activation of lymphocytes, whereas higher concentrations may cause cell damage [1–3]. Increased ROS are thought to be involved in the pathophysiology of diabetes mellitus, atherosclerosis, hypertension, or chronic inflammatory diseases, including nephritis [4–7]. ROS have been shown to induce apoptosis in glomerular mesangial cells [8] and vascular smooth muscle cells [9]. Reduced antioxidative defense mechanisms, for example, the reduced activity of superoxide dismutase (SOD), glutathione peroxidase, and catalase in erythrocytes, have been described in chronic renal failure [10]. On the other hand, changes of lymphocyte function have also been detected in chronic renal failure [11–13].

There is little evidence that increased oxidative stress in patients with chronic renal failure is associated with uremic complications [14, 15]. In particular, increased hydroxyl radicals have been associated with uremic hypertension [16]. Since lipid peroxidation products, malondialdehyde and 4-hydroxyalkenals, were found to be increased in plasma of patients with end-stage renal failure before and after hemodialysis [17], both a weakening of antioxidant defenses or an increased production of ROS during hemodialysis might be involved in the pathogenesis of the uremic syndrome or in the development of complications during long-term hemodialysis treatment.

In contrast to measurements of plasmic lipid peroxidation products or the activity of antioxidant enzymes in erythrocytes, determinations of intracellular ROS may reflect the cellular oxidative stress more directly. Therefore, in the present study, we investigated the intracellular ROS levels, for example, superoxide radical (O₂⁻) and hydrogen peroxide (H₂O₂), in patients with end-stage renal failure before and after hemodialysis using a fluorescent dye technique. It is shown that patients with end-stage chronic renal failure show increased spontaneous and stimulated levels of ROS, and intracellular ROS were not significantly changed during hemodialysis treatment.
METHODS

Patients
After giving informed consent, 27 healthy control subjects (9 males and 18 females, mean age of 68 ± 5 years, systolic blood pressure 131 ± 3 mm Hg, diastolic blood pressure 77 ± 2 mm Hg) with normal renal function, 11 patients with end-stage renal failure (4 males and 7 females, mean age of 65 ± 2 years), not yet on renal replacement therapy, and 28 stable patients with end-stage renal failure (11 males and 17 females, mean age of 72 ± 2 years, systolic blood pressure 146 ± 4 mm Hg, diastolic blood pressure 76 ± 2 mm Hg), who had been undergoing maintenance hemodialysis for 22 ± 6 months (mean ± SEM), were enrolled in this study. The cause of end-stage renal failure was chronic glomerulonephritis in 10 cases, diabetic nephropathy in 10 cases, nephrosclerosis in 7 cases, and polycystic kidney disease in 1 case. All patients were stable and free from intercurrent illness. As confirmed by the clinical examination and routine laboratory examination, patients were in a good state of health. All of the patients were routinely dialyzed for four to five hours three times weekly using biocompatible membranes (polysulfone; Fresenius Medical Care, Bad Homburg, Germany; acrylonitrile and sodium methallyl sulfonate copolymer; Hospal, Nürnberg, Germany) with no dialyzer reuse. The dialysates used were bicarbonate based. Kt/V values (the amount of plasma cleared of urea divided by the urea distribution volume) was measured according to the formula:

\[ \text{Kt/V} = -\ln (R - 0.03) + (4 - 3.5 \times R) \times \frac{\text{UF}}{\text{W}} \]

where R is the post-/pre-plasma urea nitrogen ratio; UF is the ultrafiltrate volume (L) removed, and W is the postdialysis weight (kg) [18]. Kt/V values were 1.34 ± 0.05 (N = 28). The normalized protein catabolic rate values [nPCR; measured according to this formula:

\[ \text{nPCR} = \frac{C_0}{(36.3 + 5.48 \times \text{Kt/V} + 53.5/\text{Kt/V}) + 0.168; \text{using } C_0 = \text{pre-plasma urea nitrogen}] \]

were 1.01 ± 0.07 g/kg/day (N = 28).

Preparation of lymphocytes
Blood samples were drawn from the antecubital veins of healthy control subjects and from the arterial side of the arteriovenous fistula of patients with end-stage renal failure. The lymphocytes were isolated from heparinized blood samples according to previously described procedure [20]. In brief, the blood was centrifuged at 150 × g for 15 minutes, and the supernatant was removed. The blood cells were then diluted with physiological salt solution (1:1), and 5 mL of the mixture were layered on 3 mL of lymphoprep (5.6% wt/vol; density 1.077 g/mL; Nycomed, Oslo, Norway) and then centrifuged for 15 minutes at 240 × g. The lymphocyte interphase was carefully aspirated and washed in physiological salt solution through centrifugation for five minutes at 240 × g. The cells were then resuspended in physiological salt solution ready for use. The physiological salt solution used throughout this study had the following composition (in mmol/L): NaCl 136, KCl 5.4, KH2PO4 0.44, Na2HPO4 0.34, d-glucose 5.6, CaCl2 1.0, MgCl2 1.0, and HEPES 10, pH 7.4.

Measurement of reactive oxygen species
The lymphocytes were incubated in the dark with the dye 2′,7′-dichlorofluorescin diacetate (DCF-DA, 5 mmol/L; Calbiochem, Bad Soden, Germany) for 15 minutes prior to the measurement of ROS concentrations [21–24]. DCF-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative DCFH and thereby trapped within the cells [22]. In the presence of ROS, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). The level of DCF fluorescence, reflecting the concentration of ROS, was monitored using a fluorescence spectrophotometer (F-2000 Hitachi Ltd., Tokyo, Japan) at 534 nm emission with an excitation wavelength of 488 nm. To minimize the DCFH, photo-oxidation samples were kept in the dark, and the fluorescence was rapidly collected within 60 seconds using identical parameters, such as bandwidth and brightness, for all samples. Under these circumstances, spontaneous photooxidation of the dye was less than 1%. Using H2O2, a calibration curve of the DCF fluorescence could be measured. As shown in Figure 1, the DCF fluorescence showed a nonlinear increase with increasing H2O2 concentrations.

Lymphocytes were incubated for 30 minutes with phorbol-12-myristate-13-acetate (PMA; Sigma Chemical Co., Deisenhofen, Germany). The inactive phorbol ester 4α-phorbol-12,13-didecanoate (4α-PDD) was used as a negative control. Measurements were made in the absence or presence of enzyme inhibitors and scavengers, 2-[8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrind (1,2-α-indol-3-yl)-3-(1-ethylindol-3-yl) maleimide, hydrochloride (Ro-32-0432; 140 nmol/L; protein kinase C inhibitor), 2-Amino-4-(3′,4′,5′-tri-ghydroxyphenyl)-1,1,3-tricyanobuta-1,3-diene (tyrphostin A51; 4 μmol/L; tyrosine kinase inhibitor), SOD (40 μg/mL, superoxide radical scavenger), tempol (membrane-permeable SOD-mimetic; Alexis Biochemicals, Grünberg, Germany), and catalase (5 μg/mL, H2O2 scavenger). All substances were freshly prepared from stocks for each day’s experiments, and concentrations given indicate final concentrations after all additions. All substances were purchased from Sigma or Calbiochem (La Jolla, CA, USA), if not indicated otherwise.

Statistics
Data are means ± SEM. Differences were compared with the Wilcoxon–Mann–Whitney test or t-test using the computer software GraphPad Prism 2.0 (GraphPad Software, San Diego, CA, USA).
Fig. 1. Calibration of 2',7'-dichlorofluorescin (DCF) fluorescence. The calibration curve shows the increases in DCF fluorescence after the addition of increasing amounts of \( \text{H}_2\text{O}_2 \). The fluorescence excitation was 488 nm (bandwidth, 10 nm), and the emission was collected at 534 nm (bandwidth, 10 nm). Values are the means from duplicate determinations.

Software, San Diego, CA, USA). Two-tailed values of \( P < 0.05 \) were considered significant.

RESULTS

The spontaneous production of ROS in lymphocytes was monitored over a period of 30 minutes. Within 30 minutes, ROS increased by \( 88 \pm 4\% \) \((N = 27) \) above the initial level in unstimulated lymphocytes from healthy control subjects, whereas ROS increased by \( 128 \pm 10\% \) in lymphocytes from patients with end-stage renal failure \((N = 28, P < 0.01 \) compared with healthy control subjects). Two types of biocompatible membranes (polysulfone, and acrylonitrile and sodium methylsulfonate copolymer, AN69) were used for hemodialysis. Therefore, data were analyzed for possible differences between the two dialyzers. However, the spontaneous production of ROS in lymphocytes was not significantly different between the two dialyzers (polysulfone, 143 \( \pm 17\% \); AN69, 115 \( \pm 12\%, P = 0.19 \)). In patients with end-stage renal failure who were not yet on renal replacement therapy, the spontaneous production of ROS was not significantly different compared with healthy control subjects (99 \( \pm 15\%, N = 11, \) vs. 88 \( \pm 4\%, N = 27, P = 0.32 \)).

Lymphocytes were stimulated with the phorbol ester PMA. A concentration response curve is shown in Figure 2. PMA dose-dependently induced the production of ROS, and saturation was reached at a concentration of 100 nmol/L. Additional experiments showed that the inactive phorbol ester 4\( \alpha \)-PDD did not induce the production of ROS in lymphocytes.

The PMA (100 nmol/L)-induced production of ROS was significantly higher in lymphocytes from patients with end-stage renal failure compared with healthy control subjects (252 \( \pm 33\%, N = 28, \) vs. 99 \( \pm 1\%, N = 27, P < 0.01; \) Fig. 3). The PMA-induced ROS in lymphocytes was not significantly different between the two dialyzers (polysulfone, 250 \( \pm 52\% \); AN69, 254 \( \pm 43\%, P = 0.80 \)). In patients with end-stage renal failure, not yet on renal replacement therapy, the PMA-induced ROS was significantly higher compared with healthy control subjects (631 \( \pm 149\%, N = 11, \) vs. 99 \( \pm 1\%, N = 27, P < 0.01 \)).

The PMA-induced production of ROS was significantly reduced in the presence of the \( \text{H}_2\text{O}_2 \) scavenger catalase (5 \( \mu \text{g/mL} \)) both in lymphocytes from patients with end-stage renal failure (148 \( \pm 10\%, P < 0.01 \)) and
mediated by a tyrosine kinase-dependent pathway. These pathways could both in lymphocytes from patients with end-stage renal failure and healthy control subjects. These findings support earlier reports on the regulatory effect of tyrosine kinase on production of ROS in other cells [29]. The PMA-induced production was significantly reduced both before and after hemodialysis in the presence of the specific protein kinase C inhibitor Ro-32-0432 and in the presence of the specific tyrosine kinase inhibitor tyrphostin A51.

**DISCUSSION**

In the present study, intracellular ROS were measured in lymphocytes using the fluorescent dye technique. In lymphocytes from patients with end-stage chronic renal failure, an increased spontaneous and PMA-induced ROS production could be observed. The PMA-induced ROS production was also increased in a group of patients with end-stage renal failure, not yet on renal replacement therapy, indicating that an increased ROS production is an effect of uremia rather than a long-term effect of the hemodialysis treatment. The production of ROS is part of cellular life, and in lymphocytes, ROS production may be necessary for activation and proliferation [25, 26]. On the other hand, increased ROS may be harmful to cells and cause functional disabilities. In fact, higher ROS concentrations have been shown to cause cell damage [2, 3]. The generation of ROS leads to the activation of protein tyrosine kinases followed by the stimulation of several signaling systems, including mitogen-activated protein kinases, nuclear transcription factors, caspases, and intracellular calcium concentrations [27, 28]. Impaired lymphocytic function in chronic renal failure has been described earlier [11–13]. The present study indicates that one mechanism causing impaired lymphocytic function in chronic renal failure may be due to an increased ROS production.

Using specific inhibitors, it could be shown that the PMA-induced production of ROS was mediated by a tyrosine kinase-dependent pathway. These pathways could be observed both in patients with end-stage chronic renal failure and in healthy control subjects. These findings support earlier reports on the regulatory effect of tyrosine kinase on production of ROS in other cells [29]. At present, it is not known whether the increased spontaneous production of ROS in patients with end-stage chronic renal failure may be caused by changes of activity of the tyrosine kinase caused by uremic toxins.
In the present study, lymphocytes were used to compare predialytic and postdialytic reactions, since neither the total number of lymphocytes nor the types of are markedly changed during hemodialysis [30]. The study indicates that an increased ROS production could be observed before and after hemodialysis. Therefore, the hemodialysis session using biocompatible membranes may not be the cause of the increased spontaneous ROS production. Instead, the incomplete removal of toxins accumulating in uremia may be responsible for the increased ROS production in patients with end-stage chronic renal failure. On the other hand, it has been reported that the decreased serum antioxidant activity can be improved by hemodialysis treatment [31]. Therefore, it is speculated that the improved serum antioxidant activity after the hemodialysis session cannot compensate for the increased intracellular ROS causing cellular damage. Earlier findings indicate that both biocompatible and bioincompatible hemodialysis membranes induce cytokines and activate complement, but biocompatible membranes avidly adsorb their activation products [32]. Since in the present study only biocompatible membranes were used, a similar phenomenon may be responsible for the finding that regular hemodialysis using biocompatible membranes has no effect on the elevated intracellular ROS in patients with end-stage renal failure.

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