Oxidative stress during dialysis: effect on free radical scavenging enzyme (FRSE) activities and glutathione (GSH) concentration in granulocytes

Volker Schettler¹, Eberhard Wieland², Heiko Methe¹, Peter Schuff-Werner², and Gerhard Anton Müller¹

¹Department of Nephrology and Rheumatology, ²Department of Clinical Chemistry, Georg-August-University, Göttingen, FRG

Abstract

Background. Living cells are protected by free radical scavenging enzymes against oxygen radical-mediated damage. It has been suggested that granulocytes are activated on the surface of dialyser membranes, resulting in the generation of free radicals. We have recently reported a lack of plasma lipid peroxidation and unchanged glutathione peroxidase (GSH-Px) as well as glutathione reductase (GSSG-R) activities in red blood cells of haemodialysis patients. However, because mature red cells are free of DNA and RNA, free radical scavenging enzymes (FRSE) cannot be regulated on the gene level in response to an acute oxidative stress. In contrast to erythrocytes, granulocytes are nucleated cells and FRSE protein concentrations can therefore be modulated.

Methods. GSH-Px, GSSG-R, superoxide dismutase (SOD) activities and total glutathione (GSH) were determined spectrophotometrically using a Cobas Fara semi-automatic analyser in granulocytes of 31 healthy blood donors and in 28 patients with chronic renal failure (CRF) for more than 6 months before as well as immediately after a single dialysis treatment. Patients were treated either by haemodialysis (n=17) using low-flux polysulphone membranes or by haemofiltration (n=11) using high-flux polysulphone membranes.

Results. Compared to healthy controls, SOD and GSSG-R activities were increased in granulocytes of HD and HF patients, GSH and GSH-Px were decreased before a single treatment. After dialysis SOD and GSH-Px activities were significantly induced by both HD and HF whereas GSSG-R activities and GSH were decreased.

Conclusions. These results show that the enzymatic defence against oxygen radicals can be induced in granulocytes of patients undergoing regular dialysis treatment, whereas the non-enzymatic defence is compromised as shown by decreased GSH concentrations, both suggesting increased oxidative stress.

Key words: extracorporeal circulation; free radical scavenging enzyme activity; granulocyte; haemofiltration; haemodialysis; oxygen radicals

Introduction

Oxygen radicals are toxic and are thought to be involved in the pathogenesis of a variety of diseases including chronic renal failure (CRF) [1]. Haemodialysis treatment has been suggested to impose an additional oxidative stress to patients with CRF by activation of granulocytes on dialyser membranes [1–3] or the energy of light to which blood components are usually not exposed under physiological conditions [4]. Granulocytes take part in inflammatory reactions in the body. They are activated by mediators such as cytokines or arachidonic acid metabolites to perform their phagocytic inflammatory reactions. Once activated, they undergo a respiratory burst with the concomitant release of oxygen-derived free radicals including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radicals (OH·), and hypochlorous acid (HOCl). Indiscriminate activation of granulocytes leads to tissue damage shown as oxidation of lipid membranes of adjacent cells [5,6] as well as of granulocytes themselves [7]. Former investigations indicated a dysfunction of granulocytes in patients undergoing long-term haemodialysis [8,9].

The release of oxygen radicals from granulocytes has been suggested to be involved in the formation of plasma lipid peroxidation products in haemodialysis patients [10]. However, we were not able to confirm this, as reported recently [11]. We assumed a very effective protection system provided by enzymatic and non-enzymatic antioxidants in these patients. In model systems with e.g. intestinal epithelial cells or mesangial cells it has been shown that free radical scavenging enzyme (FRSE) activities are regulated by the generation of extra- and intracellular oxygen radicals [12,13].
In the present investigation we therefore examined the FRSE activity and glutathione concentration in granulocytes of patients undergoing long-term haemodialysis or haemofiltration. FRSE were determined, by measuring the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GSSG-R).

**Subjects and methods**

**Patients and controls**

FRSE activities were determined in granulocytes of 17 patients (9 females, 8 males; mean age 70 ± 12 years) suffering from chronic renal failure who were undergoing regular haemodialysis treatment (HD patients) three times per week for more than 6 months. In 11 patients (6 females, 5 males; mean age 65 ± 12 years) who were undergoing regular haemofiltration treatment (HF patients) three times per week for more than 6 months, and in 31 healthy blood donors (11 females, 20 males; mean age 33 ± 10 years). HD-patients were treated with a low-flux dialyser (F6 HDS, DeNemours, France). Patients and controls

**Reagents**

Cytochrome C from horse heart, oxidized and reduced glutathione, glutathione peroxidase from bovine erythrocytes (EC 1.11.1.9), glutathione reductase from bakers yeast (EC 1.6.4.2), superoxide dismutase from horseradish (EC 1.15.1.1), xanthine, and xanthine oxidase from buttermilk (EC 1.1.3.22) were obtained from Sigma Chemical Co., St Louis, MO, USA. 1-Chloro-2,4-dinitrobenzol (CDNB), chloroform, hydrogen peroxide, monopotassium phosphate, dipotassium phosphate, sodium azide, monosodium carbonate, sodium carbonate, sodium chloride, sodium hydroxide solution (1.0 N), and hydrochloric acid (1.0 N) were purchased from E. Merck, Darmstadt, Germany. NADPH and haemoglobin colour kit MPR3 (Drabkin solution, cyano reagent) were from Boehringer Mannheim GmbH, Mannheim, FRG. Ethylenediamine tetraacetate acid (EDTA) was obtained from Serva Feinbiochemica GmbH & Co., Heidelberg, FRG. Ethanol was purchased from J. T. Baker B.V., Deventer, the Netherlands. Ampuwa® water, PBS-Dulbecco® were obtained from Gibco, Paisley, UK. NIM® was bought from Praesel and Lorei GmbH, Frankfurt, Germany and Lymphoprep® was purchased from Nycomed Pharma AS, Oslo, Norway. The Falcon® tubes were from Becton Dickinson, Labware, NJ, USA. All reagents were of analytical grade.

Blood specimens and isolation of granulocytes

Blood specimens (20 ml) from control individuals and patients were collected into a syringe containing 200 IU heparin. Blood specimens from the dialysis patients were collected from the arterial line of the shunt at the start of dialysis and at the end of dialysis. For granulocyte isolation, a 12-ml Falcon® tube was prepared with 4 ml NIM® overlayed with 1 ml Lymphoprep®, 5 ml of the blood specimen were carefully added on the top of the second layer. The Falcon® tube was then centrifuged at 400 g for 40 min at room temperature. After centrifugation the upper layer of the tube was decanted. The middle layer containing the granulocytes, was transferred into a new Falcon® tube containing 8 ml PBS-Dulbecco®. This tube was centrifuged at 400 g for 15 min, 4°C again. After centrifugation the supernatant above the pellet was removed and treated with ice-cold water to lyse contaminating erythrocytes. The pellet was washed twice (200 g for 15 min, 4°C) using PBS-Dulbecco®. The final pellet was dissolved in 2 ml PBS-Dulbecco® and the number of granulocytes as well as the purity of the cell preparation was determined using a cell counter (Coulter STKS, Coulter Electronics Tarrytown, NJ, USA). Cell preparations always contained > 93% granulocytes. For FRSE activity determination the granulocytes were stored at −80°C for less than 5 days.

**Determination of FRSE activities**

Granulocytes were lysed by freeze thawing and ultrasonification for 2 min. FRSE activities were measured semiautomatically using a Cobas Fara centrifugal analyser (Roche, DeNemours, France).

GSH-Px activity was measured according to Paglia and Valentine with minor modifications [14]. Reduced glutathione (52 mM) was dissolved in a reagent mixture composed of potassium phosphate buffer (50 mM, pH 7.0), EDTA (5 mM), and sodium azide (2 mM). To keep the glutathione in a reduced state, glutathione reductase was added to give a final activity of 10 U/ml. The generation of NAD(P)H was measured spectrophotometrically at 340 nm. The activity of GSH-Px was calculated using the molar extinction coefficient of NAD(P)H (6.2 × 10³ M/cm) and values are expressed as U/g protein.

GSSG-R activity was measured as originally described by Carlborg and Mannervik [15] using a potassium phosphate buffer (50 mM, pH 7.0) which contained EDTA (5 mM), NAD(P)H (1.5 mM) and oxidized glutathione (15 mM). NAD(P)H generation was followed at 340 nm. GSSG-R activity was calculated using the molar extinction coefficient of NAD(P)H (6.2 × 10³ M/cm) and data are expressed as U/g protein.

SOD activity was determined slightly modified according to McCord and Fridovich [16] using a sodium carbonate buffer (50 mM, pH 10) containing EDTA (0.1 mM), cytochrome C (10.5 μM), and xanthine (3 mM). The reaction was started by xanthine oxidase (0.14 U/ml) and data are expressed as U/g protein using an external standard curve of SOD. Cytochrome C reduction was followed at 550 nm. The glutathione concentration was determined as described by Dolphin et al. [17] using a commercially available test-kit from Bioxytech® GSH-400 (Oxis International S.A., Bonneuil sur Marne, Cedex, France) using a Beckman DU 7500 spectrophotometer (Beckman, Irvine, CA, USA).

**Other assays**

Protein was determined using the BCA protein assay reagent kit supplied by Pierce (Rockford, IL, USA).

**Statistics**

Data of patients and controls were analysed using SigmaStat 3.0 (Jandel Scientific Software, San Rafael, CA, USA) and
Results

Glutathione peroxidase activities (GSH-Px)

Before a single treatment of HD or HF was started, the GSH-Px activities in granulocytes of all patients undergoing extracorporeal treatment were significantly decreased (16.1±10.2 U/g prot. (HD); $P<0.001$ and 21.7±9.1 U/g prot. (HD); $P<0.001$) compared to controls (44.2±21.8 U/g prot.) (Figure 1). However, GSH-Px activities were significantly increased after both a single HD and HF treatment (36.1±22.8 U/g prot. (HD); $P<0.001$ and 37.2±20.7 U/g prot. (HF); $P<0.05$). HD and HF patients were not different with respect to their GSH-Px activities (Figure 1).

Glutathione reductase activities (GSSG-R)

Before a single treatment GSSG-R showed in both granulocytes of normal controls (4.7±2.4 μmol/l; controls) and GSH-Px activities were significantly increased after both a single HD and HF treatment (36.1±22.8 U/g prot. (HD); $P<0.001$ and 37.2±20.7 U/g prot. (HF); $P<0.05$). There was no significant difference of SOD activities between patients undergoing HD or HF (Figure 3).

Correlations among FRSE activities and between GSH and FRSE activities

There were no significant correlations ($P>0.05$) among SOD, GSH-Px, and GSSG-R activities or the GSH
Oxidative stress in granulocytes during dialysis

In living organisms is difficult because of the low concentration of radicals, which are usually below the detection limit of most commonly available methods [19]. Oxygen radicals are destructive to a variety of cell components including lipid membranes. Therefore, as an indirect measure of oxidative stress, lipid peroxidation has been used as an indicator [20]. Therapeutic regimens that depend on extracorporeal circuits such as haemodialysis are composed of artificial surfaces found in dialyser membranes and tubings and have been suggested to cause oxidative stress [21,22]. However, we have recently reported a lack of plasma lipid peroxidation in HD and LDL apheresis [23,24].

An alternative approach to monitoring oxidative stress is to determine the concentration of low-molecular-weight antioxidants such as glutathione or the activity of free radical scavenging enzymes such as SOD and GSH-Px [25]. In an ischaemia/reperfusion model with rat kidneys, activation of SOD has been observed due to oxidative stress [26]. Granulocytes may be particularly useful to monitor changes in FRSE activity since they are not only the source of oxygen radicals but also the target of their own endogenously produced radicals. The latter has been shown by lipid peroxidation of granulocyte cell membrane lipids upon activation of the NAD(P)H-oxidase [7]. During the contact of whole blood with artificial surfaces, granulocytes and other immune competent cells, in particular T-helper-cells, are activated, resulting in the release of radicals and other mediators such as cytokines, and chemokines (e.g. TNF-α or interleukin-1) [27,28]. This release of cytokines can further induce both the formation of oxygen radicals in granulocytes and the upregulation of FRSE activities in granulocytes [29]. We hypothesized that if haemodialysis therapy is associated with an increased production of oxygen radicals or cytokines, FRSE activity should be acutely regulated in granulocytes, thus protecting the cells against oxygen-radical-mediated injury. In addition, to assess the influence of different dialysis procedures on granulocyte FRSE activity, we compared two types of dialysis treatment: haemodialysis and haemofiltration using a polysulphone low-flux dialyser and a polysulphone high-flux dialyser respectively.

The chronic effects of uraemia and regular haemodialysis on the granulocyte antioxidant defence system were investigated by the determination of GSH-Px, GSSG-R, and SOD activity as well as by measuring intracellular GSH concentrations before a single dialysis treatment. Compared to healthy controls, reduced GSH-Px activities and GSH concentrations and increased SOD and GSSG-R activities were observed in granulocytes of patients undergoing regular HD or HF. A decrease of GSH-Px activity and GSH concentrations has been observed before in plasma of uraemic patients [30]. In addition, Koenig et al. [31] have recently reported a reduced GSH-Px activity also in erythrocytes of dialysis patients and suggested an association with plasma selenium concentrations, which were not determined in our study.

![Fig. 3. SOD activities in granulocytes of normal controls (n=31), of patients suffering from chronic renal failure undergoing regular haemodialysis treatment (HD) (n=17) and patients suffering from chronic renal failure undergoing regular haemofiltration treatment (HF) (n=17), (BD, before haemodialysis; AD, after haemodialysis; BF, before haemofiltration; AF, after haemofiltration). *P<0.05, controls vs patients before treatment; #P<0.05 before vs after treatment. Experimental conditions as described in Subjects and methods.](image)

![Fig. 4. GSH concentrations in granulocytes of normal controls (n=31), of patients suffering from chronic renal failure undergoing regular haemodialysis treatment (HD) (n=17) and patients suffering from chronic renal failure undergoing regular haemofiltration treatment (HF) (n=11), (BD, before haemodialysis; AD, after haemodialysis; BF, before haemofiltration; AF, after haemofiltration). *P<0.05, controls vs patients before treatment; #P<0.05 before vs after treatment. Experimental conditions as described in Subjects and methods.](image)

**Discussion**

Oxygen radicals released from granulocytes are able to induce oxidative stress, which can be defined as the disturbed balance between pro-oxidants and antioxidants [13,18]. The direct proof of radical production concentrations and the FRSE activities (SOD, GSH-Px, GSSG-R).

![Fig. 4. GSH concentrations in granulocytes of normal controls (n=31), of patients suffering from chronic renal failure undergoing regular haemodialysis treatment (HD) (n=17) and patients suffering from chronic renal failure undergoing regular haemofiltration treatment (HF) (n=11), (BD, before haemodialysis; AD, after haemodialysis; BF, before haemofiltration; AF, after haemofiltration). *P<0.05, controls vs patients before treatment; #P<0.05 before vs after treatment. Experimental conditions as described in Subjects and methods.](image)
A low concentration of reduced GSH in erythrocytes of haemodialysis patients has been observed by Durak et al. [32] and was explained by an inhibition of glutathione reduction. In contrast to the results of Durak et al., we measured total glutathione and therefore the decrease in granulocyte GSH cannot be explained by an impaired reduction of oxidized glutathione (GSSG). Furthermore, we found an increased activity of the glutathione-reducing enzyme GSSG-R in uraemic patients before a single dialysis treatment. The decrease of granulocyte GSH during the dialysis treatment points to either a consumption or a loss of GSH during the therapy, rather than to an effect of uraemia. Chronic haemodialysis treatment therefore obviously leads to an intracellular GSH depletion.

A changed FRSE activity pattern in granulocytes of dialysis patients has been reported and linked to hyperparathyroidism [8] which could not be confirmed in the present study. There was no correlation between plasma PTH concentrations and either SOD, GSSG-R, or GSH-Px activities (data not shown). However, no investigation regarding the acute effect of a single dialysis treatment on granulocyte FRSE activity has been performed so far.

Granulocyte FRSE activities were acutely affected by both dialysis procedures as shown in the present investigation. Immediately after a single treatment with either HD or HF we found an increase of SOD and GSH-Px activities in granulocytes of all patients, whereas the GSSG-R activity was decreased compared to the start of dialysis. Higher GSH-Px activities are associated with the formation of hydrogen peroxide or lipid hydroperoxides [33] leading to a removal of these undesired oxidation products. This may explain our previous finding that lipid hydroperoxides are not detectable in plasma of uraemic patients either before or after the dialysis procedure [23].

The reduction of GSSG-R activity can be explained either by regulation on the gene level or by the lack of the substrate GSH as mentioned above. In addition, the inhibition of enzyme activity could be caused by a depletion of NAD(P)H which is required for GSH reduction. NAD(P)H is of course consumed in granulocytes during radical generation by the NAD(P)H oxidase [34]. Furthermore, HOCl formed during the respiratory burst oxidizes GSH to GSSG and subsequently GSSG to a chloramine product, thus reducing its availability for GSSG-R [35]. These speculations cannot be substantiated by our data since we did not determine the ratio between oxidized and reduced glutathione, nor did we measure the NAD(P)H content of the granulocytes or chloramine products of GSSG.

The increased SOD activity is in contrast to Shurtz-Swirski et al. [8] who previously demonstrated a decrease of SOD activity in granulocytes of patients undergoing regular haemodialysis. However, the dialysis system used is not mentioned and may be different from those employed in the present investigation. Furthermore, these authors suggested an association between hyperparathyroidism and depressed SOD activity that was not observed in our study (data not shown).

The consumption or removal of GSH, the predominant intracellular low-molecular antioxidant, together with the induction of SOD and GSH-Px activities support the occurrence of an acute oxidative stress due to the dialysis procedure. Whether any other immunological or endocrinological deviations such as cytokine production or diabetes contribute to a modulation of FRSE activity in uraemia or haemodialysis needs further investigations.

Intracellular antioxidants such as glutathione are electron donors, which neutralize oxygen-derived radicals. Oxidized glutathione represents a second messenger, which can be involved in the regulation of FRSE gene expression. It has been shown in intestinal or mesangial cells that FRSE can be regulated by oxygen radicals responding with an upregulation of SOD on the mRNA as well as on the protein level upon challenge with H\textsubscript{2}O\textsubscript{2}. Nuclear transcription factors such as NF-\alphaB, AP-1 are known to be redox sensitive and have been shown to be involved in FRSE gene expression [13,27].

A statistical significant correlation among the activities of FRSE, GSH and the period of dialysis (HD and HF) was not observed. Even though an influence of age on FRSE in erythrocytes and plasma has been reported in the literature [36], this was not observed in the present investigation using granulocytes (data not shown). However, the number of individuals investigated here is too small to come to a final conclusion. In addition, our study was predominately aimed to see whether dialysis treatments do acutely induce a regulation of FRSE activities in granulocytes which can be assumed to be age independent.

We did not observe significant differences in FRSE activities between HD and HF, except for GSSG-R activity. This cannot be explained by different GSH concentrations, which were equal in both groups. However, there may be an effect of the membrane type used, low flux in haemodialysis and high flux in haemofiltration.

In summary, our findings show an acute effect of the extracorporeal therapy on granulocyte FRSE activities and glutathione concentrations which has not been reported before. Changes in granulocyte FRSE activities and GSH concentration may support the concept of increased oxidative stress in HD and HF and could explain the reduced bactericidal activity of granulocytes in chronic renal failure. Whether FRSE activities in granulocytes provide a tool to assess biocompatibility of dialyser membranes should be investigated by comparing different membrane types.

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References


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