Increased plasma S-nitrosothiol levels in chronic haemodialysis patients

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Abstract

Background. An impairment of nitric oxide (NO) bioavailability and/or metabolism may contribute to the excessive incidence of atherosclerotic complications observed in haemodialysis (HD) patients. Recent evidence indicates that NO metabolism involves a family of NO-related molecules that have not yet been explored in such patients. The aim of our study was to determine the plasma levels of S-nitrosothiol and nitrotyrosine in chronic HD patients, and to evaluate potential factors influencing their levels.

Methods. Plasma levels of S-nitrosothiols and nitrotyrosine were determined in 22 non-smoking HD patients and 12 healthy control subjects, together with albumin, homocysteine, haemoglobin, highly sensitive C-reactive protein (hsCRP) and various components of the oxidant–antioxidant system at the plasma and erythrocyte levels.

Results. While plasma nitrosothiol levels were significantly higher in HD patients than in controls (2.25 ± 1.17 vs 0.45 ± 0.45 μmol/l, respectively, P < 0.0001), nitrotyrosine levels were not different. HD patients also exhibited a marked deficit of ascorbate and low plasma glutathione peroxidase activity. An inverse relationship was found between plasma S-nitrosothiol and blood haemoglobin in HD patients (P < 0.005). No direct relationship was observed between plasma S-nitrosothiol levels and any of the oxidative stress markers, or hsCRP levels.

Conclusion. This study demonstrates high plasma S-nitrosothiol levels in HD patients, which are partially related to low blood haemoglobin concentrations. The pathophysiological significance of this elevation remains to be elucidated. A possible protective role against nitrosative stress is suggested in presence of normal plasma nitrotyrosine levels in such patients.

Keywords: atherosclerosis; haemodialysis; nitric oxide; nitrotyrosine; oxidant–antioxidant system; S-nitrosothiol

Introduction

Premature atherosclerosis is one of the primary causes of morbidity and mortality in patients with chronic renal insufficiency [1,2]. The mechanisms responsible for this excessive cardiovascular risk are not completely understood. Impaired endothelium-dependent vasodilatation, an early hallmark of atherosclerosis, has been reported both in patients with predialysis renal failure and in dialysis patients [3–5]. The observation of impaired endothelium-dependent vasodilatation in end-stage renal disease (ESRD) patients is presumably linked to the reduced capacity of the nitric oxide (NO) system to regulate vascular tone, particularly under conditions of NO synthase stimulation [6]. The bioavailability of NO in vivo is regulated not only by the rate of synthesis, but also by the rate of breakdown, and/or interaction with other compounds such as molecules containing functional sulph-hydryl groups (Figure 1) [7]. A reduction of whole-body NO production has been observed in ESRD patients who are not yet on dialysis [8,9], but this finding has not been studied in chronic haemodialysis (HD) patients [10]. This latter study suggested a possible extra-renal systemic production of NO [10]. On the other hand, an inhibition of NO synthase by asymmetric dimethylarginine (ADMA) remains possible at reported plasma concentrations in HD patients, and an association between plasma ADMA and overall mortality or cardiovascular outcome has been recently demonstrated in ESRD patients [11].

In contrast to the issue of NO synthesis, little information is available in ESRD patients regarding
NO breakdown and its interaction with other compounds. An elevation of plasma nitrite and nitrate ion concentrations as a result of oxidative NO inactivation has been reported in uraemic patients, but these findings have been criticized because of major limitations of the ion detection methods used [9,12]. However, there is considerable evidence indicating that NO metabolism involves a family of NO-related molecules in addition to nitrite and nitrate ions (Figure 1) [7].

First, NO can form adducts with molecules containing functional sulph-hydryl groups to yield S-nitrosothiols (Figure 1) [7]. Circulating NO is primarily complexed in S-nitrosothiol species [13]. S-nitrosothiols are considered as a NO pool, buffering NO, which is important for its storage and transport. Notably, S-nitrosothiols are potent vasodilators whose action is commonly associated with the ability to release NO at physiologically relevant sites [14]. This release may occur via a reduction by transition metal ions, ascorbate, thiol compounds, and several enzymes including plasma glutathione peroxidase (GSH-Px) [7,15].

Secondly, NO can also react with superoxide anion to form peroxynitrite (Figure 1) [7]. The latter can react with protein tyrosyl residues to form nitrotyrosine, and with thiols in proteins or in glutathione to form S-nitrosothiols [7]. The reaction with thiols may prevent the accumulation of peroxynitrite towards toxic levels.

To the best of our knowledge, no information is available at present concerning plasma levels of S-nitrosothiols and nitrotyrosine in HD patients. Therefore, the aim of our study was to determine whether plasma S-nitrosothiols and nitrotyrosine concentrations are elevated in chronic HD patients, compared with those of healthy controls, and if so, to evaluate factors potentially involved, including oxidative stress and inflammatory status.

Subjects and methods

Patients

Twenty-two chronic HD patients entered the present study after having given informed consent that conformed to the ethical recommendations of our institution. Smokers, patients with chronic hepatitis, haematological and inflammatory disorders, cancer or immunosuppressive therapy were excluded. The main characteristics of the 22 HD patients are presented in Table 1. Patients received thrice-weekly HD treatments, with a mean Kt/V urea index of 1.2 per session, using bicarbonate-buffered dialysate produced with ultra-pure water (CWP 100 system, Gambro, Sweden) with high bacteriological quality (<0.005 endotoxin units/ml). Sixteen of the patients were receiving regular subcutaneous recombinant human erythropoietin alpha (Eporex®; Janssen–Cilag, Boulogne-Billancourt, France) at a mean dose of 95 ± 8 IU/kg/week, and 10 of these were receiving parenteral iron maltate supplementation, 100 mg once weekly (Maltfer®; Lucien, Neuilly-sur-Seine, France). The remaining six patients were not being treated with erythropoietin or iron supplementation. None of the HD patients received antioxidant vitamin supplementation. Twelve non-smoking healthy subjects (5 men/7 women, 41 ± 8 years) with normal renal function were recruited as control subjects. In HD patients, blood (10 ml) was drawn from the arteriovenous fistula just before a dialysis session and collected in standard tubes containing 5 mM EDTA; 10 ml venous blood was also drawn from controls. Following centrifugation (600 times with isotonic saline solution before storage at –80°C until analysis. Erythrocytes were washed three times with isotonic saline solution before storage at –80°C until analysis of glutathione.

Biochemical determinations

Iron and transferrin were determined using routine methods on a Hitachi 917 analyser (Roche, Meylan, France). Plasma levels of albumin and highly sensitive CRP (hsCRP) were
determined using immunonephelometric procedures (Dade Behring, Paris, France). Plasma total homocysteine concentrations were measured using a radio-enzymatic method [16], and blood haemoglobin by Coulter analyser (STKS; Coultronics, Margency, France).

Plasma S-nitrosothiols levels were determined by a fluorimetric method [17]. Briefly, ammonium-sulphamate solution (50 μl of 0.1 mmol/l) was added to 100 μl of a 2-fold diluted plasma to trap nitric dioxide (NO₂). After a 10-min incubation, 50 μl of the reaction mixture (one part of 1.1 mmol/l mercuric chloride and four parts of 0.05 g/l diaminonaphthalene in 0.62 M HCl) were added. Following a 10-min incubation at room temperature and in darkness, the reaction was stopped with 20 μl of 2.8 M NaOH. Fluorescence intensity was measured in a 96-well microtitre plate (Pharmacia Biotech, Saint-Quentin en Yvelines, France) at excitation and emission wavelengths of 360 and 450 nm, respectively, and compared to a standard curve prepared using various concentrations of reduced glutathione (GSH) (50 μl of 0.15 to 10.0 μmol/l GSH in 1 M HCl), incubated with 50 μl NaNO₂ (10 μmol/l) at room temperature in darkness for 2 h, and then with 50 μl of 100 μM ammonium-sulphamate.

Plasma levels of 3-nitrotyrosine-modified proteins were measured by a commercial ELISA method (TCS Cell Works Ltd, Buckinghamshire, UK) [18]. Briefly, in a precoated microtitre plate with 4 μg/ml nitro-BSA, standards or samples were incubated in the wells with immunopurified polyclonal anti-nitrotyrosine rabbit IgG for 2 h at 37°C, followed by washing with wash buffer. Sequential incubations were then performed with biotinylated donkey anti-rabbit IgG HRP-conjugated antibody for 1 h at 37°C. After further washing, colour development was initiated by the addition of substrate, was allowed to develop for up to 30 min at room temperature, was stopped by the addition of 0.18 M sulphuric acid and was read at 450 nm on a microplate reader (MR 5000; Dynatech, France). A standard curve was constructed by incubating a serial dilution of nitro-BSA in PBS pH 7.4 (5.69–1.10 ng/ml). The concentration of nitrated proteins was estimated from the standard curve and expressed as nitro-BSA equivalent.

To eliminate the influence of plasma storage on nitrosothiol and nitrotyrosine levels, fresh plasma from healthy subjects and HD patients was evaluated and compared with that observed after storage. Mean plasma levels of nitrosothiols and nitrotyrosine were of the same magnitude in fresh and stored samples (data not shown).

Plasma levels of lipoperoxidation products, e.g. thiobarbituric acid-reactive substances (TBARS) were measured by fluorimetry, using a commercial kit from Sobioda (Grenoble, France) and those of protein oxidation, e.g. carbonyls and advanced oxidation protein products (AOPP), by spectrophotometry, as described previously [19]. Plasma ascorbate, the reduced form of vitamin C, was measured by spectrophotometry using ascorbate oxidase [20]. GSH-Px and glutathione reductase (GSSG-Red) activities were measured both in plasma and in erythrocytes, as described previously [21]. Red blood cell (RBC) enzyme activities were expressed as micromoles of NADPH oxidized per minute and per gram of haemoglobin (IU/g Hb). Erythrocyte glutathione content was simultaneously determined as reduced (GSH) and oxidized (GSGG) form by HPLC method, as reported previously [20]. The intracellular redox potential was evaluated by GSSG/GSH ratio, GSSG being expressed as GSH equivalent (one mole of GSSG corresponding to two equivalents GSH).

**Statistical analysis**

Data have been expressed as mean±SD. The Mann–Whitney U test was used to compare means between HD patients and controls. Simple regression analysis and Spearman correlation coefficient were used to determine relationships between parameters. Independent associations between one dependent variable and more than two independent variables were assessed by multiple regression analysis.

**Results**

**Plasma levels of nitrosothiols and nitrotyrosine**

Plasma nitrosothiol levels were significantly higher in HD patients than in healthy subjects (2.25±1.17 vs 0.45±0.45 μmol/l, P<0.0001). In contrast, plasma nitrotyrosine levels were not different (4.10±0.51 vs 4.12±0.50 μmol/l, P<0.0001). When HD patients were divided into two groups according to age <70 or ≥70 years, no difference could be observed for plasma nitrosothiol levels between the groups (Table 2). Moreover, no relationship was observed between age and plasma S-nitrosothiol levels or plasma nitrotyrosine levels in these patients. There was also no significant difference for plasma nitrosothiol levels when HD patients were divided into two groups according to the presence or absence of diabetes (2.66±1.22 vs 2.10±1.15 μmol/l, P=NS) or to gender (2.5±1.1 vs 1.98±1.3 μmol/l, P=NS, men/women, respectively).

**Relationship with levels of thiol substances, markers of oxidative stress and markers of inflammation**

Using regression analysis, no relationship was observed between plasma S-nitrosothiol levels and serum albumin (35.9±4.0 g/l, mean±SD) or plasma homocysteine (30.3±13.8 μmol/l) levels in HD patients. An inverse relationship was found between plasma S-nitrosothiol levels and blood haemoglobin (10.1±1.2 g/dl) concentrations in HD patients (r =−0.58, P<0.005).

Table 3 shows oxidative stress markers and antioxidant parameters in the study population. Although HD patients exhibited a combination between an increase in oxidative stress markers and a profound

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<th>Table 2. Plasma S-nitrosothiol and nitrotyrosine levels in 12 control subjects and 22 HD patients, according to age</th>
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<tr>
<td>Controls</td>
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<tr>
<td>n = 12</td>
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<tr>
<td>Mean age (years)</td>
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<tr>
<td>Sex ratio (M:F)</td>
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<tr>
<td>Nitrosothiols (μmol/l)</td>
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<td>Nitrotyrosine (μg/l)</td>
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Results are expressed as mean±SD. P values by Mann–Whitney U test for HD patients <70 years and ≥70 years vs controls. *P<0.0001.
The concentration has been observed in HD patients [23]. Elevation of predialysis plasma nitrotyrosine is normal in all HD patients. Finally, plasma levels of nitrotyrosine are normal in HD patients. The observed elevation of plasma S-nitrosothiol concentrations clearly points to the presence of circulating NO metabolites present in a potentially biological activity. Previous reports have suggested the presence of high levels of unreactive nitrate and nitrite ions, but these findings have been criticized because of limitations of the ion-detection methods used [9,12]. Collectively, these data argue against a quantitative NO deficiency in HD patients.

The cause of the elevation of plasma S-nitrosothiols cannot be fully delineated in the present study. We found a negative correlation between S-nitrosothiols and haemoglobin. Data obtained in vitro suggest that haemoglobin can scavenge the NO generated from S-nitrosothiols [22], and S-nitrosohaemoglobin may also act as a reservoir of NO [23]. Indeed, a slight elevation of predialysis S-nitrosohaemoglobin concentration has been observed in HD patients [23]. However, in the present study HD patients, who had normal Hb levels (i.e. ≥11 g/dl) also had high plasma S-nitrosothiol levels (1.81 ± 0.94 μmol/l). Notably, plasma S-nitrosothiol levels were slightly, but not significantly, different from those of HD patients who had Hb levels <11 g/dl (2.51 ± 1.23 μmol/l). Moreover, in another, non-uraemic control group with low haemoglobin levels (9.5 ± 0.9 g/dl, n = 8 subjects) due to different conditions not related to ESRD, we found only a slight elevation of S-nitrosothiol concentrations (0.75 ± 0.1 μmol/l) (our unpublished results). Therefore modification of the haemoglobin levels appears to exert only a partial influence on the level of S-nitrosothiols, and other factors could be responsible in HD patients as well.

**Discussion**

Our results demonstrate for the first time that the plasma of chronic HD patients contains significantly higher S-nitrosothiol levels than normal control plasma. Moreover, plasma S-nitrosothiol levels are negatively correlated with blood haemoglobin levels. Finally, plasma levels of nitrotyrosine are normal in HD patients.

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Increased production of NO under the influence of inflammatory stimuli is a possible mechanism, since ureaemia can be considered as an inflammatory state [20,24]. However, we observed no relationship between plasma S-nitrosothiol and hsCRP levels. An accumulation of sulph-hydryl amino acids, such as homocysteine and cysteine, has been demonstrated in ESRD patients [25,26]. However, S-nitrosothiol compounds in control plasma are present mainly as S-nitrosoalbumin [13]. Therefore it is not surprising that there was no correlation between S-nitrosothiols and homocysteine in the present study. The elevation of plasma S-nitrosothiol levels may also be related to abnormal breakdown, since HD patients have a deficit in ascorbate and low levels of plasma glutathione peroxidase activity, which is responsible for the release of NO from S-nitrosothiols [7,15]. However, we did not observe a correlation between plasma ascorbate or glutathione peroxidase activity and plasma S-nitrosothiols.

Under the oxidative stress condition encountered in HD patients, NO can react rapidly with superoxide anion to form peroxynitrite, which can attack protein, leading to nitrotyrosine. Moreover, inducible NO synthase can act as NO, as well as a peroxynitrite-producing enzyme, depending on the antioxidant capacity of the microenvironment [27]. Paradoxically, plasma nitrotyrosine levels in HD patients were not increased, compared with controls. Peroxynitrite detoxification via its reaction with thiols (e.g. glutathione) to form S-nitrosothiols could explain the high S-nitrosothiol levels observed in the present study. The observation of normal plasma nitrotyrosine levels, and of a difference of reduced glutathione red-cell levels between patients with normal and those with high plasma S-nitrosothiol levels are in favour of this explanation. Nossuli et al. [28] have demonstrated an increase in S-nitrosoglutathione when peroxynitrite was co-incubated with reduced glutathione.

S-nitrosothiols are considered as a NO reservoir and they are potent vasodilators [14]. However, in view of the marked deficit of ascorbate and the low levels of plasma glutathione peroxidase activity in HD patients, an enhanced vasodilatory function of S-nitrosothiols is questionable. A deficiency of bioactive NO has been found to be associated with arterial thrombosis in

**Table 3.** Plasma and red blood cell (RBC) biochemistry parameters in 12 control subjects and 22 HD patients

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<tr>
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<th>Controls n=12</th>
<th>HD patients n=22</th>
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<tr>
<td>Plasma TBARS (μmol/l)</td>
<td>2.26 ± 0.25</td>
<td>2.82 ± 0.45&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Plasma carbonyls (nmol mg prot)</td>
<td>0.34 ± 0.09</td>
<td>0.54 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Plasma AOPP (μmol/l)</td>
<td>49 ± 21</td>
<td>154 ± 42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma ascorbate (μmol/l)</td>
<td>67 ± 16</td>
<td>25 ± 24&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Plasma GSH-Px (IU/l)</td>
<td>80 ± 37</td>
<td>23 ± 11&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>RBC-GSH-Px (IU/g Hb)</td>
<td>8.9 ± 2.5</td>
<td>7.9 ± 2.2</td>
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<tr>
<td>RBC-GSH (μmol/g Hb)</td>
<td>4.15 ± 1.40</td>
<td>3.07 ± 1.48&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>RBC-GSSG/GSH ratio&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.37 ± 0.26</td>
<td>0.88 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
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Results are expressed as mean ± standard deviation. P values by Mann-Whitney U test for HD patients vs controls: <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.001. <sup>c</sup>P < 0.0001. GSH-Px, glutathione peroxidase; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; <sup>a</sup>GSSG was expressed as GSH equivalent; RBC, red blood cells.
animal models, in individuals with endothelial dysfunction, and in patients with a low extracellular glutathione peroxidase activity [7]. The lack of S-nitrosothiol bioavailability might participate in the generation of hypertension and thrombotic events in HD patients, and consequently favour the increased frequency of cardiovascular events. Further studies will be necessary to test whether supplementation with vitamin C or exogenous glutathione peroxidase may correct such a deficiency of bioactive NO, by enhancing the release of NO from S-nitrosothiols, and therefore decrease the frequency of cardiovascular events in these patients. Finally, it remains possible, as shown above, that elevated circulating S-nitrosothiols reflect a protective mechanism against nitrosative stress in HD patients. Again, studies with antioxidants are necessary to verify this hypothesis.

References


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