Glutathionyl hemoglobin in uremic patients undergoing hemodialysis and continuous ambulatory peritoneal dialysis

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Background. To assess the redox state in hemodialysis (HD) and continuous ambulatory peritoneal dialysis (CAPD) patients, we focused on the formation of glutathionyl hemoglobin (Hb) because the ratio of oxidized glutathione disulfide (GSSG) to reduced glutathione (GSH) is increased in uremia, and GSSG is a source of glutathionyl Hb.

Methods. Glutathionyl Hb levels were measured in 30 HD patients, 10 CAPD patients, and 20 healthy subjects by using liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS).

Results. Hbβ showed a peak at 15,686 D in a deconvoluted ESI mass spectrum. Glutathionyl Hbβ was detected at 16,173 D (15,686 + 305). The peak at 16,173 D was identified as glutathionyl Hbβ based on the following findings: (1) the peak disappeared by reducing the sample with dithiothreitol, and (2) the peak could be detected at a high level by incubating Hb in vitro with GSH in water at 37°C for seven days. Glutathionyl Hb levels expressed as the peak height ratios of glutathionyl Hbβ to intact Hbβ were significantly elevated in HD patients (8.0 ± 3.6%, mean ± SD, N = 30, P < 0.0001) and CAPD patients (5.9 ± 2.7%, N = 10, P < 0.05) as compared with normal subjects (3.0 ± 1.6%, N = 20). However, there were no significant differences in the glutathionyl Hb levels before (8.7 ± 3.2%, N = 12) and after HD (8.7 ± 2.8%, N = 12).

Conclusion. Glutathionyl Hb levels were increased in HD and CAPD patients, probably because of enhanced oxidative stress. The measurement of glutathionyl Hb may be useful to assess oxidative stress in uremic patients.

Oxidative stress, which occurs when there is excessive free radical production in the face of defective antioxidant defenses, has been reported in patients with chronic renal failure (CRF) undergoing hemodialysis (HD) [1, 2]. HD is a major cause of oxidative stress because of the activation of polymorphonuclear neutrophils through the contact of blood with dialysis membranes [3, 4]. Oxidative stress is considered to be responsible for shortened life span of erythrocytes [5, 6]. The extent of oxidative stress is exacerbated by a decreased efficiency in the antioxidant system. In fact, a low concentration of reduced glutathione (GSH) [7, 8], an increased ratio of oxidized glutathione disulfide (GSSG) to GSH [9, 10], and a decreased activity of glutathione-dependent enzymes such as glutathione S-transferase, glutathione reductase, and glutathione peroxidase [9] have been observed in the erythrocytes of HD patients.

A ratio of GSSG to GSH is increased under oxidative stress, and GSSG is a source of glutathionyl hemoglobin (Hb). Thus, we focused on the formation of glutathionyl Hb to assess the redox state in uremic patients. Glutathionyl Hb levels were measured in HD and continuous ambulatory peritoneal dialysis (CAPD) patients by using liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS).

METHODS

Patients

Blood samples were obtained under informed consent from 30 patients (21 males and 9 females, 53 ± 13 years old, mean ± SD) on maintenance HD for 8.5 ± 6.5 years, 10 patients (10 females, 53 ± 13 years old) on CAPD for 3.5 ± 1.5 years, and 20 healthy subjects (10 males and 10 females, 47 ± 10 years old). Hematocrit levels in the HD patients (30.4 ± 2.9%) and the CAPD patients (28.9 ± 3.6%) were significantly (P < 0.0001) lower than in healthy subjects (42.0 ± 4.1%). None of the HD patients were alcoholics or smokers. Furthermore, they did not suffer from diabetes, systemic lupus erythematosus, malignancy, acute infectious illnesses, chronic respiratory insufficiency, or hepatic disorders. None of them had received either blood or plasma infusions or antioxidant drugs for four months before blood collection. All of the HD patients were undergoing regular four-hour HD treatment three times per week. The HD patients were treated using bicarbonate dialysate with single-use dialyzers equipped with different types of membranes, including Cuprophan and biocompatible membranes.
The same types of membranes had been used in each patient at least for six months before the study.

Blood samples were drawn using ethylenediaminetetraacetic acid (EDTA) as an anticoagulant just before and after HD. Whole blood samples (15 μL) were immediately diluted with distilled water (485 μL). The mixture was subsequently centrifuged at 12,000 × g for 10 minutes. The supernatant was kept at −40°C until LC/ESI-MS analysis.

**In vitro preparation of glutathionyl hemoglobin**

Hemoglobin (15 mg/mL; Sigma Chemical Co., St. Louis, MO, USA) and GSH (1 mmol/L; Sigma Chemical Co.) were incubated in distilled water at 37°C for seven days. The incubation solution was subjected to LC/ESI-MS for the measurement of glutathionyl Hb.

**LC/ESI-MS**

Liquid chromatography/electrospray ionization-mass spectrometry was performed using a triple-stage quadrupole mass spectrometer (TSQ7000; Thermoquest, San Jose, CA, USA) equipped with a reversed-phase column (TSKgel Phenyl-5PW RP4.6 mm i.d. × 7.5 cm). A mobile phase consisting of solution A (2% acetonitrile in 0.2% acetic acid) and solution B (90% acetonitrile in 0.2% acetic acid) was delivered at a flow rate of 0.5 mL/min at ambient temperature. The mobile phase was linearly programmed from 15% of solution B to 45% of solution B in 30 minutes. The conditions for ESI-MS were as follows: electric field 4.5 kV, nitrogen sheath gas 70 psi, auxiliary gas 15 units, and capillary temperature 275°C. Samples (10 μL) were diluted with solvent A (90 μL) and, subsequently, were subjected to LC/ESI-MS. Molecular weights of proteins were determined by deconvoluted mass spectra of their peaks. The levels of glutathionyl Hbβ were expressed as the percents of the peak height ratios to intact Hbβ.

**Statistical analysis**

Data are expressed as means ± SD with a significance of P < 0.05. For the comparison between two groups, a paired Student t test was used. For comparison of multiple groups, analysis of variance was performed, and Scheffe's F test was used.

**RESULTS**

**Identification of glutathionyl HB**

Reconstructed ion chromatogram (RIC) of a Hb sample shows the separation of two major peaks (peak 1, Hbα, and peak 2, Hbβ; data are not shown). Figure 1A and C show the deconvoluted mass spectra of peak 1 from a normal subject (Fig. 1A) and an HD patient (Fig. 1C), in which Hbα was detected at 15,127 D and glycated Hbα at 15,289 D (15,127 + 162). However, glutathionyl Hbα could not be detected. Figure 1B and D show the deconvoluted mass spectra of peak 2 from the normal subject (Fig. 1B) and the HD patient (Fig. 1D). The Hbβ chain has a molecular weight of 15,868 D. Glycated β chain was detected at 16,030 D (15,868 + 162), while the glutathionyl β chain was detected at 16,173 D (15,868 + 305). The peak at 16,173 D was identified as the glutathionyl Hbβ chain, based on two findings: (1) the peak disappeared by reducing the sample with 1 mol/L dithiothreitol in distilled water, and (2) the peak could be detected by incubating Hb in vitro with GSH at 37°C for seven days. Relative intensity of glutathionyl Hbβ was markedly increased in the HD patient as compared with the normal subject (Fig. 1B, D).

**Glutathionyl Hb levels in HD and CAPD patients**

Glutathionyl Hb levels were measured in HD and CAPD patients and normal subjects by calculating the peak height ratios of glutathionyl Hbβ to intact Hbβ. Glutathionyl Hb levels in HD patients (8.0 ± 3.6%, N = 30, P < 0.0001) and CAPD patients (5.9 ± 2.7%, N = 10, P < 0.05) were significantly elevated as compared with normal subjects (3.0 ± 1.6%, N = 20, analysis of variance, P < 0.0001). However, there were no significant differences in glutathionyl Hb between HD patients and CAPD patients. There were also no significant differences in the glutathionyl Hb levels before (8.7 ± 3.2%, N = 12) and after HD (8.7 ± 2.8%, N = 12). Glutathionyl Hb levels before HD were correlated well with those after HD (r² = 0.925, P < 0.0001, y = 0.85x + 1.306).

**DISCUSSION**

Human adult Hb (HbA) can react in vitro with GSH with a disulfide bond formation between the cysteine 93 of Hbβ and the cysteine of GSH [11]. The glutathionyl formation is associated with β chain but not α chain, because the cysteine 93 of Hbβ provides the only accessible thiol group at the surface of the Hb molecule. Glutathionyl Hb level in normal erythrocytes was so low that it could not be detected in normal erythrocytes by using electrophoresis [12]. However, we could detect it in normal erythrocytes as well as in the erythrocytes of HD patients by using highly sensitive and specific LC/ESI-MS.

In this study, we detected glutathionyl Hb at higher levels in HD patients and CAPD patients than in healthy subjects. There were no significant differences in glutathionyl Hb levels between HD patients and CAPD patients. A single HD session did not affect the glutathionyl Hb levels in these patients. Since glutathionyl Hb is formed by oxidation of the thiol group of Hb, the increased levels of glutathionyl Hb in HD and CAPD patients may be due to enhanced oxidative stress in these patients as compared with normal subjects. Thus, the
measurement of glutathionyl Hb may be useful to assess oxidative stress in uremic patients.

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