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Determination of glutathionyl hemoglobin in hemodialysis patients using electrospray ionization liquid chromatography–mass spectrometry

Chika Naito, Mitsuharu Kajita, Toshimitsu Niwa*

Nagoya University Daiko Medical Center, 1-1-20 Daiko-minami, Higashi-ku, Nagoya 461-0047, Japan

Abstract

We first detected glutathionyl hemoglobin (Hb) β-chain in hemodialysis patients and healthy subjects using electrospray ionization liquid chromatography–mass spectrometry. The ratio of glutathionyl Hb β-chain to total β-chain was markedly increased in the hemodialysis patients as compared with healthy subjects. Glutathionyl Hb will be used as a new clinical marker of oxidative stress.

Keywords: Glutathionyl hemoglobin

1. Introduction

Oxidative stress can produce profound alterations to cellular membrane lipids, proteins and nucleic acids, impairing cell metabolism and viability, and has been considered to be involved in aging [1] and such diseases as diabetes mellitus, uremia, atherosclerosis, rheumatoid arthritis, adult respiratory distress syndrome, reoxygenation injury, human immunodeficiency virus infection and cystic fibrosis. Oxidative stress corresponds to an imbalance between the production of reactive oxygen species, mainly the superoxide anion (O_2^-), hydroxyl radical (·OH), peroxyl radical (LOO·), and hydrogen peroxide (H_2O_2), and protective mechanisms. Several enzymatic systems can detoxify reactive oxygen species: superoxide dismutase catalyzes the conversion of O_2^- to H_2O_2 and works concomitantly with catalases and a selenoprotein, glutathione peroxidase. The level of reduced glutathione (GSH) is a limiting factor in this enzymatic process, which requires the maintenance of high reduced to oxidized glutathione (GSH/GSSG) ratio as achieved by glutathione reductase. In addition, some reducing agents act as free radical scavengers to non-enzymatically detoxify reactive oxygen species: GSH, vitamin E and vitamin C.

The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) is the major intracellular thiol compound, and plays a major role in the protection of cells and tissue structures from oxidative injury. Glutathione can be reduced (GSH), oxidized (GSSG) or bound to proteins. GSH inhibits free radical-mediated injury by eliminating reactive oxygen species, and protects protein thiol groups from oxidation by serving as a biological redox agent. Intracellular and blood concentrations of GSH are in millimolar range, while plasma concentration is in the micromolar range and accounts for approximately 0.4% of total blood GSH [2,3]. Erythrocytes play

*Corresponding author. Tel.: +81-52-719-1874; fax: +81-52-719-1875.

E-mail address: tniwa@med.nagoya-u.ac.jp (T. Niwa)

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an important role in the delivery of GSH to tissues with high rate of GSH utilization, including lung, heart, gut and brain. Both the liver and kidney normally release substantial amounts of GSH in the form of precursor amino acids, which are extracted by erythrocytes, resynthesized into GSH, and then transported to the tissues.

Dialysis patients are at an increased risk from oxidative stress. Bioincompatible hemodialysis is an important source of reactive oxygen species. GSH levels are low in whole blood and erythrocytes accompanied by a decreased GSH/GSSG ratio [4–9]. Superoxide dismutase and glutathione peroxidase activities, and erythrocyte vitamin E are low, and plasma malondialdehyde (MDA), an end-product of lipid peroxidation, is elevated in dialysis patients [4–6,8,10,11].

Numerous studies investigated the markers of oxidative stress, such as MDA or thiobarbituric acid reactive substances, or antioxidant defense systems such as superoxide dismutase, glutathione peroxidase activity or free radical scavengers, e.g. GSH, vitamin E and vitamin C. Although reactive oxygen species have been detected in vitro by electron spin resonance with or without spin trapping reagents or by chemiluminescence, these methods are not yet applicable for clinical examination. Erythrocyte GSH has been measured by using the enzyme recycling method [12], the spectrophotometric assay [8,11] or high-performance liquid chromatography (HPLC) with derivatization and fluorescence detection [2,7]. These methods, except MDA, are not used for routine clinical examination, because they require complicated sample preparation, and elaborate techniques. In this study we have first demonstrated using liquid chromatography-electrospray ionization–mass spectrometry (ESI-LC–MS) that glutathionyl Hb is elevated in such diseases as diabetes and uremia, and will be used as a new sensitive marker of oxidative stress.

2. Experimental

2.1. Sample preparation

Blood samples were obtained using heparin as an anticoagulant from 10 hemodialysis patients and 20 healthy subjects. After centrifugation at 800 *g* for 10 min, the supernatant plasma was removed, and the erythrocytes were kept at −20°C. The hemolysates were obtained by thawing the frozen erythrocytes and the subsequent centrifugation at 6000 *g* for 10 min to remove erythrocyte membranes. The hemolysate (10 μl) was mixed with distilled water (490 μl), and then the mixture (10 μl) was diluted with 2% acetonitrile in 0.2% acetic acid (90 μl). After passing through a 0.45 μm filter, the diluted hemolysate sample (10 μl) was subjected to ESI-LC–MS.

2.2. ESI-LC–MS

ESI-LC–MS was performed using a triple-stage quadrupole mass spectrometer (TSQ7000; Thermoquest, San Jose, CA, USA) equipped with a reversed-phase column (TSKgel Phenyl-5PW RP 4.6 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 min. The conditions for ESI-MS were as follows; electric field 4.5 kV, nitrogen sheath gas 70 p.s.i. ($4.82 \cdot 10^5$ Pa), auxiliary gas 15 p.s.i. ($1.03 \cdot 10^5$ Pa), capillary temperature 275°C.

3. Results

Fig. 1A shows the reconstructed ion chromatogram of Hb from a hemodialysis patient, demonstrating the separation of Hb α and Hb β chains. Fig. 1B shows the the deconvoluted mass spectrum of Hb α (peak 1), and demonstrates that glycated Hb α but no glutathionyl Hb α could be detected. Fig. 1C shows the deconvoluted mass spectrum of Hb β chain (peak 2). The Hb β chain shows the molecular weight of 15 868 Da. Glycated β chain was detected at 16 030 Da (15 868+162), while glutathionyl β chain was detected at 16 173 Da (15 868+305). The peak at 16 173 Da was identified as glutathionyl β chain, based on the following findings; (1) the peak disappeared by reducing the sample with 1 *M* dithiothreitol in distilled water, accompanied by the

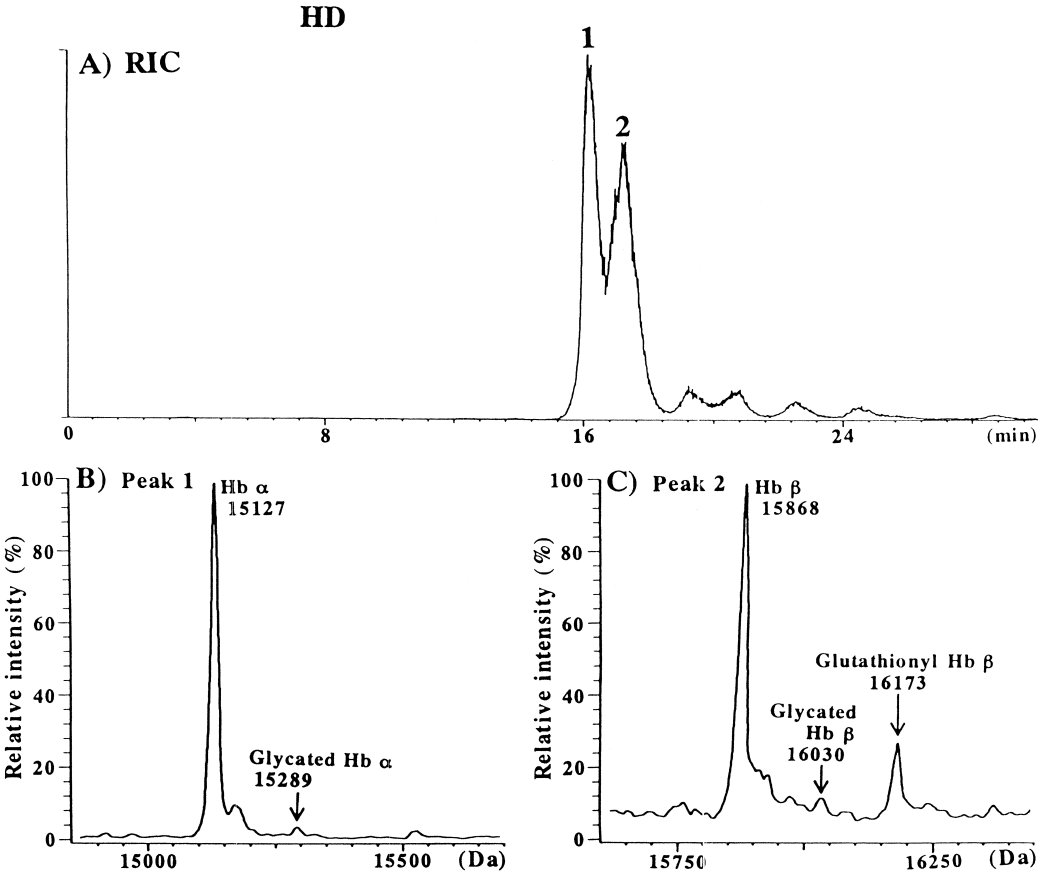

Fig. 1. Reconstructed ion chromatogram (RIC) of Hb from a hemodialysis (HD) patient (A), and deconvoluted ESI mass spectra of peak 1 (B) and peak 2 (C) in the RIC chromatogram. Glycated Hb α and Hb β could be detected, whereas glutathionyl Hb β but no glutathionyl Hb α could be detected.

simultaneous appearance of a peak of GSH at m/z 308, $(M+H)^+$, and (2) the peak could be detected by incubating Hb (15 mg/ml) (Sigma Chemical, St. Louis, MO, USA) with 1 m*M* GSH (Sigma Chemical) in distilled water at 37°C for 14 days. More notably, the synthesis of glutathionyl Hb was enhanced by adding H_2O_2 (1 m*M*), a reactive oxygen, into the incubation solution.

The levels of glutathionyl Hb β chain expressed as the percents to intact Hb β chain were markedly increased in hemodialysis patients as compared with healthy subjects, whereas the levels of glycated Hb β chain and glycated Hb α chain were not significantly increased in the hemodialysis patients. (Table 1). The level of glutathionyl Hb β chain did not significantly change after hemodialysis. Plasma MDA levels were also increased in the hemodialysis patients as compared with healthy subjects.

4. Discussion

Human adult Hb (HbA) can react in vitro with GSH with disulfide bond formation between Cys-β93 and the cysteine of GSH [13]. The glutathione adduct formation is associated with β chain but not α chain, because Cys-β93 provides the only accessible thiol group at the surface of the Hb molecule. The glutathionyl Hb produced in vitro shows increased oxygen affinity, a reduced cooperativity and a reduced alkaline Bohr effect [14]. Glutathionyl Hb was produced in vitro by thiol-disulfide exchange be-

Table 1
Glutathionyl Hb β, glycated Hb β, glycated Hb α, plasma MDA and plasma creatinine in hemodialysis patients and healthy subjects[a]

	Normal $n=20$	HD	
		Pre-HD 10	Post-HD 10
Glutathionyl Hb β (%)	3.7±0.3	18.6±0.9[b]	20.8±0.9[b]
Glycated Hb β (%)	3.4±0.2	3.2±0.2	2.6±0.1
Glycated Hb α (%)	2.5±0.1	2.2±0.1	2.2±0.1
Plasma MDA (nmol/ml)	1.5±0.07	2.0±0.07[b]	2.0±0.1[b]
Plasma creatinine (mg/dl)	0.73±0.03	9.5±0.7[b]	3.6±0.5[b]

[a] Mean±SE, HD: hemodialysis.
[b] $P<0.0001$ as compared with normal subjects by non-paired *t*-test.

tween mixed disulfides of Hb and GSH to study its anti-sickling effect. It was possible to bind most of the intracellular GSH to Hb by using a two-step reaction, the formation of a mixed disulfide, followed by a thiol-mixed disulfide exchange. By using this method, up to 25% of intracellular Hb could be obtained in the glutathionyl Hb form. However, glutathionyl Hb could not be detected in normal erythrocytes by using electrophoresis. Glutathionyl Hb levels in normal erythrocytes were so low that we could detect it by using highly sensitive and specific ESI-LC–MS. In uremia, however, the increased oxidative stress leads to increased levels of erythrocyte GSSG, which then forms a disulfide with Hb β to produce glutathionyl Hb.

Glutathione-modified α-crystalline at Cys 131 and Cys 142 was detected in the lens of uremic patients [15]. The formation of glutathionyl crystalline is also considered to be due to oxidative stress associated with uremia, and may be involved in the development of cataract. Glutathionyl Hb, which is more easily examined by using blood, may represent the glutathione modification of proteins in various tissues, including lens.

In conclusion, we first detected glutathionyl Hb in human erythrocytes, and demonstrated that its levels are markedly increased in hemodialysis patients. The enhanced oxidative stress in uremia may account for the increased glutathionyl Hb. The increased glutathionyl Hb may lead to reduced oxygen supply to peripheral tissues, due to its increased oxygen affinity.

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