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Analysis of glutathionyl hemoglobin levels in diabetic patients by electrospray ionization liquid chromatography–mass spectrometry: effect of vitamin E administration

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Abstract

By using electrospray ionization liquid chromatography–mass spectrometry, we demonstrated that glutathionyl hemoglobin (Hb)β-chain levels are markedly increased in the erythrocytes of diabetic patients as compared with healthy subjects. The administration of vitamin E to the diabetic patients for 8 weeks significantly decreased the levels of glutathionyl Hbβ, whereas it did not affect the levels of HbA1c, glycated Hbβ or glycated Hbα. Glutathionyl Hb levels can be used as a new clinical marker of oxidative stress.

Keywords: Glutathionyl hemoglobulin; Vitamins

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, and atherosclerosis. Oxidative stress corresponds to an imbalance between the production of reactive oxygen species, mainly the superoxide anion (O_2^-), hydroxyl radical ($^{\cdot}OH$), peroxyl radicals ($LOO^{\cdot}$), 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 a 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, vitamin C, and β-carotene.

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is a major intracellular nonprotein 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 the millimolar range, while plasma concentration is in the micromolar range and accounts for approximately 0.4% of total blood GSH [2,3].

Oxidative stress has been proposed as a patho-

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genic factor for diabetic complications [4–6]. Under diabetic conditions, the Maillard reaction facilitates the production of reactive oxygen species, and antioxidant defense systems are impaired, including decreased activity of superoxide dismutase and low GSH levels in the erythrocytes [6–9]. In fact, the presence of diabetic complications correlated negatively with the concentration of GSH in erythrocytes [9].

Recently, we have demonstrated that glutathionyl Hb levels are markedly increased in hemodialysis patients [10]. In the present study we determined the glutathionyl Hb levels in diabetic patients and the effects of vitamin E administration on their levels using electrospray ionization liquid chromatography–mass spectrometry (ESI-LC–MS).

2. Experimental

2.1. Sample preparation

Blood samples were obtained using heparin as an anticoagulant under informed consent from ten patients with type 2 diabetes, and 20 healthy subjects. Vitamin E (tocopherol nicotinate) was administered to the diabetic patients at a dose of 600 mg/day for 8 weeks to determine its effects on the glutathionyl Hb levels, and blood samples were obtained from the patients before and after 8 weeks.

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 subsequent centrifugation at 6000 *g* for 10 min to remove the erythrocyte membranes. The hemolysate sample (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

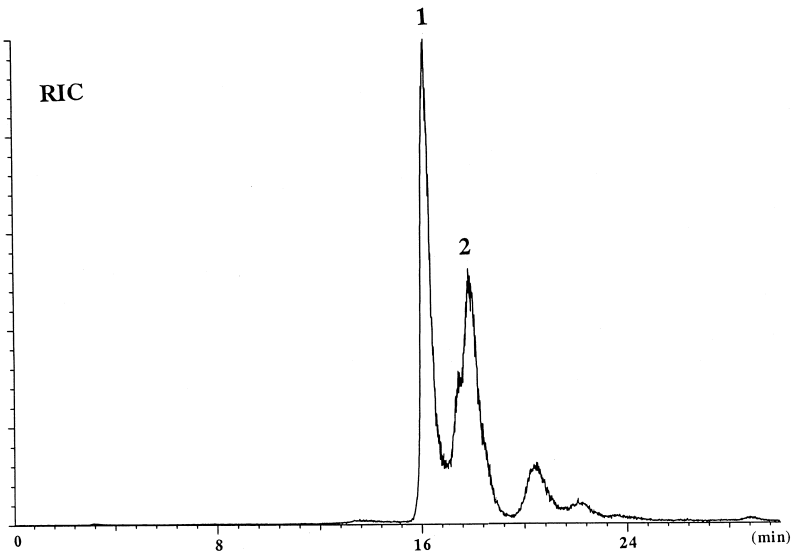

Fig. 1. Reconstructed ion chromatogram (RIC) of Hb from a diabetic patient before administration of vitamin E.

were as follows; electric field 4.5 kV, nitrogen sheath gas 70 p.s.i., auxiliary gas 15 units, capillary temperature 275°C.

The levels of glutathionyl Hbβ and glycated Hbβ were expressed as the percents of their peak height ratios to total Hbβ (intact Hbβ+glutathionyl Hbβ+glycated Hbβ). Glycated Hbα level was expressed as the percent of its peak height ratio to total Hbα (intact Hbα+glycated Hbα).

3. Results

Fig. 1 shows the reconstructed ion chromatogram (RIC) of Hb from a diabetic patient before administration of vitamin E. Fig. 2 shows the deconvoluted ESI mass spectra of peak 1 (a) and peak 2 (b) in the RIC chromatogram of Fig. 1 (before vitamin E administration), and of their respective peaks (c,d) in the same patient 8 weeks after administration of vitamin E. Glycated Hbα and Hbβ could be detected, whereas glutathionyl Hbβ but no glutathionyl Hbα could be detected. Hbβ peak showed the molecular weight of 15 868 Da. Glycated Hbβ peaks were detected at 16 030 Da (15868+162), while glutathionyl Hbβ peaks were detected at 16 173 Da (15868+305). The peak at 16 173 Da was identified as glutathionyl Hbβ, based on the following findings; (1) the peak disappeared by reducing the sample with 1 *M* dithiothreitol in distilled water, and (2) the peak could be detected by incubating Hb (15 mg/ml) with 1 m*M* GSH in distilled water at 37°C for 14 days. The peak height of glutathionyl Hbβ 8 weeks after administration of vitamin E was reduced as

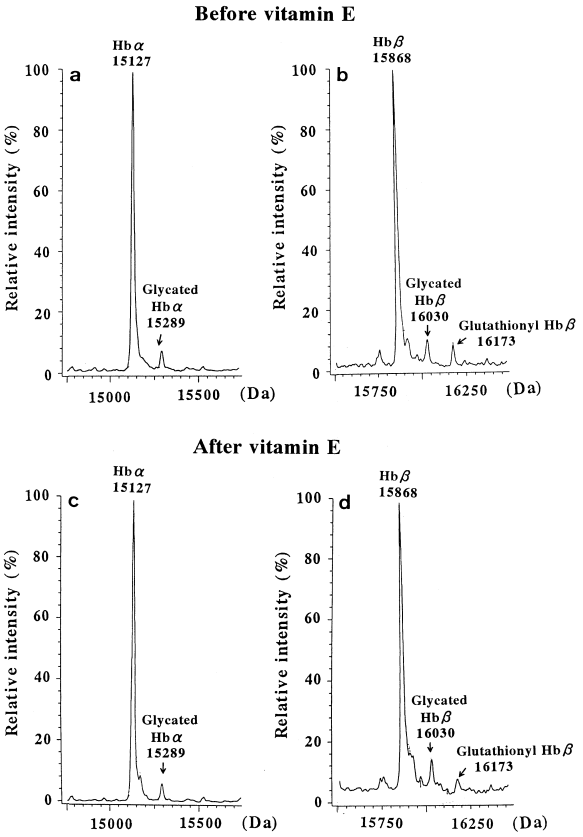

Fig. 2. Deconvoluted ESI mass spectra of peak 1 (a) and peak 2 (b) in the RIC chromatogram of Fig. 1 (before vitamin E administration), and of their respective peaks (c,d) in the same patient 8 weeks after administration of vitamin E. Glycated Hbα and Hbβ could be detected, whereas glutathionyl Hbβ but no glutathionyl Hbα could be detected. The peak height of glutathionyl Hbβ 8 weeks after administration of vitamin E was decreased as compared with that before vitamin E administration.

Table 1
Glutathionyl Hbβ before and after administration of vitamin E (600 mg/day) for 8 weeks in diabetic patients[a]

Percent (%)	Normal $n=20$	Diabetes mellitus	
		Before vitamin E $n=10$	After vitamin E $n=10$
Glutathionyl Hbβ	3.7±0.3	10.2±0.8[b]	4.1±0.4[c]
Glycated Hbβ	3.4±0.2	6.1±0.6[b]	6.6±0.5[b]
Glycated Hbα	2.5±0.1	4.5±0.4[b]	4.7±0.5[b]
HbA1c	4.8±0.04	7.6±0.3[b]	7.6±0.4[b]

[a] Mean±SE.
[b] $P<0.001$ as compared with normal by non-paired t test.
[c] $P<0.001$ as compared with before vitamin E by paired t test.

compared with that before administration of vitamin E.

The levels of glutathionyl Hbβ were markedly increased in diabetic patients as compared with healthy subjects (Table 1). The oral administration of vitamin E at a dose of 600 mg/day for 8 weeks to the diabetic patients markedly reduced the level of glutathionyl Hbβ, whereas it did not affect Hb_{A1c} levels, glycated Hbβ or glycated Hbα levels (Table 1).

4. Discussion

This is the first study to demonstrate that elevated levels of glutathionyl Hbβ in diabetic patients could be reduced by the administration of vitamin E. Human adult Hb (HbA) can react in vitro with GSH with disulfide bond formation between Cys-β93 and the cysteine of GSH [11]. The glutathione adduct formation is associated with the β chain but not the α chain, because Cys-β93 provides the only accessible thiol group at the surface of the Hb molecule. Glutathionyl Hb was produced in vitro by thiol–disulfide exchange between 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. Glutathionyl Hb level in normal erythrocytes was so low that it could not be detected in the normal erythrocytes by using electrophoresis [11]. However, we could detect it in normal erythrocytes as well as in the erythrocytes of diseased patients by using highly sensitive and specific ESI-LC–MS. In diabetes, the increased oxidative stress leads to increased levels of erythrocyte GSSG, which then forms a disulfide with Hbβ to produce glutathionyl Hb. The glutathionyl Hb produced in vitro shows increased oxygen affinity, a reduced co-operativity and a reduced alkaline Bohr effect [12].

In conclusion, glutathionyl Hb levels were markedly increased in diabetic patients, and the administration of vitamin E to the patients could significantly reduce their levels. Thus, increased oxidative stress in the erythrocytes accounts for the elevated glutathionyl Hbβ levels in diabetes mellitus.

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