Increased Glutathionyl Hemoglobin in Diabetes Mellitus and Hyperlipidemia Demonstrated by Liquid Chromatography/Electrospray Ionization-Mass Spectrometry

Toshimitsu Niwa,1* Chika Naito,1 Abdul Hassan Mohammed Mawjood,2 and Kiyohiro Imai2

Background: Erythrocytes contain a large amount of glutathione (GSH), which protects cells from oxidative injury. The purpose of this study was to examine whether hemoglobin (Hb) is modified with glutathione by oxidation of the thiol groups in diabetes mellitus and hyperlipidemia, and to determine the oxygen affinity of glutathionyl Hb.

Methods: Hb samples obtained from patients with type 2 diabetes, patients with hyperlipidemia, and healthy subjects were analyzed by liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS). Glutathionyl Hb was synthesized in vitro by incubating Hb with GSH. The oxygen affinity of glutathionyl Hb was determined by measuring its oxygen dissociation curve.

Results: We first demonstrated that the concentration of glutathionyl Hbβ chains is markedly increased in the diabetic patients and hyperlipidemic patients compared with healthy subjects. The in vitro synthesis of glutathionyl Hb by incubation of Hb with GSH was enhanced by adding H2O2, a reactive oxygen species, into the incubation solution. The glutathionyl Hb prepared in vitro by incubating Hb with GSH showed a marked increase in oxygen affinity and a marked decrease in the Hill coefficient compared with Hb incubated without GSH.

Conclusions: Glutathionyl Hb may be useful as a clinical marker of oxidative stress. The increased concentrations of glutathionyl Hb with high oxygen affinity and low cooperativity in diabetes and hyperlipidemia may lead to reduced tissue oxygen delivery.

Oxidative stress can produce profound alterations to cellular membrane lipids, proteins, and nucleic acids, impairing cell metabolism and viability, and it is thought to be involved in aging (1) and diseases such as diabetes mellitus, uremia, atherosclerosis, rheumatoid arthritis, adult respiratory distress syndrome, reoxygenation injury, HIV infection, and cystic fibrosis. Oxidative stress corresponds to an imbalance between the production of reactive oxygen species, mainly the superoxide anion (O2−), hydroxyl radical (·OH), peroxyl radicals (LOO·), and hydrogen peroxide (H2O2), and protective mechanisms. Several enzymatic systems can detoxify reactive oxygen species: superoxide dismutase catalyzes the conversion of O2− to H2O2 and works concomitantly with catalases and a selenoprotein, glutathione peroxidase. The concentration of reduced glutathione (GSH)3 is a limiting factor in this enzymatic process, which requires the maintenance of a high reduced-to-oxidized glutathione ratio as achieved by glutathione reductase. In addition, some reducing agents, such as GSH, vitamin E, vitamin C, and β-carotene, act as free radical scavengers to nonenzymatically detoxify reactive oxygen species.

The tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is the major intracellular nonprotein thiol compound, and it plays a major role in the protection of cells and tissue structures from oxidative injury. Glutathione

1 Nagoya University Daiko Medical Center, 1-1-20 Daiko-minami, Higashiku, Nagoya 461-0047, Japan.
2 Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
*Author for correspondence. Fax 81-52-719-1875; e-mail tniwa@med.nagoya-u.ac.jp.
Received August 2, 1999; accepted October 15, 1999.

© 2000 American Association for Clinical Chemistry

Nonstandard abbreviations: GSH, reduced glutathione; LC/ESI-MS, liquid chromatography/electrospray ionization-mass spectrometry; Hb, hemoglobin; and metHb, methemoglobin.
can be reduced (GSH), oxidized, 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. The intracellular and blood concentrations of GSH are in millimolar range, whereas the plasma concentration is in the micromolar range and accounts for ~0.4% of total blood GSH (2, 3).

Oxidative stress has been proposed as a pathogenic 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 concentrations in the erythrocytes (6–9). In fact, the presence of diabetic complications correlated negatively with the concentration of GSH in erythrocytes (9).

Numerous studies have investigated markers of oxidative stress such as malondialdehyde, or antioxidant defense systems such as superoxide dismutase, glutathione peroxidase activity, or free radical scavengers. 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 the enzyme recycling method (10), a spectrophotometric assay (11, 12), and HPLC with derivatization and fluorescence detection (2, 13).

In this study, we first demonstrated using liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) that glutathionyl hemoglobin (Hb) concentrations are increased in diabetes and hyperlipidemia. Furthermore, we obtained the oxygen dissociation curves of glutathionyl Hb to determine whether the modification of Hb with glutathione affects its oxygen affinity.

Materials and Methods

Subjects
Blood samples were obtained under informed consent, using heparin as an anticoagulant, from 37 patients with type 2 diabetes (24 males and 13 females), 17 patients with hyperlipidemia (6 males and 11 females), and 20 healthy subjects (10 males and 10 females). These samples were stored at −70 °C until analysis.

Sample Preparation for Quantification of Glutathionyl Hb
After centrifugation at 800 g for 10 min, the supernatant plasma was removed, and the erythrocytes were kept at −70 °C. The hemolysates were obtained by thawing the frozen erythrocytes at 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 20 mL/L acetonitrile in 2 g/L acetic acid (90 μL). After filtration through a 0.45 μm filter, the diluted hemolysate sample (10 μL) was subjected to LC/ESI-MS.

LC/ESI-MS
LC/ESI-MS was performed using a triple-stage quadrupole mass spectrometer (TSQ7000; Thermoquest) equipped with a reversed-phase column (TSKgel Phenyl-5PW RP, 7.5 cm × 4.6 mm i.d.). A mobile phase consisting of solution A (20 mL/L acetonitrile in 2 g/L acetic acid) and solution B (900 mL/L acetonitrile in 2 g/L acetic acid) was delivered at a flow rate of 0.5 mL/min at ambient temperature. The mobile phase was programmed for a linear gradient 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 psi; auxiliary gas, 15 units; capillary temperature, 275 °C. The mass spectral acquisition was performed at a scan speed of 2 s with a mass range of m/z 600–1400, using Bioworks ICIS, Ver 8.2 software (Thermoquest).

The glutathionyl Hbβ and glycated Hbβ concentrations were expressed as the percentages of their peak height ratios to those of total Hbβ (intact Hbβ + glutathionyl Hbβ + glycated Hbβ). Glycated Hbα concentrations were expressed as the percentages of their peak height ratios to those of total Hbα (intact Hbα + glycated Hbα).

Reduction of Hemolysate Samples with Dithiothreitol
Hemolysate samples were reduced with dithiothreitol (1 mol/L) at room temperature for 15 min. After reduction, the solution was centrifuged at 6000 g for 5 min, and the supernatant was subjected to LC/ESI-MS.

In Vitro Formation of Glutathionyl Hb and Effect of Added H2O2 on Its Formation
Hb (15 g/L; Sigma Chemical) and GSH (1 mmol/L; Sigma) were incubated in distilled water with or without H2O2 (1 mmol/L) at 37 °C for 7 days. The incubation was performed in water, not in buffer, because the salts in buffers disturb LC/ESI-MS analysis. The incubation solution was subjected to LC/ESI-MS for the measurement of glutathionyl Hb.

Preparation of Hb Samples for Oxygen Equilibrium Studies
A 10-mL blood sample was obtained from a healthy subject with heparin as an anticoagulant. After dilution with 9 g/L NaCl (20 mL), the blood sample was centrifuged at 800 g for 10 min. The supernatant was removed, and packed erythrocytes (4 mL) were obtained. The erythrocytes were washed three times with 9 g/L NaCl by repetition of the above procedures. After addition of distilled water (6 mL) and toluene (2 mL), the erythrocytes were hemolyzed by shaking for 10 min at 4 °C. The hemolysate was centrifuged at 15 000 g for 15 min at 4 °C, and the precipitated Hb was obtained. The Hb solution was dialyzed against 1 mmol/L Tris solution (3 L) at 4 °C overnight. After centrifugation at 15 000 g for 15 min at 4 °C, the obtained Hb was partly used as a control and
was also used to produce glutathionyl Hb by incubation with GSH for oxygen equilibrium studies. Hb (60 g/L) was incubated in 0.05 mol/L phosphate buffer (pH 7.4) with or without GSH (4 mmol/L) at 37 °C for 4 or 7 days.

MEASUREMENT OF METHEMOGLOBIN CONTENT AND REDUCTION OF METHEMOGLOBIN
After dilution of the Hb samples with 0.05 mol/L phosphate buffer (pH 7.4) and subsequent centrifugation at 2000 g for 10 min, methemoglobin (metHb) was measured by the method of Evelyn and Malloy (14). To reduce metHb and to suppress autooxidation during oxygen-equilibrium studies, the metHb reducing system prepared according to the method of Hayashi et al. (15) was added to the Hb samples (180 μmol/L), and kept at 4 °C for 2 days.

OXYGEN EQUILIBRIUM STUDIES
The oxygen dissociation curves of Hb samples were measured according to the method of Imai (16), with a Hb concentration of 180 μmol/L on a heme basis in 0.05 mol/L phosphate (pH 7.4), 25 °C.

MEASUREMENT OF HbA1c
HbA1c was measured by an HPLC method with a column (40 × 6 mm i.d.) packed with hydrous polymer (1 mL) consisting of a methacrylate-methacrylate ester copolymer, using automatic HbA1c measurement equipment, Hi-Auto A1C HA-8150 (Kyoto-Daiichikagaku).

statistical analysis
Results were expressed as mean ± SE with a significance of P < 0.05. To compare values among diabetic patients, hyperlipidemic patients, and healthy subjects, the Fisher PLSD test of ANOVA was used.

Results
MEASUREMENT OF GLUTATHIONYL Hb
The reconstructed ion chromatograms of Hb from a diabetic patient (Fig. 1A) and a hyperlipidemic patient (Fig. 2A) demonstrate the separation of Hbα and Hbβ chains. Figs. 1B and 2B show the deconvoluted mass spectra of Hbα (peak 1) and demonstrate that glycated Hbα, but not glutathionyl Hbα, could be detected. Figs. 1C and 2C show the deconvoluted mass spectra of Hbβ (peak 2). Hbβ peaks showed a molecular mass of 15 868 Da. Glycated Hbβ peaks were detected at 16 030 Da (15 868 + 162 Da), whereas glutathionyl Hbβ peaks were detected at 16 173 Da (15 868 + 305 Da). The peaks at 16 173 Da were identified as glutathionyl Hbβ on the basis of the following findings: (a) the peaks disappeared when the sample was reduced with 1 mol/L dithiothreitol in distilled water, with the simultaneous appearance of a peak for GSH at m/z 308 (M + H)+, with a retention time at 4.2 min (Fig. 3); and (b) the peaks could be detected by incubating Hb with GSH in distilled water at 37 °C for 7 days. More notably, the synthesis of glutathionyl Hbβ was enhanced by adding H2O2, a reactive oxygen species, into the incubation solution (Fig. 4).
The glutathionyl Hb\(\beta\) concentrations were markedly increased in diabetic patients and hyperlipidemic patients compared with healthy subjects (Table 1). Glutathionyl Hb\(\beta\) was not significantly correlated with the other biochemical variables. HbA1c was not correlated with glutathionyl Hb but was well correlated with glycated Hb\(\alpha\) (\(r = 0.94; P < 0.001\)) and glycated Hb\(\beta\) (\(r = 0.74; P < 0.001\)).

**Fig. 2.** Reconstructed ion chromatogram (RIC) of Hb from a hyperlipidemic patient (A), and deconvoluted ESI mass spectra of peak 1 (B) and peak 2 (C) in the RIC chromatogram. Glycated Hb\(\alpha\) and Hb\(\beta\) could be detected, whereas glutathionyl Hb\(\beta\) but no glutathionyl Hb\(\alpha\) could be detected.

**Fig. 3.** Deconvoluted ESI mass spectra of Hb\(\beta\) before (A) and after (C) reduction, and reconstructed ion chromatograms and selected-ion monitoring chromatograms of the same sample for the detection of GSH before (B) and after (D) reduction. (C and D), after reduction with dithiothreitol, glutathionyl Hb\(\beta\) has disappeared, accompanied by the simultaneous appearance of a peak of GSH at m/z 308 (M+H)+.
OXYGEN AFFINITY OF GLUTATHIONYL Hb

The oxygen dissociation curves of control Hb before incubation, and Hb samples incubated with or without GSH for 7 days are shown in Fig. 5. The Hb sample incubated with GSH showed a marked increase in oxygen affinity compared with control Hb and Hb incubated without GSH. Glutathionyl Hb was detected at markedly increased concentrations in the incubation solution of Hb with GSH. However, formation of glutathionyl Hb was not observed in the presence of 1 mol/L dithiothreitol. Table 2 summarizes the glutathionyl Hb concentrations, oxygen equilibrium parameters, and metHb contents in control Hb and Hb samples incubated with or without GSH. LC/ESI-MS analysis of Hb samples incubated with GSH demonstrated that these Hb samples were highly glutathionylated but did not show any other modifications. Hb samples incubated with GSH for 4 and 7 days were 56.9% and 79.2% glutathionylated, respectively, and exhibited 1.9- and 2.5-fold increases in oxygen affinity, and 0.72- and 0.67-fold decreases in the Hill coefficient, respectively, compared with Hb incubated without GSH. These results clearly demonstrate that glutathionyl Hb exhibits high oxygen affinity and low heme-heme interactions (low cooperativity).

Because Hb samples incubated without GSH for 4 and 7 days also showed slightly increased concentrations of glutathionyl Hb of 8.6% and 8.2%, respectively, compared with the control Hb (1.4%), they also exhibited slightly increased oxygen affinity and a slightly decreased cooperativity compared with control Hb.

Table 1. Glutathionyl Hbβ, glycated Hbβ, glycated Hbα, HbA1c, plasma creatinine, total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides in diabetic patients, hyperlipidemic patients, and healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects (n = 20)</th>
<th>Diabetes mellitus patients (n = 37)</th>
<th>Hyperlipidemic patients (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Erythrocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathionyl Hbβ, %</td>
<td>3.7 ± 0.3a</td>
<td>7.9 ± 0.5a,b</td>
<td>8.1 ± 0.8a,b</td>
</tr>
<tr>
<td>Glycated Hbβ, %</td>
<td>3.4 ± 0.2</td>
<td>6.0 ± 0.4a</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>Glycated Hbα, %</td>
<td>2.5 ± 0.1</td>
<td>4.8 ± 0.4a</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>4.8 ± 0.04</td>
<td>7.7 ± 0.3a</td>
<td>4.9 ± 0.04</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine mg/L</td>
<td>7.3 ± 0.3</td>
<td>8.4 ± 0.6</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>Total cholesterol, mg/L</td>
<td>1890 ± 60</td>
<td>2040 ± 50</td>
<td>2630 ± 60a</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/L</td>
<td>680 ± 30</td>
<td>620 ± 30</td>
<td>670 ± 40</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/L</td>
<td>1050 ± 50</td>
<td>1140 ± 60</td>
<td>1700 ± 60b</td>
</tr>
<tr>
<td>Triglycerides, mg/L</td>
<td>800 ± 80</td>
<td>1410 ± 160a</td>
<td>1280 ± 160c</td>
</tr>
</tbody>
</table>

a Mean ± SE.

abc Compared with healthy subjects by the Fisher PLSD test (ANOVA): a P < 0.001; b P < 0.01.
Table 2. Glutathionyl Hb contents, oxygen equilibrium parameters, and metHb contents in control Hb and Hb samples incubated with or without GSH.

<table>
<thead>
<tr>
<th></th>
<th>Glutathionyl Hbβ, %</th>
<th>(P_{50}\text{-}a) mmHg</th>
<th>(n_{\text{max}}\text{-}a)</th>
<th>Reducing system (−)(^{c})</th>
<th>Reducing system (+)(^{d})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.4</td>
<td>7.9</td>
<td>2.96</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>4-Day incubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH (−)</td>
<td>8.6</td>
<td>6.0</td>
<td>2.56</td>
<td>78</td>
<td>1.3</td>
</tr>
<tr>
<td>GSH (+)</td>
<td>56.9</td>
<td>3.1</td>
<td>1.85</td>
<td>77</td>
<td>1.1</td>
</tr>
<tr>
<td>7-Day incubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH (−)</td>
<td>8.2</td>
<td>5.9</td>
<td>2.52</td>
<td>94</td>
<td>1.9</td>
</tr>
<tr>
<td>GSH (+)</td>
<td>79.2</td>
<td>2.4</td>
<td>1.68</td>
<td>94</td>
<td>2.0</td>
</tr>
</tbody>
</table>

\(^{a}\) Partial pressure of oxygen at half saturation.

\(^{b}\) Hill coefficient (maximum slope of the Hill plot).

\(^{c}\) Before the addition of the reducing system.

\(^{d}\) After the measurement of the oxygen dissociation curve with the reducing system added.

Discussion

This is the first study to demonstrate that glutathionyl Hbβ concentrations are markedly increased in diabetic patients as well as hyperlipidemic patients. The enhanced oxidative stress may account for the increased glutathionyl Hb concentrations in diabetes and hyperlipidemia because the addition of \(\text{H}_2\text{O}_2\) in the incubation of Hb with GSH enhanced the synthesis of glutathionyl Hb. Because glutathionyl Hb exhibits high oxygen affinity and low cooperativity, the increased glutathionyl Hb concentrations in diabetes and hyperlipidemia may produce reduced tissue oxygen supply and thereby may contribute to tissue hypoxia.

Human adult Hb (HbA) can react in vitro with GSH with disulfide bond formation between Cys-β93 and the cysteine of GSH (17). Glutathionyl Hb was produced in vitro by thiol-disulfide exchange between mixed disulfides of Hb and GSH to study its antisickling effect. 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. The glutathionyl Hb concentration in erythrocytes of healthy subjects was so low that it could not be detected by electrophoresis (17). However, we could detect it in erythrocytes from healthy subjects as well as in the erythrocytes of diseased patients by using highly sensitive and specific LC/ESI-MS.

The \(P_{50}\) of the control (7.9 mmHg) in Table 2 was low compared with that of intact red cells (27 mmHg). This is attributable to the absence of the allosteric effector, 2,3-diphosphoglycerate, in the purified Hb sample and the low temperature (25 °C) compared with 37 °C in oxygen equilibrium measurements. Glutathionyl Hb exhibits high oxygen affinity and reduced cooperativity. The increase in oxygen affinity was similarly observed in Hb treated with other thiol reagents such as N-ethylmaleimide (18–20), iodoacetamide (18, 20), 5,5-dithiobis-3,3-nitrobenzoic acid (21), or 4,4-dithiophosphate (22). The high oxygen affinity of glutathionyl Hb, and probably also those of the other S-modified Hbs described above, is ascribed to the perturbation of the tertiary structure of the β chain and the \(\alpha_1\)-β2 contacts in the T-state of Hb (23), leading to a shift of the allosteric equilibrium toward the high affinity R-state. The marked increase in oxygen affinity of Hb incubated with GSH is primarily attributable to the formation of glutathionyl Hb and not merely by incubation at 37 °C for 7 days, because LC/MS did not show any abnormally modified Hb except glutathionyl Hb.

In conclusion, the concentrations of glutathionyl Hb with high oxygen affinity and low cooperativity were markedly increased in diabetes and hyperlipidemia, probably because of the enhanced oxidative stress.

References

9. Thornalley PJ, McLellan AC, Lo TW, Benn J, Sonksen PH. Negative


