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Increased glutathionylated hemoglobin (HbSSG) in type 2 diabetes subjects with microangiopathy

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Abstract

Objective: Protein glutathionylation is considered an important post-translational modification in the pathogenesis of complex diseases. The aim of this study was to examine whether hemoglobin (Hb) is modified by reduced glutathione (GSH) via oxidation of the thiol groups present in diabetes and its associated microangiopathy and to determine whether oxidative imbalance has any correlation with glutathionylated Hb (HbSSG) levels.

Methods: The study group consisted of a total of 130 subjects which included non-diabetic healthy control subjects ($n = 30$) and type 2 diabetic patients with ($n = 53$) and without ($n = 47$) microangiopathy. All subjects were assessed for glycemic and lipidemic status, while diabetic subjects were also assessed for the diagnosis of retinopathy and nephropathy. RBC lysates from all the subjects were analyzed by liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) for HbSSG β -globin chains. Levels of GSH and thiobarbituric acid substances (TBARS) levels were measured by spectrophotometric and fluorimetric methods, respectively.

Results: The positivity for HbSSG in diabetic subjects with microangiopathy was significantly higher (69%) compared to diabetics without microangiopathy (22%) and control subjects (14%). In univariate regression analysis, HbSSG levels were significantly associated with the duration of diabetes, HbA1c, and TBARS levels. GSH levels were negatively correlated ($r = -0.57$, $P < 0.001$) with HbSSG in diabetic subjects. A significant inverse correlation ($r = -0.42$, $P < 0.001$) between the GSH levels and HbA1c levels was also seen in diabetic subjects.

Conclusions: This is perhaps the largest LC-MS-based study to demonstrate that HbSSG levels are markedly increased in diabetic subjects with microangiopathy. Since diabetic subjects also exhibited increased lipid peroxidation and decreased GSH levels, it appears that enhanced oxidative stress may account for the increased HbSSG concentrations and altered reduction–oxidation (redox) signaling.

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Keywords: Diabetes; Glutathionylation; Oxidative stress; Redox signaling; Microangiopathy

Introduction

Several experimental, epidemiological, and clinical studies support the view that oxidative stress plays a significant role in the development of vascular complications in diabetics [1,2]. The importance of reactive oxygen

species (ROS) in the pathogenesis of diabetes and its complications are well recognized and a considerable body of evidence implicates ROS in defective cellular signaling [3,4]. However, the molecular mechanisms by which ROS alter cellular signaling are not well understood. Reactive thiols on cysteine (Cys) residues of selected proteins are among the most sensitive sites to be modified by ROS. Oxidants react with these redox-sensitive thiols to form thiyl radicals, which subsequently react with other thiols to form mixed-disulfide bonds. In mammalian cells, glutathione is

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the most abundant low molecular thiol, and hence the most likely to bind to protein thiols to form mixed-disulfides, a process termed *S*-glutathionylation [5,6].

Since a number of functionally critical proteins within the cell possess accessible Cys residues, glutathionylation may be considered an important post-translational modification in the pathogenesis of complex diseases such as diabetes. Therefore, we hypothesize that, under conditions of increased oxidative stress and changes in glutathione levels, many critical proteins are liable to undergo glutathionylation in patients with diabetes and its associated complications. In recent years, glutathionylated proteins including HbSSG have been investigated as possible biomarkers of oxidative stress using the HPLC or LC-MS detection systems [6–10]. Since the blood concentrations of glutathionylated proteins may reflect alterations in redox signaling and oxidation status [10,11], in the present study, we have used erythrocytes as a cellular model to test HbSSG as a possible biomarker of increased oxidative stress.

Methods

The study group was comprised of a total of 130 subjects, which included non-diabetic healthy control subjects ($n = 30$) and type 2 diabetic patients with ($n = 53$) and without ($n = 47$) microangiopathy. The diabetic subjects were selected from out-patients attending the Dr. Mohans' M.V Diabetes Specialties Centre (MVDSC), a tertiary referral center for diabetes care at Chennai (formerly Madras) in Southern India. Control subjects were recruited from the on-going Chennai Urban Rural Epidemiological Study (CURES), a population-based study in Chennai, the details of which have been published elsewhere [12,13]. Details such as age, sex, and, in diabetic subjects, duration of diabetes, and other details of diabetic therapy were recorded; a complete clinical examination was conducted for all subjects. Blood pressure was taken from the patient's right arm to the nearest 2 mm Hg with a mercury sphygmomanometer (Diamond Deluxe Model, Pune, India) while the patient was in the sitting position.

Biochemical and clinical parameter analysis

Biochemical analyses were done on a Hitachi-912 Autoanalyser (Mannheim, Germany) using kits supplied by Boehringer Mannheim (Mannheim, Germany). Fasting plasma glucose (glucose oxidase–peroxidase method), serum cholesterol (cholesterol oxidase–peroxidase–amidopyrine method), serum triglyceride (glycerol phosphate oxidase–peroxidase–amidopyrine method), high-density lipoprotein cholesterol (HDL-C) (direct method), and serum creatinine (modified kinetic method of Jaffe) were measured. Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula [14]. Glycosylated hemoglobin (HbA1C) was estimated by high-pressure liquid

chromatography (HPLC) using the Variant machine (Bio-Rad, Hercules, Calif., USA). Urine samples were collected in the early morning after an overnight fast. Urine creatinine was measured using Jaffe's method. Urinary protein was measured on spot urine by the sulfosalicylic acid technique and expected 24 h protein excretion was calculated as reported earlier [15]. Urine microalbumin concentration was measured using commercially available immunoturbidometric assay kits from Randox (Randox, UK).

Definitions

Microangiopathy was diagnosed if either retinopathy or nephropathy was present.

Nephropathy

Nephropathy was diagnosed if the patient had either persistent proteinuria (≥ 150 mg/day) or microalbuminuria (if albuminuria estimated by the albumin creatinine ratio (ACR) exceeded $30 \mu\text{g}/\text{mg}$ of creatinine) in the absence of a urinary tract infection.

Retinopathy

Fundus photography was done to evaluate retinopathy in the diabetic study subjects. The pupils were dilated using one drop each of phenylephrine 10% and tropicamide 1% into both eyes and drops were repeated until the best possible mydriasis was obtained. A trained photographer carried out four-field color retinal photography with a Zeiss FF 450 plus camera using 35 mm color transparencies. The photographs were graded against standard photographs of the Early Treatment Diabetic Retinopathy Study (ETDRS) grading system for severity of retinopathy as described earlier [16]. Informed consent was obtained from all study subjects and the institutional ethics committee approved the study.

Measurement of glutathionylated hemoglobin (HbSSG)

For the estimation of HbSSG, fasting blood samples were collected into vacutainer tubes containing acid citrate dextrose (ACD) solution. Whole blood was centrifuged at 2000 rpm and the plasma and the buffy coat were removed. The erythrocytes were washed three times in 0.9% NaCl and centrifuged for 5 min after each wash. The packed erythrocytes were then lysed in an equal volume of cold water (Milli-Q; Millipore, France) and processed for mass spectrometry analysis.

For electrospray experiments, an aliquot of the stock solution was diluted 500-fold with distilled water and $10 \mu\text{L}$ of this solution was injected into a single quadrupole mass spectrometer (Hewlett Packard HP 1100 MSD series) with a conventional electrospray ion source. The samples were passed through a C18 reverse phase analytical column (Zorbax, 4.6×150 mm). A linear gradient of acetonitrile from 10–95% in 40 min was employed using distilled water

and acetonitrile containing 0.1% acetic acid as the mobile phase. The flow rate was maintained at 0.2 mL/min. The acquisition was set to full scan mode and the spectra were acquired over the mass range 500 to 2000 *m/z*. Mass scale calibration was done with horse heart apomyoglobin and molecular mass information was obtained after deconvoluting the multiply-charged species using the Chemstation software supplied by the manufacturer. Deconvolution of the charged states distinctively yielded masses (± 2 Da) for the Hb α (15125 Da) and Hb β (15866 Da) chains, glycosylated Hb α (15,288 Da) and glycosylated Hb β (16028 Da) and glutathionylated Hb β (16171 Da). The glutathionyl Hb β concentration was expressed as the percentage of its peak height ratio to that of total Hb β (intact Hb β + glutathionyl Hb β + glycosylated Hb β) [6].

Measurement of glutathione level

Fasting blood samples were collected into vacutainer tubes containing acid citrate dextrose (ACD) solution and Hb concentrations were determined using a Sysmex automated blood counter. Glutathione was estimated in cell lysates (prepared by mixing 0.2 mL of packed cells to 1.8 mL of Na₂EDTA) using metaphosphoric acid as the precipitation agent. Samples were treated with 5,5'-dithio-bis-2-nitro benzoic acid (DTNB), a compound readily reduced by sulfhydryl compounds, forming a highly colored compound which has a maximum absorbance at 412 nm [17]; GSH concentrations were expressed as $\mu\text{mol/g Hb}$.

Lipid peroxidation

Plasma levels of malondialdehyde (MDA), a marker of lipid peroxidation, were estimated by measuring TBARS via fluorescence methodology [18]. Briefly, 200 μL of plasma

was precipitated with an equal volume of 8.1% SDS. The reaction mixture was acidified with 20% acetic acid and subsequently heated with thiobarbitric acid (TBA). It was then allowed to form adducts with oxidative products for 1 h at 95°C. After cooling, TBARS was extracted with butanol:pyridine (15:1) and the fluorescence of the extract was read at an excitation of 535 nm and emission of 553 nm. 1,1',3,3'-tetramethoxypropane was used as the standard for calculating the absolute MDA levels.

Statistical analysis

Statistical analysis was carried out on Windows based SPSS package (version 4.0.1, Chicago, IL). Numbers were expressed as mean \pm standard error. Comparisons between groups were performed using the unpaired Student's *t* test. Two-tailed $P < 0.05$ was considered statistically significant. Pearson correlation analysis was done between variables. The Chi-square test was used to compare proportions. Univariate regression and multiple logistic regression analysis was done using HbSSG as the dependent variable and factors such as age, duration of diabetes, HbA1c, diastolic and systolic blood pressure, serum cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, erythrocyte GSH level, and plasma TBARS levels served as the independent variables.

Results

Table 1 shows the clinical characteristics of the study groups. Diabetic subjects without microangiopathy had higher systolic blood pressure compared to control subjects. Diabetic subjects with microangiopathy had significantly higher systolic and diastolic blood pressures,

Table 1
Clinical and biochemical characteristics of the study subjects

Parameters	Control subjects (<i>n</i> = 30)	Diabetic subjects without microangiopathy (<i>n</i> = 47)	Diabetic subjects with microangiopathy (<i>n</i> = 53)
Age (years)	54 \pm 6.7	56 \pm 6.8	57 \pm 8.4
Duration (years)	Nil	8.0 \pm 7.4	13.6 \pm 7.4
Body Mass Index (kg/m ²)	25.8 \pm 6.6	24.7 \pm 3.8	25.0 \pm 4.2
Systolic BP (mm Hg)	120 \pm 18	130 \pm 15 ^a	137 \pm 14 ^a
Diastolic BP (mm Hg)	76 \pm 10	78 \pm 9	81 \pm 7 ^a
Fasting plasma glucose (mg/dL)	88 \pm 12.0	127 \pm 45.0 ^a	138.0 \pm 41.0 ^a
HbA1c (%)	5.8 \pm 0.5	7.7 \pm 2.5 ^a	8.5 \pm 1.9 ^a
Serum cholesterol (mg/dL)	158.9 \pm 36.2	167.3 \pm 40.3	169.2 \pm 38.7
Serum triglyceride (mg/dL)	110.4 \pm 69.4	129.6 \pm 56.5	156.7 \pm 81.3 ^a
HDL cholesterol(mg/dL)	47.2 \pm 14.0	39.6 \pm 14.2	42.3 \pm 11.6
LDL cholesterol (mg/dL)	113.6 \pm 33.7	129.6 \pm 56.4	156.7 \pm 81.5 ^a
Serum creatinine (mg/dL)	1.00 \pm 0.1	1.03 \pm 0.10	1.09 \pm 0.45
Glutathione (GSH) ($\mu\text{mol/g Hb}$) ^b	6.22 \pm 0.13	5.14 \pm 0.17 ^a	5.29 \pm 0.13 ^a
Thiobarbituric acid reactive substances (TBARS) (nM/mL) ^b	5.90 \pm 0.21	9.14 \pm 0.53 ^a	10.04 \pm 0.78 ^a

Values expressed as mean \pm SD.

^a $P < 0.05$ compared to control group.

^b Values expressed as mean \pm SEM.

serum triglyceride, and LDL-C levels compared to control subjects.

Fig. 1a shows an ESI mass spectrum of a non-diabetic (control) subject's hemoglobin. Deconvolution of the charge states (transformed mass scale) distinctively yielded precise masses for the α (15,125 Da; $M_{\text{calculated}}$, 15,126 Da) and β (15,866 Da; $M_{\text{calculated}}$, 15,867 Da) chains. In hemoglobin from a diabetic subject, the ESI mass spectrum revealed an additional distinct mass at 16,171 Da, apart from the normal globin chains detected at 15,125 Da (Hb α) and 15,866 Da (Hb β) (Fig. 1b). The 16,171 Da peak may be assigned to HbSSG, formed by reaction of the free thiol group of Hb $\beta^{\text{Cys}93}$ with oxidized glutathione. The assignment of HbSSG is reported in the literature [6]. To confirm our HbSSG assignment, we have recorded the ESI mass spectra of a normal Hb sample from a control subject to which oxidized glutathione was added to 100 mM NH_4HCO_3 buffer, at a pH of 8.0, and incubated at 25°C for 2 h. Under

these conditions, in vitro glutathionylation occurs, yielding 3 polypeptide chains: α (15,125 Da), β (15,866 Da), and glutathionylated β (16,171 Da) (Fig. 1c). In addition, when the reducing agent, dithiothreitol (DTT), was added to HbSSG, the 16,171 Da species disappeared (Fig. 1d), further confirming our precise assignment of HbSSG.

The percentage of samples identified as positive for HbSSG in diabetic subjects with microangiopathy (69%; 37/53) was significantly higher ($P < 0.05$) compared to those without microangiopathy (22%; 10/47) and control subjects (14%; 4/30) (Fig. 2). In all HbSSG positive cases, we were able to quantify the amount of HbSSG in terms of percentile values (see methodology), and these values (mean \pm SE) were significantly higher in diabetic subjects with (10.94 \pm 0.81%) and without (8.69 \pm 1.24%) microangiopathy compared to controls (4.85 \pm 1.12%).

In diabetic subjects, univariate regression analysis showed that after adjusting for age and sex, duration of

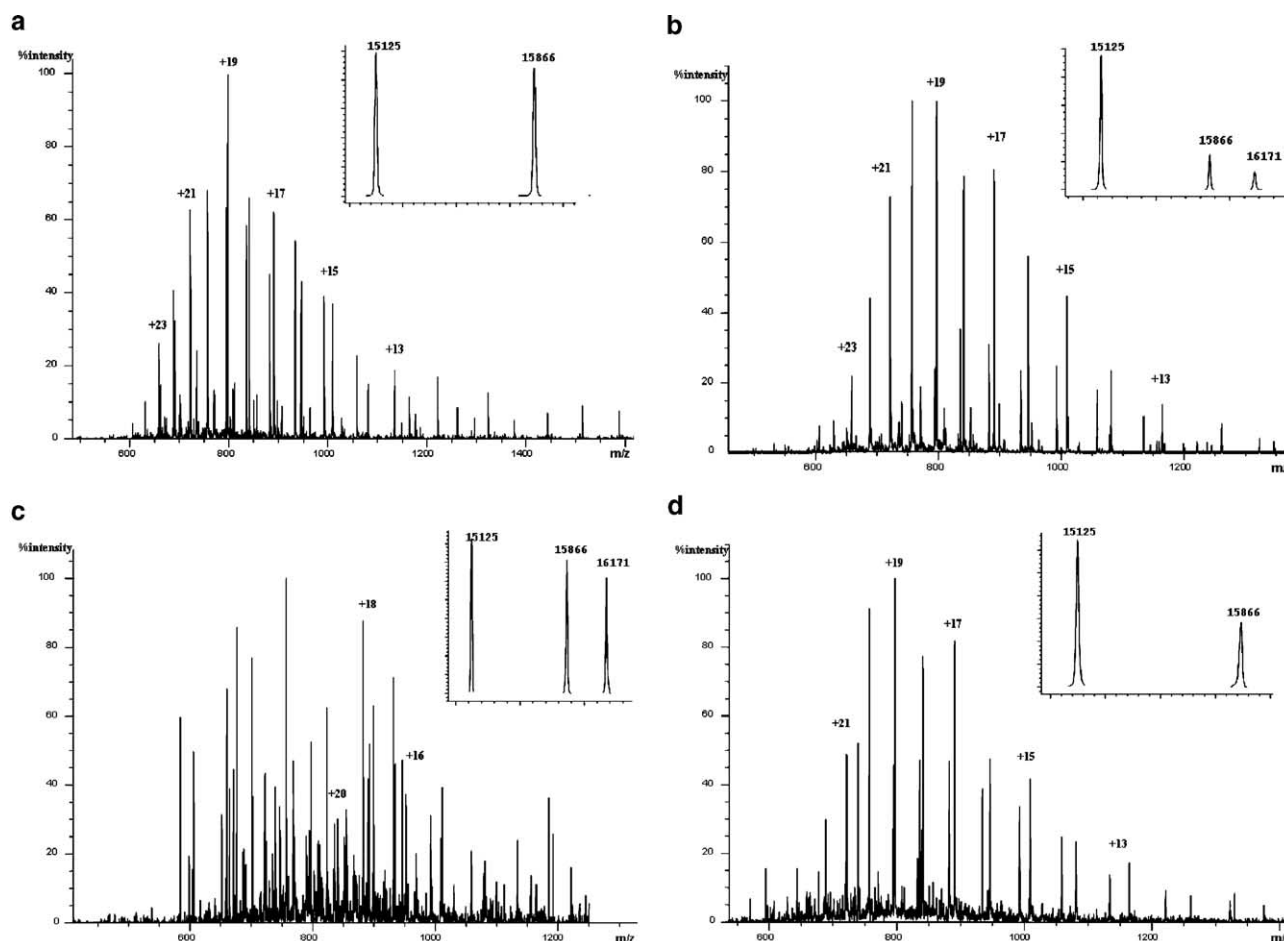


Fig. 1. (a) ESI mass spectrum of hemoglobin chains from a control non-diabetic subject. The transformed mass scale (inset) shows two typical masses detected at 15,125 Da (Hb α) and 15,866 Da (Hb β). (b) ESI mass spectrum of hemoglobin chains in a diabetic subject. The transformed mass scale (inset) shows an additional mass at 16,171 Da (glutathionylated Hb β), separate from masses detected at 15,125 Da (Hb α) and 15,866 Da (Hb β). (c) ESI mass spectrum and the transformed mass scale of globin chains (inset) from a normal hemoglobin sample after incubating with oxidized glutathione (GSSG). The peak representing HbSSG (16,171) appeared when hemoglobin was mixed with excess of GSSG in 100 mM NH_4HCO_3 , pH 8.0, and incubated at 25°C for 2 h. (d) ESI mass spectrum and the transformed mass scale (inset) of a glutathionylated hemoglobin sample after reduction with dithiothreitol. The peak representing HbSSG (16,171) disappeared when hemoglobin was incubated at 25°C for 2 h in 100 mM NH_4HCO_3 (pH 8.0) in the presence of 0.05 M dithiothreitol.

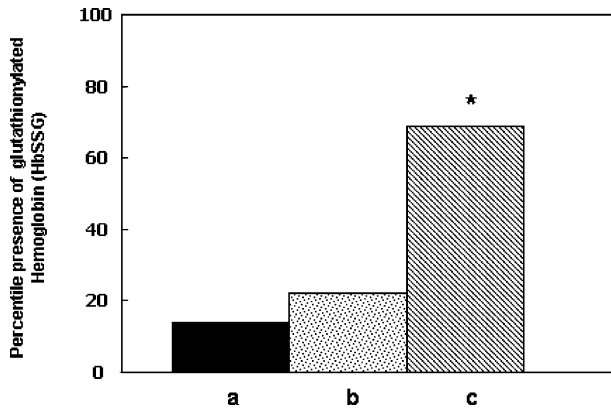


Fig. 2. Percentile presence of glutathionylated hemoglobin in (a) control subjects (14%), (b) diabetic subjects without (22%), and (c) with microangiopathy (69%) (* $P < 0.05$ when compared with diabetic subjects without microangiopathy and control subjects). The levels of glutathionylated hemoglobin computed were (a) $4.85 \pm 1.12\%$, (b) $8.69 \pm 1.24\%$, and (c) $10.94 \pm 0.81\%$.

diabetes (OR-1.07, 95% CI: 1.007–1.140; $P = 0.029$), HbA1c (OR-2.08, 95% CI: 1.502–2.871; $P < 0.0001$), and TBARS (OR-1.15, 95% CI: 1.032–1.270; $P = 0.015$) was associated with HbSSG. Multiple logistic regression analysis revealed that duration of diabetes (OR-1.15, 95% CI: 1.047–1.269; $P = 0.004$) and HbA1c (OR-1.889, 95% CI: 1.248–2.858; $P = 0.003$) was significantly correlated with HbSSG. The GSH levels in diabetic subjects with ($5.29 \pm 0.13 \mu\text{mol/g Hb}$) and without ($5.14 \pm 0.17 \mu\text{mol/g Hb}$) microangiopathy were significantly ($P < 0.001$) lower compared to control subjects ($6.22 \pm 0.13 \mu\text{mol/g Hb}$) (Table 1). There was a significant inverse correlation ($r = -0.42$, $P < 0.001$) between the GSH and HbA1c levels in diabetic subjects (Fig. 3). Interestingly, GSH values were also negatively correlated ($r = -0.57$, $P < 0.001$) with the HbSSG levels in diabetic subjects (Fig. 4). Levels of TBARS, which are a measure of lipid peroxidation, were significantly ($P < 0.001$) increased in diabetic subjects with ($10.04 \pm 0.78 \text{ nM/mL}$) and without microangiopathy ($9.14 \pm 0.53 \text{ nM/mL}$) compared to control subjects ($5.90 \pm 0.21 \text{ nM/mL}$) (Table 1). This measure of oxidative stress also correlated negatively with GSH ($r = -0.37$, $P = 0.001$)

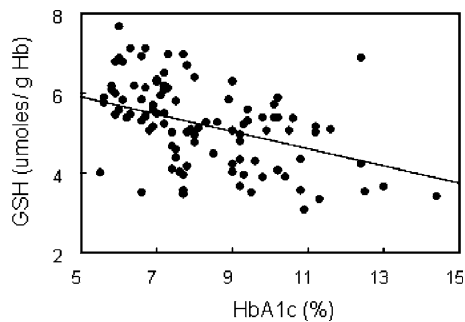


Fig. 3. Correlation between HbA1c (%) and GSH ($\mu\text{mol/g Hb}$) levels in diabetic subjects ($r = -0.42$, $P < 0.0001$).

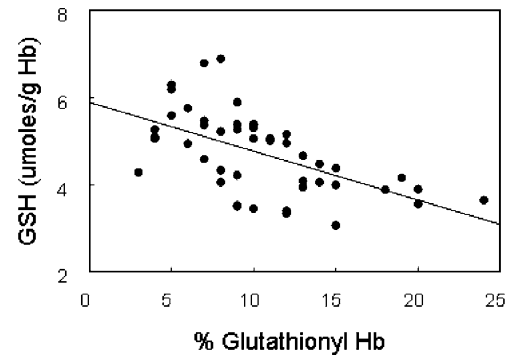


Fig. 4. Correlation of glutathionyl Hb (HbSSG) with GSH levels in diabetic subjects ($r = -0.57$; $P < 0.001$).

and positively with both HbA1c levels ($r = 0.24$, $P = 0.025$) and fasting plasma glucose ($r = 0.25$, $P = 0.018$).

Discussion

Currently, there is a growing awareness of the importance of oxidants as signaling molecules that directly impact the function of tissues in a regulatory fashion by altering the structure of proteinaceous cysteinyl thiols. Multiple modes of protein–cysteine oxidation, such as *S*-thiolation, *S*-nitrosylation, sulfenic acid, sulfinic acid, sulfenyl-amide formation, reactive oxidized lipid derivatives, and intra- and intermolecular protein disulfides are already known to be important in redox regulation [19–21]. The increasing evidence of functional changes resulting from these modifications as well as the growing number of proteins glutathionylated both in vitro and in vivo [22] indicate a role for glutathionylation in diabetes, where protein post-translational modifications might play an important role in the maintenance and progression of disease pathogenesis.

Our work is significant for two reasons. First, this is perhaps the largest LC-MS-based study to demonstrate that HbSSG levels are markedly increased in diabetic subjects with microangiopathy. Secondly, the fact that enhanced oxidative stress may account for the increased HbSSG concentrations was strongly supported by our results, which showed that diabetic subjects also exhibited increased lipid peroxidation and decreased GSH levels.

Increased oxidative stress in human diabetes has been widely reported in the literature. Diabetic microangiopathy correlated positively with lipid peroxidation levels [23,24] and negatively with the GSH concentrations in erythrocytes [25–30]. Sustained hyperglycemia facilitates the increased production of ROS and impairment of the antioxidant defense system leading to a drastic fall in GSH concentrations [31]. Niwa et al. [6] demonstrated increased HbSSG in diabetic and hyperlipidemic patients, but they did not find a correlation between HbSSG and HbA1c levels. However, our study supports the findings of Al-Abed et al. [8], who found that HbA1c levels correlated with HbSSG in diabetic subjects. While increased oxidative stress (increased lipid

peroxidation and glutathione depletion) was observed in diabetic subjects without microangiopathy, only 22% of these patients were identified with HbSSG. This implies that increased oxidative reactions could be early events in the natural history of diabetes [32–34]. In contrast, the formation of HbSSG may depend on the extent of altered glutathione turnover (GSH synthesis, its recycling, and utilization) [35]. Moreover, the extent of glutathionylation may also be related to the duration of diabetes as indicated in our study.

Apart from the increased lipid peroxidation and glutathione depletion seen in Type 2 diabetic subjects with microangiopathy, our study revealed the presence of HbSSG in a remarkable 69% of the samples. Oxidative damage has been implicated in the onset of diabetic eye complications in which the decrease in free radical scavengers was shown to be associated with the oxidation of vitreous and lens proteins [36]. GSH is present in very high concentrations (~6 mM) in the lens of the eye and maintains the eye's transparency. Indeed, one of the earliest changes identified in the diabetic rat lens, prior to opacification, was a fall in glutathione levels [37,38]. Craghill et al. [39] have recently identified a reaction site of glutathione mixed disulfide formation on gamma crystallin S in the human lens, which was claimed to play a role in cataract formation by destabilizing crystallins. Similarly, glutathionylation has also been shown to modify human beta A1/beta A3-crystallins [40]. Since glutathionyl Hb exhibits high oxygen affinity and low cooperativity [6], the increased glutathionyl Hb concentrations in diabetic patients with microangiopathy may result in a state of reduced tissue oxygen supply and thereby may contribute to tissue hypoxia. In fact, retinal hypoxia is accompanied by some pathological conditions that may promote intraocular neovascularization [41]. Mesangial cells subjected to hyperglycemic conditions exhibit elevated levels of intracellular MDA and reduced GSH [42]. The reduced GSH levels were also accompanied by decreased gene expression of gamma-glutamylcysteine synthesis, the rate-limiting enzyme in de novo synthesis of GSH. It appears that both in nephropathy and retinopathy, the decrease in GSH levels may represent an imbalance of redox signaling, favoring increased formation of GSSG and subsequent protein glutathionylation cascades. Whether these redox changes affect the functionality of the cells and tissues specifically in the retina, lens, and kidney requires further investigation.

Our study implies a role of protein glutathionylation in the pathogenesis of diabetes and its associated complications. While the increased formation of HbSSG represents a global change in the oxygen-carrying capacity of hemoglobin, tissue-specific glutathionylation cascades may have drastic cellular repercussions. In fact, the activities of several signaling molecules including PP2A [43], Ras [44], NFkB [45], RyR1 channels [46], PTP1B [47], cAMP-PK [48], aldose reductase [49], mitochondrial complex I [50], and SERCA [51] were reported to be modified

as a consequence of glutathionylation. It is interesting to note that alterations in these signals were reported in defective insulin secretion in β cells, insulin sensitization in peripheral tissues, and complication-related cell-injury and tissue damage in diabetes [22].

Despite the precise importance of oxidative stress and related signaling modifications in diabetes and its complications, studying oxidative stress in humans is limited by the lack of reproducible methods. Although ROS have been detected in vitro by electron spin resonance or by chemiluminescence/fluorescence, these methods are not yet applicable for routine clinical purposes. Our results suggest that measurement of glutathionylated hemoglobin (HbSSG), as assessed by LC-MS, can serve as a biomarker of oxidative stress. This is perhaps one of the first studies on HbSSG measurement in diabetic subjects with and without microangiopathy trying to correlate HbSSG with levels of GSH, TBARS, and glycemic and lipid markers. Recent studies indicate that the use of LC-MS is a sensitive and accurate technology for the study of oxidative stress in human subjects. In support of this, LC-MS based measurement of 8-iso-PGF₂alpha has been demonstrated as a sensitive and independent risk marker of coronary heart disease [52]. In conclusion, our study suggests that HbSSG could be used as a clinical marker of oxidative stress and altered redox regulation in diabetes and its microangiopathy.

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