Journal of Chromatography B, 877 (2009) 3467–3474

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Evidence against a role of ketone bodies in the generation of oxidative stress in human erythrocytes by the application of reliable methods for thiol redox form detection☆

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ARTICLE INFO

Article history:
Received 11 February 2009
Accepted 21 April 2009
Available online 3 May 2009

Keywords:
Oxidative stress
Diabetes
Ketone bodies
Glutathione
Thiols
Malondialdehyde
HPLC

ABSTRACT

Aim of this study was to reconsider the previously suggested contribution of ketone bodies in causing oxidative damage in human red blood cells (RBCs) in the light of our recent findings demonstrating some methodological pitfalls that can occur during detection of hematic thiols. RBCs were incubated at 37 °C with 20 mM ketone bodies and analyzed with time for their content of glutathione, glutathione disulfide and *S*-glutathionylated proteins (in both the hemoglobin and membrane skeletal protein fraction). No changes in the concentrations of glutathione and its related forms were evidenced. Differently from previous reports, our results suggest that ketone bodies do not mediate generation of oxidative stress in human RBCs.

1. Introduction

Several studies report that both type 1 and type 2 diabetic patients have increased concentrations of circulating biomarkers of oxidative stress and reduced antioxidant defenses [1–4], thus suggesting a possible contribution of reactive oxygen species (ROS) to the onset, progression, and/or vascular complications of diabetes [5,6].

Insulin-dependent or type 1 diabetes mellitus, resulting from an organ-specific, cell-mediated autoimmune attack on pancreatic β-cells, and therefore also called autoimmune diabetes, is the major example of pathological ketosis, a transient condition that is characterized by elevated serum to concentrations of ketone bodies. The term "ketone bodies" refers to three molecules: β-hydroxybutyrate (BHB), acetone, and their precursor, acetoacetate (AA). Essentially, increases in ketone bodies in pathological ketosis are similar to those occurring during fasting, but they are more pronounced. Insulin is absent or very low in abundance in the plasma and, therefore, there is no antagonistic action to restrain the opposing hormones, adrenaline, noradrenaline, and glucagon. Consequently, lipolysis in adipose tissue is greatly stimulated and plasma fatty acids increase to high concentrations. In mammals, normoketonemia is defined as a blood concentration of total ketone bodies below 0.5 mM, hyperketonemia is defined as concentrations >1 mM, and diabetic ketoacidosis as above 3 mM [7].

Diabetic patients with frequent episodes of ketosis have increased incidence of vascular diseases, neuropathy and mortality [8]. Plasma concentrations of circulating ketone bodies can be even higher than 25 mM in diabetic subjects with severe ketosis [9,10]. The immediate concern in ketotic patients is acidosis and dehydration. Current standards of clinical practice do not allow an even milder degree of ketosis in diabetic patients [11,12]. Nevertheless, ketonemia concentrations of 1–2 mM are frequently seen in type 1 diabetic patients, even at the time of routine check-up visits to the clinic [11]. It has been suggested that elevated concentrations of ketone bodies, in particular AA, can be responsible for the increased oxidative stress found in diabetes [13]. Even if the exact mechanism(s) leading to ketone bodies-mediated oxidative stress has not yet been clarified, some biomarkers of oxidative stress, such as glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) and malondialdehyde (MDA), were found to be modified after in vitro treatments of human red blood cells (RBCs) with AA [13], thus supporting the hypothesis that ketone bodies may play a role in the

☆ This paper is part of the special issue "Analysis of Thiols", I. Dalle-Donne and R. Rossi (Guest Editors).

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doi:10.1016/j.jchromb.2009.04.032

generation of some of the oxidative damages occurring in diabetic patients.

In our experience, hematic determinations of thiols and disulfides are frequently affected by analytical flaws, which may compromise the results and derived conclusions. Indeed, we have observed that hematic concentrations of GSH and in particular of glutathione disulfide (GSSG) and *S*-glutathionylated proteins (PSSG) span over a two-order-of-magnitude range even in healthy people, when measured by different research groups [14]. We have identified different pitfalls that can affect these analyses and developed and validated new methods for GSH, GSSG and PSSG assessment in blood [15,16]. Aim of this study was, therefore, to apply these new methods in order to reconsider the possible contribution of ketone bodies in causing oxidative damage in human RBCs. For this purpose, GSH, GSSG and PSSG were measured in RBCs exposed to high concentrations of AA, BHB, or acetone. In addition, other biomarkers of oxidative stress, i.e., MDA and protein carbonyls, were measured too.

2. Experimental

2.1. Chemicals

Dinitrophenylhydrazine (DNPH) and all analytical grade reagents used in this study were from Sigma–Aldrich (Milan, Italy) unless mentioned otherwise. Monobromobimane (mBrB) was obtained from Calbiochem (La Jolla, CA, USA) and HPLC grade reagents from BDH (Poole, England). HPLC column Sephasil C18 (250 mm × 4 mm, 5 μ particle size) was purchased from Pharmacia (Uppsala, Sweden). HPLC column Biosil NH_2 (250 mm × 4.6 mm, 5 μ particle size) was purchased from Biorad (Milan, Italy). Mouse monoclonal anti-GSH antibody (101-A) was obtained from Virogen (Watertown, MA, USA). Anti-dinitrophenyl-KLH antibodies, rabbit IgG fraction and anti-rabbit IgG, peroxidase conjugate were purchased from Molecular Probes (Eugene, OR, USA). Sheep anti-mouse IgG, horseradish peroxidase-conjugated was obtained from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, England). Criterion Tris–HCl (12.5% and 10%) resolving gels, and the Opti-4CN Substrate Kit, were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Blood processing and sample treatment

Blood samples were drawn from healthy volunteers (age 30–40), after informed consent, with K_3EDTA as an anticoagulant. RBCs were prepared by centrifugation at 800 × *g* for 10 min and washed three times with Na^+/K^+ phosphate buffered saline (pH 7.4) containing 6 mM glucose (PBSG). The washed RBCs were suspended in PBS containing 10 mM glucose to a hematocrit value of 15% and streptomycin (0.075 mg/ml) was added to prevent bacterial growth. RBCs were then incubated in a thermostatic bath at 37 °C, with gentle gyratory shaking (60 rpm) and treated with 20 mM (final concentration) AA, BHB or acetone.

For GSH and GSSG determinations, 1 ml of sample was washed three times with 1 ml of PBSG containing 10 mM *N*-ethylmaleimide (NEM); then 0.05 ml of packed RBCs were acidified with 0.1 ml of 10% (w/v) trichloroacetic acid (TCA). Protein carbonyls and PSSG were measured in two 0.04-ml samples of the same packed RBCs after hemolysis by addition of 0.16 ml of 5 mM Na^+/K^+ phosphate buffer, pH 8.0, containing 1 mM NEM. Samples were centrifuged (15,000 × *g* for 10 min) to separate membranes, and supernatants were passed through gel-filtration columns (PD10, Pharmacia) equilibrated with 50 mM Na^+/K^+ phosphate buffer, pH 7.4. Membranes were repeatedly washed with Na^+/K^+ phosphate buffer, pH 8.0, containing 1 mM NEM for removal of nonspecifically bound hemoglobin (Hb). In experiments in which the artifactual oxidation of GSH by omission of NEM was evaluated, 1 ml of sample was washed three times with 1 ml of PBSG without NEM; then 0.05 ml of packed RBCs were acidified with 0.1 ml of 10% (w/v) TCA.

For MDA analyses, 0.2-ml of samples were spiked with 20 μl of 0.2% (w/v) butylated hydroxytoluene and deproteinized by 5% (w/v, final concentration) TCA.

2.3. GSH, GSSG and PSSG measurements

GSH and GSSG were determined as previously described [15]. Briefly, the excess of NEM in the supernatants was extracted by addition of dichlorometane (10 volumes). 0.1 ml of samples were reacted with an equal volume of 2,4-dinitrofluorobenzene (FDNB, 1.5 vol.% in ethanol), for 3 h at room temperature in the dark, after pH alkalinization by addition of 30 μl of 2 M TRIS. Samples were then acidified with 20 μl of 37 vol.% HCl and analyzed by HPLC separation on a NH_2 column, the oven temperature was set at 30 °C. In experiments in which the artifactual oxidation of GSH was evaluated, NEM was added to 0.1 ml of acidified supernatants at a final concentration of 50 mM in the presence of 0.015 ml of 2 M TRIS; after a 30-s incubation, NEM was removed by extraction with dicholorometane and samples were derivatized for HPLC analysis as described above. Elution conditions: solvent A = methanol–H_2O (80:20, v/v); solvent B = 0.5 M acetate buffer, pH 4.6, in solvent A; 0–10 min: 70% solvent A/30% solvent B; 10–35 min linear gradient from 30% to 95% solvent B. A constant flow rate of 1.2 ml/min was applied. Detection was performed at 355 nm wavelength.

S-Glutathionylated proteins were measured in both the cytosolic and membrane fraction by a previously described method with slight modifications [17]. Briefly, samples (both eluted cytosol and membranes resuspended in Na^+/K^+ phosphate buffer, pH 7.4) were incubated with 0.5 mM dithiotreitol (DTT, final concentration) for 10 min at room temperature to reduce S–S bonds, and the released GSH was conjugated with the fluorescent label mBrB (2 mM, final concentration) by a 10-min incubation at room temperature in the dark. Finally, samples were deproteinized by adding 5% (w/v, final concentration) TCA and the mBrB–GSH derivative was separated by HPLC on C18 column, the oven temperature was held at 25 °C. Elution conditions: solvent A = sodium acetate 0.25% (v/v) pH 3.09; solvent B = acetonitrile; 0–5 min: 94% solvent A/6% solvent B; 5–10 min linear gradient from 6% to 10% solvent B. A constant flow rate of 1.2 ml/min was applied. Detection was performed at 390 nm excitation and at 480 nm emission wavelength. The hemoglobin concentration in hemolysates was determined spectrophotometrically by means of a Jasco V-530 spectrophotometer after centrifugation of samples (800 × *g*, 10 min) by recording the 500–700 nm spectra and considering the peak height at 541 nm ($\varepsilon = 13.8\ mM^{-1}\ cm^{-1}$) [18].

The applied procedures for GSH, GSSG and PSSG measurements are summarized in Fig. 1.

2.4. Membrane skeletal protein separation

The packed erythrocytes were hemolysed by addition of 1 ml of 5 mM Na^+/K^+ phosphate buffer, pH 6.5, containing 2 mM NEM and centrifuged at 20,000 × *g* for 15 min at 4 °C. For membrane skeletal protein analyses, the pellets were resuspended with a glass rod in 5 mM phosphate buffer, pH 6.5, containing 1 mM NEM and centrifuged at 20,000 × *g* for 15 min at 4 °C; this step was repeated three times.

2.5. Immunoblot analysis

Protein samples were mixed with an equal volume of modified Laemmli sample buffer, without reducing agents and supplemented with 1 mM NEM, to block eventually unreacted thiol groups, and

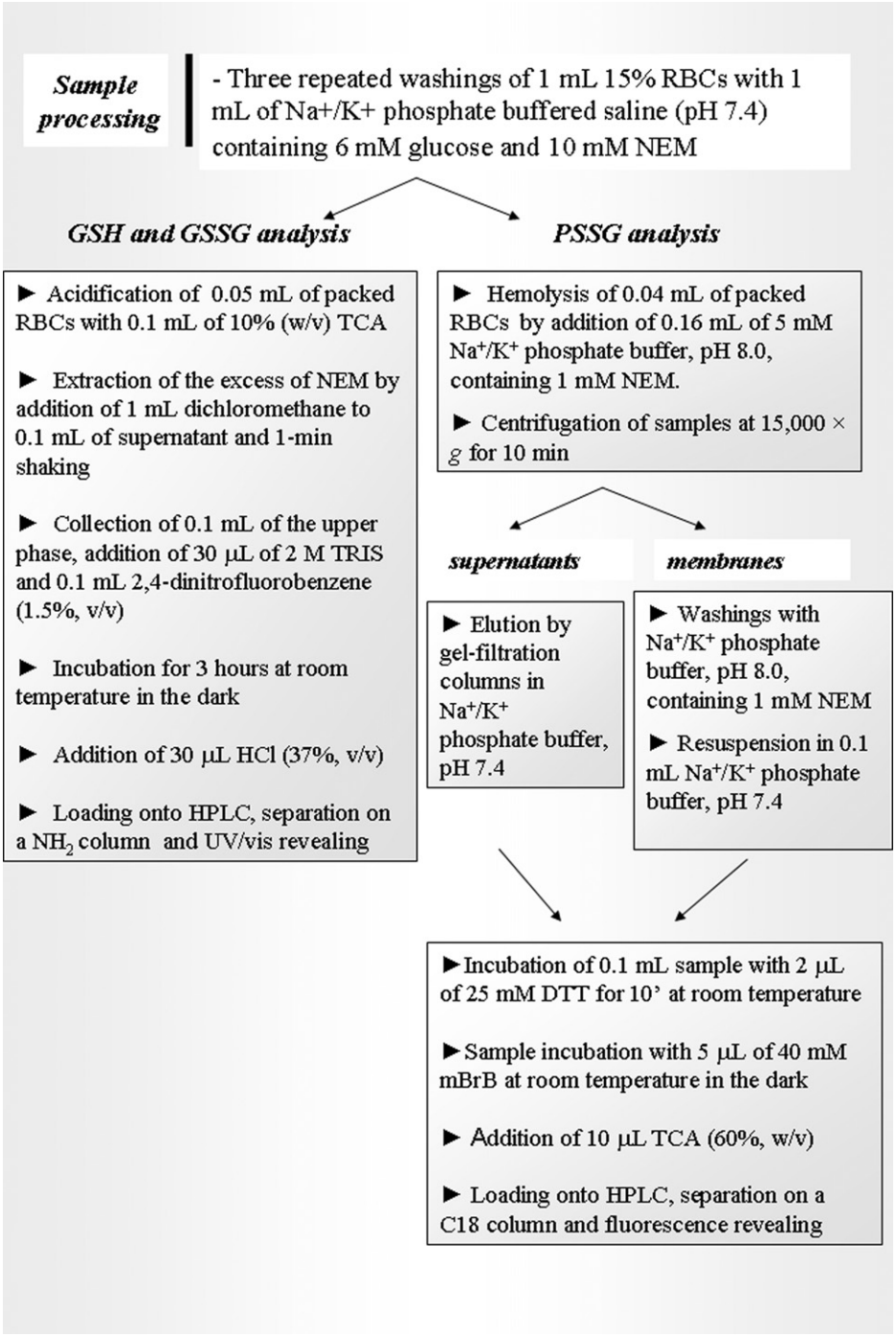

Fig. 1. Schematic of the procedural steps applied to GSH, GSSG and PSSG measurement in RBCs.

then subjected to SDS-PAGE at room temperature, using Criterion Tris–HCl 10% resolving gels for membrane skeletal proteins and 12.5% resolving gels for hemoglobin. Gels were stained with Coomassie blue. Western blotting of RBC membrane skeletal proteins and immunological detection of PSSG with monoclonal mouse anti-GSH antibody were carried out as recently reported [19–21]. Positive controls for *S*-glutathionylation of hemoglobin and membrane skeletal proteins were obtained by exposure of RBCs to diamide, as recently reported [21]. Membrane protein concentration was measured according to the Bradford assay [22].

Protein carbonyls were determined by Western immunoblotting. After SDS-PAGE separation at room temperature, samples were electroblotted to a polyvinylidene difluoride membrane by Criterion Blotter provided with sealed ice block, followed by successive incubations in 2 M HCl and DNPH (0.1 mg/ml in 2 M HCl) for 5 min each. The membrane was then washed three times in 2 M HCl and seven times in 100% methanol for 5 min each, followed by one wash in PBST {10 mM Na^+ phosphate buffer, pH 7.2, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20} and blocking for 1 h in 5% (w/v) nonfat dry milk in PBST. After three washes with PBST for 5 min each, immunologic evaluation of carbonyl formation was performed for 2 h in 5% milk/PBST containing anti-DNP-KLH antibody (1:10,000 dilution). After washing with PBST, the membrane was incubated with a 1:2000 dilution of the secondary antibody linked to horseradish peroxidase in 5% milk/PBST for 1 h. After washing with PBST, development was performed with the Opti-

4CN Substrate Kit. All washes and incubations were made at room temperature. As positive controls for protein carbonylation, isolated hemoglobin and RBC membrane skeletal proteins were treated with 1 mM HOCl for 30 min and then mixed with an equal volume of SDS sample buffer without reducing agents.

2.6. MDA measurements

MDA was determined by HPLC as previously described [23] with slight modifications. Briefly, 0.2 ml of supernatants was reacted with the same volume of 0.6% (w/v) thiobarbituric acid (TBA) for 10 min in a boiling water bath in tightly capped glass tubes. Samples were then chilled in an ice-water bath and immediately analyzed by HPLC. Calibration curves (50 nM to 1 μM range) were built by adding known amounts of the MDA precursor 1,1,3,3-tetraethoxypropane to pooled supernatants. HPLC separation was performed on a C18 column thermostated at 25 °C. Elution conditions: solvent A = 50 mM HEPES, pH 7.0; solvent B = acetonitrile; 0–5 min: 86% solvent A/14% solvent B. A constant flow rate of 1.2 ml/min was applied. Detection was performed at 532 nm wavelength. All HPLC measurements were carried out by means of an Agilent 1100 series apparatus.

2.7. Statistics

Data are expressed as mean ± SD. Analysis of variance was used to test for significant differences within experiments. Differences at the 0.05 level were considered significant.

3. Results

3.1. Artifactual oxidation of GSH during sample handling

Many different methods have been proposed for measurement of GSH and its redox forms (GSSG and PSSG) in blood as biomarkers of oxidative stress. Independently of the selected procedure, a common step is protein separation by sample acidification before analysis. We have previously observed that, during acid-induced denaturation of blood, oxyhemoglobin elicits the production of several oxidants, which induce a strong artifactual increase in both GSSG and PSSG concentration. We also demonstrated that this phenomenon can be prevented by blocking the –SH groups with alkylating agents (such as NEM) before acidification [14–16]. Taking into consideration these aspects, we developed two different methods, one applicable to GSH/GSSG measurements [16] and the other one applicable to PSSG analyses [17]. Both these methods were shown to be reliable. The accuracy and precision were assessed by measuring quality control samples at three concentrations on three different days. Intra- and inter-assay precision (RSD, %) was below 4% for all three parameters. Intra- and inter-assay relative error, which was calculated using the formula: (mean observed concentration *minus* spiked concentration)/(spiked concentration) × 100%, ranged from −2.8% to +3.3% for GSH, from −3.5% to +4.2% for GSSG and from −2.2% to +3.7% for PSSG. The artifactual GSH oxidation has also been verified here with samples constituted by washed human RBCs (15% hematocrit). As a matter of fact, when GSH was not protected from oxidation by NEM treatment before acidification, the measured concentration of erythrocytic GSH decreased by about 25%, whereas that of GSSG largely increased, as depicted by tracings reported in Fig. 2. *S*-Glutathionylated proteins too artificially increased when samples were not protected by NEM before acidification (data not shown).

It is evident from these data that the artifactual increase of both GSSG and PSSG might mask the occurrence of a slight oxidative burden. This is further supported by results reported in Table 1.

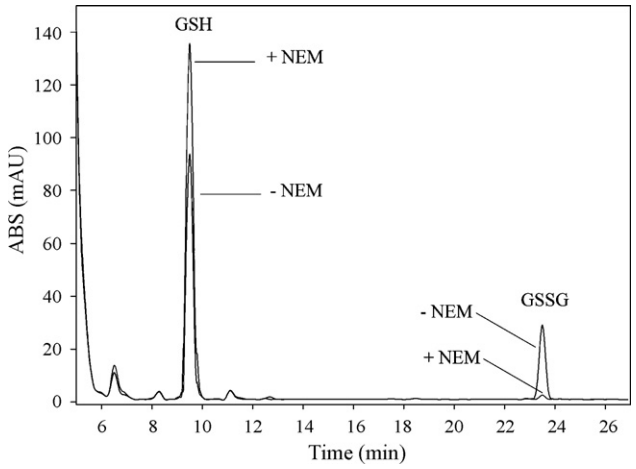

Fig. 2. Typical HPLC chromatograms obtained from RBCs (15% hematocrit) after derivatization with FDNB for GSH/GSSG analysis. Black trace: RBC sample washed with PBSG (pH 7.4) without NEM; gray trace: RBC sample washed with NEM-containing PBSG (pH 7.4). Samples were then packed, acidified with TCA and analyzed by HPLC.

In these experiments, human RBCs treated with different concentrations of *tert*-butylhydroperoxide (*t*-BOOH, an oxidizing agent) showed a significant increase of GSSG concentration depending on the amount of the added oxidant only in samples in which –SH groups had been alkylated by NEM before acidification. By contrast, non-alkylated samples induced *per se* an artifactual increase of GSSG, which masked the effect of *t*-BOOH at most of the concentrations used. Representative HPLC tracings for GSH and GSSG in human RBCs under basal conditions or after 1-min treatment with 10 μM *t*-BOOH are shown in Fig. 3. The increase in GSSG peak area after the treatment with the oxidant was evident, when artifactual thiol oxidation was avoided (panel A). By contrast, without NEM pre-treatment (panel B), we did not observe any difference with respect to basal values. Under these slight oxidative modifications, the GSH peak did not change significantly as compared to the basal values, even in the absence of artifactual oxidation, probably due to the high GSH concentration.

3.2. Effect of ketone bodies on GSH, GSSG, and S-glutathionylated proteins

Alterations in redox balance following oxidative stress can cause changes in the intracellular GSH/GSSG ratio, potentially influenc-

Table 1
GSSG concentrations in human RBCs after 1-min incubation with varying concentrations of *t*-BOOH[a].

t-BOOH (μM)	GSSG (μM)	
	With NEM	Without NEM
0 (control)	1.02 ± 0.08	31.5 ± 4.6
2.5	1.98 ± 0.24*	28.6 ± 2.6
5	5.06 ± 0.49*	29.1 ± 4.1
10	8.20 ± 1.25*	33.2 ± 4.3
25	21.6 ± 2.6*	43.8 ± 5.6*
50	40.1 ± 5.5*	63.6 ± 2.4*
100	85.3 ± 8.3*	112 ± 8.6*

[a] Human RBCs (15% hematocrit) were treated with *t*-BOOH (2 mM stock solution in saline) for 1 min at 37 °C, under gentle stirring. For GSSG measurement in the presence of NEM, 1 ml sample was washed with PBSG (pH 7.4) containing 10 mM NEM; packed RBCs were deproteinized by addition of TCA. Excess of NEM was removed by extraction with CH_2Cl_2 and samples were analyzed by HPLC. For GSSG measurement without NEM, 1 ml sample was washed with PBSG (pH 7.4, no NEM) and then packed RBCs were acidified by addition of TCA and analyzed by HPLC. See Section 2 for further details.

* $p < 0.05$ vs. control.

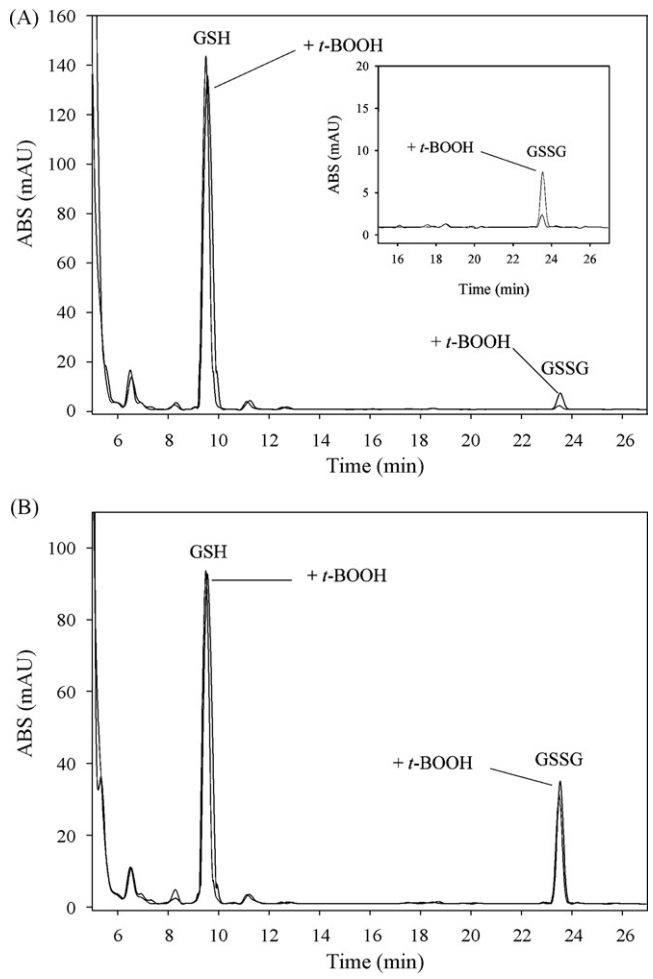

Fig. 3. Typical HPLC chromatograms obtained from RBCs (15% hematocrit) after oxidation with *t*-BOOH and derivatization with FDNB for GSH/GSSG analysis. RBCs were treated with 10 μM *t*-BOOH (2 mM stock solution dissolved in saline) at 37 °C for 1 min. (A) RBC sample washed with NEM-containing PBSG (pH 7.4). Sample was then packed, acidified with TCA and analyzed by HPLC. Gray trace: control; black trace: 10 μM *t*-BOOH. Inset: enlargement of the chromatogram in correspondence of the GSSG peak. (B) RBC sample washed with PBSG (pH 7.4) without NEM; sample was then packed, acidified with TCA and analyzed by HPLC. Gray trace: control; black trace: 10 μM *t*-BOOH.

ing a number of target proteins by causing oxidation and disulfide exchange reactions at specific protein cysteinyl residues. The results of Figs. 2, 3 and Table 1 indicate that GSH redox forms can be extremely sensitive biomarkers of oxidative stress, because their concentration can significantly change even in the presence of minimal concentrations of oxidants, if common methodological pitfalls are avoided. Therefore, we measured GSH, GSSG and PSSG concentration in human RBCs exposed to 20 mM AA, BHB or acetone to test the previously raised hypothesis that high concentrations of ketone bodies could contribute to the oxidative stress-induced alteration of GSH redox homeostasis in RBCs from diabetic patients [13]. RBCs at 15% hematocrit were treated for up to 24 h with ketone bodies. In both RBCs exposed to ketone bodies and control ones, GSH concentrations decreased very slightly with time (about 10% after 24 h), which is likely due to continuous GSH export from RBCs [24] (Fig. 4). Determination of GSSG (Fig. 5) and PSSG, both in the cytosol and in the membrane fraction (Fig. 6), showed no significant differences between RBCs exposed to ketone bodies and control ones. This indicates that ketone bodies did not cause any significant oxidation of GSH to its redox related forms.

Hemoglobin and membrane skeletal proteins (protein 4.2 and spectrin, in particular) are targets of oxidative stress-induced *S*-

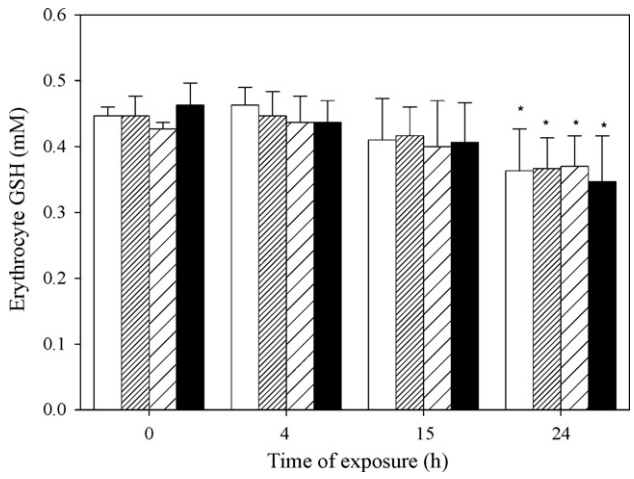

Fig. 4. Measurement of GSH in RBCs (15% hematocrit) exposed to 20 mM AA (fine stripe bars), BHB (coarse stripe bars) and acetone (black bars) for different times. White bars refer to control samples. At the indicated times, samples were washed with NEM-containing PBSG, packed, acidified and analyzed as described in Section 2. Data are referred to the 15% hematocrit RBC solution and represent the mean ± SD of five replicate measurements. $^{*}p < 0.05$ vs. "0".

glutathionylation in human RBCs [21,25]. Therefore, we tested hemoglobin and membrane skeletal protein *S*-glutathionylation in RBCs exposed to ketone bodies by using monoclonal anti-GSH antibody, after SDS-PAGE separation in the absence of reducing agents and electroblotting. None of the membrane skeletal protein bands and not even the hemoglobin band reacted with the anti-GSH antibody (Fig. 7A and C).

3.3. Effect of ketone bodies on other biomarkers of oxidative stress

The eventual occurrence of oxidative stress in samples treated with ketone bodies was finally tested by measuring MDA, which was found to be increased significantly in human blood from type 1 diabetic patients and in RBCs from hyperketonemic, but not in normoketonemic type 1 diabetic patients [13]. MDA is the end-product resulting from the interaction between ROS and polyunsaturated fatty acids of cellular membranes. Therefore, measurement of MDA is an index of ROS production and oxidative

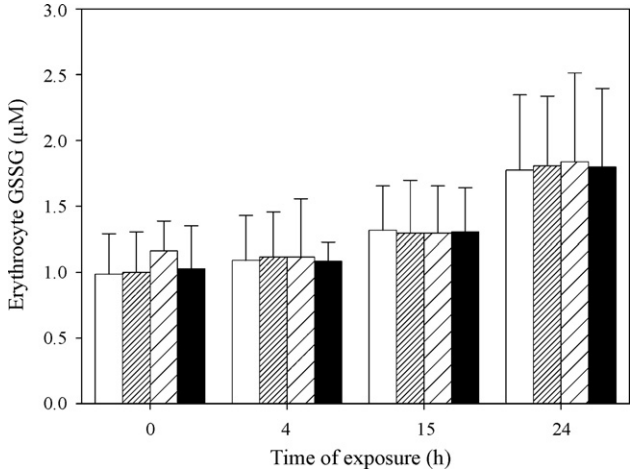

Fig. 5. Measurement of GSSG in RBCs (15% hematocrit) exposed to 20 mM AA (fine stripe bars), BHB (coarse stripe bars) and acetone (black bars) for different times. White bars refer to control samples. At the indicated times, samples were washed with NEM-containing PBSG, packed, acidified and analyzed as described in Section 2. Data are referred to the 15% hematocrit RBC solution and represent the mean ± SD of five replicate measurements.

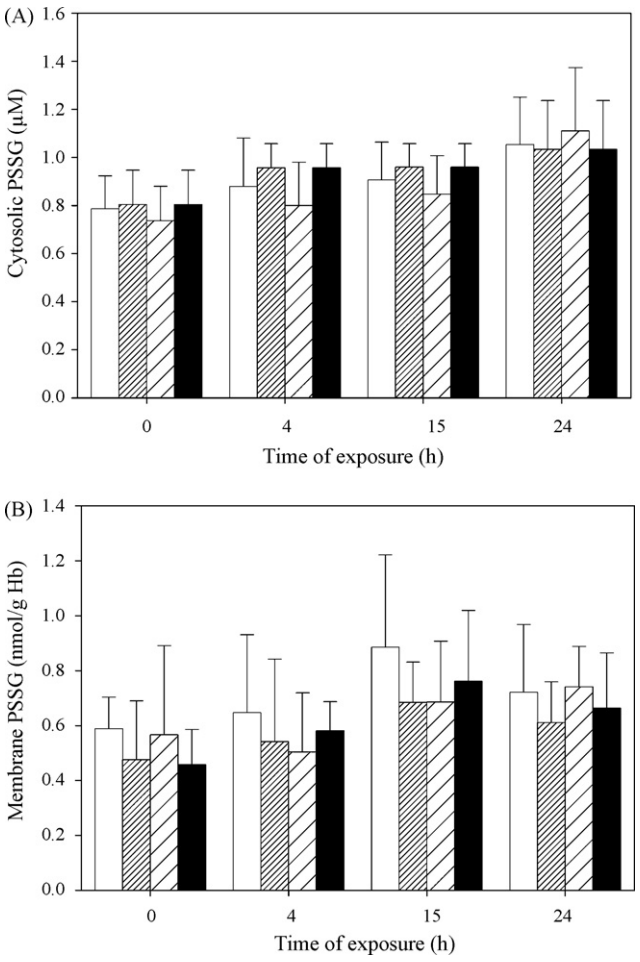

Fig. 6. Measurement of *S*-glutathionylated proteins in the (A) cytosolic and (B) membrane fraction of RBCs (15% hematocrit) exposed to 20 mM AA (fine stripe bars), BHB (coarse stripe bars) and acetone (black bars) for different times. White bars refer to control samples. At the indicated times, samples were washed with NEM-containing PBSG, hemolysed and then centrifuged to separate membranes. In both fractions, GSH released from PSSG following DTT reduction was then derivatized with mBBr and measured by HPLC as described in Section 2. Data are referred to 15% hematocrit the RBC solution and represent the mean ± SD of five replicate measurements.

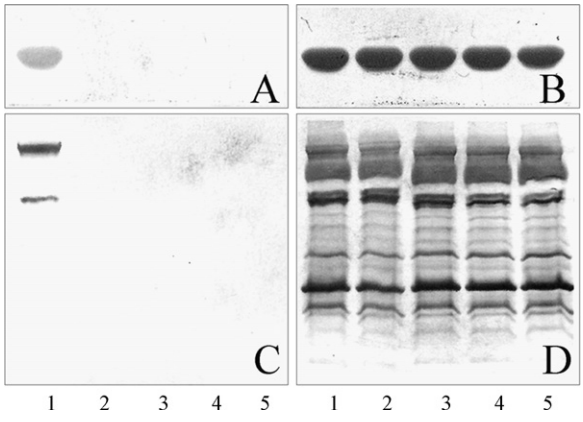

Fig. 7. Western blot analysis of *S*-glutathionylated (A and B) hemoglobin and (C and D) membrane skeletal proteins in RBCs (15% hematocrit) exposed to 20 mM AA (lane 3), BHB (lane 4) and acetone (lane 5) for 24 h. Lane 1 refers to RBCs exposed to 1.5 mM diamide for 10 min (positive control). Lane 2 refers to control RBCs. Protein samples were separated by SDS-PAGE under non-reducing conditions, blotted onto PVDF membrane and probed with anti-GSH monoclonal antibody. (A and C) Western blot probed with anti-GSH monoclonal antibody. (B and D) Blot staining with Amido Black for verifying equal protein loading. The blots shown in the figure are representative of three independent experiments.

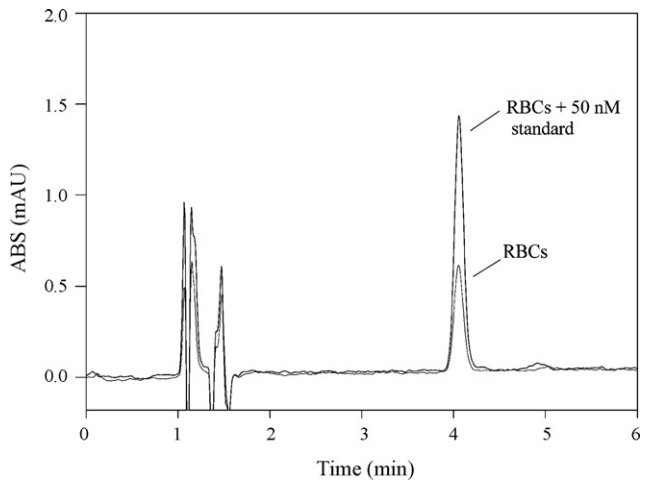

Fig. 8. Typical HPLC chromatograms obtained from analysis of MDA in RBCs (15% hematocrit) after MDA conjugation with TBA. Gray trace: RBCs analyzed without ketone bodies addition. Black trace: the same sample spiked with 50 nM of the MDA precursor 1,1,3,3-tetraethoxypropane after acid-induced deproteinization.

stress, and is one of the most frequently used indicators of lipid peroxidation. For MDA analyses we applied a previously reported HPLC method [23] with minor modifications. In Fig. 8 typical chromatograms for the adduct $(TBA)_2$–MDA in RBC samples are shown. Specifically, we report here a chromatogram obtained by analyzing RBCs before treatment with ketone bodies in comparison with the same sample spiked with 50 nM of the MDA precursor 1,1,3,3-tetraethoxypropane. The applied method was reliable with intra- and inter-assay precision (RSD) values below 2.5% and the relative error ranging from −2.8% to +2.6%. MDA content was similar in erythrocytes exposed to ketone bodies and in control RBCs (Fig. 9). Analogously, protein carbonyl concentrations, a biomarker of severe protein oxidative damage [26], did not change upon RBC exposure to ketone bodies (Fig. 10).

4. Discussion

Evidence of oxidative stress in diabetes mellitus is generally provided by demonstration of altered concentrations of biomarkers of oxidative damage, such as protein oxidative modifications and GSH

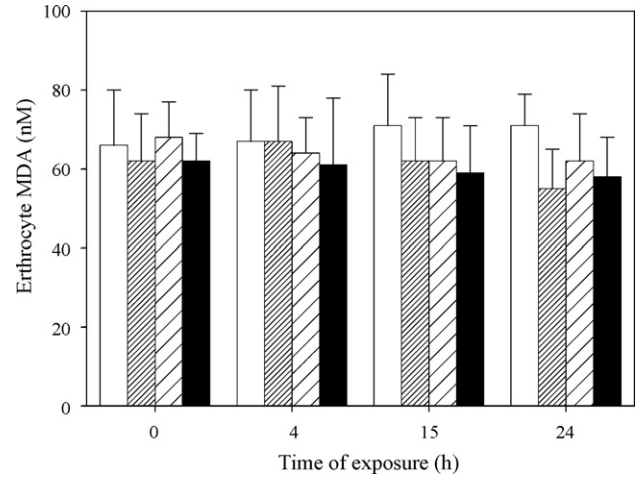

Fig. 9. Measurement of MDA concentration in RBCs (15% hematocrit) exposed to 20 mM AA (fine stripe bars), BHB (coarse stripe bars) and acetone (black bars) for different times, as detected by HPLC. White bars refer to control samples. Data refer to the 15% hematocrit RBC solution and represent the mean ± SD of five replicate measurements.

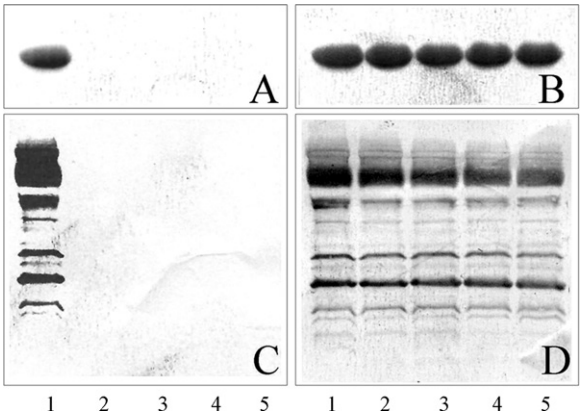

Fig. 10. Western blot analysis of carbonylation of (A and B) hemoglobin and (C and D) membrane skeletal proteins in RBCs exposed to 20 mM AA (lane 3), BHB (lane 4) and acetone (lane 5) for 24 h. Lane 2 refers to control RBCs. (A and B) Lane 1 refers to isolated hemoglobin oxidized with 1 mM HOCl for 30 min and then mixed with an equal volume of SDS sample buffer without reducing agents (positive control). (C and D) Lane 1 refers to isolated membrane skeletal proteins oxidized with 1 mM HOCl for 30 min and then mixed with an equal volume of SDS sample buffer without reducing agents (positive control). Protein samples were separated by SDS-PAGE under non-reducing conditions, blotted onto PVDF membrane and probed with anti-dinitrophenyl-KLH antibodies. (A and C) Western blot probed with anti-dinitrophenyl-KLH antibody. (B and D) Blot staining with Amido Black for verifying equal protein loading. The blots shown in the figure are representative of three independent experiments.

concentration and/or GSH/GSSG ratio [17,26]. Accordingly, previous data from our laboratory demonstrated that in patients suffering from type 1 diabetes the concentration of *S*-glutathionylated hemoglobin is significantly higher than in control subjects [17]. However, inconsistent or contradictory results have been reported by other groups [27]. For instance, the search for blood cell biomarkers of oxidative stress in type 1 diabetic subjects revealed increased [28] or decreased [29] concentrations of GSH and various antioxidant enzymes.

Notwithstanding some contradictory results, there is a close correlation between oxidative stress and diabetes mellitus [30]. Furthermore, the relationship between oxidative stress and the main diabetes-related complications – development of microangiopathy (i.e., retinopathy and nephropathy) and macroangiopathy, accelerated form of atherosclerosis leading to early coronary artery disease, increased risk of cerebrovascular disease, and severe peripheral artery disease – has been firmly established [5]. There are many hypotheses regarding the origin of oxidative stress in diabetes. These include glucose autoxidation, advanced glycosylation, end-product-mediated ROS formation, consumption of NADPH through the polyol pathway, and the activation of protein kinase C [30]. Like hyperglycemia, ketosis is a severe metabolic complication of both insulin-dependent or type 1 diabetes and "ketosis-prone type 2 diabetes" [31]. Type 1 diabetic patients frequently experience hyperketonemia caused by an increased fatty acid metabolism. The suggestion that elevated blood concentrations of ketone bodies commonly encountered in type 1 diabetic patients might relate to the observed oxidative stress is supported by studies showing a significant decrease in GSH and increase in lipid peroxidation concentrations in RBCs from hyperketonemic type 1 diabetic patients, but not in normoketonemic type 1 diabetic patients [13]. These data were confirmed by treatment of human RBCs (15% hematocrit) with AA, which resulted in an increase in both GSSG and MDA concentrations. Presumably, this finding has led to the hypothesis that ketone bodies could be responsible for the occurrence of oxidative stress in diabetic patients [13].

Blood GSSG and *S*-glutathionylated proteins are sensitive biomarkers of oxidative stress, being able to significantly increase even under slightly oxidizing conditions [15,26]. The usefulness of GSSG and PSSG as reliable biomarkers of oxidative stress has been hampered for a long time by some methodological artifacts, as highlighted by two findings: (i) in individuals with the same pathology, GSH, GSSG, and PSSG have frequently been observed to increase by few research groups but to decrease by other groups, also in diabetes (e.g., [28,29,32,33]); and (ii) measurements of GSH, GSSG and PSSG concentrations by different laboratories span over a two-orders of magnitude range in healthy subjects too [14]. Starting from these observations, we compared different procedures and finally proposed validated methodologies to measure GSH and its related redox forms. These methods avoid most of the analytical and pre-analytical pitfalls that can lead to erroneous overestimation of GSSG and PSSG, particularly in the case of hemoglobin and blood samples. Other authors too have identified artifacts that compromise glutathione analyses, and elimination of these methodological problems have finally resulted in measurements close to the actual concentrations of GSH, GSSG and PSSG [34–39].

By using our validated procedures, we examined the possible contribution of ketone bodies to the oxidative damage observed in RBCs from diabetic patients or exposed to ketone bodies. By performing experiments similar to those previously carried out by other authors regarding type of analyzed sample, final concentration of ketone bodies used, and the length of the treatment [13], we obtained in the present study diverging results. In fact, we did not find any significant increase in GSSG (paralleled by the decrease in GSH) upon RBC treatment with AA, BHB or acetone, unlike previous reports by others [13]. We only observed a slight decrease in GSH concentrations after a 24-h treatment, without any detectable rise in GSSG, in all tested samples, including controls. In addition, the concentrations of PSSG both in the cytosol and in the membrane fraction, of MDA and protein carbonyls did not change significantly upon RBC incubation with ketone bodies. Therefore, on the basis of these results it is unlikely that ketone bodies contribute to oxidative damage to RBCs in vitro. The reasons that have led to such a discrepancy with previous studies [10,13] are unknown. One possibility could be methodological artifacts. Analogous to the observations made for thiols, detection of lipid peroxidation can produce artifacts in many assays [40]. Therefore, some guidelines have been suggested to minimize methodological problems when using the TBA assay. One effective solution is coupling the TBA assay with HPLC separation and sample pre-treatment with butylated hydroxytoluene. By applying the HPLC-based TBA assay, researchers found MDA values below 0.1 μM in fresh blood [40]. Nevertheless, many discrepancies in reference to the measured MDA concentrations in human body fluids are still evident.

AA, but not BHB, has been shown to increase lipid peroxidation in cultured human endothelial cells [41], as well as the in vitro susceptibility to oxidation of low density and very low density plasma lipoproteins [42]. However, our data indicate that ketone bodies at 20 mM concentration do not induce oxidative stress in human RBCs.

In conclusion, previous studies included some of our groups, suggested that biomarkers of oxidative stress such as *S*-glutathionylated proteins are significantly increased in RBCs from type 1 diabetic patients in comparison with age-matched healthy subjects [13,17,43,44]. However, in the present study and differently from previous results [10,13], we have not found a causative role for AA, BHB, and acetone (ketone bodies) in the generation of oxidative stress in RBCs of healthy humans in vitro. These contradictory results are probably due to application of methods in the past which had been inadequately validated regarding quantitative determination of these biomarkers of oxidative stress. Our present results suggest that ketosis would not be the cause for oxidative stress prevailing in RBCs from type 1 diabetic subjects. Taking into consideration that we have analyzed a relatively small number of possible oxidative modifications that may occur in proteins and lipids in

RBCs, our results do not exclude the occurrence of other types of oxidative modifications in our samples. Nevertheless, our results suggest that previous findings [10,13] should be considered with caution. In the present study we investigated potential oxidative effects of ketone bodies on RBCs from healthy humans. Additional studies on RBCs from patients with diabetes, a condition associated with altered erythrocyte GSH redox state, remain to be performed.

Acknowledgments

This work was supported by FIRST 2007 (Fondo Interno Ricerca Scientifica e Tecnologica), University of Milan, and Fondazione Monte dei Paschi di Siena (Siena, Italy).

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