Turk J Chem
27 (2003) , 433 – 443.

The Effect of *tert*-Butylhydroperoxide on the Thiol Redox Status in Human Erythrocytes and the Protective Role of Glucose and Antioxidants

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Received 18.03.2002

For survival, living cells maintain their thiol redox status within acceptable limits by three different mechanisms: i. glutathione disulfide export, ii. reduction of glutathione disulfide by pentose phosphate pathway and, iii. reduction of glutathione disulfide by Protein-SH. To assess the relative contribution of each one of the systems, intracellular [glutathione], [glutathione disulfide] and their export, in fresh and aged erythrocytes subjected to oxidative stress, in ±glucose and ±antioxidants, were measured. Glutathione was rapidly oxidized by *tert*-butylhydroperoxide in ±glucose in both groups. The regeneration of glutathione, in both groups, in ±glucose was about 100 and 50%, respectively. In parallel, intracellular glutathione disulfide concentrations were increased by about 200-350%.

The protective effects of ascorbate and α-tocopherol were similar and they behaved like radical scavengers.

In the absence of glucose, glutathione regeneration depends solely on the reduction of glutathione disulfide by protein-SH, and so it remained at about 50%. In the presence of glucose, the pentose phophate pathway was also involved and the regeneration approached 100%.

Since glutathione or glutathione export correspond from 0 to 1% of total cellular glutathione content, glutathione export makes no contribution to the establishment of the intracellular thiol redox status.

Key Words: Human erythrocytes, GSH, GSSG, Thiol redox status, *tert*-Butylhydroperoxide, Ascorbate, α-Tocopherol.

Introduction

The tripeptide glutathione (GSH) and its oxidized form glutathione disulfide (GSSG) comprise the major, low-molecular-weight thiol/disulfide redox buffer of most cells and their levels are strictly controlled[1,2]. Other thiol compounds such as cysteine, lipoic acid, coenzyme A (CoA) and glutamylcysteine make a negligible contribution to the total thiol pool of erythrocytes[2,3]. The redox status of the cell is defined by the total cellular concentration of glutathione and its derivatives: GSH, GSSG and mixed disulfides (RSSG), where R stands mainly for a protein or to a lesser extent for cysteine, CoA etc. Reported values for RSSG range from <1 to 50% of the total GSH content of a cell[1−5].

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GSH concentration is about 2.5 mM in human eythrocytes. It constitutes 90% of the nonprotein sulfur and plays a central role in several cellular processes[1−6]. Its depletion potentiates oxidative injury since exogenous reducing agents such as ascorbate and α-tocopherol need GSH to perform their reductive action[7−12].

In a viable cell, the GSH/GSSG ratio, reflecting the redox status, is maintained between 10 and 100[1,2,7−15] by the following systems: i. pentose phosphate pathway[12,13] ii. GSSG export[2,14], and iii. reduction of GSSG by protein-SH[15−17].

In this study, the relative contribution of each of the above mechanisms to thiol redox status was investigated in fresh and aged human erythrocytes under oxidative stress in the presence and absence of glucose, ascorbate and α-tocopherol.

Materials and Methods

Materials: Ascorbic acid, α-tocopherol and *tert*-butylhydroperoxide (t-BOOH) were purchased from Merck, Germany; N-ethymaleimide (NEM), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), glutathione disulfide, glutathione disulfide reductase (GSSGGR), sodium lauryl sulfate (SDS), nicotinamide adenine dinucleotide phosphate (NADPH), glucose, bovine serum albumin (BSA) and ethylenediamine tetraacetic acid (EDTA) were obtained from Sigma, U.S.A.

All other chemicals were standard products of Sigma or Aldrich, U.S.A.

Methods: Blood from healthy subjects was drawn onto citrate phosphate dextrose adenine (CPDA). Samples were used either directly (fresh erythrocytes) or after 20 days of incubation at 4 °C (aged erythrocytes). The erythrocytes were washed three times with 4-5 volumes of phosphate buffered saline containing 1 mM EDTA (PBSE). Two sets of preincubation mixtures, composed of four tubes each containing 1 mL erythrocyte pellet + 9 mL of PBSE, were prepared. A 9 mM sample of glucose was added to the second set of tubes. The third and fourth tubes of each set also contained 1 mM ascorbate and 0.6 mM α-tocopherol, respectively. All the tubes were preincubated for 30 min at 37 °C. Following the preincubation, all the tubes, except for the first tubes of both sets (controls), were supplemented with 0.3 mM t-BOOH and aliquots were drawn at certain intervals. The cells were pelleted by centrifugation at 2500 rpm for 2 min using a microfuge. The pellet (intracellular medium) and supernatant (extracellular medium) were treated as described below and analyzed for reduced thiol (RSH), GSH, GSSG and total thiol[18−21].

The pellet was hemolyzed by the addition of 9 volumes of distilled water. The protein-free supernatant was obtained by adding trichloroacetic acid (TCA, final concentration, 5% (w/v)) and centrifuging at 13,000 rpm for 5 min. The supernatant was analyzed for: i. Total RSH, determined by titration with DTNB[13]; ii. Total thiol (GSH + GSSG), determined by the enzymatic recycling method[19−21]; and iii. GSSG, also measured by the enzymatic recycling method following removal of reduced thiols by treatment with 20 mM NEM and extraction of excess NEM with ether[19−21].

In hemolyzates hemoglobin was measured by Drabkin's reagent[22].

The extracellular fluid was obtained by centrifugation of the erythrocyte suspension at 2500 rpm for 2 min using a microfuge. It was divided into two fractions. One of the fractions was treated with TCA (final concentration, 5% (w/v); TTF: TCA treated fraction) and the second fraction was not treated with TCA (TNF: TCA nontreated fraction). In both fractions the following parameters were measured as described above: i. Total RSH[17]; ii. GSSG; and, iii. Total thiol[19−21].

Protein determinations in the TCA nontreated fractions were carried out by the method of Lowry et al.[23]. In TCA treated fractions protein concentrations were also determined by the method of Lowry et al., but in this case after solubilization of the TCA precipitate with 1% (w/v) SDS. BSA used as a standard was also prepared in 1% SDS.

Results

The effect of t-BOOH on intracellular [GSH] in fresh erythrocytes in ±glucose and the protective effects of ascorbate and α-tocopherol: The GSH level in fresh erythrocytes in the absence of glucose was 5.57 ± 0.83 μmol/gHb and the same value was maintained throughout the incubation period of 2 h (Figure 1A). In the presence of t-BOOH, [GSH] showed a sharp decrease reaching zero by the end of the first minute; a gradual recovery, up to 51% at the end of 2 h, was then observed (Figure 1A).

In the presence of glucose the general patterns of GSH oxidation and recovery did not change, but extents of the oxidation and recovery were different. Oxidation proceeded more slowly; GSH was not completely depleted (the minumum level observed was about 5% of the initial value) and recovery at the end of 2 h of incubation was 78% (Fig. 1A).

In ± glucose both ascorbate and α-tocopherol counteracted the oxidative effect of t-BOOH. GSH levels did not decrease to zero and recovery started earlier than in the presence of t-BOOH alone. The extent of recoveries, at the end of 2 h of incubation, were also higher than in the absence of antioxidants: in the absence of glucose 65 and 50%, and in the presence of glucose 92 and 82% for ascorbate and α-tocopherol, respectively (Fig. 1A).

The effect of t-BOOH on intracellular [GSH] in aged erythrocytes in ±glucose and the protective effects of ascorbate and α-tocopherol: The GSH level in aged erythrocytes in the absence of glucose was 2.94 ± 0.32 μmol/gHb and the same value was maintained throughout the incubation period of 2 h (Fig. 1B). In the presence of t-BOOH, [GSH] showed a sharp decrease, reaching zero by the end of the 30 s. A gradual recovery, up to 60% at the end of 2 h, was then observed (Fig. 1B).

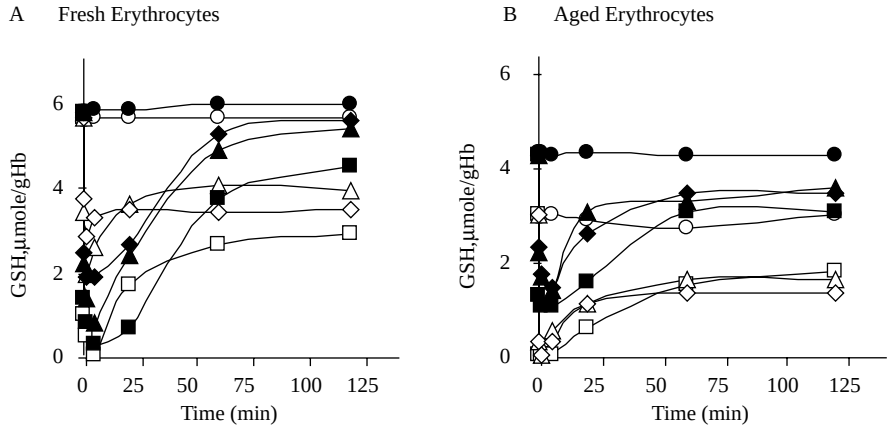

Figure 1. Oxidation of the human intra-erythrocyte GSH by t-BOOH. **A**: Fresh erythrocytes; **B**: Aged erythrocytes. In the absence of glucose: (—○—), Control; (—□—), t-BOOH; (—Δ—), t-BOOH + Ascorbate; (—◇—), t-BOOH + α-Tocopherol. In the presence of glucose: (—●—), Control; (—■—), t-BOOH; (—▲—), t-BOOH + Ascorbate; (—♦—), t-BOOH + α-Tocopherol. Values are means ± S.E.M., n = 6.

In the presence of glucose the zero-time GSH level was about 1.5 times higher than in the absence of glucose. Oxidation proceeded more slowly; GSH was not completely depleted (24% left) and recovery at the end of 2 h of incubation was 72% (Fig. 1B).

Although, as in fresh erythrocytes, both ascorbate and α-tocopherol counteracted the oxidative effect of t-BOOH and showed similar patterns of GSH oxidation and regeneration, the efficiency of protection was lower in aged erythrocytes: GSH levels did not decrease to zero and recovery started earlier than in the presence of t-BOOH alone, but the extent of recovery at the end of 2 h (ca. 50%) of incubation was the same as in the absence of antioxidants (Fig. 1B).

In the presence of antioxidants the disappearance and regeneration of GSH followed the same general pattern in both groups, but the absolute values were different (Figs. 1A and B). In the absence of glucose, the antioxidant capacity of ascorbate was better than α-tocopherol in fresh and aged erythrocytes. GSH recoveries in the presence of ascorbate and α-tocopherol were 70 vs. 55% and 62 vs. 45% for fresh vs aged erythrocytes, respectively (Figs. 1A and B). In the presence of glucose, the antioxidant capacity of ascorbate and α-tocopherol in fresh and aged erythrocytes was greater than 90 and 80%, respectively (Figs. 1A and B).

The effect of t-BOOH on the intracellular [GSSG] in fresh and aged erythrocytes in the absence and presence of glucose and the effects of ascorbate and α-tocopherol: GSSG levels in fresh erythrocytes in ±glucose, were 0.69 ± 0.08 μmol/gHb, and they were maintained throughout the incubation period of 2 h (Fig. 2A). Oxidation with t-BOOH caused a sharp increase, with the GSSG level reaching a maximum of 200 and 350% of the initial value at around the first minute in the absence and presence of glucose, respectively (Fig. 2A).

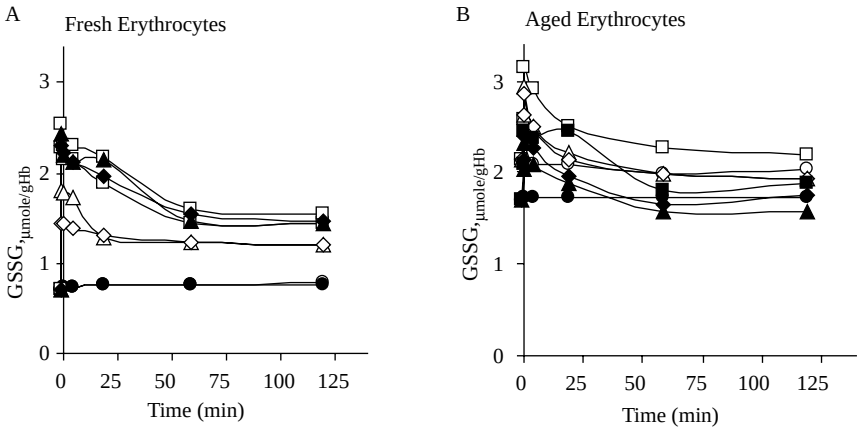

Figure 2. Intracellular GSSG in human erythrocytes. **A**: Fresh erythrocytes; **B**: Aged erythrocytes. In the absence of glucose: (—○—), Control; (—□—), t-BOOH; (—△—), t-BOOH + Ascorbate; (—◇—), t-BOOH + α-Tocopherol. In the presence of glucose: (—●—), Control; (—■—), t-BOOH; (—▲—), t-BOOH + Ascorbate; (—♦—), t-BOOH + α-Tocopherol. Values are means ± S.E.M., n = 6.

In aged erythrocytes the maximum GSSG level was <150% under all conditions (in ±glucose and/or ±antioxidants) (Fig. 2B). Neither glucose nor antioxidants had appreciable effects on GSSG formation. The reduction of the GSSG was much slower than the regeneration of the GSH in both fresh and aged erythrocytes (cf. Figs. 1A and B with Figs. 2A and B). Surprisingly, GSSG levels in aged erythrocytes

returned to control levels at the end of 2 h of incubation in ±glucose (Fig. 2B). In constrast, GSSG levels in fresh erythrocytes were still at about 200% of control levels (Fig. 2A).

It seems that antioxidants, ascorbate and α-tocopherol had no effect on intracellular GSSG status in both fresh and aged erythrocytes (Figures 2A and B).

Intracellular total thiol in fresh and aged erythrocytes in the absence and presence of glucose and antioxidants: Intracellular total thiol values were 6.58 ± 0.82/7.42 ± 0.91 vs. 6.83 ± 0.78/7.58 ± 0.66 μmol/gHb in fresh vs. aged erythrocytes (in the presence vs. absence of glucose). The presence of antioxidants (ascorbate and α-tocopherol) did not have any appreciable effect on total thiol values and they remained constant throughout the incubation period of 2 h in the absence and presence of glucose (Figures 3A and B).

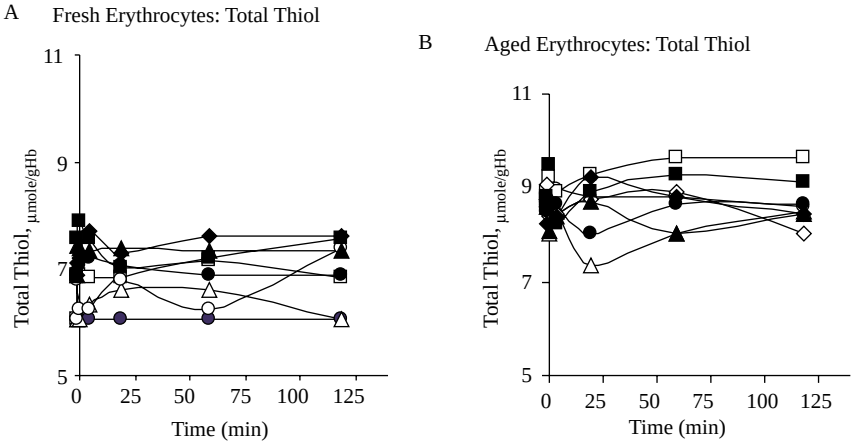

Figure 3. Intracellular total thiol in human erythrocytes.**A**: Fresh erythrocytes; **B**: Aged erythrocytes. In the absence of glucose: (—○—), Control; (—□—), t-BOOH; (—△—), t-BOOH + Ascorbate; (—◇—), t-BOOH + α-Tocopherol. In the presence of glucose: (—●—), Control; (—■—), t-BOOH; (—▲—), t-BOOH + Ascorbate; (—♦—), t-BOOH + α-Tocopherol. Values are means ± S.E.M., n = 6.

Extracellular thiols in fresh and aged erythrocytes in ±glucose: Extracellular fluids collected at 0, 15, 30, 45, 60 and 120 min were divided into two fractions. One fraction was treated with TCA as described in the methods section; in both fractions RSH and protein concentrations were determined. In the absence of glucose, DTNB-titratable thiols were measured in TCA nontreated fractions (TNF) at 0 min, for both groups, were 44, 27, 33 and 254 nmol/mL for the control, t-BOOH control, t-BOOH + ascorbate, and t-BOOH + α-tocopherol, respectively, and following 1 h incubation at 37 °C, these values increased 2-3 times (Table 1). When the TCA precipitate dissolved in 1% SDS and titrated with DTNB, the same values as in TNF were obtained (Table 1). In the supernatants of the TCA treated fractions (TTF), DTNB-titratable thiol values were ≤13 nmol/mL in all fractions including the α-tocopherol fraction. The same patterns were also observed for the protein and the recoveries were ≥95% (Table 1).

In the presence of glucose, DTNB-titratable thiol values for the control and t-BOOH samples at zero time were comparable with the values obtained in the absence of glucose (Table 2), but the presence of the antioxidants decreased zero time values to 21 and 172 nmol/mL with ascorbate and α-tocopherol, respectively (Table 2).

Table 1. Extracellular RSH and protein in the absence of glucose in fresh and aged erythrocytes.

Fresh Erythrocytes		0 min incubation				60 min incubation			
		Control	t-BOOH	t-BOOH+A	t-BOOH+T	Control	t-BOOH	t-BOOH+A	t-BOOH+T
RSH (nmole/ml)	TNF*	44 ± 3	27 ± 3^{a}	33 ± 4	254 ± 30	53 ± 6	84 ± 9^{a}	146 ± 16^{b,c}	482 ± 53^{b,c}
	TTF/SDS♣	42 ± 3	25 ± 3^{a}	32 ± 3	252 ± 30	50 ± 5	80 ± 9^{a}	146 ± 15^{b,c}	468 ± 53^{b,c}
	Difference♦	2.5	2.2	0.9	1.8	2.7	4.1	0.2	13.3
Protein (μg/ml)	TNF*	31 ± 4	33 ± 4^{a}	35 ± 5	124 ± 14^{b,c}	39 ± 6	50 ± 7^{a}	35 ± 5	174 ± 24^{b,c}
	TTF/SDS♣	30 ± 3	31 ± 4^{a}	34 ± 4	123 ± 13^{b,c}	38 ± 5	50 ± 7^{a}	34 ± 4	172 ± 23^{b,c}
	Difference♦	0.9	1.5	1.4	1.6	0.5	0.1	1.4	2.5

Fresh Erythrocytes		0 min incubation				60 min incubation			
		Control	t-BOOH	t-BOOH+A	t-BOOH+T	Control	t-BOOH	t-BOOH+A	t-BOOH+T
RSH (nmole/ml)	TNF*	43 ± 6	45 ± 6^{a}	21 ± 3^{b}	172 ± 19^{b,c}	77 ± 10	115 ± 13^{a}	118 ± 14	379 ± 40^{b,c}
	TTF/SDS♣	43 ± 5	42 ± 6^{a}	20 ± 3^{b}	170 ± 18 ^{b,c}	75 ± 9	109 ± 11^{a}	115 ± 14	365 ± 37^{b,c}
	Difference♦	0	2.8	0.9	2.6	1.6	6.2	3.7	13.3
Protein (μg/ml)	TNF*	30 ± 4	32 ± 4^{a}	44 ± 5^{b}	90 ± 12^{b,c}	44 ± 6	55 ± 7^{a}	71 ± 9^{b}	161 ± 20^{b,c}
	TTF/SDS♣	30 ± 3	30 ± 4^{a}	42 ± 5^{b}	89 ± 12^{b,c}	43 ± 6	55 ± 7^{a}	70 ± 9^{b}	157 ± 19^{b,c}
	Difference♦	0.7	1.6	2.5	1.7	1.5	0.1	0.4	3.8

*TNF, TCA-nontreated fraction; ♣TTF/SDS, TCA-treated; precipitate redissolved in 1% SDS; ♢Difference, TNF – TTF/SDS; A, Ascorbate; T, α-Tocopherol.

Student's unpaired t test: $^{a}P < 0.05$, with respect to control; $^{b}P < 0.05$, with respect to t-BOOH; $^{c}P < 0.05$ with respect to t-BOOH + A . Values are means ± S.E.M., n = 6.

Table 2. Extracellular RSH and protein in the presence of glucose in fresh and aged erythrocytes.

Fresh Erythrocytes		0 min incubation				60 min incubation			
		Control	t-BOOH	t-BOOH+A	t-BOOH+T	Control	t-BOOH	t-BOOH+A	t-BOOH+T
RSH (nmole/ml)	TNF*	72 ± 9	51 ± 6^{a}	39 ± 5^{b}	174 ± 18^{b,c}	84 ± 9	187 ± 19^{a}	162 ± 18^{b}	375 ± 42^{b,c}
	TTF/SDS♣	66 ± 8	46 ± 6^{a}	35 ± 4^{b}	165 ± 13^{b,c}	80 ± 6	183 ±15^{a}	156 ± 16^{b}	359 ± 36^{b,c}
	Difference♦	6.0	5.5	3.9	8.2	3.2	3.5	5.8	15.4
Protein (μg/ml)	TNF*	47 ± 6	47 ± 5^{a}	46 ± 6	96 ± 11^{b,c}	50 ± 7	97 ± 11^{a}	90 ± 11^{b}	163 ± 20^{b,c}
	TTF/SDS♣	44 ± 6	43 ± 4^{a}	47 ± 5	92 ± 10^{b,c}	47 ± 6	94 ± 10^{a}	89 ± 9^{b}	154 ± 18^{b,c}
	Difference♦	2.5	4.0	-0.4	4.2	3.7	2.6	1.7	8.9

Fresh Erythrocytes		0 min incubation				60 min incubation			
		Control	t-BOOH	t-BOOH+A	t-BOOH+T	Control	t-BOOH	t-BOOH+A	t-BOOH+T
RSH (nmole/ml)	TNF*	44 ± 5	35 ± 4^{a}	39 ± 5	160 ± 17^{b,c}	117 ±15	140 ±17^{a}	148 ± 19^{b}	376 ± 52^{b,c}
	TTF/SDS♣	44 ± 4	35 ± 6^{a}	35 ± 5	162 ± 16^{b,c}	114 ±15	135 ±15^{a}	148 ± 17^{b}	375 ± 51^{b,c}
	Difference♦	-0.4	0.6	4.8	-2	2.9	5.6	-0.7	0.3
Protein (μg/ml)	TNF*	42 ± 5	43 ± 4^{a}	44 ± 6	89 ± 12^{b,c}	50 ± 6	72 ± 9^{a}	98 ± 13^{b}	174 ± 24^{b,c}
	TTF/SDS♣	42 ± 5	43 ± 4^{a}	40 ± 6	84 ± 12^{b,c}	50 ± 6	72 ± 9^{a}	96 ± 12^{b}	171 ± 22^{b,c}
	Difference♦	0.6	-0.1	4.5	4.8	-0.2	0.7	2	3.1

*TNF, TCA-nontreated fraction; ♣TTF/SDS, TCA-treated; precipitate redissolved in 1% SDS; ♦Difference, TNF – TTF/SDS; A, Ascorbate; T, α-Tocopherol.

Student's unpaired t test: $^{a}P < 0.05$, with respect to control; $^{b}P < 0.05$, with respect to t-BOOH; $^{c}P < 0.05$ with respect to t-BOOH + A. Values are means ± S.E.M., n = 6.

It was observed that in the presence of glucose, ascorbate was much more effective than α-tocopherol. Although the amounts of proteins released in aged erythrocytes were comparable with the proteins released by fresh erythrocytes, the amounts of DTNB-titratable thiols were less in the aged erythrocytes than in the fresh erythrocytes (cf. Tables 1 and 2).

GSSG export in fresh and aged erythrocytes incubated in the presence or absence of glucose and antioxidants: GSSG levels in the extracellular fluids, at 0 min and after 60 min of incubation at 37 °C, were measured using an enzymatic recycling assay after blocking RSH as described for intracellular GSSG measurement [19-21]. In the absence of glucose, in controls, 10 and 18 nmol GSSG/gHb/h were exported from fresh and aged erythrocytes, respectively (Fig. 4, control groups without glucose: first bars in both groups). In the presence of t-BOOH these values increased to 34 and 87 nmol/gHb/h (Fig. 4, t-BOOH groups without glucose: second bars in both groups). In the absence of glucose, GSSG export was more efficient in aged erythrocytes than in fresh erythrocytes; both ascorbate and α-tocopherol inhibited GSSG export (Fig. 4; t-BOOH + A and t-BOOH + α-tocopherol groups without glucose: third and fourth bars in fresh and aged erythrocytes).

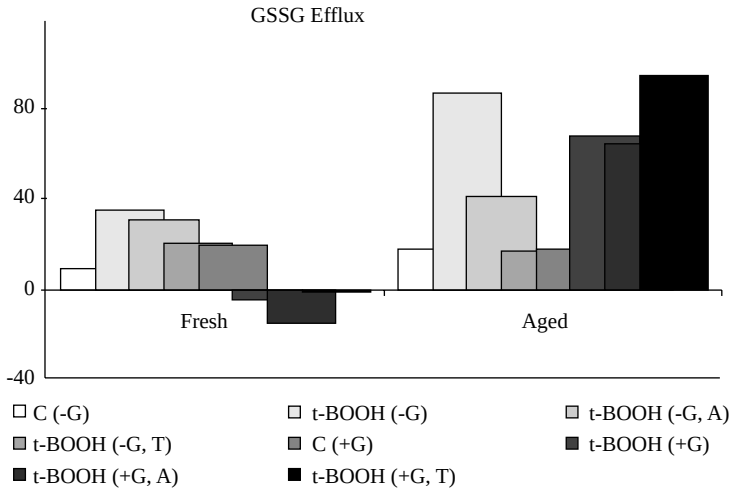

Figure 4. GSSG export in fresh and aged erythrocytes. C, Control; -G, in the absence of glucose; +G, in the presence of glucose; t-BOOH, t-BOOH; A: Ascorbate; T: α-Tocopherol.

In the presence of glucose, in controls, the amounts of GSSG exported (about 17 nmol/gHb/h) from fresh and aged erythrocytes were comparable (Fig. 4; control groups with glucose: fifth bars in both groups). In fresh erythrocytes, lower rates of GSSG export in the absence of glucose were observed and this export was completely blocked, and even reversed, by the presence of glucose and antioxidants (Fig. 4; fresh erythrocytes with glucose: 6-8 bars).

Both in the presence and absence of glucose, GSSG export was higher in aged erythrocytes than in fresh erythrocytes (Fig. 4; cf. first and second panels). In aged erythrocytes, in the absence of glucose, GSSG export was decreased by ascorbate and α-tocopherol, whereas in the presence of glucose GSSG export was increased (Fig. 4; aged erythrocytes: cf. 2-4 bars with 6-8 bars).

Discussion

During oxidative stress GSH is rapidly oxidized to GSSG, which in turn may be reduced by GSSGGR in the presence of NADPH[16]. In living cells to keep the ratio of GSH/GSSG within acceptable limits three different mechanisms (GSSG export, GSSG reduction by pentose phosphate pathway and by PSH) are used[1,2,7−17]. The relative contribution of each system was investigated in fresh and aged erythrocytes subjected to oxidative stress in ± glucose and antioxidants.

In the absence of glucose the concentrations of GSH were about 6 and 3 μmol/gHb in fresh and aged erythrocytes, respectively (Figs. 1A and B). The presence of glucose did not cause an appreciable effect on the GSH level in fresh erythrocytes, but an approximate 40% increase in the GSH content in aged erythrocytes was observed (Fig. 1B). The oxidation and regeneration of GSH were very rapid in ±glucose both in aged and fresh erythrocytes, but the extent of regeneration was different: about 50 vs. 90% in the absence vs. presence of glucose (cf. Figs. 1A with B). The values obtained for GSH were in good correlation with literature values[1−4,10−17].

The amount of exported GSSG from human erythrocytes has been found by several investigators to be in the range of 18-130 nmol/h/mL erythrocytes (corresponding to a range of 6.7-17.8 nmol/h/gHb assuming that Hb concentration was 15%)[2,24]. Oxidative stress increased this range to 24-94 nmol/gHb/h[2,24]. In this study, in the absence of glucose, GSSG export values of 10 and 18 nmol/gHb/h were obtained for fresh and aged erythrocytes (Fig. 4). These control values were increased to 37 and 87 nmol/gHb/h in the presence of t-BOOH. Both antioxidants decreased GSSG export (Fig. 4). There was no export of GSH (Tables 1 and 2). All exported RSH was protein and was precipitated by 5% TCA (Tables 1 and 2)[2,24]. The protein-associated SH in the extracellular medium appeared to arise from hemolysis rather than an export process; the absolute values obtained for RSH at zero and 60 minutes were similar in magnitude (Tables 1 and 2). Ninety nine percent of the RSH found in the extracellular medium was protein in nature and SDS-PAGE analysis (not shown) indicated the presence of hemoglobin. The hemolysis was much higher in the presence of α-tocopherol because it was dissolved in ethanol, and so the absolute values for RSH and GSSG in the presence of α-tocopherol were higher (Fig. 4; 8^{th} bars in fresh and aged erythrocytes). GSSG found in the extracellular medium at zero time was also due to hemolysis. Indeed, in the presence of glucose in fresh erythrocytes during incubation at 37 °C, a decrease in GSSG level, and even a slight import, was observed (Fig. 4, 6-8^{th} bars). Due to extensive membrane oxidation, the malonyldialdehyde level in aged erythrocytes was twice that in fresh erythrocytes (not shown). This membrane damage probably caused aged erythrocytes to be unsuccessful in GSSG import both in the absence and presence of glucose (Fig. 4, second panel).

The results presented here for the intracellular concentration of GSH and GSSG efflux correlate with the literature[2−6,10−17,24]. However, due to the very low export rate of GSSG, the contribution of export to the establishment of GSH/GSSG (thiol redox status) was assumed to be negligible. The high levels of PSH in erythrocytes reflect a large capacity for transforming GSSG to GSSP and GSH[15]. Simplicio et al. showed that the maximum amount of GSSP generated after oxidative stress in fresh rat red blood cells is about 65% of total GSH, but this was not true for human erythrocytes[4,15]. Exchange reactions between thiols are very fast and may involve thiols with different reactivities and enzymes such as GSSGGR and thioredoxin reductase[18,25−28]. These multiple reactions have not been considered previously at the intracellular level.

Whatever the reduction reaction, the ultimate source of reducing power is NADPH derived from glucose oxidation in the pentose phosphate pathway.

GSH was rapidly oxidized by t-BOOH in ± glucose in both fresh and aged erythrocytes (Figs. 1A and B). GSH regeneration in the presence of glucose was almost 100% in both fresh and aged erythrocytes, whereas in the absence of glucose it remained around 50% (Figs. 1A and B). On the other hand, the oxidative action of t-BOOH led to an increase in GSSG of 200-350% (Figs. 2A and B). The rate of decrease in GSSG was much slower than the rate of regeneration of GSH in the presence and absence of glucose (cf. Figs. 1A and B with 2A and B). The regeneration of GSH in the absence of glucose remained at around 50%, indicating that reduction by RSH (protein-SH) was the major pathway (Fig. 1A). On the other hand, in the presence of glucose the regeneration of GSH was about 100% both in aged and fresh erythrocytes, indicating the contribution of the pentose phosphate pathway to GSSG reduction by protein-SH (Figs. 1A and B). The export of GSSG per hour, in both systems ±glucose, was only about 1/100 the amount of GSSG generated in the same time period (Fig. 4). On the other hand, the regeneration of GSH was very fast under all conditions studied (Figs. 1A and B). All data clearly indicate that, independent of erythrocyte age, the reduction of GSSG by protein-SH (RSH) constitutes the major pathway for reestablishing the thiol redox status (GSH/GSSG) in the absence of glucose, and that reduction of GSSG by protein-SH and by GSSGGR constitutes the major pathway in the presence of glucose. Under all conditions studied the contribution of GSSG export to the maintenance of the thiol redox status is negligible. The protective effects of ascorbate and α-tocopherol against oxidative stress were similar and limited by their capacity to act as radical scavengers.

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