Eur. J. Biochem. **267**, 684–689 (2000) © FEBS 2000

Energy metabolism and lipid peroxidation of human erythrocytes as a function of increased oxidative stress

Barbara Tavazzi[1], Donato Di Pierro[1], Angela Maria Amorini[2], Giovanna Fazzina[2], Michele Tuttobene[3], Bruno Giardina[4] and Giuseppe Lazzarino[2]

[1]*Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Italy;* [2]*Department of Chemical Sciences, Laboratory of Biochemistry, University of Catania, Italy;* [3]*Transfusional Center, 'Garibaldi' Hospital, Catania, Italy;* [4]*Institute of Chemistry and Clinical Chemistry and 'Centro C.N.R. per lo Studio della Chimica dei Recettori', Catholic University of Rome 'Sacro Cuore', Italy*

To study the influence of oxidative stress on energy metabolism and lipid peroxidation in erythrocytes, cells were incubated with increasing concentrations (0.5–10 mM) of hydrogen peroxide for 1 h at 37 °C and the main substances of energy metabolism (ATP, AMP, GTP and IMP) and one index of lipid peroxidation (malondialdehyde) were determined by HPLC on cell extracts. Using the same incubation conditions, the activity of AMP-deaminase was also determined. Under nonhaemolysing conditions (at up to 4 mM H_2O_2), oxidative stress produced, starting from 1 mM H_2O_2, progressive ATP depletion and a net decrease in the intracellular sum of adenine nucleotides (ATP + ADP + AMP), which were not paralleled by AMP formation. Concomitantly, the IMP level increased by up to 20-fold with respect to the value determined in control erythrocytes, when cells were challenged with the highest nonhaemolysing H_2O_2 concentration (4 mM). Efflux of inosine, hypoxanthine, xanthine and uric acid towards the extracellular medium was observed. The metabolic imbalance of erythrocytes following oxidative stress was due to a dramatic and unexpected activation of AMP-deaminase (a twofold increase of activity with respect to controls) that was already evident at the lowest dose of H_2O_2 used; this enzymatic activity increased with increasing H_2O_2 in the medium, and reached its maximum at 4 mM H_2O_2-treated erythrocytes (10-fold higher activity than controls). Generation of malondialdehyde was strictly related to the dose of H_2O_2, being detectable at the lowest H_2O_2 concentration and increasing without appreciable haemolysis up to 4 mM H_2O_2. Besides demonstrating a close relationship between lipid peroxidation and haemolysis, these data suggest that glycolytic enzymes are moderately affected by oxygen radical action and strongly indicate, in the change of AMP-deaminase activity, a highly sensitive enzymatic site responsible for a profound modification of erythrocyte energy metabolism during oxidative stress.

Keywords: AMP-deaminase; energy metabolism; human erythrocytes; IMP; oxidative stress.

Reactive oxygen species (ROS) are produced in living organisms from the partial reduction of molecular oxygen under physiological and pathological conditions. Because ROS has the capability of reacting nonspecifically with a vast number of compounds, all living organisms contain several enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic (α-tocopherol, ascorbic and uric acids, glutathione and other thiol protein groups) antioxidants with the specific purpose of protecting the functional and structural integrity of biologically fundamental macromolecules (nucleic acids, proteins, phospholipids). Imbalance between ROS production and antioxidant cell defences has been reported to occur in several physiopathological conditions of both animals and man: for example tissue ischemia and reperfusion [1], nervous trauma [2], etc. Formation of modified DNA bases [3], oxidation of protein thiol groups [4], decrease of nonenzymatic antioxidants [5] and lipid peroxidation of membrane phospholipids [6] have been recorded following increased ROS generation.

Many *in vitro* and *in vivo* studies showed that several parameters of red blood cell function and integrity are negatively affected by increased oxidative stress. In fact, changes of erythrocyte membrane ionic permeability [7], increase of lipid peroxidation [8], oxidation of protein sulfihydryl groups [9] and activation of proteolysis [10] have all been described following the challenge of red blood cells with different oxygen radical-generating systems. However, data referring to modifications of erythrocyte energy metabolism under these experimental conditions are not abundant, and thus it is not very clear which metabolites, and hence enzymes, are more sensitive to oxidative stress.

The aim of this study was to determine the effects of increased oxidative stress on the main parameters representative of erythrocyte energy metabolism, looking also for a correlation with the onset of lipid peroxidation and haemolysis. On the basis of these results, we were prompted to evaluate the

Correspondence to G. Lazzarino, Department of Chemical Sciences, Laboratory of Biochemistry, University of Catania, Viale A. Doria 6, 95125 Catania, Italy. Fax: + 39 095337036, Tel.: + 39 0957384095, E-mail: lazzarig@mbox.unict.it

Abbreviations: MDA, malondialdehyde; ROS, reactive oxygen species; Hpx, hypoxanthine; Ino, inosine; Σ_{NT}, sum of nucleotides; Xan, xanthine.

Enzymes: AMP-deaminase (EC 3.5.4.4); catalase (EC 1.11.1.6); glutathione peroxidase (EC 1.11.1.9); hexokinase (EC 2.7.1.1); nucleoside phosphorylase (EC 2.4.2.1); 5′-nucleotidase, 5′-ribonucleotide phosphorylase, 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5); superoxide dismutase (EC 1.15.1.1); xanthine oxidase (EC 1.1.3.22).

(Received 12 October 1999, revised 23 November 1999, accepted 24 November 1999)

role of AMP-deaminase as a potential 'highly sensitive site' towards ROS action and its involvement in the metabolic derangement of erythrocytes subjected to oxidative stress.

MATERIALS AND METHODS

Induction of oxidative stress to human erythrocytes

Peripheral venous blood samples were obtained from healthy volunteers and collected into heparinized tubes. After 10 min centrifugation at 1853 ***g*** at 4 °C, carried out within 15 min of withdrawal, erythrocytes were washed twice with a large volume of 10 mM glucose-supplemented NaCl/P_i. After the second wash, packed erythrocytes were gently resuspended with NaCl/P_i-glucose to obtain a 5% haematocrit and preincubated at 37 °C for 10 min in presence of 1 mM NaN_3 (to inhibit catalase activity). Subsequently, they were divided into eight aliquots: seven were challenged with increasing concentrations of H_2O_2 (0.5, 1, 2, 4, 6, 8 and 10 mM) and one was untreated (control). After 60 min at 37 °C, cells were immersed for 60 s in an ice bath and then centrifuged at 1853 ***g*** for 10 min, at 4 °C. Supernatants were divided in to two parts: one was used for determining haemoglobin concentration, the other was deproteinized using 70% $HClO_4$ (10 : 1, v/v) for measuring the concentration of metabolites released in the incubation medium. Packed erythrocytes were either deproteinized by adding ice-cold 1.2 M $HClO_4$ (1 : 2, w/w), or haemolysed by adding ice-cold double-distilled H_2O (1 : 5, w/w). An aliquot of each haemolysate (200 μL) was then used to determine AMP-deaminase activity. Both deproteinized red blood cells and supernatants were centrifuged at 20 690 ***g*** for 10 min at 4 °C, neutralized by adding 5 M K_2CO_3 in the cold, filtered through a 0.45-μM Millipore-HV filter and then analysed by HPLC (50 μL) for the simultaneous direct determination of malondialdehyde (MDA) and adenine nucleotide derivatives [11].

In other experiments, erythrocytes were incubated (after pretreatment with sodium azide, as described previously) with the maximal haemolysing dose of H_2O_2 (10 mM) and with increasing concentrations of mannitol (1, 5, 10, 25, 50, 100 mM). At the end of incubation, erythrocytes were processed as described above.

Assay of AMP-deaminase

Haemolysates of both control and H_2O_2-treated erythrocytes (200 μL) were incubated for 45 min at 37 °C in the presence of 2 mM AMP, 10 mM KCl, 50 mM Mes pH 6.5. After deproteinization with 70% $HClO_4$ and further processing as described above, 10 μL were loaded onto an HPLC column for IMP determination. Activity of AMP-deaminase was calculated on the basis of the total amount of IMP produced in the reaction mixture during the incubation with AMP.

Biochemical analysis

Concentrations of MDA, oxypurines, nucleosides, nicotinic coenzymes and high-energy phosphates (either in erythrocytes or in the suspending medium) were determined on 50 μL of perchloric acid extract of both erythrocytes and the suspending medium by an ion-pairing HPLC method [11] using a Kromasil 250 × 4.6 mm, 5-μm particle size column, with its own guard column (Eka Chemicals AB, Bohus, Sweden), and using tetrabutylammonium hydroxide as the pairing reagent. Briefly, separation of different metabolites was obtained by forming a step gradient (adapted to the column length increase with respect to the original method [11]), with two buffers of the following composition: buffer A, 10 mM tetrabutylammonium hydroxide, 10 mM KH_2PO_4, 0.25% methanol pH 7.00; buffer B, 2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, 30% methanol pH 5.50. The gradient was: 10 min 100% buffer A; 3 min up to 90% buffer A; 10 min up to 70% buffer A; 12 min up to 55% buffer A; 15 min up to 45% buffer A; 10 min up to 25% buffer A; 5 min up to 0% buffer A. The flow rate throughout chromatographic runs was 1.2 $mL \cdot min^{-1}$ and the column temperature was kept at a constant 23 °C by using water-jacketed glassware. The HPLC apparatus consisted of a Constametric 3500 dual pump system (ThermoQuest Italia, Rodano, Milan, Italy) connected to a SpectraSystem UV6000-LP diode array detector (ThermoQuest Italia) at 200–300 nm. Acquisition and analysis of data were performed using the CHROMQUEST program (ThermoQuest Italia). Comparison of areas, retention times and absorbance spectra of the peaks of sample chromatograms with those of freshly prepared ultrapure standards allowed calculation of the concentration of the various compounds at 267 nm (the maximum of MDA absorbance spectrum) and identification of the different metabolites. The same apparatus and chromatographic conditions were used to determine IMP in samples used for evaluating AMP-deaminase activity, except that IMP was calculated at 250 nm.

Haemoglobin and percentage haemolysis were calculated by standard haematological techniques [12] using a Jasco-685 double beam spectrophotometer.

Statistical analysis

Two-way analysis of variance was used to determine significant differences between control and H_2O_2-treated erythrocytes, as well as differences between H_2O_2-treated erythrocytes and H_2O_2-treated erythrocytes preincubated with mannitol. Analysis of variance of regression coefficients of H_2O_2 dose–response curves (MDA and haemolysis) was also performed.

RESULTS

As reported in Table 1, the main phosphorylated compounds necessary for all the energy-requiring reactions of erythrocyte metabolism, i.e. ATP and GTP, were significantly reduced when the H_2O_2 concentration exceeded 1 mM. After 1 h incubation at 37 °C in the presence of the highest nonhaemolysing H_2O_2 concentration (4 mM), a 44% ATP depletion with a concomitant not significant ADP increase (data not shown) was observed. The AMP concentration increased less than expected so that its concentration varied from 17.69 $\mu mol \cdot L^{-1}$ in control erythrocytes to $\approx$ 42.16 $\mu mol \cdot L^{-1}$ in 4 mM H_2O_2-treated erythrocytes. Consequently, the sum of ATP + ADP + AMP (Σ_{NT}) of these erythrocytes was almost half that of control erythrocytes ($\approx$ 1200 and $\approx$ 2020 $\mu mol \cdot L^{-1}$, respectively) indicating that metabolites other than ADP and AMP were produced. Unexpectedly, the IMP concentration increased with increasing H_2O_2 in the suspending medium; the maximum was in the presence of 4 mM H_2O_2, when IMP reached levels 22-fold higher than those of control erythrocytes. The increase in IMP was already significant in 0.5 mM H_2O_2-treated erythrocytes, suggesting that even the lowest H_2O_2 concentration tested was able to cause a marked metabolic derangement of erythrocyte energy metabolism.

Table 1. Effect of increasing hydrogen peroxide concentrations on erythrocyte energy metabolism. Fresh human erythrocytes were incubated for 1 h at 37 °C in NaCl/P_i-glucose supplemented with increasing nonhaemolysing concentrations of H_2O_2, at a final 5% haematocrit. Determination of high-energy phosphates was carried by HPLC on 50 μL of neutralized perchloric acid extracts of packed erythrocytes. Each value represents the mean (SD) of five different blood samples and is expressed as $\mu mol \cdot L^{-1}$. Only those compounds significantly affected by oxidative stress are reported.

	ATP	AMP	IMP	GTP
Control	1776.15 (208.76)	17.69 (3.29)	10.05 (1.96)	105.02 (13.17)
H_2O_2 0.5 mM	1630.83 (177.80)	21.03 (3.15)	66.24[a] (10.54)	91.32 (8.44)
H_2O_2 1 mM	1562.47 (183.00)	37.21[a] (4.73)	177.47[a] (32.86)	86.04 (6.85)
H_2O_2 2 mM	1313.98[a] (168.31)	39.43[a] (6.49)	186.28[a] (28.67)	73.17[a] (10.41)
H_2O_2 4 mM	987.51[a] (115.24)	42.16[a] (7.32)	222.86[a] (36.03)	62.40[a] (9.07)

[a] Significantly different from control ($P < 0.05$).

In order to determine whether AMP-deaminase was activated by the increased oxidative stress (thus explaining the marked change in IMP concentration of H_2O_2-treated erythrocytes) we measured the activity of this enzyme at different concentrations of H_2O_2. Fig. 1 shows the dose–response curve of AMP-deaminase activity of intact fresh human erythrocytes as a function of increasing H_2O_2 concentrations. A highly remarkable AMP-deaminase activation was recorded at up to 4 mM H_2O_2, starting from the lowest H_2O_2 concentration tested which already doubled the enzymatic activity. In erythrocytes treated with 4 mM H_2O_2 maximal enzymatic activity was recorded, corresponding to an almost 10-fold increase with respect to the value determined in control erythrocytes incubated in the presence of buffer only. Concentrations of H_2O_2 greater than 4 mM (i.e. 6, 8 or 10 mM) provoked a slight but progressive decrease of AMP-deaminase activation; however, even at the maximal dose of H_2O_2 tested ≈ sixfold higher enzymatic activity with respect to control erythrocytes was still observed.

As far as a change of metabolism is concerned, it should be recalled that not even the addition of IMP concentration to the calculated Σ_{NT} was sufficient to reach the Σ_{NT} value of control erythrocytes. In fact, the correct energy balance of H_2O_2-treated erythrocytes was obtained only by taking into consideration concentrations of dephosphorylated metabolites, either those detected in erythrocyte extracts or those determined in the suspending medium. For instance, according to this calculation, the value of Σ_{NT} + IMP + intra- and extra-cellular oxypurines and nucleosides was ≈ 2030 $\mu mol \cdot L^{-1}$ and 1990 $\mu mol \cdot L^{-1}$ in control and 4 mM H_2O_2-treated erythrocytes, respectively. As reported in Fig. 2, maximal production of hypoxanthine (Hpx), xanthine (Xan), inosine (Ino) and uric acid was determined, intracellularly and extracellularly, in 4 mM H_2O_2-treated erythrocytes.

Separate analysis of extracellular and intracellular MDA concentrations showed that 80–90% of this compound was localized outside erythrocytes and that its total concentration (i.e. intracellular + extracellular MDA) progressively augmented by increasing the concentration of H_2O_2 in the medium. Dose–response trends of total MDA and percentage haemolysis as a function of increasing H_2O_2, are reported in Fig. 3 and indicated that total MDA was correlated to H_2O_2 concentration according to a power curve ($r = 0.994$). The same relationship was obtained by plotting the percentage of haemolysis as a function of corresponding total MDA ($r = 0.994$). In contrast with MDA, a significant degree of haemolysis was observed only if the H_2O_2 concentration was > 4 mM. Because at high H_2O_2 concentrations haemoglobin might undergo denaturation and aggregation, it is possible that we underestimated its supernatant concentration in H_2O_2-treated erythrocytes, thus underevaluating the actual value of haemolysis. It should be recalled that MDA and haemolysis of control erythrocytes, at the end of 60 min incubation at 37 °C with the suspending medium only, were undetectable and energy metabolism was maintained very close that of fresh erythrocytes analysed immediately after withdrawal.

Table 2 shows the results of experiments carried out by incubating erythrocytes with the highest H_2O_2 dose tested (10 mM) and with increasing concentrations of the membrane permeable free radical scavenger mannitol (25–100 mM), in which high-energy phosphates (ATP, AMP, IMP and GTP), AMP-deaminase activity, lipid peroxidation and haemolysis were monitored. Mannitol was ineffective at < 25 mM (1–10 mM mannitol did not produce significant changes of any parameter reported in Table 2; data not shown). Maximal efficacy was obtained with 100 mM mannitol, as demonstrated by the reduction of high-energy phosphate depletion, less pronounced AMP-deaminase activation and decrease of both lipid peroxidation and

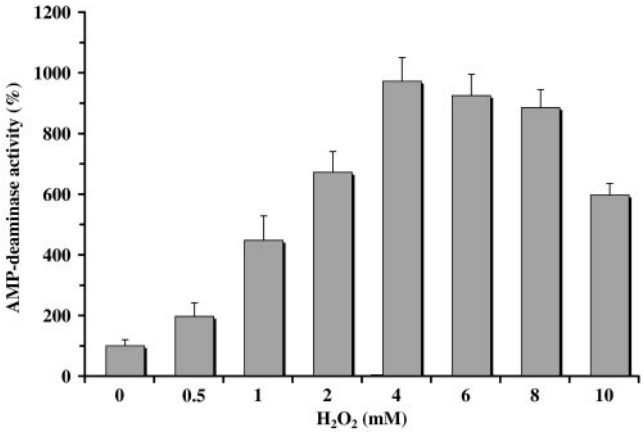

Fig. 1. AMP-deaminase activity of human erythrocytes as a function of increased oxidative stress obtained by incubating erythrocytes with 1 mM NaN_3 and different concentrations of H_2O_2. Each column shows the mean of five different blood samples assayed in duplicate and expressed as percentage AMP-deaminase activity. SD values are represented by vertical bars; all values of H_2O_2-treated erythrocytes were significantly different from those of controls ($P < 0.001$). AMP-deaminase activity of control erythrocytes was considered equal to 100% (mean absolute value of AMP-deaminase activity in controls = 2.09 $U \cdot L^{-1}$; SD = 1.17).

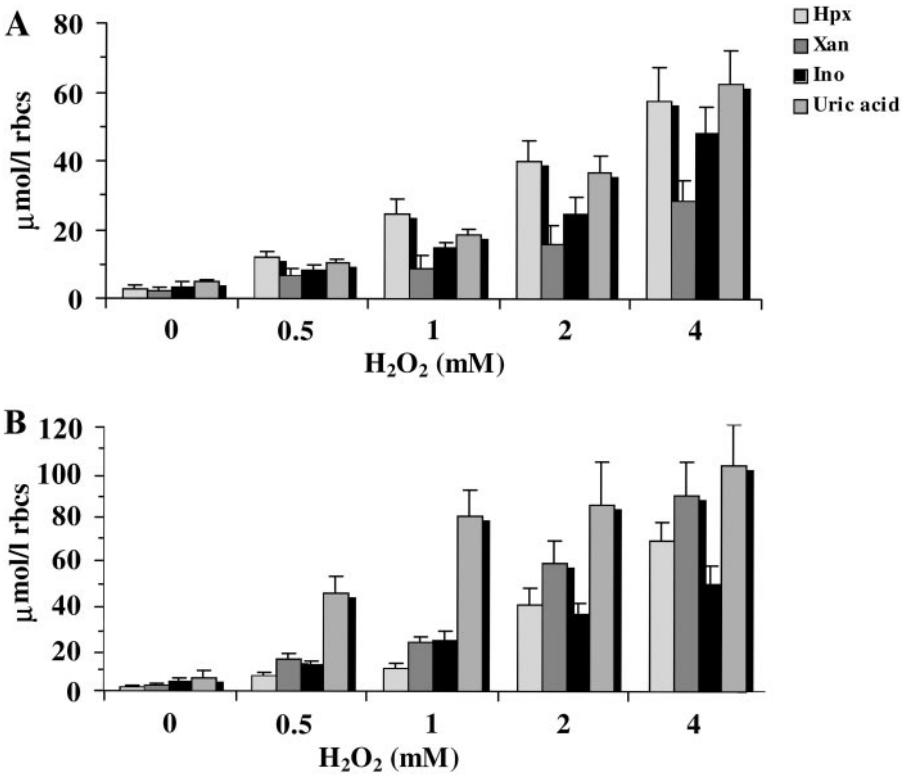

Fig. 2. Effect of increasing nonhaemolysing H_2O_2 concentrations on the formation of dephosphorylated compounds derived from erythrocyte ATP catabolism. Determination of intracellular (A) and extracellular (B) oxypurines (Hpx, Xan and uric acid) and nucleosides (Ino) was carried out by HPLC on 50 µL of neutralized perchloric acid extracts of packed erythrocytes and suspending medium. Each column represents the mean of five different blood samples. SD values are represented by vertical bars. Concentrations of intra- and extracellular compounds are expressed as $\mu mol \cdot L^{-1}$. To obtain the same unit, values of extracellular catabolites were initially calculated as $\mu mol \cdot L^{-1}$ and then multiplied by 20 for haematocrit correction (in a 5% haematocrit the erythrocyte volume is 1/20 of the total volume). All values of H_2O_2-treated erythrocytes were significantly different from those of controls ($P < 0.01$). Rbcs, red blood cells.

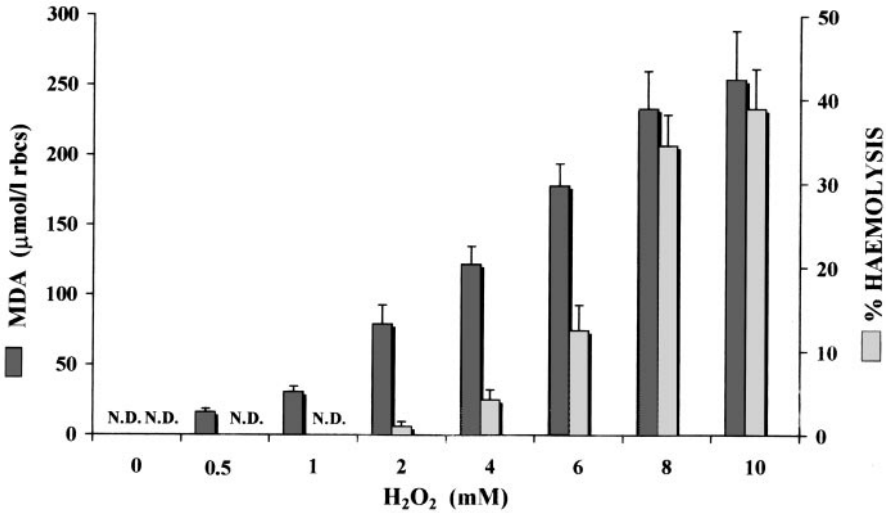

Fig. 3 Dose–response effects of increasing H_2O_2 concentrations on total MDA (i.e. intracellular MDA + extracellular MDA) and percentage haemolysis of fresh erythrocytes incubated for 1 h at 37 °C with 1 mM NaN_3 and different concentrations (0–10 mM) of H_2O_2. Values are the mean of five different blood samples assayed in duplicate. SD values are represented by vertical bars. All values of H_2O_2-treated erythrocytes were significantly different from controls ($P < 0.001$). ND = Not detectable. Rbcs, red blood cells.

Table 2. Effect of mannitol on energy metabolism, activation of AMP-deaminase, lipid peroxidation (MDA) and haemolysis of 10 mM H_2O_2-treated erythrocytes. Fresh human erythrocytes (5% haematocrit) were incubated for 1 h at 37 °C in NaCl/P_i-glucose + 1 mM NaN_3 + 10 mM H_2O_2 and each of the reported mannitol concentrations. Values are the mean (SD) of five different blood samples. ATP, IMP and MDA (corresponding to the sum of intra- and extracellular MDA concentrations) are expressed as $\mu mol \cdot L^{-1}$; AMP-deaminase is expressed as percentage activity with respect to the value of control erythrocytes (mean absolute value of AMP-deaminase activity in controls = 2.09 $U \cdot L^{-1}$; SD = 1.17); percentage haemolysis was calculated on the basis of haemoglobin amount released in to the erythrocyte suspending medium.

Mannitol(mM)	MDA	% Haemolysis	ATP	IMP	AMP-deaminase
0	248.15 (36.21)	38.92 (5.26)	348.22 (58.37)	381.15 (56.70)	611.00 (81.17)
25	184.66[a] (22.45)	26.88[a] (3.65)	453.46[a] (57.98)	298.44 (71.66)	509.57 (74.78)
50	140.74[a] (25.61)	17.03[a] (3.00)	634.78[a] (69.14)	294.98 (50.03)	461.33[a] (58.71)
100	111.67[a] (18.86)	13.62[a] (1.92)	821.33[a] (100.62)	277.13[a] (33.48)	403.00[a] (57.70)

[a] Significantly different from the corresponding value of 10 mM H_2O_2-treated erythrocytes incubated without mannitol ($P < 0.05$).

haemolysis. Nevertheless, even with 100 mM mannitol, high-energy phosphates, AMP-deaminase activity, MDA and haemolysis were significantly different from control erythrocytes, thus indicating the link between oxidative stress, inhibition of energy metabolism and lipid peroxidation.

DISCUSSION

Potential ROS toxicity towards several biologically important macromolecules has been demonstrated in several *in vitro* and *in vivo* studies and still many efforts are devoted to find possible new targets of ROS-damaging activity. Although one of the first exhaustive experiments in human erythrocytes showing the ability of oxidative stress to induce lipid peroxidation and haemolysis was carried out in 1971 by Stocks and Dormandy [13], little it is known about the effects on erythrocyte energy metabolism. Only recently, data were reported by Stocchi *et al*. who studied the influence of iron-ascorbate on compounds and enzymes of glycolysis [14]. In their study, modest effects were shown, except for a partial inhibition of hexokinase after 1 h exposure to their ROS-generating system.

Data reported in our study indicated that the adverse effect on erythrocyte energy state was more evident than that reported previously [14], probably because of the much higher efficiency of the system we chose for generating intracellular oxidative stress. We demonstrated that the effects of H_2O_2 on high-energy phosphates (Table 1) were significant at concentrations > 1 mM and that, among the adenine nucleotides, only ATP was markedly affected. In fact, only a modest increase of AMP was observed at any nonhaemolysing H_2O_2 concentration tested, although the Σ_{NT} recorded with the highest nonhaemolysing H_2O_2 concentration (4 mM) was 50% lower than that of control erythrocytes.

As under these experimental conditions IMP increased up to 20-fold with respect to the control value, and because there was a massive release of Ino, Hpx, Xan and uric acid (derived from IMP catabolism) into the medium, we hypothesized that high IMP and low AMP levels were due to the activation of AMP-deaminase. Experiments conducted to determine variations in the activity of this enzyme (Fig. 1) demonstrated clearly that AMP-deaminase was dramatically activated by oxidative stress, even at the lowest H_2O_2 concentration. Therefore, AMP was rapidly converted to IMP and, as soon as oxidative stress started to deplete the ATP concentration, more substrate (AMP) was available for the activated enzyme. As a consequence, the energy state of erythrocytes was profoundly imbalanced, as IMP cannot be used by erythrocytes for adenine nucleotide resynthesis [15]. Moreover, our data showed that excess IMP was partly accumulated inside the erythrocytes and partly lost in to the suspending medium as Ino, Hpx, Xan and uric acid. These uncharged catabolites are capable of permeating freely the erythrocyte membrane and are generated by the activity of 5′-nucleotidase, nucleoside phosphorylase and xanthine oxidase [16]. The hypothesis that AMP-deaminase activity is modulated by oxidative stress was supported by incubating 10 mM H_2O_2-treated erythrocytes with increasing concentrations of the free radical scavenger mannitol. Under these conditions, a reduction of AMP-deaminase activation was observed and it was mirrored by a lower IMP concentration and a higher ATP level. Hence, it might be postulated that secondary ROS, generated in the reaction of H_2O_2 with haemoglobin, could act either directly on the enzyme or through the generation of some positive activator with high affinity for the enzyme itself. Regardless of the mechanism of AMP-deaminase activation, the final result is loss of control of this delicate enzymatic activity by erythrocytes.

As already observed in the post-ischemic myocardium [17,18], increasing levels of lipid peroxidation (MDA) were determined in H_2O_2-treated erythrocytes without any appreciable detection of cell death (haemolysis) at up to a certain level of oxidative stress. This suggests the existence of a level of tolerance of erythrocytes to lipid peroxidation beyond which erythrocyte membrane modifications would irreversibly alter the structure and function of the membrane, thus allowing escape of macromolecules and, finally, to haemolysis. However, it should be noted that several studies pointed out that, in erythrocytes, lipid peroxidation is not the sole modification of the plasma membrane. In fact, oxidation of SH-groups [9], leakage of K^+ ions [19,20], formation of haemoglobin–spectrin adducts [21] and activation of proteolysis [10] have been described using different ROS-generating systems. Not all of the aforementioned studies showed a strict correlation with lipid peroxidation, thereby suggesting that lipid peroxidation itself might have a secondary role in the subsequent deterioration of the cell that leads to haemolysis. It should be recalled that, in one study, such a conclusion was drawn because of a nonlinear correlation between MDA production and K^+ leakage [20]. In the present study, we found a similar nonlinear correlation between MDA and haemolysis and, in our opinion, results of both studies may be interpreted by assuming the existence of a threshold value of lipid peroxidation, below which no dramatic changes either of several cell functions or of cell survival occur. It cannot even be excluded that in determining haemolysis, under the conditions of oxidative stress used here, a critical role was played by the profound changes of energy metabolism, in great part due to the loss of regulation of AMP-deaminase. It has been suggested that this enzymatic activity might be accelerated in dying erythrocytes with the specific purpose of depleting the energy state of the erythrocyte and facilitating haemolysis [22].

In summary, this study indicated that AMP-deaminase is a highly sensitive site of ROS action, and may be the main ROS-modifiable enzymatic activity in human erythrocytes. To the best of our knowledge, this is the first report showing that oxidative stress induces the activation (rather than the inhibition) of an enzyme directly involved in energy metabolism whose regulation might closely be related to the maintenance of cell integrity. In addition, our data show that energy metabolism and lipid peroxidation of human erythrocytes are both deeply affected by oxidative stress which is also responsible for haemolysis, but only if lipid peroxidation is higher than the threshold tolerance compatible with cell integrity. Further studies to characterize AMP-deaminase activation by oxidative stress and to gain insight into the mechanism of such an activation are in progress.

AKNOWLEDGEMENTS

This work was supported in part by a grant ‘ex-quota 40%: Nuovi approcci valutativi in tossicologia’ from Ministero dell’Università e della Ricerca Scientifica e Tecnologica of Italy (MURST) and by Research Funds of Catania University.

REFERENCES

1. Forman, M.B., Virmani, R. & Puett, D.W. (1990) Mechanisms and therapy of myocardial reperfusion injury. *Circulation* **81**, IV69–IV78.
2. Roof, R.L., Hofmann, S.W. & Stein, D.G. (1997) Progesterone protects

against lipid peroxidation following traumatic brain injury in rats. *Mol. Chem. Neuropathol.* **3**, 1–11.

3. De Flora, S., Izzotti, A., Randerath, K., Randerath, E., Bartsch, H., Nair, J., Balansky, R., van Schooten, F., Degan, P., Fronza, G., Walsh, D. & Lewtas, J. (1996) DNA adducts and chronic degenerative disease. Pathogenetic relevance and implications in preventive medicine. *Mut. Res.* **366**, 197–238.
4. Ravindranath, V. & Reed, D.J. (1990) Glutathione depletion and formation of glutathione-protein mixed disulfide following exposure of brain mitochondria to oxidative stress. *Biochem. Biophys. Res. Commun.* **169**, 1075–1079.
5. Tavazzi, B., Lazzarino, G., Di Pierro, D. & Giardina, B. (1992) Malondialdehyde production and ascorbate decrease are associated to the reperfusion of the isolated postischemic rat heart. *Free Rad. Biol. Med.* **13**, 75–78.
6. Di Pierro, D., Tavazzi, B., Lazzarino, G. & Giardina, B. (1992) Malondialdehyde is a biochemical marker of peroxidative damage in the isolated reperfused rat heart. *Mol. Cell. Biochem.* **116**, 193–196.
7. Maridonneau, I., Braquet, P. & Garay, R.P. (1983) Na+ and K+ transport damage induced by oxygen free radicals in human red cell membranes. *J. Biol. Chem.* **258**, 3107–3113.
8. Rohn, T.T., Nelson, L.K., Waeg, G. & Quinn, M.T. (1998) U-101033E (2,4-diaminopyrrolopyrimidine), a potent inhibitor of membrane lipid peroxidation as assessed by the production of 4-hydroxynonenal, malondialdehyde, and 4-hydroxynonenal-protein adducts. *Biochem. Pharmacol.* **56**, 1371–1379.
9. Snyder, L.M., Fortier, N.L., Leb, L., McKenney, J., Trainor, J., Sheerin, H. & Mohandas, N. (1988) The role of membrane protein sulfhydryl groups in hydrogen peroxide-mediated membrane damage in human erythrocytes. *Biochim. Biophys. Acta* **937**, 229–240.
10. Davies, K.J.A. & Goldberg, A.L. (1987) Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J. Biol. Chem.* **262**, 8220–8226.
11. Lazzarino, G., Di Pierro, D., Tavazzi, B., Cerroni, L. & Giardina, B. (1991) Simultaneous separation of malondialdehyde, ascorbic acid, and adenine nucleotide derivatives from biological samples by ion-pairing high-performance liquid chromatography. *Anal. Biochem.* **197**, 191–196.
12. Miki, M., Tamai, H., Mino, M., Yamamoto, Y. & Niki, E. (1987) Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Arch. Biochem. Biophys.* **258**, 373–380.
13. Stocks, J. & Dormandy, T.L. (1971) The autoxidation of human red cell lipids induced by hydrogen peroxide. *Br. J. Hematol.* **20**, 95–111.
14. Stocchi, V., Biagiarelli, B., Fiorani, M., Palma, F., Piccoli, G., Cucchiarini, L. & Dachà, M. (1994) Inactivation of rabbit red blood cell hexokinase activity promoted in vitro by an oxygen-radical-generating system. *Arch. Biochem. Biophys.* **311**, 160–167.
15. Bontemps, F., van der Berghe, G. & Hers, H.G. (1986) Pathway of adenine nucleotide catabolism in erythrocytes. *J. Clin. Invest.* **77**, 824–830.
16. Paglia, D.E., Valentine, W.N., Nakatani, M. & Brockway, R.A. (1986) Mechanisms of adenine 5′-monophosphate catabolism in human erythrocytes. *Blood* **67**, 988–992.
17. Giardina, B., Penco, M., Lazzarino, G., Romano, S., Tavazzi, B., Fedele, F., Di Pierro, D. & Dagianti, A. (1993) Effectiveness of thrombolysis is associated with a time-dependent increase of malondialdehyde in peripheral blood of patients with acute myocardial infarction. *Am. J. Cardiol.* **71**, 788–793.
18. Tavazzi, B., Di Pierro, D., Bartolini, M., Marino, M., Distefano, S., Galvano, M., Villani, C., Giardina, B. & Lazzarino, G. (1998) Lipid peroxidation, tissue necrosis, and metabolic and mechanical recovery of isolated reperfused rat heart as a function of increasing ischemia. *Free Rad. Res.* **28**, 25–37.
19. Sugihara, T., Rawicz, W., Evans, E.A & Hebbel, R.P. (1991) Lipid hydroperoxides permit deformation-dependent leak of monovalent cation from erythrocytes. *Blood* **77**, 2757–2763.
20. Deuticke, B., Heller, K.B. & Haest, C.W.M. (1987) Progressive oxidative membrane damage in erythrocytes after pulse treatment with *t*-butylhydroperoxide. *Biochim. Biophys. Acta* **899**, 113–124.
21. Snyder, L.M., Fortier, N.L., Trainor, J., Jacobs, J., Leb, L., Lubin, B., Chiu, D., Shoet, S. & Mohandas, N. (1985) Effect of hydrogen peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics, and spectrin-hemoglobin cross-linking. *J. Clin. Invest.* **76**, 1971–1977.
22. Valentine, W.N., Paglia, D.E., Clarke, S., Morimoto, B.H., Nakatani, M. & Brockway, R. (1985) Adenine ribo- and deoxyribonucleotide metabolism in human erythrocytes, B- and T-lymphocyte cell lines, and monocyte-macrophages. *Proc. Natl Acad. Sci. USA* **82**, 6682–6686.