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INHIBITION OF POLY(ADP-RIBOSE) POLYMERIZATION PRESERVES THE GLUTATHIONE POOL AND REVERSES CYTOTOXICITY IN HYDROGEN PEROXIDE-TREATED LYMPHOCYTES

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Abstract—DNA damage caused by oxygen radicals activates poly(ADP-ribosyl) polymerase (pADPRP), a nuclear enzyme that utilizes NAD^+ as substrate. It has been demonstrated that pharmacological inactivation of pADPRP rescues human lymphocytes damaged by oxygen radicals, but not those damaged by equitoxic doses of ionizing radiation. In the present paper we demonstrate that the NAD^+ pool decreases after both damaging treatments and is preserved in a similar fashion by pADPRP inhibition. On the contrary, the ATP pool, cell energy charge and reduced thiols are decreased only by the administration of oxygen radicals, and are preserved if poly(ADP)ribosylation is inhibited. In fact, treatment with oxidant agents depletes the cell energy pools owing to the simultaneous demands of the glutathione (GSH)/NADPH cycle and pADPRP-driven NAD^+ consumption, while in irradiated cells only the latter mechanism operates. We suggest that, when pADPRP is inhibited, enough energy is available for the preservation of cell thiols, thereby allowing oxidant-treated cells to survive and undergo mitosis. Thus, GSH and energy shortage appear to be the main cause of cell death in oxidant-injured cells.

Activated oxygen species are well-documented mediators of cell injury under a variety of pathological [1] and physiological [2] conditions, and have been involved in tumor initiation and promotion [3]. Hydrogen peroxide is a relatively stable compound, naturally occurring as a product of superoxide anion dismutation as well as of other oxidation-related processes, and can participate in the iron-catalysed generation of hydroxyl radicals, a highly reactive oxygen species. Among the biological mechanisms evolved to protect the cell against oxidative injuries, thiol groups, and notably glutathione (GSH‡), play a central role in endogenous defences [4]. Thiol action is particularly relevant in peripheral blood lymphocytes, which are subjected to a variety of oxidative challenges, including those at inflammatory sites, and where glutathione is also involved in the regulation of cell cycle progression and proliferation [5]. Lymphocyte integrity is a requisite for proper immune function and defence against cancer. Moreover, lymphocyte impairment has been observed in individuals with genetic predisposition to cancer, such as colon cancer, who have been suggested to carry abnormalities in their oxidative stress responses [6]. All these reasons make lymphocytes a useful tool for the study of oxidative injury.

DNA damage caused by free radicals activates poly(ADP-ribosyl) polymerase (pADPRP, EC 2.4.2.30), a nuclear enzyme that utilizes NAD^+ as substrate. Previous work in our laboratory [7] demonstrated that pharmacological inactivation of poly(ADP)-ribosylation is able to rescue lymphocytes damaged by oxygen radicals, but not those damaged by equitoxic doses of ionizing radiation. We concluded that ionizing radiation, at doses that impair lymphocyte survival by 80–90%, does not cause DNA damage requiring a massive activation of pADPRT activity. In the present work, we measured the reduced thiols and the nucleotide pools of peripheral blood lymphocytes after oxidative and ionizing radiation damage in order to find a satisfactory explanation of such behaviour. We found a dose-dependent decrease in -SH content after hydrogen peroxide treatment, a parallel decrease in ATP and NAD^+ pools, and restoration of both -SH groups and nucleotide pools following treatment with pADPRP inhibitors only in oxygen radical-damaged lymphocytes.

MATERIALS AND METHODS

Materials. All reagents were analytical grade. Xanthine oxidase (XOD), cell culture-tested nicotinamide (NAM), 3-aminobenzamide (3ABA) and 3-aminobenzoic acid (3AAB) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.),

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‡ Abbreviations: pADPRP, poly(ADP-ribosyl) polymerase; 3ABA, 3-aminobenzamide; 3AAB, 3-aminobenzoic acid; 3MBA, 3-metoxybenzamide; XOD, xanthine oxidase; HYP, hypoxanthine; GSH, glutathione; NAM, nicotinamide.

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while 3-methoxybenzamide (3MBA) was obtained from Aldrich (Germany). HPLC standards were from Sigma also, except for NAD^+, NADH, NADP and NADPH, which were obtained from Boehringer-Mannheim (Germany). LC18-T column was purchased from Supelco Inc. (Bellefonte, PA, U.S.A.).

Cell preparation. Peripheral blood mononuclear cells were obtained after Ficoll gradient separation of buffy coats from blood donations of non-smoker healthy males, aged 25 to 45. Cell suspensions were deprived of platelets; then lymphocytes were separated from monocytes by centrifugation in isotonic Percoll gradient [8]. Lymphocytes thus obtained were over 97% pure as judged by morphological criteria.

Cell survival. [^{3}H]TdR incorporation on the 3rd day after phytohaemagglutinin stimulation of mononuclear cell cultures was chosen as a measure of cell survival [7].

Treatments. Cells were treated with 50 or 500 mM H_2O_2 or with 100 mU/mL of xanthine oxidase plus 100 μM hypoxanthine (XOD/HYP) for 1 hr at 37° at the cell concentration of 1×10^6 cells/mL in Saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose, pH 7.3). γ Radiation from a ^{60}Co cell was administered at the same cell concentration and in the same medium at a dose rate of 4 Gy/min. Where required, 3ABA, 3MBA, NAM or 3AAB was added to the cell suspension just before damage. The drugs were freshly dissolved in Saline A; the pH of 3AAB solutions was adjusted to 7.3. At the end of treatment, cells were cooled in an ice bath, then washed by centrifugation and resuspended in proper cold medium, according to the biochemical analysis to be performed. In kinetic studies, part of the samples was resuspended in culture medium, allowed to recover at 37° for 2 hr, and then cooled and processed as required. Kinetic studies were limited to the first 2 hr after the damaging treatment since we feared that, beyond this time, the different modes of cell death would influence the biochemical comparison between oxygen radical-treated and irradiated cells. In fact, after treatment with oxygen radicals the number of Trypan blue-positive cells steadily increased from the first few hours after damage; during this time, the cell membrane of injured or dying cells is likely to be fragile and to leak cytoplasmic components. On the other hand, after irradiation causing comparable inhibition of proliferative capacity, the number of Trypan blue-positive cells was much smaller [9].

Determination of GSH and of thiol groups. After treatment, cells were suspended in cold phosphate-buffered saline containing 2×10^{-4} M ascorbic acid to reduce spontaneous oxidation and washed by centrifugation. Total protein and non-protein thiols and their oxidation products were evaluated by an original spectrophotometric method [10] based on the development of yellow water-soluble complexes of ammonium tetrachloropalladate with -SH or -S-S-. Total thiols were determined in the 100,000 *g* supernatant obtained from cells disrupted by freeze–thawing. To evaluate non-protein sulphydryls, cell pellets were suspended in ice-cold 2.5% sulphosalicylic acid in 0.2% Triton X-100 and centrifuged to remove precipitated proteins. Corresponding to 8×10^6 cells, 800 μL were employed in the assay. Optimal molar ratio Pd/-SH ranged between 20 and 48; these values were chosen in order to accelerate the complexation kinetics. Thiol concentration was calculated by extrapolation after addition of standard solutions of GSH. Disulphures were measured in the same sample after heating at 65° for 1 hr. GSH was evaluated by means of the *GSH-400* kit supplied by Bioxytech S.A. (Bonneuil sur Marne, France), following the suggested experimental procedures. Corresponding to 2×10^7 ceils, 250 μL were employed in the assay.

Evaluation of nucleotide pools. Nucleotide pools were evaluated by HPLC on an LC18-T column 25 cm × 4.6 mm developed with a phosphate buffer–methanol gradient at pH 6.0, according to the method of Stocchi *et al.* [11], with minor modifications. Beckman Gold HPLC apparatus and software were used. Cells to be evaluated were suspended in cold phosphate buffer, washed by

Table 1. Reduced thiol pools in peripheral blood lymphocytes

Sample	Total -SH	NPSH	GSH
Untreated undamaged control	100	100	100
Undamaged control + 5 mM 3ABA	100	106.7	100
Undamaged control + 2 mM 3MBA	96.2	ND	ND
Undamaged control + 10 mM NAM	103	ND	ND
Undamaged control + 5 mM 3AAB	87	ND	ND
50 μM H_2O_2	71	ND	ND
500 μM H_2O_2	56.9	76.6	63
500 μM H_2O_2 + 5 mM 3ABA	86.9	101.9	97.4
500 μM H_2O_2 + 2 mM 3MBA	75	ND	ND
500 μM H_2O_2 + 10 mM NAM	91	ND	ND
500 μM H_2O_2 + 5 mM 3AAB	34	ND	ND

Data are shown as per cent of control values. Each point is the average of at least three independent evaluations on different subjects. SEM never exceeded 10%.

Total -SH = total thiols; NPSH = non-protein thiols; ND = not determined.

Treatment of mononuclear cells with 50 and 500 μM H_2O_2 brought cell survival to 30 and 1% of control values, respectively (not shown).

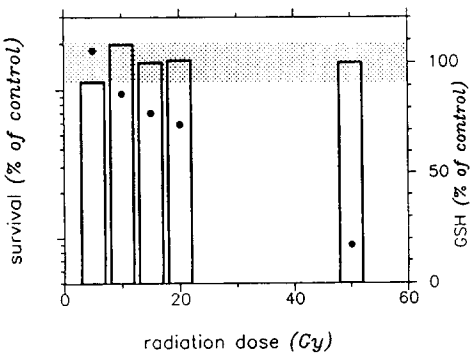

Fig. 1. Lymphocyte survival and GSH content as per cent of undamaged control values. Lymphocytes were irradiated with γ-rays, then stimulated with phytohaemagglutinin, and DNA synthesis (as [^{3}H]TdR incorporation) was evaluated after 3 days. GSH was measured immediately after irradiation. Shaded area represents the normal range of GSH concentration, bars are GSH concentrations evaluated in samples administered with the indicated radiation doses, which resulted in the survival rate shown by the dots. Data are the means of two independent experiments, which displayed very similar results.

centrifugation and then resuspended in the same medium at a concentration of 20×10^6/mL. Macromolecules were then precipitated with 3% perchloric acid and removed by centrifugation. The supernatant was neutralized with potassium carbonate and 1 M KH_2PO_4 was added equivalent to 20% vol. In a typical chromatographic run, the sample volume was 100 μL, corresponding to 1×10^6 cells.

RESULTS

In human peripheral lymphocytes, total (protein and non-protein) reduced thiols were found to amount to 12.06 ± 1.00 nmol/10^6 cells, while non-protein thiols were 3.7 ± 0.2 nmol/10^6 cells, and GSH was 1.36 ± 0.15 nmol/10^6 cells. Taking into account that quiescent lymphocytes are rather small cells, these concentrations are comparable to those recently reported for V79 fibroblasts [12]; comparison of the tetrachloropalladate method and the commercial kit will be the object of a separate study. In control samples, oxidized thiols amounted to less than 1% or were undetectable. As shown in Table 1, treatment with 50 or 500 μM hydrogen peroxide caused a decrease in total reduced thiols to 71 and 56.9% of control values, respectively, and a similar, although smaller, decrease in non-protein thiols and GSH. This may reflect a delay in the mutual equilibration of the different pools of cellular mercaptans. The GSH pool was found to be further decreased 2 hr after treatment. One hour exposure of control cells to 5 mM 3ABA, 2 mM 3MBA or 10 mM NAM, three pADPRP inhibitors [13], did not alter the content of reduced thiols of the sample, whereas the non-inhibitory analogue 3AAB at a concentration of 5 mM caused a slight decrease in -SH concentration, perhaps owing to inadequate buffering. On the other hand, the presence of pADPRP inhibitors during the damaging treatment resulted in statistically significant ($P = 0.02$) preservation of GSH and reduced thiol content. Again, the addition of 3AAB caused a further decrease in sulphydryls. In contrast, experimental data shown in Fig. 1 demonstrate that the GSH pool is not reduced at all by ionizing radiation, even when cell survival falls to values lower than 1% of control.

Figure 2 allows comparison of the original chromatograms of two lymphocyte samples, a control sample and one treated with XOD/HYP, and shows that both the ATP and NAD^+ contents are severely depleted as a result of treatment with an oxygen radical generating system. In Table 2 the effects of the oxidant agent are compared to those of ionizing radiation. As can be seen, both DNA damaging agents caused depletion of the NAD^+ pool. However, only when DNA damage is due to the action of XOD/HYP do the ATP pool and the cell energy charge [14] decrease.

The addition of a pADPRP inhibitor during the damaging treatment preserves the NAD^+ pool in both XOD/HYP-treated and γ-irradiated lymphocytes, thus indirectly demonstrating that in both cases NAD^+ consumption is due to activation of poly(APD-ribose) polymerization. As a result of the preservation of NAD^+ content, in XOD/HYP-treated lymphocytes the ATP pool and the total energy charge of the cell are also maintained, whilst they are unaffected by irradiation in both the presence and absence of 3ABA. Two hours after the damaging treatment, both the ATP and NAD^+ pools were further decreased in XOD/HYP-treated cells, while they were substantially unchanged in irradiated cells (not shown). This suggests that such an extent of NAD^+ depletion, though long-lasting, does not lower the ATP pool *per se*, and that the differences in the NAD^+ and ATP pools of irradiated and oxygen radical-treated cells are not merely quantitative or related to kinetics.

DISCUSSION

The effect of pADPRP inhibition on the GSH content of hydrogen peroxide-treated lymphocytes apparently contrasts with the conclusions reached by Schraufstatter *et al.* [15–17], who ruled out possible interactions between the GSH cycle and pADPRP activity. However, they found that: (i) addition of 3ABA does not alter the hexose monophosphate shunt activation that occurs upon stimulation of the GSH/NADPH cycle; (ii) inhibition of the hexose monophosphate cycle does not affect the NAD^+ concentration and pADPRP activity. Therefore, there is no real contradiction between their results and ours. In turn, Pero *et al.* [6] observed a correlation between the decrease in both constitutive and hydrogen peroxide-activated pADPRP activity and treatments resulting in GSH depletion, such as administration of buthionine sulphoximine and exposure to different prooxidant agents.

Although GSH plays a central role in radioprotection [18, 19], as well as in protection against

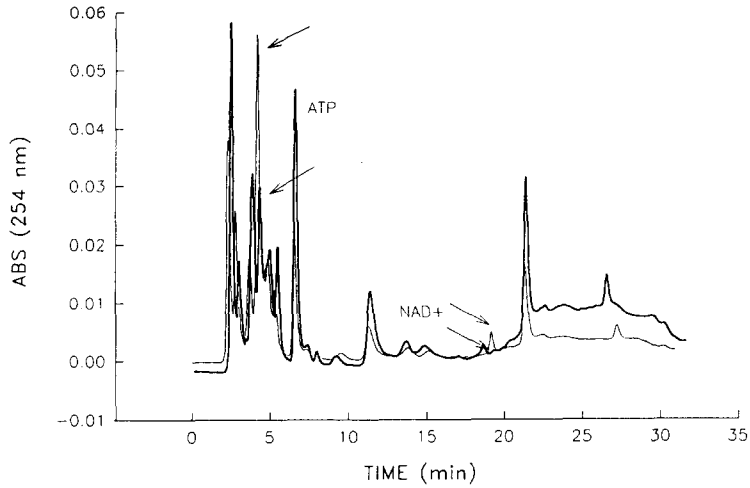

Fig. 2. Superimposed original chromatograms of control (thin line) and XOD-HYP-treated (thick line) lymphocyte samples from the same subject. The samples were separated by HPLC in successive runs; diagrams were aligned and superimposed by means of a software method. Both arrows on the left indicate ATP peaks; both arrows on the right indicate NAD^+ peaks.

Table 2. Nucleotide pools and energy charge of peripheral blood lymphocytes

Sample	NAD^+ concentration (nmol/10^6 cells)	ATP concentration (nmol/10^6 cells)	Energy charge
Undamaged control	0.171 ± 0.025	1.389 ± 0.184	10.095
XOD/HYP-treated	0.101 ± 0.013	0.744 ± 0.112	6.622
Irradiated (10 Gy)	0.080 ± 0.012	1.324 ± 0.150	9.272
Undamaged control + 3ABA	0.122 ± 0.030	1.191 ± 0.298	8.759
XOD-HYP-treated + 3ABA	0.101 ± 0.011	1.134 ± 0.143	7.845
Irradiated (10 Gy) + 3ABA	0.130 ± 0.022	1.114 ± 0.106	8.493

Each point is the mean ± SEM of at least seven independent evaluations on different subjects. Energy charge was calculated from ATP, ADP and AMP concentrations using the following algorithm [14]:

$$[ATP] + 0.5 \cdot [ADP]/[ATP] + [ADP] + [AMP].$$

At the indicated doses, XOD/HYP brought mononuclear cell survival to about 3% and γ-rays to about 20% of control values. For a discussion of similarities between the damaging action of hydrogen peroxide and of XOD/HYP on the cell, and its DNA and for a comparison between the administration of oxidant agents and of ionizing radiations, see Ref. 9. 3ABA was added during the damaging period at a concentration of 5 mM.

other oxidative injuries [4], it was predictable that ionizing radiation would not drastically reduce the GSH/GSSG ratio. The following stoichometric considerations may aid an understanding of this statement: (i) a dose of 50 Gy (which, according to the conditions of irradiation, reduces lymphocyte proliferative capacity to 0.5–2% of control) originates, in aqueous solution, a concentration of primary water radicals of about 33 μM; (ii) cellular GSH concentration is millimolar; should all radicals due to a radiation dose of 50 Gy be scavenged by GSH, that would reduce its concentration by a few per cent only. This means that, under otherwise normal conditions, scavenging of radiation-elicited radicals by GSH is not likely to entail a heavy energy burden on the cell. On the other hand, upon treatment with oxidant agents, ATP and energy pools are depleted because of the contemporary demands of the GSH/NADPH cycle and of pADPRP-driven NAD^+ consumption.

Although other, still not fully understood, side effects of pADPRP inhibitors cannot be ruled out [20], we suggest an interpretation of the present results that takes into account the occurrence of rapid interconversion of pyridine nucleotides consequent to oxidative damage [21]. On the basis of the present data and of other available information, we suggest that pADPRP inhibition may affect the GSH cycle by: (i) reducing NAD^+ consumption, thus allowing a comparatively greater conversion of NAD^+ to $NADP^+$, and (ii) saving valuable energy for $NADP^+$ reduction to NADPH. When the

NADPH demand is high and the GSH pool may very well be depleted because of the action of oxidant agents, pADPRP inhibition results in cell rescue [22] because it allows the redox balance of the cell to be maintained. The damaging effects of ionizing radiation [7] or of other agents [23] are not counterbalanced by pADPRP inhibition because they are not mediated by the shortage of both ATP and GSH. We are currently investigating whether GSH cycle preservation is involved also in other instances where pADPRP inhibitors rescue damaged cells.

The relevance of preservation of reduced thiols in a given subcellular compartment rather than of the overall sulphydryl content has been pointed out [24]; in fact, GSH preservation is a prerequisite for the preservation of thiol-containing enzymes, among which are some enzymes in the glycolytic pathway needed for ATP resynthesis [25] and repair enzymes [4], including pADPRP itself [6]. Defense mechanisms, including the neo-synthesis of stress proteins [26] and DNA [27], require full GSH availability. Although even a huge loss of GSH may be reversible [28], it should be pointed out that damage to subcellular compartments or to given pathways and enzymes may not be. For this reason, we are presently investigating the mechanisms by which the GSH content of the cell may be preserved or increased [29] and what protection such an increase may provide.

The present results suggest that energy shortage and thiol depletion is the chief mechanism by which hydrogen peroxide kills cells. Ward *et al.* [30] and Bump and Brown [19] pointed out that hydrogen peroxide produces about 400 times more DNA single-strand breaks than an equitoxic dose of ionizing radiation. In our view, such overwhelming DNA damage may not be the main cause of cell death, since pADPRP inhibition rescues hydrogen peroxide-treated cells without preventing DNA damage and even delaying its repair [6]. The above-discussed mode of cell death may be defined as acute or necrotic; experiments from our laboratory suggest that a limited number of oxygen radical-damaged cells die in a distinct mode: programmed cell death or apoptosis. However, our preliminary data suggest that 3ABA does not rescue hydrogen peroxide-treated cells from such a mode of cell death (manuscript in preparation) [31], at variance with reports of cells damaged by tumour necrosis factor [32] or etoposide VP16 [33].

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