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Development and characterization of hydrogen preoxide-resistant Chinese hamster overy (CHO) cell variants—II. Relationships between non-protein sulfydryl levels and the induction/stability of the oxidant-resistant phenotype

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Abstract—Hydrogen peroxide sensitive and resistant sublines of Chinese hamster ovary (CHO) cells were tested for their non-protein sulfydryl (NPSH) content in an attempt to establish whether a relationship exists between resistance to growth inhibition elicited by the oxidant and the NPSH pool. Cell variants characterized by increasing levels of resistance to hydrogen peroxide displayed a significant increase in cellular NPSH (expressed on a per million cell basis). Growth of resistant cells for various lengths of time in the absence of H_2O_2 decreased resistance, whereas the NPSH content did not vary (at least up to 127 days of growth in peroxide-free medium). The NPSH pool returned to control levels after an additional 82 days. These changes, however, were probably related to differences in cell size/amount of total cell proteins in the sublines. Indeed, when NPSH levels were expressed on a per milligram protein basis, essentially no variations were observed in sensitive and resistant sublines. It is important to note that, even by expressing the NPSH content on a per million cell basis, no correlation was found with the degree of resistance to growth inhibition elicited by the oxidant. Further experiments have demonstrated that, under conditions of reduced NPSH content (obtained by growing the cells in the presence of a glutamylcysteine synthetase inhibitor), the cytotoxic action of hydrogen peroxide was very slightly, if at all, augmented in both wild type and resistant cells. We may therefore conclude that cellular NPSH do not afford significant protection against growth inhibition induced by hydrogen peroxide in wild type cells, and that the same lack of effect occurs in cells with an increased NPSH content and carrying the oxidant-resistant phenotype.

Key words: hydrogen peroxide; resistant cells; toxicity; NPSH

Evidence in the literature demonstrates that the exposure of cultured mammalian cells (CHO*, HA-1) to progressively higher concentrations of hydrogen peroxide results in the development of cell variants resistant to the insult elicited by the oxidant [1]. These cells were characterized by a very high stability of the oxidant-resistant phenotype, and the degree of resistance was associated with an increased catalase activity [1]. Consistently, highly resistant cell lines clonally isolated from these variants were also found to display an enormous increase (50 times or more) in catalase [2–4] and, in addition, had increased glutathione content [5, 6] and glutathione peroxidase activity [3, 4]. Taken together, these results suggest that resistance to hydrogen peroxide occurs via induction of an increased detoxification system. This conclusion, however, does not appear univocal, since the hydrogen peroxide resistant CHO cells that we have recently developed displayed very minor changes in their catalase content (less than 1.5 times above the control value) and the oxidant-resistant phenotype was only partially based on the increased catalase activity [7]. Obviously, differences in the isolation procedures utilized in the two studies may have accounted for the differential mechanisms involved in acquisition of the oxidant-resistant phenotype.

In order to gain a better understanding of the molecular mechanisms governing cellular resistance to oxidative stress in the resistant cells that we have developed [7], it is important to obtain additional information on the status of the non-protein low molecular weight thiols (NPSH). Indeed, the NPSH pool, of which GSH represents 90% or more, is known to play a protective role against the insult generated by an array of cytotoxic agents, including reduced oxygen species [8, 9].

In this study we have measured the NPSH levels in sensitive and resistant cells and attempted to establish their role in protecting the cells against growth inhibition induced by hydrogen peroxide.

Materials and Methods

Materials. Hydrogen peroxide was purchased as a 30% solution from J. T. Baker Chemicals B.W. (Deventer, Holland). L-buthionine sulfoximine and most reagent-grade biochemicals were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Mc Coy's 5a medium, foetal bovine serum and trypsin were from Gibco (Grand Island, NY, U.S.A.).

Cells and cell culture conditions. Cell lines were grown in Mc Coy's 5a medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2 in air, at 37°.

Isolation of hydrogen peroxide-resistant cells. The procedure utilized for isolating hydrogen peroxide resistant variants has been described elsewhere [7]. Briefly, cells (2×10^6 cells/75 cm^2 flask) were adapted to 850 μM H_2O_2 (treatment was for 1 hr followed by 3 days of growth) by stepwise increases from 150 to 850 μM H_2O_2 over a period of 4.5 months. These cells will be hereafter called V 150, V 250, V 450, V 650 and V 850 (depending on the oxidant concentration to which cells had been adapted). V 850 cells were further cultivated for 50, 100, 127 and 209 days and never exposed to the oxidant, in which case they are referred to as R 50, R 100, R 127 and R 209, respectively.

NPSH assay. Cells were seeded at a density of 2×10^6 cells/75 cm^2 flask and harvested after 48 hr of growth. Cell

* Abbreviations: CHO, Chinese hamster ovary; NPSH, non-protein sulfydryl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

pellets, obtained after centrifugation at 400 *g* for 5 min were washed twice with Saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$ and 5 mM glucose). NPSH were extracted by incubating the cells in 0.3 M orthophosphoric acid at ice temperature for 15 min. Cell extracts were then centrifuged at 12×10^3 rpm for 5 min and an aliquot of the supernatant was transferred to a tube containing a phosphate buffer (0.121 M NaCl, 3 mM KCl, 0.01 M Na_2HPO_4 and 1.5 mM KH_2PO_4). To each tube 100 μL of 0.04% DTNB in 1% Na-citrate were added, the contents were mixed and the absorbance was measured at 412 nm. The concentration of NPSH in the samples was determined by comparing the optical density readings to a standard curve constructed using reduced glutathione.

Proteins were assayed as described by Lowry *et al.*, using bovine serum albumin as a standard [10].

Cell growth inhibition studies. Cells (4.5×10^5) were inoculated into 60 mm tissue culture dishes and after 6 hr the monolayers were exposed for 1 hr (at 37°) to increasing concentrations of hydrogen peroxide (added from a freshly prepared solution in distilled water). In other experiments the cells were inoculated at a density of 2.5×10^5 cells/60 mm dish and allowed to attach. After 6 hr the original medium was removed and replaced with fresh culture medium containing 0 or 7.5 μM L-buthionine sulfoximine. Following incubation for 16 hr the cells reached a density of approx. 4.5×10^5/60 mm dish and were exposed for 1 hr to increasing concentrations of hydrogen peroxide. In both types of experiments, after treatments with the oxidant, the cells were allowed to grow for 48 hr and then counted with a Coulter counter particle-size analyser. Cell number present in the oxidant-treated dishes was expressed as a function of the number of cells in untreated dishes to assess the influence of hydrogen peroxide on cell reproduction.

Results

NPSH levels in sensitive and resistant cells. We have previously reported the isolation and partial characterization of CHO cell variants resistant to hydrogen peroxide [7]. By using a treatment protocol involving exposure to mildly cytotoxic levels of the oxidant, we were able to develop resistant cells characterized by an oxidant-resistant phenotype which only partially depended on over-expression of catalase [7]. Since cellular NPSH were previously shown to play an important protective role against oxidative stress [8, 9], we decided to investigate whether a correlation exists between the NPSH content and resistance to the growth inhibitory action elicited by hydrogen peroxide. In particular, our interest was to determine the levels of cellular NPSH in cells isolated at different stages during the adaptation process and during growth for various time intervals with no hydrogen peroxide. Obviously the sublines under investigation were characterized by different levels of resistance to the oxidant [7].

Since sensitive and resistant cells displayed large variations in their total protein content [7], we have normalized the experimental results obtained in NPSH assays both on a per million cell and per milligram protein basis.

Figure 1A indicates that the adaptation process increased NPSH levels when results were expressed on a per 10^6 cell basis. Indeed, V 150, V 250 and V 850 cells had a higher NPSH content than wild type cells (7 ± 0.1 nmol NPSH/10^6 cells) and the increase (1.47-, 1.77- and 1.83-fold, respectively) was statistically significant (V 150, $P = 0.003$; V 250, $P < 0.003$; V 850, $P < 0.001$). NPSH levels in R 127 cells were not significantly different from those of the V 850 subline and were 1.74-fold higher compared to wild type cells ($P < 0.001$). Further growth in peroxide-free medium, however, resulted in a progressive decline in the NPSH content and, after 82 days (R 209), NPSH levels returned to control values. This change was statistically

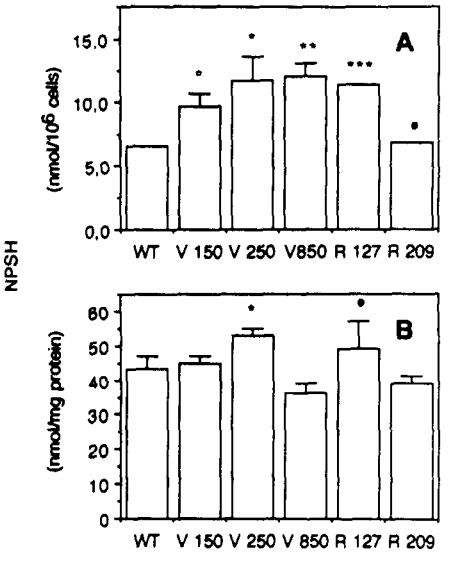

Fig. 1. Non-protein sulfydryl content in sensitive and resistant cells. NPSH levels were determined spectrophotometrically using DTNB (as described in Materials and Methods) 2 days after splitting. The results are means ± SEM calculated from four to seven determinations in separate cultures, in three different experiments. Data are expressed both on a per million cells basis (A) and per milligram protein basis (B). (A) Significantly different from wild type cells: $P < 0.003$ (*); $P < 0.001$ (**); $P < 0.0001$ (***). Significantly different from V 850 cells: $P < 0.001$ (•) (B) Significantly different from wild type cells: $P < 0.05$ (*). Significantly different from V 850 cells: $P < 0.01$ (•).

significant vs V 850 cells ($P < 0.001$). When comparison of the NPSH content in the various sublines was made on a per milligram protein basis (Fig. 1B), no significant change was observed in wild type (43 ± 1 nmol/mg) and resistant sublines, except for V 250 cells which had 1.24 times more NPSH with respect to the parental cell line ($P = 0.05$). In addition, R 127 cells had 1.44 times more NPSH than V 850 cells ($P = 0.05$).

In summary, results presented in this section suggest that the NPSH levels of cells acquiring or losing resistance to oxidative stress, are basically a function of the total cell proteins, which become progressively higher during the adaptation process and decrease upon growth in the absence of the oxidant.

Relationship between resistance to hydrogen peroxide and NPSH levels. We next attempted to correlate the NPSH content of the various sublines with their level of resistance to growth inhibition induced by H_2O_2. Experimental results obtained in growth inhibition studies were essentially superimposable to those obtained in the previous work describing the isolation and preliminary characterization of the variants [7].

The relationship existing between NPSH levels and the ID_{50} values (the drug concentration inhibiting cell growth by 50%) for growth inhibition by H_2O_2 in sensitive and resistant cells is shown in Fig. 2. Cellular NPSH expressed on a per million cell basis show a very weak correlation ($r = 0.732$) with resistance of the sublines isolated at different stages of the adaptation process (Fig. 2A), whereas no apparent relationship ($r = 0.339$) is detectable by comparing the same parameters in cells that had been grown for various time intervals with no hydrogen peroxide (Fig. 2C). The lack of relationship between NPSH levels

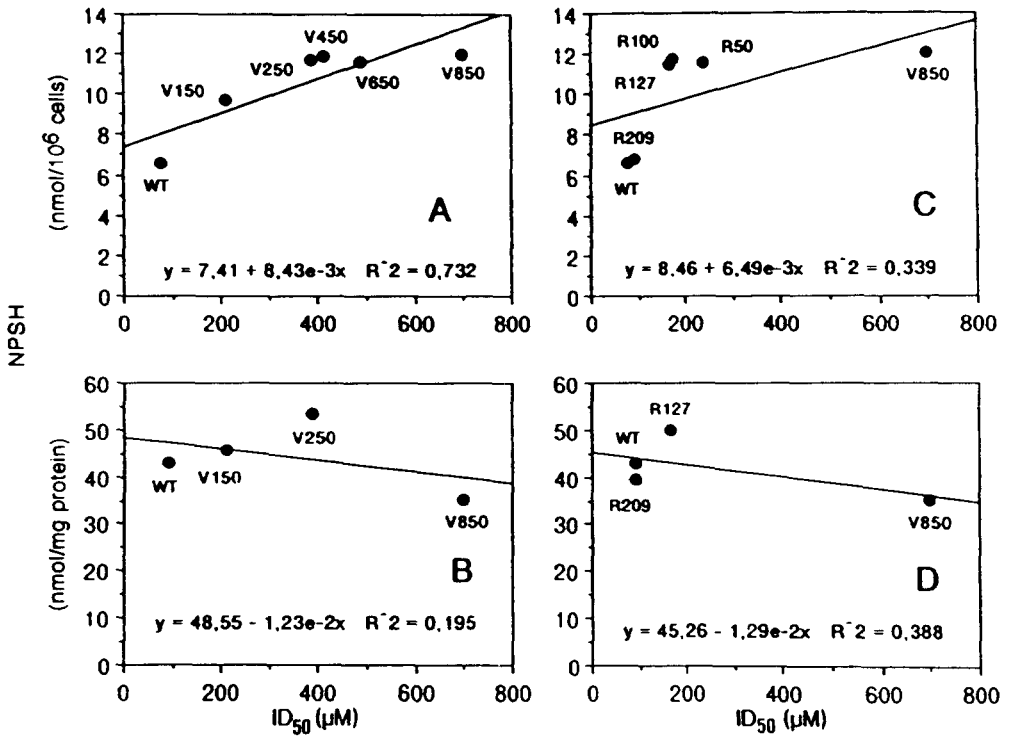

Fig. 2. Correlation between NPSH content and cytotoxicity induced by hydrogen peroxide in sensitive and resistant cells. NPSH values expressed either on a per million cells basis (A and C) or per milligram proteins basis (B and D) have been plotted against the ID_{50} values for growth inhibition induced by H_2O_2 in sublines obtained during the adaptation process (A and B) or following growth of V 850 cells in the absence of the oxidant for various lengths of time (C and D).

and growth inhibition induced by H_2O_2 was even more obvious when the NPSH content was expressed on a per milligram protein basis (Fig. 2B and D). It is informative to note that, by using this normalization procedure, V 850 cells, the most resistant subline, had the lowest NPSH content, and all the other sublines, although displaying large variations in resistance to hydrogen peroxide, had very similar NPSH levels. This lack of relationship was found both in the sublines isolated during the process of adaptation (Fig. 2B; $r = 0.195$) and in those obtained by growing resistant cells with no hydrogen peroxide for various lengths of time (Fig. 2D; $r = 0.388$). It should be noted that in Fig. 2 data from additional sublines to those shown in Fig. 1 have been included. These cells were adapted to 450 and 650 μM H_2O_2 and were named V 450 and V 650, respectively (Fig. 2A), or consisted of V 850 cells grown with no H_2O_2 for 50 or 100 days in which case they were named R 50 and R 100, respectively (Fig. 2C). These experimental data for cellular NPSH have not been reported in Fig. 1 since they were obtained in a single determination performed in duplicate.

Additional experiments comparing the cytotoxic response of wild type and V 850 cells to hydrogen peroxide, under conditions of normal and reduced NPSH levels, have further demonstrated that cellular NPSH does not account for resistance to the oxidant (Fig. 3). Indeed, treatment for 16 hr of wild type and V 850 cells with L-buthionine sulfoximine (7.5 μM) although resulting in a marked decrease in cellular NPSH (80% in wild type cells; 88% in V 850 cells) only marginally increased the growth inhibitory response elicited by increasing concentrations of hydrogen peroxide in both wild type and V 850 cells.

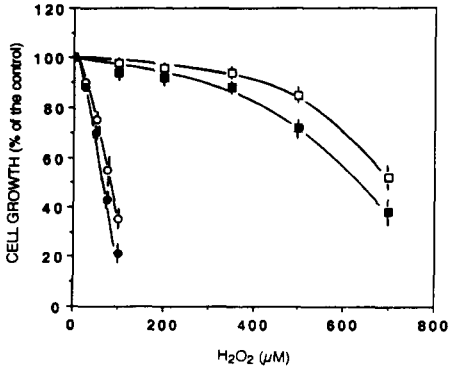

Fig. 3. Lack of an effect of NPSH depletion on the sensitivity of wild type and V 850 cells to hydrogen peroxide. Wild type (○, ●) and V 850 cells (□,■) were exposed for 1 hr to increasing concentrations of hydrogen peroxide following growth for 16 hr either in the absence (open symbols) or presence (closed symbols) of 7.5 μM L-buthionine sulfoximine. After treatment with oxidant the cells were allowed to grow for 48 hr and then assayed for cell number. Pretreatment with L-buthionine sulfoximine lowered the cellular NPSH levels to 80 and 88% of control levels in wild type and V 850 cells, respectively.

Discussion

The results presented in this paper indicate that the NPSH levels of CHO cells undergoing an adaptation process to oxidative stress, or resistant cells grown in the absence of the oxidant for various time intervals, display large fluctuations, depending on the normalization procedure utilized to express the experimental results. Indeed, when the data were expressed on a per million cells basis, the NPSH content increased during the adaptation process and decreased upon removal from H_2O_2. Cellular NPSH, however, remained essentially the same by expressing the results on a per milligram protein basis. These results clearly indicate that the NPSH follow the increase and then the decrease in cell mass accompanying the development/loss of the oxidant-resistant phenotype [7]. Nevertheless, no relationship was found between the NPSH pool and cytotoxicity mediated by the oxidant. These findings, along with the observation that the toxicity of hydrogen peroxide is only marginally increased in NPSH-depleted sensitive and resistant cells, suggest that cellular NPSH are not critical in determining resistance to the growth inhibitory action elicited by hydrogen peroxide both in normal cells and in cell variants adapted to the oxidant.

It is important to note, however, that under our experimental conditions, L-buthionine sulfoximine treatment did not completely deplete the NPSH pool, since about 12% of the initial content was still present at the time of oxidative injury. Therefore, it is possible that residual NPSH might suffice in supporting the activity of glutathione peroxidase, and indeed previous work has demonstrated that the enzyme levels, and not those of the substrate, are rate-limiting as far as the activity of the system is concerned [11]. This would explain why L-buthionine sulfoximine treatment did not significantly alter the cytotoxic response of both sensitive and resistant cells. Our results, however, are in marked contrast with those obtained by other authors [8, 9] who, under similar conditions, found a significant potentiation of the toxicity elicited by the oxidant. The reason for this discrepancy is not immediately apparent although the fact that in this work cytotoxicity was assayed in terms of growth inhibition might be of great importance. Importantly, in Refs. 8 and 9 acute cytotoxic responses were measured by using conventional assays like LDH or radioactive hexavalent chromium release. With these two approaches markedly different levels of the oxidant have to be utilized and this could explain the differential response brought about by thiol depletion in oxidatively injured cells.

In order to give an answer to some of these unsolved problems, and to gather more information on the mechanism for the acquired resistance in our variants, more work is necessary. As an example, it will be of interest to measure, and compare, the GSH-peroxidase activity of sensitive and resistant cells. Also of importance will be to measure the levels of the enzyme superoxide dismutase which, by scavenging the superoxide anion, could impair the efficiency of the cell to reduce trivalent iron to the divalent state. This step of the so-called Haber–Weiss reaction does indeed appear as rate limiting for the formation of a highly cytotoxic intermediate, the hydroxyl radical, which results from the reaction between hydrogen peroxide and divalent iron and is thought to mediate most of the deleterious effects elicited by the oxidant.

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