Effects of variation in glutathione peroxidase activity on DNA damage and cell survival in human cells exposed to hydrogen peroxide and t-butyl hydroperoxide

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The selenium-dependent glutathione peroxidase activities of two human cell lines, the colon carcinoma HT29 and the mesothelioma P31, cultured in medium containing 2% serum, increased from 195 to 541 and from 94 to 361 units/mg of protein respectively after supplementation with 100 nM-selenite. The catalase activity remained unchanged by this treatment. The effects of the obtained variation in glutathione peroxidase activities were investigated by exposing cells to H_2O_2 and t-butyl hydroperoxide. Selenite supplementation resulted in a decrease in H_2O_2 -induced DNA single-strand breaks in both HT29 and P31 cells. A small, but significant, decrease in the number of DNA single-strand breaks for low doses (10–50 μM) of t-butyl hydroperoxide was found only in P31 cells and not in HT29 cells. We could detect neither induction of double-strand breaks (detection limit approx. 1000 breaks per cell) nor DNA–protein cross-links after exposing the cells to the two peroxides. In spite of the apparent protective effect of increased glutathione peroxidase activity on DNA single-strand break formation, there were no differences between selenite-supplemented and non-supplemented cells in cell survival after exposure to peroxide.

INTRODUCTION

Glutathione peroxidase (GSH: H_2O_2 oxidoreductase, EC 1.11.1.9) is a tetrameric selenoenzyme [1] dependent on one selenocysteine residue per subunit for its activity [2]. The 'classical' intracellular enzyme is mainly a cytosolic enzyme, but is also found in the mitochondrial matrix of mammalian cells [3]. Findings in recent years indicate the existence of at least three additional selenium-dependent GSH peroxidases, two apparently different monomeric phospholipid hydroperoxide GSH peroxidases [4,5] and a tetrameric, glycosylated, secretory GSH peroxidase [6]. The enzymes catalyse the reduction of H_2O_2 to water or a variety of organic hydroperoxides to the corresponding alcohols [3–6] with a specific requirement for the reductant substrate, GSH [3]. Together with catalase, which also reduces H_2O_2 , and the superoxide dismutases, which degrade superoxide radicals, GSH peroxidases provide the main intracellular enzymic defence against reactive oxygen metabolites in the mammalian body.

We have previously shown that, by culturing cells in medium containing little serum (2%) with or without addition of 100 nM-selenite, large differences in selenium-dependent GSH peroxidase can be induced [7,8]. The activities of catalase, Mn-superoxide dismutase or CuZn-superoxide dismutase were essentially unchanged by the selenite supplementation [8]. The selenium-independent activity given by GSH S-transferases has been shown to increase in selenium-deficient tissues [9], but we could not find that this activity was influenced by our selenite supplementation procedure [8]. When we compared the radiation response, measured as cell survival and DNA single-strand break formation, of three cell lines we could not find any differences between selenite-supplemented cells and non-supplemented cells [8]. Hydrogen peroxide, an efficient DNA single-strand break inducer [10–22], is generated by ionizing radiation, but in relatively small amounts [10]. To extend our studies concerning variable GSH peroxidase activity we wanted to know whether

larger doses of H_2O_2 would lead to any changes in the response of our end points. Since H_2O_2 is also a substrate for catalase, we were interested in adding a more clear-cut GSH peroxidase substrate for comparison. Organic hydroperoxides cannot be disproportionated by catalase, and among the hydroperoxides t-butyl hydroperoxide in particular is a poor substrate for GSH S-transferases [23]. t-Butyl hydroperoxide thus seemed to us a good choice as a complementing agent in this study.

The aim of this study was to elucidate the role of GSH peroxidase in protection against hydroperoxides by comparing the effects of variation in GSH peroxidase activity on H_2O_2 - and t-butyl hydroperoxide-induced geno- and cyto-toxicity. We investigated the effects at the molecular level, measured as DNA single-strand and double-strand break formation, and DNA–protein cross-link induction by using the DNA precipitation assay [24,25], and at the cellular level, measured as cell survival (viability) with the use of a number of different assays.

MATERIALS AND METHODS

Cell culture

Detailed descriptions of cell lines, culture conditions and harvesting of cells, here reported briefly, have been published elsewhere [7]. We used two human cell lines in this study: HT29, a colon carcinoma, and P31, a mesothelioma of probable asbestos-induced origin. Stock cultures of HT29 and P31 were maintained in Ham's F-10 medium (NordVacc, Skärholmen, Sweden) at 37 °C in 5% CO_2 in humidified air. The medium was supplemented with 10% (v/v) fetal-calf serum, antibiotics and L-glutamine (NordVacc). At 2 weeks before each experiment the fetal-calf serum content was lowered to 2%. A single serum batch was used throughout the study. Its selenium content, determined by a fluorimetric assay employing diaminonaphthalene [26], was 0.29 μM , resulting in a selenium concentration of 6 nM in the control medium. To half of the culture dishes we added

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100 nM of selenite (Na_2SeO_3 ; cell-culture-tested, Sigma Chemical Co., St. Louis, MO, U.S.A.). The remainder of the cells were used as non-supplemented control cells. We changed the medium three times a week and subcultured the cells weekly to maintain exponentially growing cultures.

DNA single-strand break assay

DNA damage was detected with the DNA precipitation assay [24] modified by the use of double radiolabelling according to the principles outlined by Rydberg [27] and Sandström & Johanson [28] for the detection of DNA strand breaks with improved precision. Exponentially growing selenite-supplemented and control cell populations were labelled either with 3.7 kBq (0.1 μCi) of [^3H]thymidine/ml or with 0.74 kBq (0.02 μCi) of [^{14}C]thymidine/ml; specific radioactivities 74 GBq (2Ci)/mmol and 2.2 GBq (59 mCi)/mmol respectively (Amersham International, Amersham, Bucks., U.K.). The labelling medium was removed after 42 h and replaced with non-radioactive medium for 1–3 h. The cells were then trypsinized and ^3H -labelled selenite-supplemented cells were mixed with ^{14}C -labelled control cells and vice versa. The cell suspensions were diluted to contain 4×10^4 cells/ml and exposed to different amounts of H_2O_2 and t-butyl hydroperoxide in Hepes-buffered (20 mM, pH 7.4) Ham's F-10 medium for 30 min at 37 °C under repeated agitation. After centrifugation the medium was discarded and the cells were washed once with ice-cold Hepes-buffered medium. Then individual samples of about 3×10^5 cells in 100 μl of medium were lysed and processed as described by Olive [24]. The mean percentage precipitated DNA was calculated on an equal number of mixtures of ^3H -labelled selenite-supplemented cells and ^{14}C -labelled control cells and mixtures of ^{14}C -labelled selenite-supplemented cells and ^3H -labelled control cells.

DNA double-strand break assay

The DNA precipitation assay was run with double radiolabelling under neutral conditions, pH 8.3, to detect double-strand breaks. γ -Irradiated samples served as controls of the assay. About 25 Gy (1000 double-strand breaks per cell; [29]) was found to be necessary to induce a detectable decrease in precipitated DNA.

Detection of DNA-protein cross-links

The DNA precipitation procedure described by Olive *et al.* [25] was essentially followed. Peroxide-exposed and non-exposed control cells were irradiated with 5, 10, 15 and 20 Gy, as well as left non-irradiated, and the irradiation-induced slope was used to detect cross-links. Cross-links induce a decrease in slope. The amounts of precipitated DNA were corrected for effects of saturation of the assay, so that untreated samples represented 100% precipitated DNA and sonicated samples 0% precipitated DNA. Cross-links induced by 5 μM -2,2'-dichlorodiethyl sulphide (mustard gas) were used to control the validity of the assay.

Clonogenic survival experiments (HT29 cells)

Selenite-supplemented and control HT29 cells were trypsinized, counted and exposed at 37 °C under repeated agitation to various amounts of freshly prepared H_2O_2 or t-butyl hydroperoxide (Merck, Darmstadt, Germany) in Hepes-buffered medium at 4×10^4 cells/ml. After 60 min, the cells were centrifuged, washed free of peroxide and plated in 10 ml of Ham's F-10 medium containing 10% fetal-calf serum. After 9–11 days the colonies were stained with Methylene Blue and colonies consisting of 50 or more cells were scored. The mean plating efficiency was 23% for non-supplemented cells and 28% for supplemented cells.

Cell growth assay (P31 cells)

We seeded 8×10^4 P31 cells per dish in 5 ml of non-supplemented or selenite-supplemented medium and allowed them to attach for 20–24 h. The cells were then exposed to 3 ml (at about 4×10^4 cells/ml) of peroxide in Hepes-buffered medium for 60 min at 37 °C. After removal of the peroxide, the cells were washed with 5 ml of Hepes-buffered medium and cultured for another 72 h in 5 ml of non-supplemented or selenite-supplemented medium. Then the cells were trypsinized and counted in a Coulter model D counter (Coulter Electronics, Luton, Beds, U.K.). Cell growth was calculated as the number of cells at each dose point in comparison to the number of untreated control cells.

Determination of cell viability by using the MTT assay (HT29 and P31 cells)

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [30], which involves the reduction of a tetrazolium salt (MTT) to coloured formazan by viable cells, was used as another way of determining cell growth/viability. We distributed 5×10^3 cells in respective medium at 200 μl per well on to 96-well low-evaporation microtitre plates (Costar, Cambridge, MA, U.S.A.). The medium in each well was replaced by 200 μl of H_2O_2 or t-butyl hydroperoxide in Hepes-buffered medium 24 h later. After 60 min at 37 °C the peroxide-containing medium was discarded and replaced by 200 μl of the original medium for 72 h. Then 25 μl of a 5 mg/ml stock solution of MTT (Sigma Chemical Co.) was added. After an additional 2 h the MTT-containing medium was substituted by a lysing buffer [31] consisting of 20% (w/v) SDS (Sigma Chemical Co.) dissolved in a 50% (v/v) dimethylformamide (BDH Chemicals, Poole, Dorset, U.K.) solution, pH 4.7. The optical density was measured at 540 nm (570 nm optimal; [31]) in a Titertek Multiskan MCC/340 photometer (Eflab, Helsinki, Finland) after overnight lysing. Viable fraction was calculated as the percentage of uptake of control cells.

Preparation of cells for enzymic analysis

Cells, from cultures parallel to the ones used in each experiment, were harvested and stored as pellets at -80 °C for enzyme and protein analysis. After thawing, the cells were suspended in 2 ml of 10 mM-potassium phosphate buffer, pH 7.4, containing 30 mM-KCl, sonicated under cooling with ice for 1 min, and then shaken for 30 min at 4 °C. The homogenates were centrifuged at 20000 g for 15 min and the enzymic analyses were performed on the supernatants.

GSH peroxidase assay

The analysis was performed at 37 °C in 0.1 M-Hepes/NaOH buffer, pH 7.4, containing 1 mM-EDTA, 2 mM-GSH, 1 unit of GSH reductase/ml, 0.16 mM-NADPH and 0.88 mM-t-butyl hydroperoxide. (One unit of enzyme activity is defined as the activity that reduces 1 nmol of hydroperoxide/min.) The GSH concentration used was comparatively high and the t-butyl hydroperoxide concentration low. Under these conditions selenium-dependent GSH peroxidase provides the main contributions to the observed activity [23].

Catalase assay

The activity was analysed with a Clark oxygen electrode, essentially as described by Del Rio *et al.* [32]. Homogenates were added to 3 ml of de-aerated 10 mM-Hepes/NaOH buffer, pH 7.4, containing 0.1 mM-EDTA and also 15 mM- H_2O_2 . The catalase activity was calculated from the initial rate of O_2 liberation. (One unit is defined as the activity that disproportionates 1% of the H_2O_2 in 1 min.)

Table 1. Effect of selenite supplementation on activities of peroxide-reducing enzymesActivities are given as means $\pm$ S.D.

Cell line	GSH peroxidase activity (units/mg of protein)	Catalase activity (units/mg of protein)
HT29		
-Se	195 $\pm$ 113	23.1 $\pm$ 9.0
+Se	541 $\pm$ 150	25.7 $\pm$ 8.7
P31		
-Se	94 $\pm$ 66	8.3 $\pm$ 5.9
+Se	361 $\pm$ 248	6.9 $\pm$ 3.2

* Total number of experiments.
† Number of determinations.

Protein determination

Protein content was determined by employing the Coomassie Brilliant Blue G-250 technique [33], for which human serum albumin was used as a standard.

Statistics

Statistical differences between selenite-supplemented and non-supplemented cells in DNA single-strand break induction were determined with Student's paired-comparisons *t*-test, for which SAS software (SAS Institute, Cary, NC, U.S.A.) was used.

RESULTS

We were able to increase the selenium-dependent GSH peroxidase activity about 3-fold in HT29 cells and 4-fold in P31 cells (Table 1). The exact GSH peroxidase activities are given in the legends of each relevant Figure. Catalase activity in cells from several experiments were analysed, but remained relatively unchanged by the selenite supplementation (Table 1). During the course of this work we made an interesting observation con-

Fig. 1. DNA strand breakage in relation to exposure time

A mixture of selenite-supplemented (■ and ●) and non-supplemented (□ and ○) P31 cells were exposed to an initial concentration of 100 μ M- H_2O_2 (□ and ■) or 100 μ M-*t*-butyl hydroperoxide (○ and ●) at 4×10^4 cells/ml and the amount of DNA single-strand breaks (decrease in DNA precipitation) was determined after the indicated times. Results are the means $\pm$ S.D. of double determinations in triplicate. GSH peroxidase activity: non-supplemented cells, 113 units/mg of protein; selenite-supplemented cells, 659 units/mg of protein. The error bars refer to inter-sample variation.

cerning P31 cells. These cells lost as much as 80 % of their initial GSH peroxidase activity during 5 months of culture. The relative difference between non-supplemented and selenite-supplemented cells, however, remained the same. There was no similar effect on catalase activity of P31 cells (cf. S.D. values in Table 1), and both the GSH peroxidase and catalase activities of HT29 cells remained constant during the same time, showing that the variation was not due to analytical problems.

The resistance to induction of DNA single-strand breaks in cells exposed to H_2O_2 at 37 °C is dependent on the combined

Fig. 2. H_2O_2 -induced DNA strand breakage

A mixture of selenite-supplemented (● and ■) and non-supplemented (○ and □) P31 cells (a) and HT29 cells (b) were exposed to initial concentrations of 0–200 μ M- H_2O_2 at 4×10^4 cells/ml, and the amount of DNA single-strand breaks was determined after 30 min. Results are the means $\pm$ S.D. of double determinations in triplicate from (a) one and (b) two experiments. The different symbols in (b) refer to different experiments and are arranged on each side of actual concentration used. GSH peroxidase activity: non-supplemented P31 cells, 19 units/mg of protein; non-supplemented HT29 cells, 222 (○) and 194 (□) units/mg of protein; selenite-supplemented P31 cells, 144 units/mg of protein; selenite-supplemented HT29 cells, 637 (●) and 628 (■) units/mg of protein. The error bars show inter-sample variation. Statistical significance: $P < 0.003$ for all exposures except 200 μ M in (a) where $P < 0.02$.

activities of catalase and GSH peroxidase, and in the case of t-butyl hydroperoxide of GSH peroxidase and GSH S-transferases. The peroxide consumption leads to problems in interpreting the results, and to overcome these problems we exposed the cells at as low a density as was practically feasible (4×10^4 cells/ml) with the present DNA strand break assay. We found that even at this comparatively low cell density there was a marked peroxide consumption, 35–50 % for H_2O_2 and about 10 % for t-butyl hydroperoxide during 30 min [11]. Fig. 1 shows that, when P31 cells were exposed to $100 \mu\text{M}$ - H_2O_2 , there was a rapid induction of DNA single-strand breaks that reached a maximum after 10 min, whereas with $100 \mu\text{M}$ -t-butyl hydroperoxide the number of DNA strand breaks apparently increased during 60 min. The relatively rapid peroxide consumption during experiments also make comparisons of effects of reduced or increased enzyme activity hard to interpret if the analyses are made separately, i.e. is an effect dependent upon extra- or intra-cellular peroxide concentration. To prevent analytical problems with a gradually disappearing agent such as H_2O_2 we have developed a double-labelling assay for detection of DNA damage. Selenite-supplemented cells were prelabelled with [^3H]thymidine and non-supplemented cells with [^{14}C]thymidine and vice versa. The cells were mixed before each experiment and exposed to the peroxides together. By using this assay we were able to avoid the problem of cells being exposed to different amounts of extracellular peroxide. We were also able to minimize statistical variation. This allowed us to find subtle differences between selenite-supplemented and non-supplemented cells in DNA single-strand break induction that otherwise would have been difficult to detect. Fig. 2 shows that, when cells were exposed to H_2O_2 and analysed in this way, selenite-supplemented P31 cells (Fig. 2a), as well as HT29 cells (Fig. 2b), contained fewer single-strand breaks than did non-supplemented cells. The catalase and GSH per-

oxidase activities of the two cell lines were inversely correlated with the amount of DNA damage, as expected. Selenite-supplemented P31 cells also showed a significant decrease in the number of single-strand breaks when the cells were exposed to 10–50 μM -t-butyl hydroperoxide for 30 min (Fig. 3). There was an unexpected change between selenite-supplemented and non-supplemented cells when exposed to increasing amounts of t-butyl hydroperoxide, and at 300 μM non-supplemented cells were significantly ($P = 0.034$) less damaged. There was no significant difference between supplemented and non-supplemented HT29 cells when exposed to any concentration of t-butyl hydroperoxide (results not shown). The HT29 cells were also more resistant than P31 cells to t-butyl hydroperoxide-induced DNA single-strand breakage, in agreement with their respective GSH peroxidase activities (results not shown). If t-butyl hydroperoxide, in addition to inducing DNA single-strand breaks, also induced DNA–protein cross-links, this could explain why exposure to 300 μM -t-butyl hydroperoxide resulted in more apparent DNA single-strand breaks in selenite-supplemented P31 cells than in non-supplemented cells. The increased GSH peroxidase activity would decompose more t-butyl hydroperoxide, and, given that the concentration-dependences of DNA–protein cross-link and DNA single-strand break induction were different, the amount of precipitated DNA could decrease (i.e. an increase in the number of strand breaks). The possibility of DNA–protein cross-link induction was tested for t-butyl hydroperoxide, and also for H_2O_2 , but we found no evidence for the existence of DNA–protein cross-links for either P31 cells (Fig. 4) or HT29 cells (results not shown), although we tested doses as high as 1 mM in the case of t-butyl hydroperoxide.

The protective effects of selenite-supplementation were in most cases small, but we were still interested to see if we could detect any significance of the decrease in single-strand breaks on cell survival/viability after peroxide exposure. However, although several sets of experiments were performed, we could not detect any changes in the surviving fractions when P31 cells (Fig. 5) or HT29 cells (Fig. 6) were exposed to H_2O_2 or t-butyl hydro-

Fig. 3. t-Butyl hydroperoxide-induced DNA strand breakage

A mixture of selenite-supplemented (● and ■) and non-supplemented (○ and □) P31 cells were exposed to initial concentrations of 0–300 μM -t-butyl hydroperoxide at 4×10^4 cells/ml, and the amount of DNA single-strand breaks was determined after 30 min. Results are the means $\pm$ s.d. of double determinations in triplicate from two experiments. The different symbols refer to different experiments and are arranged on each side of actual concentration used. GSH peroxidase activity: non-supplemented cells, 34 (○) and 52 (□) units/mg of protein; selenite-supplemented cells, 132 (●) and 194 (■) units/mg of protein. The error bars show inter-sample variation. Statistical significance: $P < 0.0005$ for 10–50 μM exposures and $P = -0.034$ for 300 μM .

Fig. 4. Lack of peroxide-induced cross-links

P31 cells at 4×10^4 cells per ml were exposed for 30 min to 1 mM-t-butyl hydroperoxide (□), 200 μM - H_2O_2 (▲), 5 μM of the positive control, mustard gas (△), or left unexposed (●). The ability of ^{137}Cs γ -rays to degrade DNA normally was taken as an indication that the peroxides did not induce significant amounts of DNA–protein cross-links. Results are the means of double determinations in duplicate from one of two similar experiments.

Fig. 5. P31 cellular growth after peroxide exposure

About 1.2×10^5 selenite-supplemented (●) or non-supplemented (○) P31 cells in monolayer culture were exposed for 1 h to 3 ml of medium containing different concentrations of H_2O_2 (a) and t-butyl hydroperoxide (b). Cell growth in relation to growth of non-exposed control cells was determined 72 h later by counting the number of attached cells. Results are the means $\pm$ S.D. of four dishes per experiment from (a) three experiments and (b) two experiments. GSH peroxidase activity: non-supplemented cells, 109 (a) and 113 (b) units/mg of protein; selenite-supplemented cells, 221 (a) and 217 (b) units/mg of protein.

Fig. 6. HT29 cell survival after peroxide exposure

About 4×10^4 HT29 cells were exposed for 1 h to different concentrations of H_2O_2 (a) and t-butyl hydroperoxide (b), and their clonogenic survival was determined. Results are means and maximal variation of selenite-supplemented (●) and non-supplemented cells (○) from (a) two experiments and (b) one experiment. GSH peroxidase activity: non-supplemented cells 130 (a) and 51 (b) units/mg of protein; selenite-supplemented cells, 266 (a) and 322 (b) units/mg of protein.

peroxide. Generally 0.1–0.3 mM- H_2O_2 was found to be less cytotoxic than corresponding doses of t-butyl hydroperoxide (including MTT results; results not shown) and in contrast with the respective patterns of DNA single-strand break induction. As can be seen (cf. Figs. 2 and 3), peroxide doses inducing a massive number of DNA single-strand breaks did very little harm in terms of cell killing. Indeed, single-strand breaks, in contrast with double-strand breaks, are considered to be a relatively insignificant molecular damage in cell killing. However, our double-strand break assay, which has a detection limit for

ionizing radiation-induced double-strand breaks of about 25 Gy (1000 double-strand breaks per cell [29]), detected neither H_2O_2 - nor t-butyl hydroperoxide-induced DNA double-strand breaks. Although we also tested the assay at 0 °C to prevent DNA repair, we still could not detect any signs of double-strand break induction after doses as high as 10 mM of both peroxides (results not shown).

DISCUSSION

The maximum number of DNA single-strand breaks in P31 cells exposed to 100 μM - H_2O_2 at 37 °C was detected after 10 min (Fig. 1). At this point there is apparently a steady state between the induction and rejoining of single-strand breaks. Since H_2O_2 is decomposed by the cells and DNA single-strand break formation is concentration-dependent (cf. Fig. 2), the rate of DNA strand-break induction will decrease whereas the DNA rejoining rate is likely to persist, explaining the approach to control levels in number of DNA single-strand breaks at 60 min. The literature concerning rejoining of H_2O_2 -induced DNA single-strand breaks in repair-proficient cells generally give a $t_{1/2}$ in the 4–7 min range [12–14]. The results in Fig. 1 fit well with such a $t_{1/2}$ value if the induction of additional breaks by the decreasing amounts of H_2O_2 is taken into consideration.

Exposure of P31 cells to 100 μM -t-butyl hydroperoxide at 37 °C did not produce the same pattern of induction and rejoining of DNA single-strand breaks as exposure to H_2O_2 (Fig. 1). Whereas 100 μM - H_2O_2 brought down the amount of precipitated DNA from 95% (control level) to 30–40% in 5–10 min, t-butyl hydroperoxide only decreased the amount precipitated by 12–13% in 10 min and by about 30% in 60 min. Unlike the case with H_2O_2 there was no net rejoining effect during 60 min with t-butyl hydroperoxide. This is consistent with t-butyl hydroperoxide being decomposed more slowly than H_2O_2 by GSH peroxidase [34] and that the decomposition of H_2O_2 is further aided by catalase. The apparent K_m of GSH peroxidase is 7.8 μM with H_2O_2 and 60 μM with t-butyl hydroperoxide as the substrate (calculated from ref. [34]), assuming a GSH concentration of 1 mM.

Selenite-supplemented cells were more resistant than non-supplemented cells to DNA single-strand break induction by H_2O_2 (Fig. 2), and, in the case of P31 cells, to breaks induced by low concentrations of t-butyl hydroperoxide (Fig. 3). This is certainly due to the faster peroxide decomposition in supplemented cells, since they contain 3–4 times as much GSH peroxidase activity as non-supplemented cells. However, the protection against peroxide-induced DNA damage, brought about by the increase in GSH peroxidase activity, did not significantly alter cell survival. One explanation could possibly be the fact that the assays measuring cell survival carry less precision than the DNA single-strand break assay. Examining the kinetic parameters for GSH peroxidase gives additional explanations for the lack of observed effect on cell survival. From the kinetic data [34] it can be deduced that under physiological conditions removal of H_2O_2 is close to $V_{\max}$ at 100 μM . Decomposition by catalase, on the other hand, is a first-order process and thus this enzyme will at any H_2O_2 concentration reduce the same fraction of the H_2O_2 over a given time. The result is that at increasing H_2O_2 concentrations catalase will become increasingly important and the effect of variation in GSH peroxidase activity more difficult to discern. As to t-butyl hydroperoxide, an 8-fold increase in dose is necessary to reach the same turnover as with H_2O_2 . The reaction approaches $V_{\max}$ at 0.5–1 mM (assuming 1 mM-GSH), and the fraction of t-butyl hydroperoxide reduced during a given time will thus decrease with increasing t-butyl hydroperoxide concentration. Any pro-

protective effect of an increase in GSH peroxidase activity on cell killing (mainly seen at 0.3–10 mM) is thus likely to be more difficult to discern than an effect on single-strand breaks (seen at 10–50 μ M). Finally, at high hydroperoxide concentrations the reduction rate may be increasingly dependent on the GSSG-reduction rate, diminishing the effects of variation in GSH peroxidase activity.

GSH depletion has been shown to lead to increased cytotoxicity after exposure to peroxide [35,36]. Several other mechanisms have also been suggested to be involved in peroxide cytotoxicity. These include activation of poly(ADP-ribose) polymerase [15], involvement of a Ca^{2+} -dependent endonuclease [16], production of DNA double-strand breaks [17] or locally multiply damaged sites in DNA [14], maintenance of cellular reductive processes [37] and onset of lipid peroxidation [38]. Consensus is reached on the involvement of free radicals in the onset of cytotoxicity whatever the suggested mechanism behind it. Hydroxyl radicals are formed from H_2O_2 [18] and, probably, t-butyloxy radicals from t-butyl hydroperoxide [39], in transition-metal-ion-catalysed reactions and indeed, chelation of iron or copper ions has been found to both prevent H_2O_2 - [16,18–20,35] and t-butyl hydroperoxide-induced [40] DNA damage and increase cell survival [16,35–38,41] after exposure to H_2O_2 , but not after exposure to t-butyl hydroperoxide [41]. An involvement of radicals in peroxide-induced genotoxicity [10,12,18–20] and cytotoxicity [35,36,41] is further supported by results with radical scavengers.

A good correlation between DNA single-strand break yield and cell killing effect of H_2O_2 in cells of different species was obtained by Hoffmann *et al.* [21]. This is contrasted by the results of the present experiments, where the induction of DNA single-strand breaks is seemingly not correlated with cell killing, at least for low doses (0.1–0.3 mM), for the two peroxides. At these doses the induction of single-strand breaks was much higher for H_2O_2 than for t-butyl hydroperoxide (Figs. 1–3), but H_2O_2 generally killed less cells than t-butyl hydroperoxide [Figs. 5 and 6, and results of the MTT assay (results not shown.)]. This suggests the existence of at least one peroxide cell-killing mechanism separate from DNA damage, with the assumption that other types of DNA damage also are produced by the same mechanism that produces single-strand breaks. A cell-killing mechanism separate from DNA damage is further supported by our other results as we, unlike Prise *et al.* [17], were unable to detect double-strand breaks (results not shown). Our results agree with previous reports in the literature showing lack of DNA double-strand breakage by H_2O_2 at doses of biological interest [10,13,22]. We could not detect DNA–protein cross-links after exposure to H_2O_2 , in agreement with available data on cross-link formation in intact cells [12,13,22]. In contrast, the occurrence of DNA–protein, as well as DNA inter-strand, cross-links after exposure to H_2O_2 has been reported by Lesko *et al.* working with isolated chromatin [42]. Our interest in DNA–protein cross-links originated from the pattern of DNA single-strand break formation after exposure to t-butyl hydroperoxide, an agent for which we have not found results of cross-link induction previously reported.

In conclusion, we have shown that increasing the GSH peroxidase activity of cultured cells 3–4-fold resulted in significant protection against H_2O_2 -induced DNA single-strand breaks. There was less significant protection against t-butyl hydroperoxide-induced strand breaks, only at low peroxide concentrations and only in one cell line, P31. We were unable to show any significance of the decrease in DNA single-strand breaks on cell killing, and, whereas H_2O_2 was much more efficient than t-butyl hydroperoxide in inducing DNA single-strand breaks at doses up to 0.3 mM, t-butyl hydroperoxide

generally killed more cells than H_2O_2 at these doses. This indicates that, at low peroxide doses, at least one of the two peroxides may kill cells by a mechanism separate from DNA damage. The nature of the mechanism is currently unknown.

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