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# Cytotoxicity and metabolism of 4-hydroxy-2-nonenal and 2-nonenal in $H_2O_2$-resistant cell lines

## Do aldehydic by-products of lipid peroxidation contribute to oxidative stress?

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Toxic aldehydes, such as 4-hydroxy-2-nonenal (4HNE) and 2-nonenal (2NE), formed during lipid peroxidation have been isolated and implicated in the cytotoxic effects of oxidative stress. We have investigated the cytotoxicity and metabolism of 4HNE and 2NE in control (HA-1) cells and in two $H_2O_2$-resistant Chinese hamster fibroblast cell lines. The $H_2O_2$-resistant cells were found to be significantly more resistant than HA-1 cells to the cytotoxicity of 4HNE, as determined by clonogenic cell survival (dose-modifying factors at 10% isosurvival of 2.0–3.0). The $H_2O_2$-resistant cells demonstrated a significant 2–3-fold increase in the amount of 4HNE removed (mol/cell) from culture media containing 72 $\mu$M-4HNE when compared with HA-1 cells. The enhanced ability of $H_2O_2$-resistant cells to metabolize 4HNE was abolished by heating the cells at 100 °C for 45 min. Similar results were obtained with 2NE. Total glutathione and glutathione transferase activity, believed to be involved in cellular detoxification of 4HNE, were found to be significantly increased (2–3-fold) in the resistant cells when compared with the HA-1 cells. These results show that cell lines adapted and/or selected in a highly peroxidative environment are also resistant to the cytotoxicity of aldehydes formed during lipid peroxidation. This resistance appears to be related to increased cellular metabolism of these aldehydes, possibly through the glutathione transferase system. These findings suggest that the formation of aldehydes due to lipid peroxidation may contribute significantly to the mechanisms of oxidant-induced injury and the selective pressure exerted by $H_2O_2$-mediated cytotoxicity in culture.

## INTRODUCTION

Lipid peroxidation reactions, initiated in biological systems by the interaction of free radicals with polyunsaturated fatty acids (PUFA), have been hypothesized to contribute significantly to many types of cellular damage [1–11]. 4-Hydroxy-2-nonenal (4HNE) and related aldehydes have been identified as by-products of the peroxidation of linoleic and arachidonic acids, microsomes and cells [12–14]. These aldehydes are capable of damaging functional proteins, inhibiting protein synthesis, causing DNA damage and inhibiting DNA synthesis, as well as causing cell lysis and loss of cellular reproductive integrity [15–19]. Since 4HNE and related aldehydes are toxic and are produced by lipid peroxidation, it has been suggested that they contribute significantly to the cytopathological effects in biological systems exposed to oxidizing agents capable of producing lipid peroxidation. However, direct evidence that these aldehydes contribute to the cytotoxicity of biologically relevant oxidants is lacking.

We have isolated and characterized Chinese hamster fibroblast cell lines that are stably resistant to the cytotoxic effects of hydrogen peroxide in culture [20,21]. These $H_2O_2$-resistant cell lines were derived from the HA-1 parental line following continuous exposure to progressively increasing concentrations of $H_2O_2$. $H_2O_2$ is believed to react with metal ions (such as iron) to produce highly reactive species such as hydroxyl radicals [22–25], which contribute to $H_2O_2$-mediated cytotoxicity in culture [26,27], as well as being initiators of lipid peroxidation reactions [24,27]. Since metal ions are prevalent in culture media, and since another report has shown that exposure of cultured cells to $H_2O_2$ is accompanied by increases in lipid peroxidation [27], the $H_2O_2$-resistant cell lines were adapted and/or selected for growth in a highly peroxidative environment in which the production of lipid aldehydes may occur. We reasoned that if 4HNE and/or related aldehydes were produced and contributed significantly to the cytotoxic selection pressure exerted by chronic exposure to $H_2O_2$ in culture, then $H_2O_2$-resistant cell lines should also have become resistant to the cytotoxicity of 4HNE.

The objective of these studies was to investigate the possible role of lipid-peroxidation-derived aldehydes in the cytotoxic selection process initiated by exposure of mammalian cells to oxidant injury. The cytotoxicity of 4HNE and related aldehydes was determined by using a clonogenic cell survival assay in the HA-1 and two $H_2O_2$-resistant cell lines. The cellular metabolism of 4HNE and related aldehydes, as assayed by their disappearance from culture media exposed to HA-1 and $H_2O_2$-resistant cells, was also determined. Conjugation reactions involving glutathione and glutathione transferases (GSTs) have been suggested as a major pathway leading to 4HNE detoxification [28,29]. Therefore total GSH and GST activity were also determined in the HA-1 and $H_2O_2$-resistant cell lines.

## MATERIALS AND METHODS

### Cells and culture conditions

Chinese hamster fibroblasts designated HA-1 [30] and the $H_2O_2$-resistant cell lines (OC5 and OC14) were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (Hyclone) and penicillin/streptomycin (100 units/ml and 0.1 mg/ml respectively; Sigma). The OC5 and OC14 cell lines were clonally isolated from a population of HA-1 cells that were adapted and/or selected for growth in 800 $\mu$M-$H_2O_2$ for 200 days [21]. Cell cultures were grown at 37 °C in a humidified incubator with air/$CO_2$ (19:1). Cell cultures were

Abbreviations used: 4HNE, 4-hydroxy-2-nonenal; 2NE, 2-nonenal; 2,4-NDAL, 2,4-nonadienal; 2,4-DDAL, 2,4-decadienal; 2,4-NDOL, 2,4-nonadienol; 2,4-DDOL, 2,4-decadienol; MEM, Eagle's minimal essential medium; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione transferase; PUFA, polyunsaturated fatty acids; DMF, dose modification factor.

checked routinely for mycoplasma contamination (University of Virginia, Clinical Labs., Division of Microbiology). $CO_2$ concentrations were adjusted using a Beckman LB-2 medical gas analyser calibrated to a certified gas standard ($\pm 0.1\%$).

### Aldehydes and alcohols

(*E*)4-Hydroxy-2-nonenal (4HNE) was provided by Dr. Hermann Esterbauer, University of Graz, Austria, (*E*)2-nonenal (2-NE), (*EE*)2,4-nonadienal (2,4-NDAL). (*EE*)2,4-decadienal (2,4-DDAL), 2,4-nonadienol (2,4-NDOL) and 2,4-decadienol (2,4-DDOL) were obtained from Alfa Products (Danvers, MA, U.S.A.). The identity and purity of all aldehydes and alcohols were verified, by comparison with published spectra, using g.c.–m.s without derivatization. This was carried out by Dr. Michael T. Kinter, Division of Clinical Chemistry, Pathology Department, University of Virginia. Standard solutions containing a known molarity of aldehyde were prepared in 100% methanol as well as in a 1:1 mixture of h.p.l.c. grade $H_2O$ and acetonitrile/acetic acid (24:1, v/v) (either method of preparation yielded similar results) and stored at −20 °C.

### Survival experiments

Cells [$(1–2) \times 10^5$] were plated in 60 mm dishes or 25 $cm^2$ flasks and grown exponentially for 2 days, at which time cell density was $(3–7) \times 10^4$ cells/$cm^2$. Special care was taken to ensure that all cultures within an experiment were at the same cell density at the time of treatment. For aldehyde survival experiments, the cultures were treated in 4 ml of MEM without serum or antibiotics, because serum has been shown to facilitate the disappearance of aldehydes from culture media [12]. For survival experiments with 2-NE, 2,4-NDAL and 2,4-DDAL, the cells were treated in sealed 25 $cm^2$ flasks gassed with air/$CO_2$ (19:1) as these aldehydes are volatile and disappear rapidly from culture dishes. 4-HNE was stored in methylene chloride at −20 °C (10 mg/ml). Samples for experiments were evaporated under $N_2$ in sterile conditions and dissolved in sterile water, and the concentration was determined spectrophotometrically at 223 nm ($\epsilon$ 13750 $M^{-1} \cdot cm^{-1}$). This aqueous solution was added to MEM immediately before treating the cells. All other aldehydes were dissolved in 95% ethanol and added to MEM immediately before treating the cells. The cultures were exposed to aldehydes at 37 °C for 30–180 min. Following treatment, each culture was washed three times with sterile Puck's saline, and trypsin-treated. The resulting single-cell suspension was counted (Coulter counter), diluted and plated for colony survival. Each dilution was made such that 100–300 cells were plated for survival analysis in the most diluted replicate, 1000–3000 in the next dilution, 10000–30000 in the next dilution and 100000–300000 in the least diluted replicate. Each replicate dilution was plated into three separate cloning dishes. After 8–10 days of incubation at 37 °C, colonies were fixed (70% ethanol), stained with Crystal Violet and counted under a dissecting microscope. The dilution replicates which yielded 50–250 surviving colonies were used for survival analysis. A colony had to contain 50 cells to be considered as a survivor. Plating efficiencies were 60–90% for all untreated cell lines. The ethanol concentration in MEM used to add dissolved aldehydes (0.02%) demonstrated no cytotoxic effects. Survival data following treatment were normalized to appropriate control plating efficiencies.

### Aldehyde removal experiments

Immediately before and immediately after treatment of cells, a 1 ml portion of MEM containing the aldehyde was mixed with 1 ml of acetonitrile/acetic acid (24:1, v/v) to stop the consumption of aldehyde by the media [31]. The samples so collected were stable for at least 7 days at −20 °C. Those collected immediately before treatment were assayed to determine the amount of aldehyde (mol) available to the cells at zero time of exposure. The samples collected from cell cultures after exposure to aldehyde-containing MEM were assayed to determine the amount of aldehyde (mol) consumed during the exposure interval. For each time point during the exposure of cell cultures to aldehyde, a sample handled identically, but not exposed to cells, was collected to determine the amount of aldehyde consumed by non-cell-mediated processes. This amount was subtracted from the total amount consumed by the cell cultures during each interval to determine the amount of aldehyde (mol) removed by the cells in each culture during each treatment interval. The results were calculated as mol of aldehyde removed/cell.

### Assay of aldehydes in MEM

Aldehyde concentrations in MEM (no serum or antibiotics) mixed 1:1 with acetonitrile/acetic acid (24:1, v/v) were assayed by h.p.l.c. using an isocratic Beckman System Gold (u.v. detector model 166) with a ALTEX Ultrasphere ODS (particle size 5 $\mu$m; internal diam. 4.6 mm; length 4.5 cm) precolumn and a ALTEX Ultrasphere ODS (particle size 5 $\mu$m, 4.6 mm × 15 cm) column. A Varian 4270 integrator was used to determine the areas under the h.p.l.c. peaks corresponding to the retention times of the standard aldehyde solutions. Standard curves for each aldehyde were constructed by plotting the areas under the peaks obtained on injection of 25 $\mu$l of each standard solution (at 223 nm for 4HNE and 238 nm for 2NE) versus the concentration of aldehyde in the standard solution. A linear regression line was then fitted to the standard curve. Correlation coefficients of the standard curves (from 1 $\mu$M- to 50 $\mu$M-aldehyde) were always greater than 0.996, and the slopes of the linear regression lines as well as the *y*-intercepts were stable throughout the experiments. Unknown aldehyde concentrations, in 25 $\mu$l sample injections, were then determined by comparison of the sample peak heights (corresponding to the aldehyde retention time) with the standard curve.

### Total GSH and GST assays

Cell pellets were collected from culture dishes by washing exponentially growing monolayers with Puck's saline (4 °C), scraping into cold Puck's saline, refrigerated centrifugation (500 ***g***) and aspiration of the saline. Identical co-cultures were trypsin-treated and counted to obtain cell number/dish. The cell pellets were subjected to one freeze–thaw, then homogenized in hypo-osmotic 50 mM-phosphate buffer, pH 7.8, with 1.34 mM-diethylenetriaminepenta-acetic acid (DETAPAC), and a 50 $\mu$l portion was assayed for total GSH by the method of Anderson [32]. A sample of cell homogenate was then assayed for protein content by the method of Lowry *et al.* [33]. The cell homogenate was then frozen at −20 °C until assayed for GST activity.

GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate was determined by the method of Simons & Vander Jagt [34]. GST activity with ethacrynic acid as substrate was determined by the method of Habig *et al.* [35]. Total GSH and GST activity were expressed on a per mg of protein as well as a per cell basis. Each sample measurement was the mean of three replicate assays, and at least six separate samples were measured for each cell line in this fashion.

### Statistical analysis

Differences between three population means were determined with analysis of variance (ANOV), and comparisons of individual means with controls were accomplished with Dunnett's two-tailed test [36]. Significance was accepted when $P < 0.05$.

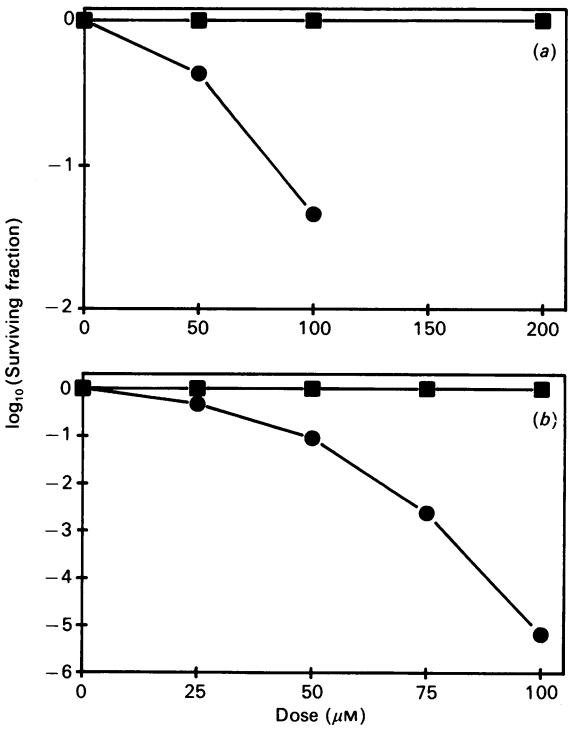


**Fig. 1. Survival curves following exposure of HA-1 cells to aldehydes and alcohols**

Exposure was for 1 h at 37 °C in 25 $cm^2$ sealed culture flasks gassed with air/$CO_2$ (19:1). (*a*) ●, 2,4-NDAL; ■, 2,4-NDOL; (*b*) ●, 2,4-DDAL; ■, 2,4-DDOL.

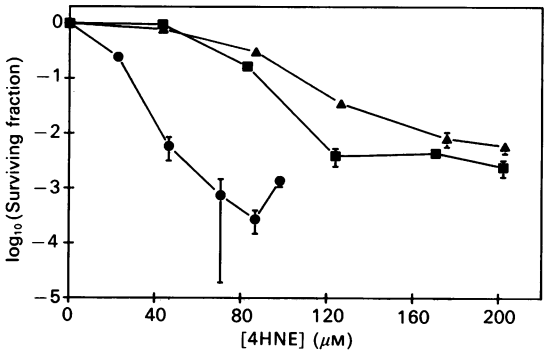


**Fig. 2. Survival curves following exposure of HA-1 (●), OC14 (■) and OC5 (▲) cell monolayers to increasing concentrations of 4HNE**

Incubations were carried out for 1 h at 37 °C in 60 mm culture dishes. Results are shown as means ± S.D. of three separately treated cultures. (S.D. values are shown when larger than the symbol). The surviving fractions of the OC14 and OC5 cells were significantly different from those of HA-1 cells at the 40 and 80 μM doses (ANOV, Dunnett's two-tailed test, $P < 0.01$).

## RESULTS

### Cytotoxicity of aldehydes versus alcohols

An experiment comparing the cytotoxicity of 2,4-NDAL and 2,4-DDAL to that of 2,4-NDOL and 2,4-DDOL was done in order to determine if the aldehyde functional group was important to the cytotoxicity of lipid aldehydes. Figs. 1(*a*) and 1(*b*) show that the aldehydes were cytotoxic to HA-1 cells in the dose range of 25 μM to 200 μM. The alcohols, which were identical in structure except for the aldehyde moiety, were non-toxic in the dose ranges tested. These results indicate that the aldehyde moiety was necessary for the cytotoxic effect in these dose ranges.

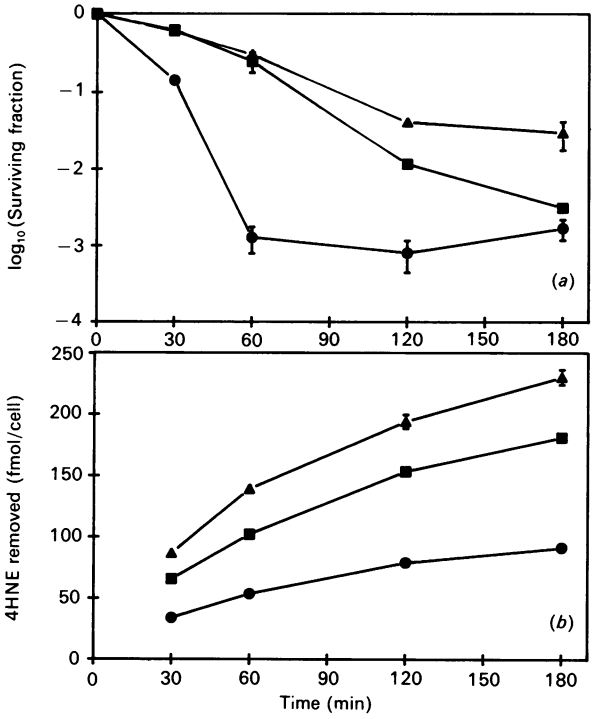


**Fig. 3. Survival (*a*) and removal (*b*) curves following exposure of HA-1 (●), OC14 (■) and OC5 (▲) cell monolayers to 4HNE**

Exposure was to 72 μM-4HNE for the indicated times at 37 °C in 60 mm culture dishes. Results are means ± S.D. for three separately treated and assayed cultures (S.D. values are shown when larger than the symbol). The surviving fractions for the OC14 and OC5 cells are significantly different from those of HA-1 at 30 and 60 min, and the 4HNE removed (fmol/cell) by the OC14 and OC5 cells is significantly different from that for HA-1 at all time points (ANOV, Dunnett's two-tailed test, $P < 0.01$). The h.p.l.c. conditions for the assay of 4HNE were: mobile phase, acetonitrile/water, (2:3, v/v); flow rate, 0.7 ml/min.; wavelength, 223 nm. With these conditions, the retention time of 4HNE was 9.25 min.

### Cytotoxity of 4HNE in $H_2O_2$-resistant cell lines

Fig. 2 shows the survival curves obtained when HA-1 and $H_2O_2$-resistant cell lines (OC14 and OC5) were exposed to increasing concentrations of 4HNE. The HA-1 cells were inactivated exponentially (clonogenic assay) at doses of 20–80 μM-4HNE. The OC5 and OC14 cells were inactivated exponentially at doses of 40–200 μM-4HNE, and were significantly more resistant than HA-1 cells to the cytotoxic effects of 4HNE at doses of 40 and 80 μM. The OC5 cells appeared to be slightly more resistant to 4HNE than the OC14 cells. Dose modification factors at 10% isosurvival (DMFs) were calculated using the following equation:

$$\text{DMF} = \frac{\text{dose to reach 10\% survival in the resistant cell line}}{\text{dose to reach 10\% survival in the HA-1 cell line}}.$$

The OC14 DMF was 3.3 and the OC5 DMF was 3.9. This means that in this experiment, 3.3 and 3.9 times as much 4HNE was required to clonogenically inactivate 90% of the OC14 and OC5 cell populations respectively as was required to clonogenically inactivate 90% of the HA-1 cell populations. Similar dose–response survival experiments were performed using 2NE. OC14 and OC5 cells were also found to be resistant to the cytotoxic effects of this aldehyde relative to HA-1 cells, and the OC5 cells appeared to be slightly more resistant than the OC14 cells (results not shown).

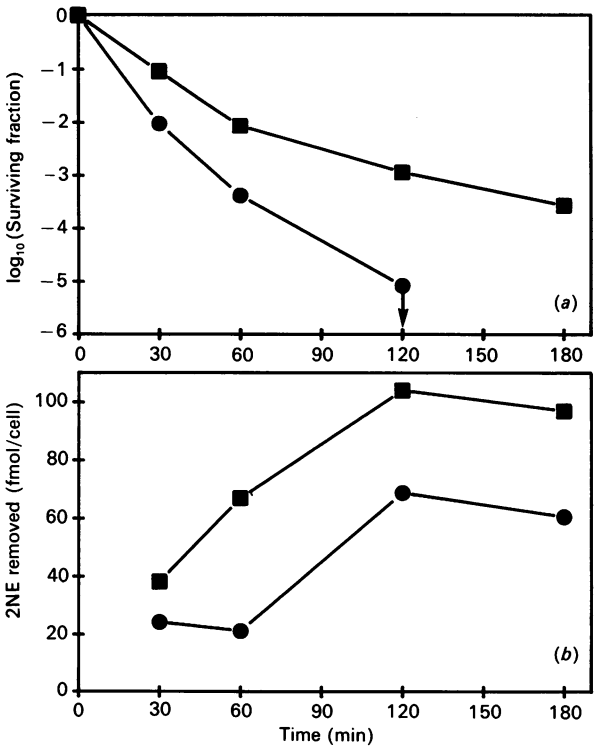


**Fig. 4. Survival (*a*) and removal (*b*) curves following exposure of HA-1 (●) and OC14 (■) cell monolayers to 2NE**

Exposure was to 90 μM-2NE for the indicated times at 37 °C in 25 $cm^2$ sealed culture flasks. Each point represents separately treated and assayed cultures. The arrow in (*a*) at 120 min indicates that no surviving colonies were counted from 140000 cells plated into each of three replicate cloning dishes. The h.p.l.c. conditions for the assay of 2NE were: solvent, acetonitrile/water, (3:1, v/v); flow rate, 0.7 ml/min.: wavelength, 238 nm. With these conditions, the retention time of 2NE was 7.10 min.

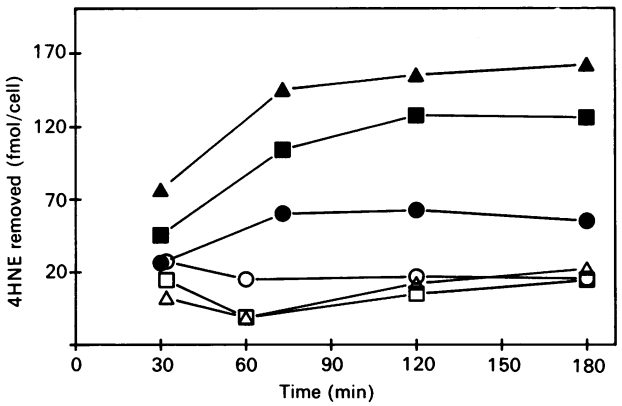


**Fig. 5. Removal of 4HNE from the medium surrounding heat-inactivated or non-heat-inactivated HA-1, OC14, and OC5 cells following exposure to 4HNE**

Closed symbols represent non-heat-inactivated cells and open symbols represent heat-inactivated cells. ●, ○, HA-1 cells; ■, □. OC14 cells; ▲, △, OC5 cells. Cells were exposed to 65 μM-4HNE for the indicated times at 37 °C. Each point represents the mean of three h.p.l.c. injections of one sample collected at each time point. H.p.l.c. conditions were the same as those used in Fig. 3.

### Cell-mediated removal of 4HNE and 2NE from the culture media

Known amounts of aldehydes in MEM were placed on to cell monolayers, and the cytotoxicity and cell-mediated removal of the aldehydes were quantified as a function of time of exposure. Fig. 3(*a*) shows the clonogenic cell survival curves obtained when 72 μM-4HNE in MEM was placed on HA-1, OC5 and OC14 cell cultures for 30–180 min. When the times to reach 10% isosurvival were used to calculate DMFs, the OC14 and OC5 cell lines demonstrated DMFs of 2.4 and 2.9 respectively. Therefore the OC14 and OC5 cells required 2.4- and 2.9-fold longer time periods than HA-1 cells for the clonogenic inactivation of 90% of the population by 72 μM-4HNE. These survival results are similar to those obtained in the dose–response experiment in Fig. 2. The $H_2O_2$-resistant cell lines were significantly more resistant, relative to the HA-1 cells, to the cytotoxic effects of 4HNE (at 30 and 60 min time points), and the OC5 cells also appeared to be slightly more resistant than the OC14 cells.

When the removal of 4HNE from the media surrounding the different cell lines was quantified and corrected for the amount of 4HNE removed by non-cell-mediated processes (Fig. 3*b*), the $H_2O_2$-resistant cells removed significantly more 4HNE (on a per cell basis) at all time points tested, relative to the HA-1 cells. The amount of 4HNE removed/cell at all time points (relative to HA-1) was (1.94±0.03)-fold greater for the OC14 cells and (2.53±0.06)-fold greater for the OC5 cells. These data indicate that greater quantities of 4HNE were removed from the media by cell lines which were more resistant to the cytotoxicity of 4HNE. Further, the magnitude of the increase in removal of 4HNE by the resistant cells was similar to the magnitude of increased resistance to the cytotoxicity of 4HNE (relative to HA-1, as determined by DMF).

Fig. 4(*a*) shows the clonogenic cell survival curves obtained when 90 μM-2NE in MEM was placed on HA-1 and OC14 cell cultures for 30–180 min. When the times to reach 10% isosurvival were used to calculate DMF, the OC14 cell line demonstrated a DMF of 1.90. These survival results are similar to those obtained with 4HNE in the experiment in Fig. 3(*a*).

When the removal of 2NE from the media surrounding the HA-1 and OC14 cell lines was quantified and corrected for the amount of 2NE removed by non-cell-mediated processes (Fig. 4*b*), the $H_2O_2$-resistant cells removed more 2NE (on a per cell basis) at all time points tested compared with the HA-1 cells. The average increase in the amount of 2NE removed/cell by OC14 cells for all time points (relative to HA-1) was 1.96-fold. The results of the survival and removal studies with 2NE (Figs. 4*a* and 4*b*) were remarkably similar to the results obtained from the same studies with 4HNE (Figs. 3*a* and 3*b*).

The next series of experiments were designed to determine if the cells had to be metabolically intact, with active GST, in order to retain the ability to remove aldehydes from the culture medium. Equal numbers ($7.5 \times 10^5$) of freshly trypsin-treated cells from each cell line were placed in 15 ml centrifuge tubes each containing 4 ml of MEM (controls without cells were also prepared). These samples were then placed in a 100 °C water bath for 45 min. Identical samples were prepared but were not subjected to the 100 °C water bath. Following the heating procedure, the cells were examined visually with a haemocytometer and phase-contrast microscopy, as well as with a Coulter counter, which had a visual display of particle size. The cells appeared to have intact plasma membranes (visual inspection) and greater than 80% of the cells were also recovered with particle sizes approximately equivalent to those in non-heated cells (as determined by Coulter counting) for up to 3 h after heating. However, more than 90% of the cells could not exclude Trypan Blue dye and none of the cells were clonogenic. Furthermore, heating GST for 45 min at 100 °C abolished the transferase activity as determined by the CDNB assay. These results indicated that the cells were metabolically inactivated by

**Table 1. Total GSH and GST activity in $H_2O_2$-resistant and control cells**

Results are means ± S.E.M. *Significantly different from HA-1 cells (ANOV, Dunnett's 2-tailed comparison, $P < 0.05$).

| | | Total GSH | |
|---|---|---|---|
| Cell line | *n* | (nmol of GSH/ mg of protein) | (fmol of GSH/ cell) |
| HA-1 | 9 | 7.8 ± 0.3 | 1.66 ± 0.10 |
| OC14 | 10 | 23.4 ± 2.3* | 3.38 ± 0.36* |
| OC5 | 9 | 19.9 ± 2.0* | 4.49 ± 0.55* |
| | | GST activity (using CDNB) | |
| Cell line | *n* | (munits/mg of protein) | (munits/ $10^6$ cells) |
| HA-1 | 6 | 169 ± 8 | 37 ± 3 |
| OC14 | 7 | 484 ± 30* | 71 ± 5* |
| OC5 | 7 | 560 ± 50* | 132 ± 14* |

the heat treatment and that the enzymes associated with 4HNE metabolism (GST) were inactivated.

With all samples at room temperature, equal quantities of aldehydes were added to all tubes and zero time aliquots were collected. The samples were then incubated at 37 °C and the removal of the aldehyde was quantified as a function of time from 30 to 180 min. Fig. 5 shows the removal of 4HNE/cell plotted versus time of exposure to 65 μM-4HNE for the HA-1, OC14 and OC5 cells which were heat-inactivated (open symbols) or not heat-inactivated (closed symbols). The cells that were not inactivated showed a similar pattern of 4HNE removal to that seen with attached monolayers of cells in Fig. 3(*b*). The average increase in the amount of 4HNE removed/cell for all time points (relative to HA-1) for the OC14 cells was (1.95 ± 0.28)-fold, and for the OC5 cells it was (2.69 ± 0.28)-fold. When the same cell lines were heat-inactivated (Fig. 5), the $H_2O_2$-resistant cells did not show increased removal of 4HNE relative to HA-1 cells. Furthermore, none of the heat-inactivated cells removed 4HNE to the same extent as did non-heat-inactivated cells (60–180 min). This experiment was repeated using HA-1 and OC14 cells exposed to 85 μM-2NE, and similar results were obtained (results not shown).

### Total GSH and GST activity

The cellular mechanism suggested by other authors [28,29] to be responsible for the detoxification of 4HNE is the GSH/GST system. GST has been shown to catalyse the conjugation of GSH to 4HNE [28,29]. The GSH–4HNE conjugate does not demonstrate significant absorbance at 223 nm, and therefore the formation of this conjugate would result in the disappearance of the unconjugated 4HNE peak from an h.p.l.c. profile [29]. Since the $H_2O_2$-resistant cell lines were resistant to the cytotoxicity of 4HNE and also removed the 4HNE peak from the medium h.p.l.c. profile more effectively than for control cells, the levels of total GSH and of GST activity were quantified in these cell lines relative to the values in HA-1 cells.

The results of the total GSH assays in Table 1 indicate that the $H_2O_2$-resistant cell lines contained significantly greater quantities of GSH regardless of the normalization procedure (per mg of protein or per cell). On a per cell basis, the OC14 and OC5 cells contained 2.0-fold and 2.7-fold more GSH respectively than did HA-1 cells. GST activity assays with CDNB as substrate showed that the $H_2O_2$-resistant cell lines had significantly greater activity than the HA-1 cells, regardless of the normalization procedure (Table 1). On a per cell basis, the OC14 and OC5 cells demonstrated 1.9-fold and 3.6-fold greater GST activities respectively relative to HA-1 cells. Both total GSH and GST activity appeared to be slightly higher in the OC5 cells than in the OC14 cells (Table 1). The GST isoenzyme with the greatest reactivity toward 4HNE also shows the greatest reactivity toward ethacrynic acid [28]. GST activity with ethacrynic acid as the substrate was 1–2% of the CDNB-metabolizing activity in HA-1, OC14 and OC5 cells (results not shown). This indicates that $H_2O_2$-resistant cells did not demonstrate a selective increase in the activity of the GST isoenzyme with the highest reactivity toward ethacrynic acid. Furthermore, it appears that GST isoenzymes, other than the isoenzyme with the highest reactivity toward ethacrynic acid, account for the greater part of the increased CDNB activity.

## DISCUSSION

Lipid peroxidation of PUFA in cell membranes has been implicated as a damaging reaction in a wide variety of pathological conditions induced by exposure of mammalian systems to presumptive oxidizing agents. Ischaemia–reperfusion injury, adriamycin cardiotoxicity, carbon tetrachloride hepatotoxicity, the process of carcinogenesis, the process of atherogenesis and the toxicity of hyperoxia are some of the pathological conditions that have been suggested to involve free radicals capable of producing lipid peroxidation [1–11]. However, the extent to which lipid peroxidation is causally related *in vivo* to the aforementioned pathologies is unknown.

Aldehydes have been isolated following peroxidative breakdown of PUFA containing more than one methylene-interrupted double bond, such as linoleic and arachidonic acids, as well as after stimulation of lipid peroxidation in various biological materials including mammalian cells [12,14,29]. These aldehydes inhibit biologically important cellular functions such as cell division and DNA and protein synthesis. They also damage DNA, cause cell lysis, damage membranes (as assayed by Trypan Blue dye exclusion and leakage of lactate dehydrogenase), and cause a loss of clonogenic potential [12,17–19,37]. 4HNE is one of the major aldehydes formed by lipid peroxidation that might contribute significantly to the toxicity of agents capable of initiating peroxidation [29].

Kaneko *et al.* [37] have compared the relative cytotoxicities of the aldehydes tested in the present paper. They found that 2,4-DDAL was toxic at 10 μM whereas 4HNE, 2NE and 2,4-NDAL were toxic at 25 μM when endothelial cells were exposed for 3 h and assayed by growth capability [37]. In the present study, the doses necessary to reduce the clonogenicity of HA-1 cell populations by 90% following 1 h exposures at 37 °C were: 4HNE, 28 μM; 2,4-DDAL, 48 μM; 2,4-NDAL, 82 μM; 2NE, 100 μM. It would appear that the rank order of toxicity of the aldehydes tested was: 4HNE > 2,4-DDAL > 2,4-NDAL > 2NE. However, 4HNE, 2,4-DDAL, 2,4-NDAL and 2NE are not equally soluble in the media and the non-hydroxylated aldehydes are highly volatile. It was found by h.p.l.c. analysis that only 40–50% of the 2NE remained in the medium after 60 min of incubation at 37 °C in the absence of cells. More than 90% of 4HNE added to medium remained after incubation for 60 min at 37 °C with no cells in the culture. Based on the above observations, it is not appropriate to make comparisons between the toxicities of these aldehydes using the data presented in this paper. However, differences in the media formulation could account for the apparent differences between the observations in this report (MEM + 0.02% ethanol) versus the observations of Kaneko *et al.* [37] (Earle's solution or MCDB-104 with 0.1% ethanol).

GSTs are enzymes that catalyse efficiently the conjugation of GSH to 4HNE and related aldehydes [28,29]. The GSH–4HNE conjugate is then believed to be exported from the cell [29]. The relatively high activity of GST towards 4HNE combined with the cellular export of the GSH–4HNE conjugate have been suggested to provide an efficient cellular detoxification mechanism for aldehydic products of lipid peroxidation [28,29].

Our group has isolated and characterized cell lines capable of growth in 800 $\mu$M-$H_2O_2$ [20,21]. The greater resistance to $H_2O_2$-mediated cytotoxicity seen in these cell lines, as determined by the clonogenic cell survival assay, most closely correlates with greater catalase activity, which was accompanied by similarly increased quantities of immunoreactive catalase protein [20,21]. Since $H_2O_2$ can react with metal ions to produce highly reactive species which are strongly implicated as initiators of lipid peroxidation [22–25,27], the $H_2O_2$-resistant cells were adapted and/or selected for growth in a highly peroxidative environment. One might speculate that 4HNE and/or related aldehydes contribute significantly to the cytotoxic selection process created by this peroxidative environment. If this were true, the $H_2O_2$-resistant phenotype should also be accompanied by resistance to the cytotoxicity of these by-products of lipid peroxidation. Furthermore, if GST and GSH provide the cells with an efficient means of detoxifying 4HNE and related aldehydes produced by peroxidation, then cells which are resistant to the cytotoxic effects of $H_2O_2$ should demonstrate increases in GST and GSH. This increase should correlate with increased resistance to 4HNE as well as to increased disappearance of 4HNE from the environment.

The present study utilized the quantitative clonogenic cell survival assay to determine the responses of $H_2O_2$-resistant cell lines to the cytotoxicity mediated by 4HNE and related aldehydes. Clonogenic potential is a particularly relevant end-point because the ability of single cells to undergo cell division is integrally related to the ability of regenerating systems to survive exposure to a cytotoxic insult. Furthermore, this assay is more sensitive in detecting cytotoxic events at lower concentrations of aldehyde exposure than the Trypan Blue dye exclusion assay [18]. When dose–response survival curves for 1 h exposures of HA-1 cells were constructed, all aldehydes tested were cytotoxic at concentrations of 20–200 $\mu$M (Figs. 1 and 2), and the aldehyde moiety appeared to be necessary for the cytotoxic effect. 4HNE caused exponential cell killing in HA-1 cells at concentrations of 20–80 $\mu$M (Fig. 2). This is consistent with other reports [12,18]. 4HNE caused exponential cell killing in OC5 and OC14 $H_2O_2$-resistant cells at concentrations of 40–200 $\mu$M (Fig. 2). OC14 and OC5 cell lines were significantly more resistant to the cytotoxic effects of 4HNE at doses of 40 and 80 $\mu$M, and they demonstrated DMFs of 3.3 and 3.9 respectively relative to the HA-1. The OC5 line (DMF = 3.9) appeared to be slightly more resistant than the OC14 line (DMF = 3.3). Similar results were obtained with 2NE (results not shown).

In the next series of experiments, cultures of HA-1 and $H_2O_2$-resistant cells were exposed to 72 $\mu$M-4HNE and 90 $\mu$M-2NE, and cytotoxicity and removal from the media of the aldehydes were quantified as a function of exposure time (30–180 min, Figs. 3, 4 and 5). The resistance seen in $H_2O_2$-resistant cells to the cytotoxicity of both aldehydes was accompanied by significantly increased removal of the aldehydes from the culture media by a cell-mediated process(es) that was heat-labile (Figs. 3, 4 and 5). Furthermore, the magnitude of the increased removal of aldehydes by $H_2O_2$-resistant cells (2–3-fold) was remarkably similar to the magnitude of the increased resistance to the cytoxicity of the aldehydes. These data suggest that the increased removal of the aldehydes might be related to the increased resistance to the cytotoxicity of the aldehydes. Therefore the resistance to the cytotoxicity of the aldehyde might be explained if we assume that the increased removal of aldehydes by resistant cells was a consequence of increased enzyme-catalysed detoxification of aldehydes. The activity of the cellular enzyme system believed to be responsible for the detoxification of 4HNE and related aldehydes (GST and GSH) was elevated by 2–3-fold in the $H_2O_2$-resistant cells relative to HA-1 cells (Table 1). Also, the OC5 cells, which appeared to be slightly more resistant to 4HNE and better capable of removing 4HNE from culture media than the OC14 cells, appeared to have more total GSH and GST activity per cell than OC14 cells (Table 1). The magnitudes of the increases in total GSH and in GST activity seen in the $H_2O_2$-resistant cells relative to the HA-1 cells were remarkably similar to the relative increases in the removal of, and resistance to, 4HNE and 2NE seen in these cell lines (Figs. 3 and 4). However, when the amounts of GSH (mol/cell) (Table 1) are compared with the amounts of 4HNE removed (mol/cell) (Fig. 3), the amounts of 4HNE removed in the first 30 min are approx. 10–20-fold greater than the amounts of GSH in all cell lines. This suggests the cells have a considerable capacity for the synthesis of GSH and/or that other pathways for 4HNE metabolism exist.

In conclusion, these results are consistent with the hypothesis that chronic exposure to 800 $\mu$M-$H_2O_2$ produces a peroxidative environment, containing 4HNE and/or related aldehydes, in which cells increase the cellular detoxification of aldehydes through the GSH/GST system in order to survive. The OC14 $H_2O_2$-resistant cell line is also resistant to the cytotoxic effects of hyperoxia [38], which have been suggested to involve lipid peroxidation [11]. One could further speculate that the more efficient detoxification of aldehydes produced as a consequence of exposure to hyperoxia may contribute to the $O_2$-resistance seen in this $H_2O_2$-resistant cell line.

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