Peroxide-induced cell death and lipid peroxidation in C6 glioma cells

Arne Linden, Michael Gülden *, Hans-Jörg Martin, Edmund Maser, Hasso Seibert

Institute for Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Campus Kiel, Brunsbücker Strasse 10, 24105 Kiel, Germany

Received 23 October 2007; accepted 7 February 2008
Available online 14 February 2008

Abstract

Peroxides are often used as models to induce oxidative damage in cells in vitro. The aim of the present study was to elucidate the role of lipid peroxidation in peroxide-induced cell death. To this end (i) the ability to induce lipid peroxidation in C6 rat astroglioma cells of hydrogen peroxide (H₂O₂), cumene hydroperoxide (CHP) and tert-butyl hydroperoxide (t-BuOOH) (ii) the relation between peroxide-induced lipid peroxidation and cell death in terms of time and concentration dependency and (iii) the capability of the lipid peroxidation chain breaking α-tocopherol to prevent peroxide-induced lipid peroxidation and/or cell death were investigated. Lipid peroxidation was characterised by measuring thiobarbituric acid reactive substances (TBARS) and, by HPLC, malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and hexanal.

Within 2 h CHP, t-BuOOH and H₂O₂ induced cell death with EC₅₀ values of 59 ± 9 μM, 290 ± 30 μM and 12 ± 1.1 mM, respectively. CHP and t-BuOOH, but not H₂O₂ induced lipid peroxidation in C6 cells with EC₅₀ values of 15 ± 14 μM and 130 ± 33 μM, respectively. The TBARS measured almost exclusively consisted of MDA. 4-HNE was mostly not detectable. The concentration of hexanal slightly increased with increasing concentrations of organic peroxides. Regarding time and concentration dependency lipid peroxidation preceded cell death. Pretreatment with α-tocopherol (10 μM, 24 h) prevented both, peroxide-induced lipid peroxidation and cell death. The results strongly indicate a major role of lipid peroxidation in the killing of C6 cells by organic peroxides but also that lipid peroxidation is not involved in H₂O₂ induced cell death.

© 2008 Elsevier Ltd. All rights reserved.

Keywords: Peroxides; Cell death; Lipid peroxidation; Oxidative stress

1. Introduction

In many studies dealing with oxidative stress in vitro and the role of antioxidants peroxides are used as model compounds to generate radicals in cultured cells. Oxygen radicals can cause severe damage to cellular structures and functions, which in the end can lead to cell death.

One of the consequences of oxidative stress is the peroxidation of lipids. This phenomenon has been investigated extensively. Not quite clear is, however, which relation exists between cell death and lipid peroxidation, e.g. is lipid peroxidation causally related to cell death or is it just an accompanying phenomenon or even a consequence of cell death.

The aim of the present study was to elucidate the role of lipid peroxidation in peroxide-induced cell death. To this end, the ability to induce lipid peroxidation in C6 rat astrogloma cells of hydrogen peroxide (H₂O₂) and of the organic hydroperoxides, cumene hydroperoxide (CHP) and tert-butyl hydroperoxide (t-BuOOH), and the relation between peroxide-induced lipid peroxidation and cell death in terms of time and concentration dependency was
investigated. Furthermore, it was examined whether the lipid soluble \( \alpha \)-tocopherol, known to partition into lipid membranes and to break the lipid peroxidation chain due to reaction with lipid peroxyl radicals, prevents lipid peroxidation and/or cell death induced by CHP.

During lipid peroxidation various reactive aldehydes are generated with malondialdehyde (MDA) as the most abundant species (Esterbauer and Cheeseman, 1990). Determination of MDA by thiobarbituric acid (TBA) is one of the most common assays used to assess lipid peroxidation. However, this assay is not specific for MDA because other aldehydes can also react with TBA (Draper and Hadley, 1990). Therefore, additionally to the measurement of thiobarbituric acid reactive substances (TBARS) lipid peroxidation induced by peroxides was characterised by using HPLC to determine MDA as the MDA–TBA complex and two other aldehydes, 4-hydroxynonenal (4-HNE) and hexanal, as cyclohexadione derivatives. The unsaturated aldehyde 4-HNE is exceptionally reactive and belongs to the most cytotoxic products of lipid peroxidation (Esterbauer et al., 1991).

2. Materials and methods

2.1. Chemicals

Hydrogen peroxide (30%), cumene hydroperoxide (88%), \( \epsilon \)-butyl hydroperoxide, 4-hydroxynonenal (98%) and 1,1,3,3-tetraethoxypropane (97%) were purchased from Sigma–Aldrich, DL-\( \alpha \)-tocopherol from Calbiochem. Stock solutions of \( \alpha \)-tocopherol were prepared in dimethyl sulfoxide and diluted in culture medium. The final concentration of dimethyl sulfoxide was always 0.2\% (v/v). The peroxides were dissolved directly in culture medium or Hank’s balanced salt solution (HBSS).

2.2. Cell culture and experimental design

The rat C6 glioma cell line was purchased from the American type culture collection (ATCC, No. CCL-107, passage no. 37). Frozen stocks were routinely thawed and grown in 25 cm\(^2\) tissue culture flasks in humidified atmosphere of 10\% CO\(_2)/90\%\) air at 37\(^\circ\)C and passaged once a week. The culture medium was pyruvate free DMEM, supplemented with 5\% foetal bovine serum (FBS), gentamycin (100 \(\mu\)g/ml), fungizone (1.25 \(\mu\)g/ml) and 20 \(\text{mM}\) 2-hydroxyethylpiperazine-\( \text{N}^\prime \)-2-ethansulfonic acid (HEPES). For the experiments the cells were harvested and seeded into 35 mm dishes at high density (2 \(\times\) 10\(^6\) cells/dish). In some experiments the culture medium (1 ml) contained 10 \(\mu\)M \( \alpha \)-tocopherol initially dissolved in dimethyl sulfoxide (final concentration: 0.2\% (v/v)). After 24 h when the cells exhibiting a differentiated phenotype formed a semiconfluent layer the medium was replaced by 1 ml of either FBS-free culture medium or Hank’s balanced salt solution (HBSS) supplemented with glucose (1 g/l) and the cells were exposed to the peroxides. At the end of exposure the following parameters were determined: (1) cell protein content per dish, total number of cells and percentage of living cells, (2) the amount of TBARS by means of a colorimetric assay, and (3) the amount MDA, 4-HNE and hexanal by means of HPLC.

2.3. Cytotoxicity assay

Cytotoxicity was determined as loss of living cells from the monolayer. For cell counting the culture medium was aspirated and the cells were harvested by trypsinisation. The cells were suspended in culture medium and 100 \(\mu\)l of the cell suspension was mixed with an equal volume of 0.2\% (w/v) Trypan Blue in phosphate buffered saline. After 30 s of incubation, living (unstained) and dead cells (stained blue) were counted with a hemocytometer. The number of living cells in the peroxide treated cultures was related to the number of living cells in the control cultures of the same experiment and expressed as percentage of control. Control cultures contained about 2.25 \(\pm\) 0.09 \(\times\) 10\(^6\) cells/dish (mean \(\pm\) SEM, \(n = 14\)).

2.4. Protein assay

For determination of cellular protein the medium was aspirated and the cell layers were washed two times with 1 ml phosphate buffered saline. Cells were lysed by incubation with 1 ml 0.5 N NaOH for 45 min (37\(^\circ\)C). Protein content was determined by the method of Lowry et al. (1951) modified for microtiter plates. Bovine serum albumin served as standard. Absorption at 630 nm was read with a microtiter plate photometer. Untreated control cultures contained about 0.39 \(\pm\) 0.02 mg cell protein per dish (mean \(\pm\) SEM, \(n = 14\)).

2.5. TBA assay

Lipid peroxidation products were quantified by measuring thiobarbituric acid reactive substances (TBARS, Draper and Hadley, 1990). One ml of 20\% (w/v) trichloroacetic acid containing 0.8\% (w/v) thiobarbituric acid was added to each culture dish. The cells were scratched off with a rubber policeman, the suspensions were transferred to glass centrifuge tubes and boiled for 45 min. After cooling to room temperature and centrifugation the absorbance of the supernatant at 535 nm was determined. Using the molar extinction coefficient of the MDA–TBA complex of 1.49 \(\times\) 105 \(\text{M}^{-1}\) \(\text{cm}^{-1}\) (Draper and Hadley, 1990) the amount of TBARS was expressed as nmol MDA equivalents formed per mg cell protein measured in a further set of control cultures not used for cytotoxicity or TBA assay.

2.6. HPLC

The exact amount of the MDA–TBA complex in the TBARS containing supernatant was determined by HPLC using a LiChrospher column (RP-18, 5 \(\mu\)m, Merck),
25 mM Na₂HPO₄–methanol in a ratio of 58/42 (v/v) as mobile phase, a flow rate of 1 ml/min and a fluorescence detector (515 nm excitation, 553 nm emission). The MDA–TBA complex was eluted in 4.8 min. For calibration defined concentrations of the MDA–TBA complex were made from aqueous MDA solutions prepared by acid hydrolysis (1% H₂SO₄) of 1,1,3,3-tetraethoxy-propane at room temperature.

4-HNE and hexanal were quantified by HPLC detection of their cyclohexandione derivatives. Cells were scraped off in the culture medium (1 ml) and added to 2 ml of an aqueous 1,3-cyclohexandione (CHD) solution. CHD was purified by recrystallization from ethyl acetate. Aqueous solutions contained CHD (0.25%, w/v) and ammonium sulphate (10%, w/v). The mixture was incubated for 60 min at 60 °C, cooled down and 1 ml of methanol was added to precipitate cellular proteins. Proteins were pelleted by centrifugation and 3 ml of the supernatant was applied to a LiChroCART 250-4 column (Agilent Technologies) for solid phase micro extraction. The CHD derivatives were eluted by 2 ml of methanol and separated by HPLC on a LiChrospher column (RP-18, 5 μm, Merck), using a linear gradient mobile phase system (tetrahydrofuran(THF)/water) under following conditions: a flow rate of 1 ml/min, THF was increased from 5% to 50% over 50 min, followed by 50% THF for 10 min and again 5% THF for 10 min. CHD derivatives were detected using a fluorescence detector (380 nm excitation, 415 nm emission).

The amount of peroxidation products determined by HPLC were expressed as nmol per mg cell protein measured in a further set of control cultures not used for determination of cytotoxicity or lipid peroxidation.

2.7. Presentation of results

With a few exceptions, experiments were repeated at least three times with cultures of different passages. Data are given as mean ± SEM if n ≥ 3. Median effective concentrations (EC₅₀) were determined by fitting a Hill equation \(Y = \text{bottom} + (\text{top} - \text{bottom}) \left/ \left(1 + 10^{\left[\log(\text{EC}_{50}/X)\times\text{HillSlope}\right]}\right)\right.\) to the concentration-effect data using GraphPad Prism. The EC₅₀ values are reported as best fit value ± SE. Experimental data were analysed by means of one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. The p-value had to be <0.05 to be considered significant. All statistical analyses were made by means of GraphPad InStat.

3. Results

3.1. HPLC of cyclohexandione derivatives of 4-hydroxynonenal and hexanal

During exposure to peroxides C6 cells were originally cultured in serum free DMEM supplemented with antibiotics and 20 mM HEPES. For calibration various concentrations of 4-HNE and hexanal, respectively, were prepared with this culture medium, derivatised with cyclohexandione, extracted and determined by HPLC as described in the Section 2. 4-HNE was eluted at about 34 min and hexanal at about 38.5 min. The hexanal peak increased with increasing hexanal concentration while the 4-HNE peak stayed constant, irrespective of the different 4-HNE concentrations used (data not shown). This phenomenon was not observed if 4-HNE was dissolved in water or in HBSS (Fig. 1). Obviously the quantification of 4-HNE by HPLC
was disturbed by components of the culture medium. To avoid this interference, in general, cells were cultured in HBSS during exposure to peroxides. Fig. 1 shows that a concentration of 4-HNE as low as 36.2 nM equivalent to an amount of 0.0362 nmol per culture dish could be well detected.

3.2. Cytotoxicity of peroxides

All peroxides investigated induced cell death within 2 h (Fig. 2). With EC50 values of 59 ± 9 μM (best fit value ± SE) and 290 ± 30 μM the organic peroxides CHP and t-BuOOH were considerably more potent than H2O2 (EC50 = 12000 ± 1100 μM).

3.3. Induction of lipid peroxidation

Lipid peroxidation, measured as an increased TBARS production during the exposure period, was induced by the organic peroxides CHP and t-BuOOH but not by H2O2 (Fig. 2). Regarding the concentration dependencies CHP (EC50 = 15 ± 14 μM) and t-BuOOH (EC50 = 130 ± 33 μM) started to cause lipid peroxidation and induced its maximum at lower concentrations than those necessary to kill the cells.

3.4. Products of lipid peroxidation

Quantitatively, the most important product of lipid peroxidation found in untreated as well as in peroxide treated C6 cells were MDA (Fig. 3). Apparently, the TBARS measured almost exclusively consist of MDA. The amount of 4-HNE was very low (≤0.01 nmol/mg protein) and mostly not detectable. The amount of hexanal, also mostly not detectable in untreated C6 cells, was slightly increased with increasing concentrations of organic peroxides.

3.5. Time dependency of cytotoxic action and induction of lipid peroxidation

The time dependencies of cell death and of lipid peroxidation induced by 200 μM CHP in C6 cells cultured in DMEM are shown in Fig. 4. After 2 h of exposure this concentration of CHP resulted in an almost total loss of viable cells, while the maximum amount of TBARS was already reached after 1 h. No further TBARS were produced later on indicating that lipid peroxidation does not proceed in dead cells. The process of lipid peroxidation rather appears to precede the loss of viable cells.

3.6. Cytoprotection by α-tocopherol

When CHP was used to induce oxidative stress pre-incubation of the cells with 10 μM α-tocopherol for 24 h strongly inhibited lipid peroxidation determined by accumulation of TBARS, MDA and hexanal, respectively (Fig. 5A). Concomitantly, pre-incubation with α-tocopherol almost completely prevented cell death caused by 200 μM CHP (Fig 5B).

4. Discussion

Hydrogen peroxide as well as the organic hydroperoxides CHP and t-BuOOH can be activated in the iron dependent Fenton reaction to highly reactive hydroxyl and alkoxyl radicals, respectively (Cohen, 1985). Additionally, various radicals analogous to those generated from fatty acids during lipid peroxidation can be generated via cytochrome P450 mediated metabolism of t-BuOOH (Karlsson et al., 2000). C6 rat glioma cells can be rendered more resistant against the cytotoxic action of H2O2 (unpublished data) and CHP (Zielinska et al., 2003), if the cells are trea-
mented with the ferric iron chelator deferoxamine or the water soluble radical scavenger dimethyl thiourea. This indicates that the cytotoxic action of these peroxides depends on the generation of oxygen radicals. A similar result was obtained with t-BuOOH in cultured rat hepatocytes (Masa-

The organic peroxides CHP and t-BuOOH were shown to induce lipid peroxidation in C6 cells. In the first instance lipid peroxidation was quantified by measuring TBARS. The determination of the MDA–TBA complex by HPLC. Data given are means of 2–5 independent experiments ± SEM if n ≥ 3. * Significantly different from control, p < 0.05.

Fig. 3. Products of lipid peroxidation induced in C6 cells by CHP (A), t-

Fig. 4. Time dependence of cytotoxicity and lipid peroxidation induced in C6 cells by CHP. Cells were exposed to 200 μM CHP in FBS-free culture medium. Cytotoxicity was determined as loss of attached viable cells, lipid peroxidation as increase in thiobarbituric acid reactive substances (TBARS). Viability was determined by Trypan Blue staining. Data given are means of 4 independent experiments ± SEM.

Fig. 5. Protection of C6 cells by pre-incubation with 10 μM α-tocopherol for 24 h from lipid peroxidation (A) and cytotoxicity (B) induced by 200 μM CHP. Cells were exposed to CHP in HBSS for 2 h. Cytotoxicity was determined as loss of attached viable cells, lipid peroxidation as increase in thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA) and hexanal. Viability was determined by Trypan Blue staining. MDA and hexanal were determined by HPLC. Data given are means of 3 independent experiments ± SEM. * Significantly different from control, + significantly different from CHP alone, p < 0.05.

A. Linden et al. / Toxicology in Vitro 22 (2008) 1371–1376 1375

H2O2 for 2 h. Lipid peroxidation was determined as increase in thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), 4-hydroxy-

Hydrogen peroxide at concentrations being cytotoxic to C6 cells after 2 h of exposure did not induce lipid peroxidation. The inability of H2O2 to induce lipid peroxidation, its cytotoxicity in the absence of lipid peroxidation or divergent cytotoxic and lipid peroxidating potencies have also been observed in other cell types in vitro (Sheridan et al., 1996; Weidauer et al., 2004; Erba et al., 2003). Taken together, these observations indicate, that lipid peroxida-

The organic peroxides CHP and t-BuOOH were shown to induce lipid peroxidation in C6 cells. In the first instance lipid peroxidation was quantified by measuring TBARS. The determination of the MDA–TBA complex by HPLC
revealed that the TBARS measured almost exclusively consist of MDA.

When performing the TBA assay with the whole acidified sample (cells + culture medium), as it was done here, not only free MDA but also protein-bound MDA and even MDA generated from oxidised lipids during heating the sample is captured. Since all the MDA measured is a secondary product of lipid peroxidation this method is considered to provide a reliable measure of lipid peroxidation in terms of MDA equivalents (Draper and Hadley, 1990).

Free aldehydes can be determined by HPLC as reaction products with 1,3-cyclohexanedione (Esterbauer and Cheeseman, 1990). By this method hexanal was identified as another product of peroxide-induced lipid peroxidation in C6 cells although to a much lower amount than MDA. 4-Hydroxynonenal, however, in most cases was not detected. This is not to say that no 4-HNE was produced in the course of lipid peroxidation. 4-HNE is known to react rapidly with cellular nucleophiles, preferentially thiol groups, and, additionally, to be efficiently detoxified by various enzymatic pathways which, taken together, may result in a very short half life of 4-HNE in the cellular environment (Petersen and Doorn, 2004). This may be the reason why 4-HNE was not found as product of lipid peroxidation in C6 cells.

Regarding time and concentration dependency lipid peroxidation induced by CHP and tert-BuOOH preceded cell death. This rules out that lipid peroxidation is a consequence of cell death. Furthermore, pre-incubation with α-tocopherol, known to break the lipid peroxidation chain due to reaction with lipid peroxyl radicals (Traber and Atkinson, 2007), prevented both lipid peroxidation and cell death induced by CHP. This strongly indicates a major role of lipid peroxidation in the killing of C6 cells by organic peroxides supporting a previous study which provided experimental evidence that the cell death induced by tert-BuOOH in cultured hepatocytes is causally related to lipid peroxidation (Masaki et al., 1989).

It remains to be elucidated why radicals produced from H₂O₂ (mainly hydroxyl radicals) cause cell death without any lipid peroxidation, while alkoxylradicals produced from CHP and tert-BuOOH induce lipid peroxidation preceding cell death. In any case, the results presented here support the view that “there is a specificity to the location of damage produced in cells by particular reactive oxygen-generating species” (Vroegrop et al., 1995).

References


