Original Contribution

Cytotoxic potency of H2O2 in cell cultures: Impact of cell concentration and exposure time

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ABSTRACT

Using C6 glioma cells in this study we investigated in detail how exposure time and cell concentration affect the cytotoxic potency of H2O2 in vitro. Median cytotoxic concentrations (EC50) decreased from 500 to 30 μM with increasing incubation time from 1 to 24 h. Twenty-four hours proved to be sufficient to determine incipient cytotoxic concentrations of H2O2. The incipient EC50 values were linearly related to the cell concentration. A cell concentration-independent median cytotoxic cell dose (ED50) of 430 nmol/mg cell protein or 860 nmol/10^7 cells was derived. Median cytotoxic H2O2 concentrations were completely eliminated from the culture medium at a rate proportional to both the H2O2 and the cell concentrations. In contrast to EC50 values the corresponding areas under the concentration versus time curve (AUC) were independent of the cell concentration and amounted to 1800 μM×min. With decreasing cell concentration the H2O2 elimination decelerates and, thus, exposure to H2O2 applied as a bolus approaches a continuous exposure to a steady H2O2 concentration. Taken together, our results indicate that the cytotoxic potency of H2O2 administered to cultured cells as a bolus is characterized by the AUC, which depends on its initial concentration, the ability of the cells to eliminate H2O2, and the cell concentration. We recommend expressing the toxic potency of H2O2 in vitro by the incipient toxic cell dose (e.g., nmol H2O2/mg cell protein or nmol H2O2/10^7 cells), in particular for comparative purposes.

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Hydrogen peroxide is a physiological constituent of living cells and is continuously produced via diverse pathways. The intracellular concentration of H2O2 is tightly controlled by various enzymatic and nonenzymatic antioxidant systems and is assumed to vary between 1 and 700 nM [1–3]. Intracellular steady-state concentrations of H2O2 above 1 μM are considered to cause oxidative stress inducing growth arrest and cell death [2,3].

In experimental models used to investigate physiological functions and toxic effects of H2O2, oxidative stress responses of cells, or cytoprotection by antioxidant agents, cultured cells are often exposed to H2O2 added as a bolus into the culture medium. In these experimental settings substantial variations in the concentrations of H2O2 determined to be cytotoxic can be found, ranging from less than 10 μM to more than 1000 μM [e.g., 4–14]. In proliferating mammalian cell lines the following pattern of responses to H2O2 has been described [5,7]: very low levels (3 to 15 μM) cause growth stimulation, higher levels (120 to 150 μM) induce a temporary growth arrest, intermediate concentrations (250 to 400 μM) cause a permanent growth arrest, and high concentrations (≥ 1 mM) produce necrotic cell death. Thus, the extracellular concentrations of H2O2 applied in vitro and found to be cytotoxic in most cases are considerably higher than the intracellular concentrations of H2O2 assumed to be cytotoxic. The objective of this study was to investigate to what extent the level and the variability of extracellular cytotoxic concentrations can be attributed to experimental conditions such as exposure time and cell concentration.

Cultured cells remove H2O2 from the culture medium [15–21] at a rate depending on the concentration of cells in the in vitro system [17,20]. Hence, the cell concentration affecting the extracellular H2O2 concentration must be an important determinant of the cytotoxic potency of H2O2. Spitz et al. [4] were the first to report that the cytotoxicity of H2O2 added as a bolus was cell-density dependent but became independent of the cell density if the survival data were plotted as a function of μmol H2O2/cell. Wiese et al. [5], using the same cell line (HA-1), also found that H2O2 toxicity was related to the cell density, but only at lower cell concentrations (<10^6 cells/60-mm dish). At higher cell concentrations they found that cell density was not an important factor. In these and later works [4,5,7,22] both research groups expressed the amount of H2O2 added to cell cultures as a dose (μmol/10^7 cells) “to minimize cell density artefacts” [22]. Although some others [23,24] also reported a cell-density dependence of H2O2 cytotoxicity, this has not been generally acknowledged.
Quantitative information about the relation between elimination and cytotoxicity of H$_2$O$_2$ concentrations added to cell cultures could not be found in the literature.

Using growth-arrested C6 glioma cells, we therefore reexamined the impact of the cell concentration on the cytotoxicity of H$_2$O$_2$ added as a bolus. To this end we first determined the influence of incubation time on the cytotoxicity of H$_2$O$_2$ to ensure that we assessed the incipient cytotoxicity of H$_2$O$_2$, the cytotoxicity after an infinite time of exposure to H$_2$O$_2$. Furthermore, the concentration of H$_2$O$_2$ in the culture medium was monitored and the dependency of the H$_2$O$_2$ elimination rate on the H$_2$O$_2$ and the cell concentration, respectively, was investigated. The median cytotoxic concentrations (EC$_{50}$) of H$_2$O$_2$ as well as the H$_2$O$_2$ elimination rates were found to be linearly related to cell concentration over the whole range of cell concentrations investigated. Not the initial concentration (μM) added, but the concentration versus time relationship, the area under the curve (AUC; μM × min), and the administered cell dose (nmol/mg cell protein) were found to determine the incipient cytotoxicity of H$_2$O$_2$ added as a bolus to cell cultures. The results show that concentrations of H$_2$O$_2$ that are cytotoxic in cell cultures can vary widely with changing incubation time and cell concentration. Differences in these experimental conditions very probably contribute to the large variation in reported cytotoxic concentrations of H$_2$O$_2$ in cell cultures.

Materials and methods

Cell culture and experimental design

The rat C6 astroglioma cell line was purchased from the American Type Culture Collection (No. CCL-107, passage 37). Frozen stocks were routinely thawed, grown in 25-cm$^2$ tissue culture flasks in an incubator with a humidified atmosphere of 10% CO$_2$/90% air at 37 °C, and passaged once a week. The maintenance culture medium was pyruvate-free DMEM supplemented with 5% fetal bovine serum, 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, gentamicin (100 μg/ml), and Fungizone (1.25 μg/ml). Experiments were performed with cells from passages 44 to 60.

For the experiments the cells were harvested and subcultured in the maintenance culture medium in 96-well microtiter plates (growth area: 0.32 cm$^2$/well) by inoculating 4 × 10$^4$ cells in 200 μl/well, in 24-well tissue culture plates (1.91 cm$^2$/well) by inoculating 2 × 10$^4$ to 1.2 × 10$^6$ cells in 1 ml/well, and in 35-mm culture dishes (8.55 cm$^2$/dish) by inoculating 10$^5$ to 8 × 10$^6$ cells in 1.5–2 ml/dish. After at least 24 h of growth the maintenance culture medium was replaced, after two washes, with H$_2$O$_2$ containing serum- and pyruvate-free DMEM to determine the cytotoxic potency of H$_2$O$_2$ (96- and 24-well plates) or the time course of H$_2$O$_2$ removal from the culture medium (35-mm dishes). Preceding experiments in our laboratory had shown that serum deprivation strongly reduces or even arrest the growth of C6 cells.

Determination of the cytotoxic potency of H$_2$O$_2$

To determine the time dependence of the cytotoxic potency of H$_2$O$_2$, C6 cells grown for 24 h in 96-well microtiter plates were exposed to various H$_2$O$_2$ concentrations (six concentrations per plate, 6 wells per concentration) in a volume of 200 μl/well. Two groups of 6 wells served as controls, receiving medium without H$_2$O$_2$. Using parallel microtiter plates after 1, 2, 4, 24, and 48 h of incubation (37 °C, 10% CO$_2$/90% air) we determined cytotoxicity as a decrease in the number of attached cells compared to controls quantified by means of the cell protein content per well.

For determination of the protein content the medium was aspirated and the cell layers were washed twice with phosphate-buffered saline. Cells were lysed with 0.5 N NaOH and the protein content was determined by the method of Lowry et al. [25] modified for microtiter plates. Bovine serum albumin served as standard.

Absorption at 630 nm was read with a microtiter plate photometer (Bio-Tek ELx800UV).

The mean protein content of 6 culture wells per concentration was related to the mean protein content of the control cultures (12 wells per plate) and expressed as the percentage of control. Experiments were replicated three times with cultures of different passages. The EC$_{50}$ values were determined from the mean concentration–effect relationships by fitting a Hill equation using GraphPad Prism.

To determine the influence of the cell concentration on the cytotoxic potency of H$_2$O$_2$, cultures with various cell concentrations were prepared either by inoculating and exposing different cell numbers per well of 24-well tissue culture plates in a constant volume of medium (0.5 ml) or by exposing cultures with a constant cell number to H$_2$O$_2$ in different volumes (0.25, 0.5, 1 ml) of culture medium per well. Each plate received six H$_2$O$_2$ concentrations (3 wells per concentration). Three wells served as controls, receiving medium without H$_2$O$_2$. Cytotoxicity was assessed 24 h after the start of exposure by means of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [26].

MTT is reduced by mitochondrial dehydrogenases of living cells into purple insoluble formazan. MTT was dissolved in phosphate-buffered saline (7 mg/ml) and added to each well at the end of exposure (50 μl MTT solution per 250 μl culture medium). After incubation (37 °C, 10% CO$_2$/90% air) for 20–60 min, depending on the cell density, 0.25, 0.5, or 1 ml solubilization solution, depending on the culture medium volume, was added. The solubilization solution was 50% (v/v) dimethyl sulfoxide containing 20% (w/v) sodium dodecyl sulfate, 2.5% (v/v) 1 N hydrochloric acid, and 2.5% (v/v) acetic acid (80%). After a further 2 h of incubation the formazan production was assessed by measuring the absorbance at 570 nm with a microtiter plate photometer (Bio-Tek ELx800UV). The mean absorbance of the three culture wells per H$_2$O$_2$ concentration was related to the mean absorbance of the control cultures of the same plate and expressed as the percentage of control. Using GraphPad Prism a Hill equation was fitted to the concentration–effect data to derive the EC$_{50}$ value.

To measure the cell concentration during exposure to H$_2$O$_2$, the cell protein content per well in parallel 24-well tissue culture plates of the same cell densities that were not exposed to H$_2$O$_2$ was determined by the Lowry method as described above. The cell protein concentration during exposure to H$_2$O$_2$ was calculated from the amount of cell protein (mg/well) divided by the volume of culture medium (ml/well) during the incubation with H$_2$O$_2$. Using a protein content of C6 cells of 0.2 mg/10$^6$ cells from preceding measurements in our laboratory, the cell concentration could be calculated from the cell protein concentration.

Measurement of H$_2$O$_2$ concentration in the medium of C6 cell cultures

C6 cell cultures grown in 35-mm dishes were used to measure the concentration of exogenously applied H$_2$O$_2$ in the culture medium over time. After the maintenance medium was removed, the cells were washed twice with serum-, pyruvate-, and phenol red-free DMEM (37 °C), 2 ml of this medium containing 100 μM H$_2$O$_2$ was added, and the cells were incubated in a humidified atmosphere of 10% CO$_2$/90% air at 37 °C. After various incubation times three 10-μl samples of culture medium were collected, each time from the same dish, for the measurement of the peroxide concentration. The cell protein content of parallel culture dishes of the same cell densities that were not exposed to H$_2$O$_2$ was determined by the Lowry method as described above.

The peroxide concentrations were measured by a modification of the ferrous oxidation–xylene orange assay [27] adapted to microtiter plates by Dringen et al. [28]. This assay is based on the ability of peroxides to oxidize the ferrous Fe$^{2+}$ ions to ferric Fe$^{3+}$ ions, which react with xylene orange to a colored complex. The 10-μl samples were added to microtiter plate wells containing 140 μl of 25 mM

\[ \text{H}_2\text{O}_2 + 2\text{Fe}^{2+} \rightarrow \text{H}_2\text{O} + 2\text{Fe}^{3+} \]
H₂O₂. One hundred fifty microliters of the reaction solution (0.5 mM (NH₄)₂Fe(SO₄)₂, 200 μM xylene orange, 200 mM sorbitol in 25 mM H₂SO₄) was added to each well, the microtiter plates were gently shaken for 45 min, and the absorption at 570 nm was read with a microtiter plate reader. The concentrations of peroxides were determined using standard peroxide solutions in the same microtiter plate. Standard peroxide solutions (3.125–100 μM) were always freshly prepared solutions of hydrogen peroxide (30%; Sigma–Aldrich) in the same culture medium that was used to incubate the cells with hydrogen peroxide. The hydrogen peroxide concentration in culture medium cannot be determined photometrically at 240 nm. Instead diluted solutions of hydrogen peroxide in phosphate buffer were repeatedly measured photometrically, establishing the stability of the 30% hydrogen peroxide solution and the validity of the dilution procedure.

Using GraphPad Prism the initial exponential parts of the concentration vs time relationships were fitted to a first-order rate function,

\[ C = C_0 \times e^{-kt}, \]

where \( C_0 \) is the initial concentration and \( k \) (min⁻¹) the first-order rate constant. The rate constant was used to calculate elimination half-life, \( t_{1/2} = \ln 2 / k \),

and was converted into an elimination rate, \( v \) (nmol/ml×min), by multiplying by the initial H₂O₂ concentration. To determine the specific elimination rate, \( v_e \), it was related to the cell protein concentration during exposure to H₂O₂ (nmol/min×mg cell protein).

Results

Influence of incubation time on the nominal cytotoxic H₂O₂ concentrations

Nominal concentration–effect relationships for the cytotoxic action of H₂O₂ in C6 cells were obtained after various incubation times ranging from 1 to 48 h. Cytotoxicity was determined as loss of protein from the cell layer compared to the untreated control cultures. Fig. 1 shows that the concentration–effect curves were shifted to lower concentrations with increasing the incubation time from 1 to 24 h. A further prolongation of the incubation time to 48 h did not increase the activity any further. The EC₅₀ values derived from the concentration–effect relationships (Table 1) reveal that the cytotoxic potency of H₂O₂ increased about 17-fold by prolonging the incubation time.

The relationship between EC₅₀ values and incubation time (Fig. 2) can be described using the equation

\[ EC_{50} = EC_{50,\text{ref}} + kt^{-n}, \]

which is an extended version of the potency/time relationship known as Haber’s rule [29]. EC₅₀,ref represents the time-independent threshold or “incipient” EC₅₀ value, i.e., the minimal concentration necessary to elicit 50% of the cytotoxic effect after an infinite incubation time. The incipient EC₅₀ value for the cytotoxic action of H₂O₂ was calculated to be 26 μM.

Influence of cell concentration on the nominal cytotoxic H₂O₂ concentrations

Using an incubation time of 24 h, shown above to be sufficient to determine the incipient cytotoxicity of H₂O₂ in C6 cells, the influence of the cell concentration on the cytotoxicity of H₂O₂ was investigated. The cell concentration is given by the number of cells per milliliter of culture medium.

Cultures with differing cell concentrations during exposure to H₂O₂ were prepared in two ways, by altering either the cell number or the medium volume per well. Raising the cell concentration by increasing the cell number resulted in a shift of the nominal effect curves for the cytotoxic action of H₂O₂ toward higher concentrations (Fig. 3A). The EC₅₀ values derived from these concentration–effect curves were related to the cell concentration during exposure to H₂O₂ as given by the cell protein concentration (Fig. 4). Additionally Fig. 4 presents EC₅₀ values measured with cultures of constant cell number but varying medium volume. The figure reveals no difference between both modes of achieving

![Fig. 1. Influence of incubation time on the concentration dependency of the cytotoxic action of hydrogen peroxide in C6 glioma cells.](image1)

![Fig. 2. Potency/time relationship of the cytotoxic action of hydrogen peroxide in C6 glioma cells.](image2)
and the median cytotoxic cell doses (ED50 values) are almost the same and were estimated using GraphPad Prism to derive (A) EC50 values of 20 (μM) for C6 cells, ED50 = 430 ± 26 nmol/mg cell protein.

Fig. 3 shows that, although the nominal concentration of H2O2 in the medium of C6 cell cultures starts to decrease immediately after administration of the peroxide. At least for the first 10–15 min of incubation H2O2 disappears from the culture medium following first-order kinetics (exponential decay). The half-life of elimination (t1/2) decreased from 26 to 7 min with increasing cell concentration. In the absence of cells but otherwise the same conditions the peroxide concentrations in the culture medium remained almost constant for at least 60 min.

Different cell concentrations, indicating that it is indeed the cell concentration and not the cell number nor the medium volume alone that affects the cytotoxicity of H2O2. A linear relationship between the EC50 values and the cell protein concentration can be seen. Using a protein content of 0.2 mg/10⁶ cells the range of 0.036 to 0.49 mg cell protein/ml investigated is equivalent to cell concentrations of 0.18–2.5 × 10⁶ cells/ml. The slope of the line has the dimension of a dose (μmol/mg cell protein) and can be defined as the median cytotoxic cell dose of H2O2 for C6 cells, ED50 = 430 ± 26 nmol/mg cell protein (best fit ± SE), i.e., the amount of H2O2 that has to be added to C6 cell cultures per milligram of cell protein to produce 50% cytotoxicity. Based on cell number the median cytotoxic cell dose of H2O2 amounts to 860 nmol/10⁶ cells.

The cell dose of H2O2 can be calculated from the nominal concentration of H2O2 applied and the cell protein concentration during exposure to H2O2. Fig. 3 shows that, although the nominal concentration–cytotoxicity relationships (Fig. 3A) and the EC50 values change with varying cell number, the dose–cytotoxicity relationships and the median cytotoxic cell doses (ED50 values) are almost independent of the cell number (Fig. 3B). This indicates that it is the dose rather than the nominal concentration that determines the cytotoxicity of H2O2 in cell cultures.

### Elimination of H2O2 from the culture medium

The concentration–effect relationships determined for H2O2 shown above are based on nominal concentrations representing the amount of H2O2 added per volume of culture medium. Fig. 5 shows that the concentration of H2O2 in the medium of C6 cell cultures starts to decrease immediately after administration of the peroxide. At least for the first 10–15 min of incubation H2O2 disappears from the culture medium following first-order kinetics (exponential decay). The half-life of elimination (t1/2) decreased from 26 to 7 min with increasing cell concentration. In the absence of cells but otherwise the same conditions the peroxide concentrations in the culture medium remained almost constant for at least 60 min.

Experiments like that shown in Fig. 5 were performed several times by exposing C6 cell cultures of various cell densities and various passages to 100 μM H2O2. The initial exponential parts of the H2O2 elimination curves were fitted to a first-order rate equation (Eq. (1))

\[ \text{H}_2\text{O}_2 \text{ concentration (μM)} \times \text{time (min)} = \text{ED50} / \text{t}_1/2 \]

where ED50 is the amount of H2O2 added per volume of culture medium, and t1/2 is the half-life of elimination. The concentration of cells was quantified by exposing C6 cell cultures of various cell densities and various passages to 100 μM H2O2. The initial exponential parts of the H2O2 elimination curves were fitted to a first-order rate equation (Eq. (1))

\[ \text{H}_2\text{O}_2 \text{ concentration (μM)} \times \text{time (min)} = \text{ED50} / \text{t}_1/2 \]

where ED50 is the amount of H2O2 added per volume of culture medium, and t1/2 is the half-life of elimination. The concentration of cells was quantified by exposing C6 cell cultures of various cell densities and various passages to 100 μM H2O2. The initial exponential parts of the H2O2 elimination curves were fitted to a first-order rate equation (Eq. (1))

\[ \text{H}_2\text{O}_2 \text{ concentration (μM)} \times \text{time (min)} = \text{ED50} / \text{t}_1/2 \]
to derive the first-order rate constant and to determine the elimination rate of 100 μM H₂O₂. As shown in Fig. 6 the rate of elimination was linearly related to the cell concentration represented by the cell protein concentration (mg cell protein/ml medium).

An initial exponential decline of the H₂O₂ concentration in the culture medium was observed with H₂O₂ concentrations between 20 and 1000 μM (Fig. 7). In general, concentrations of H₂O₂ ≤ 100 μM were completely eliminated during the first 60 min after administration. At initial H₂O₂ concentrations of ≥ 200 μM, however, after the initial rapid phase the elimination became slower and the concentration of H₂O₂ leveled off. This developing inability of C6 cells to eliminate H₂O₂ very probably reflects the onset of cytotoxic effects that manifest earlier with increasing peroxide concentration (see Fig. 2).

Elimination of H₂O₂ was measured in C6 cells grown in 35-mm culture dishes. In the majority of the experiments performed the cell concentration in these cultures was in the range of 0.1–0.3 mg cell protein/ml. Using the above-derived ED₅₀ of 430 ± 26 nmol H₂O₂/mg cell protein, EC₅₀ values of 40 to 130 μM can be estimated for the incipient cytotoxic action of H₂O₂ in these C6 cell cultures.

Elimination rates (nmol/ml × min) for 20 to 1000 μM H₂O₂ were derived as described above from the concentration versus time curves shown in Fig. 7 and from similar experiments with C6 cell cultures of various passages and related to the respective cell protein concentration to give the specific elimination rates (nmol/min × mg cell protein). Fig. 8 shows that the elimination rate is linearly dependent on the H₂O₂ concentration. From the slope of the regression line in Fig. 8 a specific first-order elimination rate constant, kₑ = 0.24 ml/min × mg protein, was derived. In a single experiment no deviation from linearity was observed up to a concentration of 5000 μM (data not shown).

**AUC to quantify toxic exposure of cells to H₂O₂**

The area under the concentration versus time curve is a common measure used in pharmacokinetics to quantify internal exposure to changing concentrations of a substance. Fig. 5 shows that using the same initial concentration of H₂O₂ the AUC decreases with increasing cell concentration. This is the reason the same nominal concentration of H₂O₂ becomes less cytotoxic when the cell concentration is increased (see Figs. 3A and 4).

If a substance is completely eliminated during the observation and the concentration changes exponentially with time the AUC is given by the integral of the first-order rate equation (Eq. (1)),

\[
\text{AUC} = \frac{C_0}{k_e}.
\]

where C₀ is the initial concentration and kₑ is the first-order rate constant.

We have calculated the concentration versus time curves and the AUC for the half-maximum cytotoxic H₂O₂ concentrations determined in the experiments of Fig. 3A in C6 cultures with various cell numbers using Eqs. (1) and (4). The EC₅₀ values determined were used as the initial concentration C₀ and values of the first-order rate constants were derived by multiplying the specific first-order rate constant kₑ = 0.24 ml/min × mg protein by the cell protein concentration of the cultures used. Fig. 9 shows that the increase in the EC₅₀ values from 20 to 190 μM when increasing the cell concentration, and thereby the rate of H₂O₂ elimination, results in an almost constant AUC of 1800–2300 μM × min.

Dividing the ED₅₀, the median cytotoxic cell dose of H₂O₂, by kₑ, the specific rate constant for elimination of H₂O₂, a median cytotoxic AUC₅₀ = 1800 μM × min can be determined.

**Discussion**

In this study it was shown that the nominal concentration of H₂O₂ that exerts cytotoxicity in C6 cell cultures is largely dependent on the incubation time and the cell concentration. The minimal incubation
time necessary to determine the time-independent threshold or incipient EC$_{50}$ value for the cytotoxic action of H$_2$O$_2$ was approximately 24 h (see Fig. 2). A similar result was obtained in cultures of vascular smooth muscle cells from rats [30]. When the incubation time was shorter, the nominal H$_2$O$_2$ concentration had to be increased up to 17-fold to exert the same cytotoxic effect. Differences in the incubation time may explain a large part of the variation found in cytotoxic concentrations of H$_2$O$_2$ reported in the literature. Quincozes-Santos et al. [12], for instance, reported 1000 μM H$_2$O$_2$ to be not cytotoxic in C6 cells after an incubation time of 30 min, whereas Wätjen and Beyersmann [10] measured an EC$_{50}$ value of 50 μM for the cytotoxic action of H$_2$O$_2$ in the same cell line after an incubation time of 24 h.

In the case of H$_2$O$_2$, incubation time is not equivalent to exposure time. The clearance measurements reveal that H$_2$O$_2$ is rapidly eliminated from the culture medium. Using the specific elimination rate constant, $k_s = 0.24$ ml/min × mg cell protein, derived from the relationship between the specific elimination rate and the peroxide concentration (Fig. 8), it can be estimated that the incipient EC$_{50}$ of 26 μM H$_2$O$_2$ in the microtiter plate cultures (cell concentration about 30 μg cell protein/ml medium) disappears from the culture medium with a half-life of about 60 min. That means that H$_2$O$_2$ is almost completely removed after about 4 h of incubation. Obviously (see Fig. 1), the manifestation of the incipient cytotoxicity of H$_2$O$_2$ takes several hours longer than the exposure to H$_2$O$_2$. In line with this, Antunes and Cadenas [2] observed that Jurkat cells needed 12 h after a 1-h exposure to a steady-state concentration of 25 μM H$_2$O$_2$ to develop maximum cytotoxicity. Whittemore et al. [23] showed that the cytotoxic effect of H$_2$O$_2$ in primary cultures of neuronal cells needed at least 12 h to develop fully.

The incipient EC$_{50}$ value of H$_2$O$_2$ was linearly related to the cell concentration over the whole range of cell concentrations (0.18 to 2.5 × 10$^6$ cells/ml) tested, irrespective of whether the cell concentration was varied by changing the cell number or the medium volume. In the experiments with cultures of various cell numbers we measured cell protein contents between 0.018 and 0.2 mg/well. Using a protein content of 0.2 mg/10$^6$ cells and a growth area of 1.9 cm$^2$/well this is equivalent to cell densities between 5.2 × 10$^3$ and 4.7 × 10$^4$ cells/cm$^2$. Spitz et al. [4] found a cell-density dependence of H$_2$O$_2$ cytotoxicity in a similar range of cell densities (4.4 × 10$^4$ to 3.2 × 10$^5$ cells/cm$^2$). Using the same cell line (HA-1) Wiese et al. [5] reported a cell-density dependence of the cytotoxicity of H$_2$O$_2$ at low cell densities (<10$^5$ cells/60-mm dish) but not at those (≥10$^5$ cells/60-mm dish) investigated by Spitz et al. [4] and us. One million cells/60-mm dish is equivalent to 5 × 10$^4$ cells/cm$^2$. This discrepancy cannot be solved. Our results, however, indicate that there is no principle difference between cultures of low and of high cell densities and that it is the cell concentration, the relation between cell number and culture medium volume, that affects the cytotoxicity of H$_2$O$_2$.

If H$_2$O$_2$ is quantified as a cell dose (nmol/mg cell protein) the dependency of its cytotoxic potency from the cell concentration disappears. An ED$_{50}$ of 430 nmol/mg cell protein, equivalent to 860 nmol/10$^7$ cells, was derived from the slope of the regression line for the relation between the EC$_{50}$ values and the cell protein concentration. From the data presented by Spitz et al. [4] a comparable ED$_{50}$ value of 1–2 μmol/10$^7$ cells can be identified for the cytotoxic action of H$_2$O$_2$ in HA-1 cells. The colony formation assay used in that study to assess cytotoxicity, however, cannot discriminate between induction of growth arrest and cell death. In the same cell
line the following pattern of responses to increasing H2O2 cell doses was observed [5]: 0.1–0.5 μM/10^7 cells stimulated cell growth, 2–5 μM/10^7 cells induced temporary growth arrest, 9–14 μM/10^7 cells caused permanent growth arrest, and doses of 30 μM/10^7 cells and above caused cell death. In this study neither stimulation nor arrest of growth could occur in C6 cells because they were exposed under serum-free conditions and, thus, growth arrested. In this case a reduction in viability measured is definitely caused by cell death. It seems that the C6 glioma cells are much more sensitive to the cytotoxic action of H2O2 than at least the HA-1 cells in the studies of Wiese et al. [5]. It must, however, be recognized that the maintenance culture conditions used in this study render the C6 cells very sensitive to oxidative stress induced by peroxides [31].

Differences in the cell concentration very probably contribute to the large variation found in the cytotoxic concentrations of H2O2 reported in numerous papers. Such differences, however, are difficult to judge, as relevant information on cell number or cell protein content and culture medium volume most often is not published. Therefore, in agreement with previous studies [4,5,7] we recommend expressing the in vitro toxic potency of H2O2 or the sensitivity of cells to H2O2 by the cell density-independent cytotoxic cell dose instead of the nominal cytotoxic concentration. This would enable meaningful comparisons, for example, of the sensitivities of different cell types and the impact of exposure conditions.

It has to be noted that different sensitivities of the cells to H2O2, due to cell type [32], adaptation to oxidative stress [4,5,7,33], or different supplementation of culture medium with antioxidants and precursors [31,34], as well as different contents of peroxide-consuming additives in the culture medium such as pyruvate [17,35] and serum albumin [36], certainly will also add to the observed variability in the cytotoxic potency of peroxides.

Nominal concentrations of H2O2 in cell cultures in fact are initial concentrations that are eliminated rapidly from the culture medium. This has long been known [15–21]. The H2O2 elimination rate in C6 cell cultures, as shown here, was proportional to the cell concentration, as has been described earlier [17,20] with cultures of human fibroblasts and endothelial cells. Elimination of H2O2 in C6 cell cultures followed first-order kinetics over a wide range of H2O2 concentrations (20–1000 μM) for which elimination kinetics can be described by a single specific first-order rate constant (k = 0.24 ml/min × mg cell protein, see Fig. 8). This indicates that even the highest tested concentration of H2O2 is well below the half-saturating concentration (K50) for this elimination pathway and that C6 cells have a high elimination capacity (Vmax) for H2O2 far beyond 300 nmol/min×mg protein. The regression line (Fig. 8) intersects the y axis at 9±6.5 nmol/min×mg cell protein (best fit±SE), indicating a residual second elimination pathway with a rather small capacity (Vmax ≈ 9 nmol/min×mg) already saturated at H2O2 concentrations of 20 μM. This is in line with the observations of Makino and co-workers of a biphasic concentration dependency of the H2O2 removal rate from the H2O2 concentration in cultures of various cell types [17,19,20], including primary rat astrocytes and C6 astroglia cells [21]. The first phase is attributed to the glutathione peroxidase reaction following Michaelis–Menten kinetics, which is saturated at low H2O2, whereas the second phase is attributed to the practically nonsaturable catalase reaction following first-order kinetics [19,21]. From Fig. 1 of Makino et al. [21] a specific elimination rate constant of about 0.27 ml/min×mg cell protein for the second phase, a maximum rate of the first phase of 38 nmol/min×mg cell protein, and saturation of the first phase at approximately 40 μM H2O2 can be derived for H2O2 elimination by C6 cells. Our measurements with C6 cells indicate a similar rate for the second phase but in the case of the first phase saturation at lower H2O2 concentrations and a lower elimination capacity (see above). The latter might be due to the low glutathione content and glutathione peroxidase activity of our C6 cells maintained in low-serum growth medium [31].

The data obtained for the kinetics of H2O2 elimination were used to calculate concentration vs time curves and corresponding AUC for half-maximum nominal cytotoxic concentrations (EC50) of H2O2 in C6 cell cultures of various cell densities. The AUC can be used to quantify exposure to changing concentrations with time. This analysis revealed an approximately constant AUC, whereas the EC50 changed with the cell concentration (Fig. 9). Obviously, short exposures to high concentrations of H2O2 are equipotent to long exposures to lower H2O2 concentrations as long as the AUC is equal. The median cytotoxic AUC50 corresponding to the ED50 (430 nmol/mg cell protein) of H2O2 was calculated to be 1800 μM min.

As noted above the elimination of H2O2 by cells is essentially biphase. The calculation of the AUC was based on the kinetic data obtained for the second phase dominating at higher concentrations of H2O2. Thus, the calculated AUC may be more erroneous at lower H2O2 concentrations. In the case of C6 cells the magnitude of the first phase is rather small; therefore the error may be small, too.

This analysis further reveals that with decreasing cell density a bolus administration of H2O2 changes its character from a transient exposure to rapidly declining concentrations into continuous exposure to slowly declining or almost constant concentrations of H2O2. Fig. 9 shows that the half-life of H2O2 in the culture medium increased from 7 to 80 min when the cell concentration was decreased from 0.4 to 0.036 mg cell protein/ml. With decreasing cell concentrations the EC50 values become lower and the half-life of elimination longer. Hence, with decreasing cell concentration the EC50 values approximate the extracellular concentrations of H2O2 which, if continuously present, exert 50% cytotoxicity in C6 cells. Fig. 4 reveals that this half-maximum cytotoxic steady extracellular concentration of H2O2 cannot be higher than a few micromolar.

Similar values were found by Antunes and Cadenas [2], who administered glucose oxidase together with H2O2 to attain various steady-state concentrations of H2O2 for at least 1 h in Jurkat cell cultures. They found an extracellular steady-state concentration of 5 μM H2O2 not to be cytotoxic but 20 μM H2O2 to exert maximum cytotoxicity by inducing apoptosis. Using a factor of 7 for the difference between extracellular and intracellular (cytosolic) steady-state concentrations of H2O2 in Jurkat cells [18], intracellular steady-state concentrations of ≤0.7 μM were estimated to be not cytotoxic [2]. Following this line of argument our results indicate that steady intracellular concentrations of around 1 μM are cytotoxic in C6 cells.

In conclusion, the nominal cytotoxic concentrations of H2O2 administered as a bolus into the medium of cultured cells largely are determined by the incubation time as well as the cell concentration and can be varied widely by changing these conditions. The time-independent incipient cytotoxicity of a transient exposure to H2O2 after bolus administration depends on the concentration versus time relationship; the area under the curve (μM×min), which depends on the initial concentration of H2O2, the ability of the cultured cells to eliminate H2O2, and the cell concentration, whereas the cytotoxicity of a constant exposure to H2O2 depends on the steady extracellular concentration. These relations largely explain the wide variation in cytotoxic concentrations of H2O2 reported in cell cultures.

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References


