ELSEVIER

Biochemical Pharmacology 62 (2001) 705–712

Biochemical Pharmacology

tert-Butyl hydroperoxide-induced lipid signaling in hepatocytes: involvement of glutathione and free radicals

César Martín, Rosa Martínez, Rosaura Navarro, José Ignacio Ruiz-Sanz, Mercedes Lacort, M. Begoña Ruiz-Larrea*

Department of Physiology, University of the Basque Country, E-48080 Bilbao, Spain

Received 31 October 2000; accepted 26 April 2001

Abstract

tert-Butyl hydroperoxide (TBHP) mobilizes arachidonic acid (AA) from membrane phospholipids in rat hepatocytes under cytotoxic conditions, thus leading to an increase in intracellular AA, which precedes cell death. In the present work, the involvement of lipid peroxidation, thiol status, and reactive oxygen species (ROS) in the intracellular AA accumulation induced by 0.5 mM TBHP was studied in rat hepatocytes. Cells treated with TBHP maintained viability and energy status at 10 min. However, TBHP depleted GSH, as well as inducing lipid peroxidation and ROS formation, detected by dichlorofluorescein (DCF) fluorescence. TBHP also significantly increased (32.5%) the intracellular [^{14}C]-AA from [^{14}C]-AA-labelled hepatocytes. The phospholipase A_2 (PLA_2) inhibitor, mepacrine, completely inhibited the [^{14}C]-AA response. The addition of antioxidants to the cell suspensions affected the TBHP-induced lipid response differently. The [^{14}C]-AA accumulation correlated directly with ROS and negatively with endogenous GSH. No correlation between [^{14}C]-AA and lipid peroxidation was found. Promethazine prevented lipid peroxidation and did not affect the [^{14}C]-AA increase. We conclude that TBHP stimulates the release of [^{14}C]-AA from membrane phospholipids through a PLA_2-mediated mechanism. Endogenous GSH and ROS play a major role in this effect, while lipid peroxidation-related events are unlikely to be involved. Results suggest that specific ROS generated in iron-dependent reactions, different from lipid peroxyl radicals, are involved in PLA_2 activation, this process being important in TBHP-induced hepatocyte injury.

Keywords: Rat hepatocyte; Antioxidants; *tert*-butyl hydroperoxide; Lipid peroxidation; Gluthatione; ROS

1. Introduction

There exists a great interest in the mechanisms by which cells respond to ROS. ROS have diverse effects on cells and elicit a series of responses, including the up-regulation of adhesion molecules, inflammatory responses, cell growth, and cell death [1–5]. The knowledge of the signaling events that take place under ROS exposure to target cells is important for a clearer understanding of the ROS-induced pathological consequences and their prevention. Lipid signaling implicates rapid events through phosphorylation reactions triggered by kinases, PLA_2 being a possible target, which ultimately leads to AA release from membrane phospholipids. The mechanisms involved in the regulation of PLA_2 and the subsequent AA release are still not completely understood. However, alterations in the lipid bilayer and increases in the intracellular Ca^{2+} levels have been postulated to mediate these effects [6]. It has been reported recently that several types of PLA_2 can also be activated in the presence of peroxidized lipids [7]. In isolated rat hepatocytes, lipid peroxidation is associated with increased cytosolic free Ca^{2+} [8], this cation being required for full activation of several PLA_2 isoforms [9].

The pro-oxidant compound TBHP is a hepatotoxic agent frequently used as a model to study the mechanism of cellular alterations resulting from free radical action. In particular, the hydroperoxide induces the release of AA in different cell types [6,10–13]. We have recently reported enhanced AA liberation into the medium and intracellular

* Corresponding author. Tel.: +34 944 601 2829; fax: +34 946 01 5662.

E-mail address: ofprulam@lg.ehu.es (M.B. Ruiz-Larrea).

Abbreviations: [^{14}C]-AA, [^{14}C]-arachidonic acid; DCF, 2′,7′-dichlorofluorescein; DCFDA, 2′,7′-dichlorofluorescin diacetate; DTT, 1,4-dithiothreitol; MDA, malondialdehyde; PLA_2, phospholipase A_2; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; and TBHP, *tert*-butyl hydroperoxide.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00704-3

accumulation of both AA and diacylglycerol in hepatocytes treated with TBHP under cytotoxic conditions [14]. The present study was designed to determine the involvement of lipid peroxidation, thiol status, and ROS in the intracellular AA accumulation induced by TBHP in liver cells, which precedes cell death. Diverse compounds that inhibit oxidation by different mechanisms were used for this purpose.

2. Materials and methods

2.1. Materials

[1-^{14}C]-AA (55 mCi/mmol) was obtained from Radiochemical Center (Amersham). Fetal bovine serum was purchased from Biochrom K.G. Bovine serum albumin (essentially fatty acid-free), deferoxamine mesylate, 17β-estradiol, diethylstilbestrol, TBHP, α-tocopherol, phospholipid, and neutral lipid standards were purchased from Sigma. Collagenase A, GSH, and DTT were from Boehringer Mannheim. DCFDA and DCF were from Molecular Probes.

2.2. Preparation of hepatocytes

Liver cells were isolated from male Sprague–Dawley rats (180–200 g) by the collagenase perfusion method as previously described [15]. The hepatocyte viability was determined by means of the Trypan blue exclusion test and was typically greater than 90%.

2.3. Incubation conditions

Hepatocytes were resuspended ($2 \cdot 10^6$ cells/mL) in fresh Krebs–Henseleit buffer plus 2% fatty acid-free bovine serum albumin, pH 7.4. After a 20-min equilibration at 37°, hepatocytes were incubated with TBHP (0.5 mM) for 10 min. Cells were pretreated with different compounds for 10 min before adding TBHP. DTT and deferoxamine were dissolved in water and added to hepatocyte suspensions at the final concentrations of 1, 0.5, and 0.025 mM, respectively. 17β-Estradiol, diethylstilbestrol, α-tocopherol, and promethazine were dissolved in ethanol and added to cells at the final concentrations indicated in the text. The final concentration of ethanol was 0.1% (v/v). This concentration of ethanol had no effect on the parameters studied.

2.4. Determination of GSH, protein thiols, ATP, and lipid peroxidation

Cell aliquots were removed in order to analyse the different cytotoxic parameters. Lipid peroxidation was measured in the supernatants of deproteinized cells by the thiobarbituric acid method [16] as previously described [17]. Protein thiol groups were determined spectrophotometrically using Ellman's reagent [18]. ATP was quantified by enzyme assays according to Jaworek *et al*. [19] and performed as in Leal *et al*. [17]. Cellular protein was assayed by Peterson [20], using BSA as standard. GSH was measured according to Brigelius [21]. For this purpose, aliquots of cell suspensions were added to ice-cold 20% $HClO_4$ containing 2 mM EDTA and centrifuged. Supernatants were used for measuring GSH.

2.5. TBHP-stimulated accumulation of [^{14}C]-AA

Hepatocytes were labelled with [^{14}C]-AA, as previously described [14]. Briefly, cells ($3 \cdot 10^6$ cells/mL) were resuspended in Krebs–Henseleit buffer, containing 2.5 mM $CaCl_2$, 20% fetal bovine serum, and 20 mM HEPES, pH 7.4. Typically, 20 mL of cell suspensions was incubated with 1 μCi [^{14}C]-AA at 37° under an atmosphere of carbogen (95% O_2, 5% CO_2) for 60 min. The [^{14}C]-prelabelled hepatocytes were washed thoroughly 3 times with Krebs–Henseleit medium containing 0.5% fatty acid-free bovine serum albumin. Cells were then resuspended in fresh Krebs–Henseleit buffer plus 2% fatty acid-free bovine serum albumin, pH 7.4 and incubated with TBHP as described above. The labelling procedure did not affect thiol status, ATP levels, or lipid peroxidation, which were comparable to those observed with non-labelled hepatocytes. After incubating [^{14}C]-AA-labelled cells, aliquots were removed and centrifuged. Cell pellets were extracted for their total lipids with chloroform-methanol (2:1, by vol.), according to Folch *et al*. [22], and the chloroform phase was evaporated under vacuum. The lipid residue was dissolved in toluene. An aliquot of the toluene solution was counted directly to determine radioactivity incorporated into total lipids. The rest of the lipid sample was spotted on TLC plates. Plates were developed in *n*-heptane/diisopropyl ether/acetic acid (70:30:2, by vol.). The band corresponding to free fatty acids was scraped off the TLC plate and assayed for radioactivity, using a commercially available scintillation cocktail (Biogreen 3). Under the conditions used for labelling the cells, 55.8 $\pm$ 2.8% of the total [^{14}C]-AA added to cell suspensions was incorporated into cell lipids.

2.6. DCF assay

DCFDA was used to evaluate the rate of TBHP-induced ROS formation, essentially as described in Royall and Ischiropoulos [23]. Hepatocytes ($4 \cdot 10^6$ cells/mL) were resuspended in Krebs–Henseleit buffer containing 2% BSA. Cell suspensions were incubated with 20 μM DCFDA for 30 min. Cleavage of acetate moieties by endogenous esterases causes the 2,7-dichlorofluorescin to be trapped inside the cell and to be available for oxidation by ROS to the fluorescent DCF. After loading, the cells were washed at 0–4° by centrifugation at 50 *g* for 3 min and resuspended (10^6 cells/mL) in Krebs–Henseleit buffer. Aliquots of cell suspensions were placed in a quartz cuvette in a Perkin Elmer LS-50B fluorimeter, equipped with cell stirring, and

Table 1
Effect of different compounds on lipid peroxidation, GSH-, and protein-SH depletion induced by TBHP[a]

Treatment	nmol MDA/10^6 cells	nmol GSH/10^6 cells	nmol SH/mg protein
Untreated control	0.28 ± 0.01	44 ± 2	75 ± 5
TBHP	1.33 ± 0.08***	28 ± 2***	51 ± 2***
+ 17β-Estradiol (5 μM)	1.15 ± 0.02***	21 ± 2***	67 ± 4##
+ 17β-Estradiol (10 μM)	0.77 ± 0.02**,###	N.D.	N.D.
+ 17β-Estradiol (50 μM)	0.39 ± 0.04###	39 ± 2###	62 ± 3##
+ Diethylstilbestrol (5 μM)	1.24 ± 0.14*	36 ± 1###	63 ± 6#
+ Diethylstilbestrol (10 μM)	1.01 ± 0.08**,#	N.D.	N.D.
+ Diethylstilbestrol (50 μM)	0.35 ± 0.02###	41 ± 2###	69 ± 4##
+ α-Tocopherol (0.1 mM)	1.27 ± 0.12*	33 ± 2**,#	62 ± 3**,##
+ α-Tocopherol (0.5 mM)	1.23 ± 0.06**	N.D.	N.D.
+ α-Tocopherol (1 mM)	0.62 ± 0.10###	40 ± 3###	65 ± 6##
+ Promethazine (0.25 μM)	0.97 ± 0.12*,#	28 ± 4***	59 ± 2***
+ Promethazine (0.50 μM)	0.87 ± 0.11*,##	N.D.	N.D.
+ Promethazine (1 μM)	0.47 ± 0.08###	24 ± 3***	59 ± 6***
+ DTT (1 mM)	0.49 ± 0.06###	N.D.	69 ± 8##
+ Deferoxamine (25 μM)	0.47 ± 0.05###	26 ± 5***	55 ± 11**

[a] Hepatocytes ($2 \cdot 10^6$ cells/mL) were incubated for 10 min without (untreated control) or with 0.5 mM TBHP either alone or in the presence of the compounds indicated in the table. The incubation conditions are described in Materials and Methods. The treatments of cells with the compounds alone had no effect on the intracellular parameters assayed. Results are the means ± SEM of three separate hepatocyte preparations. N.D., not determined.

* $P < 0.05$; ** $P < 0.025$; *** $P < 0.01$, different from untreated cells. # $P < 0.05$; ## $P < 0.025$; ### $P < 0.01$, different from TBHP-treated cells.

thermostatted at 37°. Hepatocytes were exposed to TBHP. The oxidation of 2,7-dichlorofluorescin was monitored at λex = 502 nm and λem = 525 nm. Data points were taken every 30 sec for 30 min and the data were exported to Excel spreadsheet software for analysis.

2.7. Statistical analysis

All values are given as means ± SEM from at least three separate hepatocyte preparations with triplicate values. Statistical differences were determined by analysis of the variance using the ANOVA test. Values were considered statistically significant when $P < 0.05$. Kendall's correlation was used to determine the relationship between covariates.

3. Results

3.1. TBHP effects on markers of oxidative cellular damage (GSH, protein thiols, lipid peroxidation, ROS formation)

Time-course experiments of viability showed that 0.5 mM TBHP induced hepatocyte death at 40 min (data not shown). No loss of viability could be detected at 10 min, with the ATP content also remaining unchanged (untreated controls 23 ± 1; TBHP 23 ± 2 nmol/10^6 cells). However, the intracellular GSH and protein -SH groups were dramatically depleted in the presence of the hydroperoxide (Table 1). The pro-oxidant also stimulated lipid peroxidation significantly (Table 1). TBARS could be detected right from the first minutes assayed (2.5 min) (data not shown). Under our experimental conditions, no correlation between GSH and TBARS could be found.

We then studied the *in vitro* effects of a variety of compounds which interfere with lipid peroxidation at different stages on the TBHP-induced actions. Estrogens at concentrations beyond 5 μM inhibited lipid peroxidation significantly (Table 1). The peroxyl radical scavenger promethazine, the thiol compound DTT, and the iron chelator deferoxamine all significantly decreased the TBHP-induced TBARS formation. α-Tocopherol at concentrations below 1.0 mM was not efficient in protecting hepatocytes from lipid oxidation (Table 1). By contrast, α-tocopherol at the lowest concentration of 0.1 mM was able to partially restore GSH and protein -SH groups depleted by TBHP. Both diethylstilbestrol and estradiol also protected cells from thiol oxidation. On the other hand, neither promethazine nor deferoxamine prevented the depletion of GSH or protein thiols (Table 1).

The generation of ROS was monitored by loading hepatocytes with dichlorofluorescin. Non-fluorescent dichlorofluorescin reacts with ROS to produce highly fluorescent DCF. Fig. 1 shows the concentration–response relationship of cells exposed to TBHP. The increase in fluorescence is linearly correlated ($r^2 = 0.93$) with the concentration of TBHP in the range of 0.05 to 1.0 mM. Fig. 2 shows the time-course of DCF fluorescence in hepatocytes with or without 0.5 mM TBHP. In the absence of the hydroperoxide, no increase in DCF fluorescence was observed. A rapid increase in DCF fluorescence could be detected 5.4 ± 0.2 (N = 6) min after the addition of TBHP. The ROS detected by DCF fluorescence in TBHP-treated cells were probably not lipid peroxyls, because promethazine at concentrations

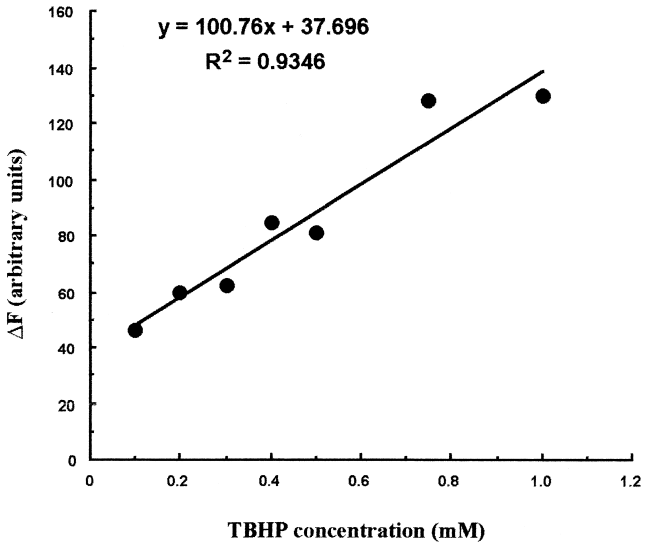


Fig. 1. Concentration–response curve for the increase in DCF fluorescence in hepatocytes after a 10-min exposure to various concentrations of TBHP. $\Delta F = F_{10} - F_0$, where F_{10} is the fluorescence at 10 min and F_0 is the fluorescence at time 0 min. Each data point represents the mean of two separate hepatocyte preparations. The line was created by curve fitting using linear regression. R is the Pearson correlation coefficient.

that inhibited lipid peroxidation (Table 1) only significantly reduced TBHP-enhanced DCF fluorescence (Table 2). A pretreatment with diethylstilbestrol, DTT, or deferoxamine totally prevented the TBHP-induced DCF fluorescence increase. α-Tocopherol and 17β-estradiol reduced the TBHP-stimulated ROS formation in a dose-dependent manner (Table 2). A negative correlation ($P < 0.05$) between ROS and the intracellular GSH levels in the presence of different antioxidants seems to come out (Fig. 3), thus suggesting a role for GSH depletion in TBHP-induced ROS formation.

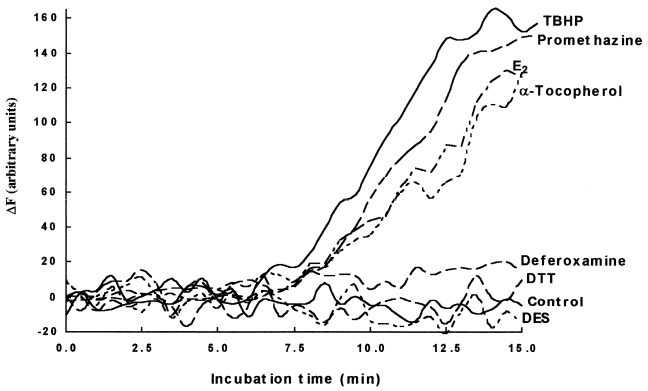


Fig. 2. Effect of different compounds on the time-course of intracellular ROS formation induced by TBHP. Cells were loaded with dichlorofluorescin and then exposed to 0.5 mM TBHP alone or with 5 μM 17β-estradiol (E_2), 5 μM diethylstilbestrol (DES), 0.25 μM promethazine, 1 mM α-tocopherol, 25 μM deferoxamine, and 1 mM DTT. The incubation conditions are described in Materials and Methods. $\Delta F = F_t - F_0$, where F_t is the fluorescence at the time measured (in min) and F_0 is the fluorescence at time 0 min. The compounds alone had no effect on the fluorescence respecting untreated controls. The figure shows data from a representative experiment of three.

Table 2
Effects of different compounds of TBHP-induced ROS formation in hepatocytes[a]

Addition	ROS formation (%)
TBHP	100
+ 17β-Estradiol (5 μM)	54 ± 15
+ 17β-Estradiol (10 μM)	40 ± 13
+ 17β-Estradiol (50 μM)	14 ± 7
+ Diethylstilbestrol (5 μM)	14 ± 3
+ Diethylstilbestrol (10 μM)	9 ± 3
+ Diethylstilbestrol (50 μM)	7 ± 3
+ α-Tocopherol (0.1 mM)	67 ± 13
+ α-Tocopherol (0.5 mM)	67 ± 8
+ α-Tocopherol (1 mM)	44 ± 7
+ Promethazine (0.25 μM)	84 ± 11
+ Promethazine (0.5 μM)	83 ± 14
+ Promethazine (1 μM)	65 ± 10
+ DTT (1 mM)	5 ± 1
+ Deferoxamine (25 μM)	7 ± 8
+ Diethylmaleimide (0.5 mM)	100 ± 8

[a] Dichlorofluorescin-loaded hepatocytes were incubated for 10 min with 0.5 mM TBHP alone or in the presence of the indicated compounds, as described in Materials and Methods. The increase in fluorescence = F_{10}–F_0 was calculated, where F_{10} is the fluorescence at 10 min and F_0 the fluorescence at time 0 min. The fluorescence increase in cells with no TBHP added was 0. Fluorescence increments are referred to those of TBHP-treated cells, the mean of which is considered to be 100 (232 ± 16 arbitrary units for TBHP-treated cells at 10 min). Values are expressed as the means ± SEM of at least three hepatocyte preparations.

3.2. TBHP-stimulated accumulation of [^{14}C]-AA

The effect of TBHP on the intracellular [^{14}C]-AA accumulation is shown in Table 3. The hydroperoxide substan-

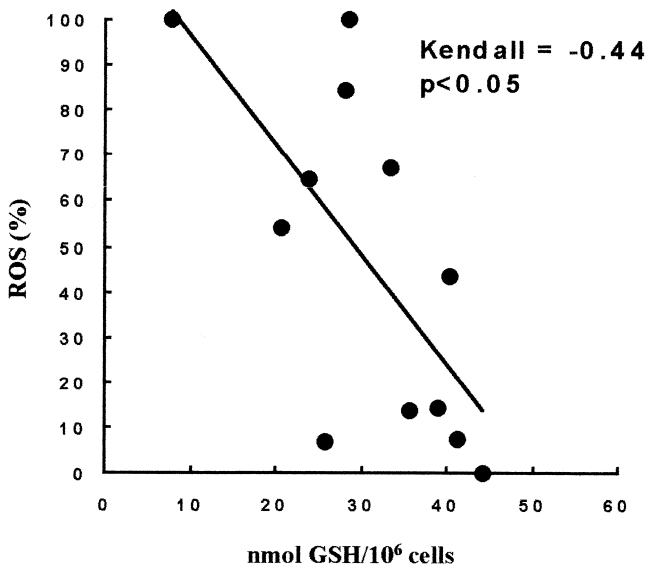


Fig. 3. Correlation between intracellular GSH and ROS formation induced by TBHP in the presence of different compounds at 10 min. Data were collected from Tables 1 and 2 for GSH and ROS values, respectively. Results obtained with 0.5 mM diethylmaleimide are also included (8 ± 2 nmol GSH/10^6 cells). Kendall coefficient and significance are indicated in the figure.

Table 3
Effect of different compounds on the intracellular accumulation of [^{14}C]-AA[a]

Treatment	[^{14}C]-AA (%)
Untreated control	0.44 ± 0.08
TBHP	0.61 ± 0.09**
+ 17β-Estradiol (5 μM)	0.48 ± 0.04**,###
+ 17β-Estradiol (10 μM)	0.46 ± 0.03###
+ 17β-Estradiol (50 μM)	0.39 ± 0.02###
+ 17β-Estradiol (100 μM)	0.41 ± 0.03###
+ Diethylstilbestrol (5 μM)	0.43 ± 0.01##
+ Diethylstilbestrol (10 μM)	0.35 ± 0.04##
+ Diethylstilbestrol (50 μM)	0.32 ± 0.10###
+ α-Tocopherol (0.1 mM)	0.53 ± 0.07#
+ α-Tocopherol (0.5 mM)	0.39 ± 0.02###
+ α-Tocopherol (1 mM)	0.44 ± 0.06###
+ Promethazine (0.25 μM)	0.60 ± 0.05**
+ Promethazine (0.5 μM)	0.59 ± 0.06*
+ Promethazine (1.0 μM)	0.59 ± 0.02**
+ DTT (1 mM)	0.43 ± 0.04##
+ Deferoxamine (25 μM)	0.49 ± 0.05##
+ Diethylmaleimide (0.5 mM)	0.61 ± 0.03**

[a] [^{14}C]-Loaded hepatocytes ($2 \cdot 10^6$ cells/mL) were incubated for 10 min without (untreated controls) or with 0.5 mM TBHP either alone or in the presence of the compounds indicated in the table. The incubation conditions are described in Materials and Methods. The treatments of cells with the compounds alone had no effect on the intracellular [^{14}C]-AA accumulation. Results are expressed as the percentage of the [^{14}C]-AA incorporated into cells. Values are the means ± SEM of three separate hepatocyte preparations.

* $P < 0.025$; ** $P < 0.01$, different from untreated cells. # $P < 0.05$; ## $P < 0.025$; ### $P < 0.01$, different from TBHP-treated cells.

tially increased the intracellular levels of [^{14}C]-AA (32.5% over control) at 10 min. This effect was time-dependent, being significant from 7.5 min (data not shown).

We investigated the effects of antioxidants on the TBHP-stimulated increase in [^{14}C]-AA (Table 3). Most of their preventive effects on lipid peroxidation paralleled their effects on [^{14}C]-AA accumulation. However, the potent antioxidant promethazine at concentrations that inhibited significantly lipid peroxidation failed to prevent TBHP-induced [^{14}C]-AA release. Fig. 4 outlines the correlations between [^{14}C]-AA and a) lipid peroxidation, b) GSH, and c) ROS in the presence of the compounds used. As can be seen, [^{14}C]-AA did not correlate with lipid peroxidation. However, a significant negative correlation between [^{14}C]-AA levels and GSH could be observed ($P < 0.01$). Despite this GSH dependence, GSH does not seem to be the only factor involved in the [^{14}C]-AA response. Thus, treating the cells with deferoxamine at concentrations that failed to restore basal GSH or thiol status completely reverted the TBHP-induced [^{14}C]-AA increase. These data suggest that mechanisms other than GSH oxidation are also involved in the responsiveness of hepatocytes treated with TBHP.

[^{14}C]-AA also correlated significantly ($P < 0.05$) with ROS (Fig. 4c). These findings suggest a role for specific ROS in the AA response.

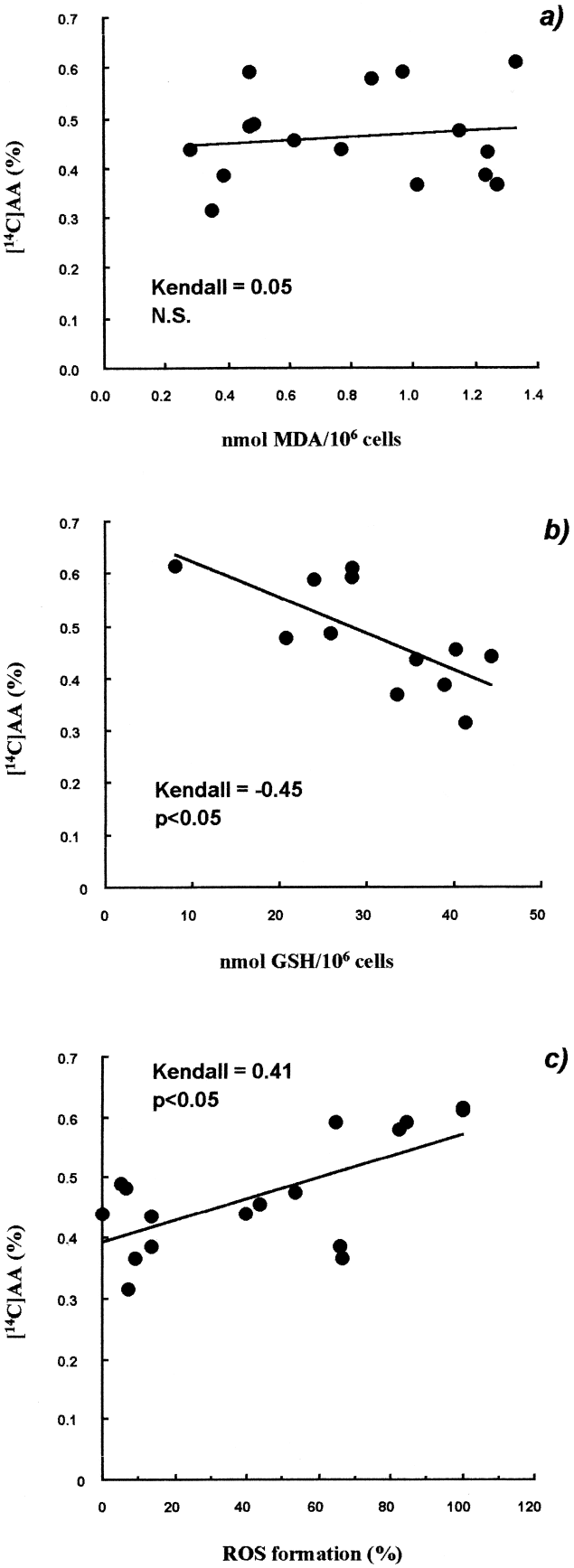
The principal pathway for AA release from membrane phospholipids is via PLA_2. We then studied the involvement of PLA_2 in the oxidant-mediated AA increase by using the PLA_2 inhibitor mepacrine. Mepacrine reduced to basal levels the [^{14}C]-AA increase induced by TBHP (Fig. 5).

4. Discussion

The present study was carried out in order to establish the involvement of lipid peroxidation and thiol status in TBHP-induced AA accumulation in hepatocytes, which precedes cell death. TBHP (0.5 mM) did not produce viability loss or ATP depletion at 10 min. This time was chosen to study the initial changes taking place in the hepatocyte irrespective of any alterations due to cell killing.

TBHP clearly induced lipid peroxidation and depleted GSH and protein thiol groups. Inside the hepatocyte, TBHP is metabolized by different pathways. On the one hand, TBHP reacts with GSH via GSH peroxidase, generating GSSG [24,25]. GSSG is reduced back to GSH at the expense of NADPH by GSSG reductase. Depletion of GSH and NADPH oxidation are associated with altered Ca^{2+} homeostasis, thus leading to cell viability loss [26]. Alternatively, TBHP is converted into free radicals (*tert*-butoxyl radicals) by iron-dependent reactions, thus initiating lipid peroxidation [27]. In addition to the detoxicant function of cellular GSH, the potential of this thiol to modulate signal transduction processes has recently become evident [28–30]. Thiol homeostasis determines critical aspects of cell function and response. Oxidative stress results in the oxidation of thiols to disulfides, and such redox changes are also involved in the regulation of signal transduction processes, which activate phospholipases. In our system, TBHP appeared to increase [^{14}C]-AA through a PLA_2-mediated mechanism, as could be seen in experiments with the phospholipase inhibitor, mepacrine (Fig. 5). A significant inverse correlation between intracellular [^{14}C]-AA and GSH could be observed (Fig. 4b), the data indicating the involvement of endogenous GSH depletion in the AA mobilization response to the oxidant. It is interesting to note that deferoxamine completely inhibited the TBHP-induced [^{14}C]-AA accumulation (Table 3), despite failing to prevent intracellular GSH loss (Table 1). Deferoxamine also inhibited lipid peroxidation (Table 1) and was found to prevent cell killing under cytotoxic conditions [17]. E_2 and diethylstilbestrol prevented the accumulation of [^{14}C]-AA efficiently (Table 3). In a previous work, we showed that these estrogenic compounds prevented *in vitro* the toxicity induced by 1 mM TBHP in liver cells [17]. Several reports have described similar beneficial effects of E_2 in intact cells exposed to oxidative stress, these effects being ascribed to the non-receptor-mediated antioxidant activity of the hormone [31–33].

Respecting the possible involvement of lipid peroxidation in TBHP-induced AA increase, results showed the following:



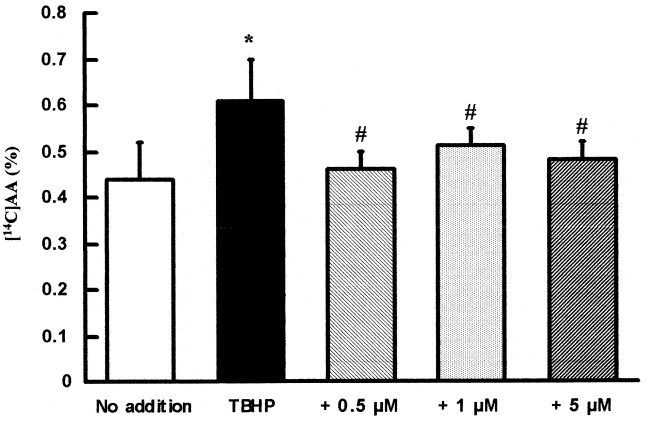


Fig. 5. Effect of mepacrine on the intracellular [^{14}C]-AA accumulation induced by TBHP in hepatocytes. [^{14}C]-Loaded hepatocytes ($2 \cdot 10^6$ cells/mL) were incubated for 10 min without (untreated controls) or with 0.5 mM TBHP either alone or in the presence of mepacrine at the concentrations indicated in the figure. Results are expressed as the percentage of the [^{14}C]-AA incorporated into cells. Values are the means ± SEM of three separate hepatocyte preparations. * $P < 0.01$, different from untreated cells. # $P < 0.01$, different from TBHP-treated cells.

1. Promethazine completely blocked TBARS formation without altering [^{14}C]-AA accumulation;
2. The compounds diethylstilbestrol (5 μM), estradiol (5 μM), and α-tocopherol (0.1–0.5 mM) prevented the TBHP-induced AA-response without affecting TBARS formation; and
3. There was no correlation between lipid peroxidation estimated by TBARS formation and the intracellular accumulation of [^{14}C]-AA.

Therefore, these data do not support a major role for lipid peroxidation in the mechanism of [^{14}C]-AA increase induced by TBHP in hepatocytes.

Promethazine is selective toward the inhibition of the peroxidative decomposition of membrane lipids [26,34], but was a poor scavenger of TBHP-induced reactive species, as could be observed in experiments with the fluorescent probe DCF (Fig. 1). The DCF assay is widely used for measuring overall ROS formation in biological systems [35]. The method is based on the formation of fluorescent DCF from non-fluorescent dichlorofluorescin by oxidizing species. TBHP clearly induced in a dose-dependent manner an increase in DCF fluorescence in rat hepatocytes (Fig. 1), although it did not oxidize dichlorofluorescin directly (data not shown). These results suggest the formation of reactive species derived from TBHP metabolism inside the cell. The

Fig. 4. Correlations between the intracellular [^{14}C]-AA accumulation induced by TBHP and a) lipid peroxidation, b) intracellular GSH, and c) ROS formation in the presence of different compounds. Data were collected from Tables 1 (lipid peroxidation and GSH), 2 (ROS formation), and 3 ([^{14}C]-AA values). Results obtained with 0.5 mM diethylmaleimide were also included (8 ± 2 nmol GSH/10^6 cells) (Fig. 4b). Kendall coefficients and significances are indicated in the figure. N.S. = not significant.

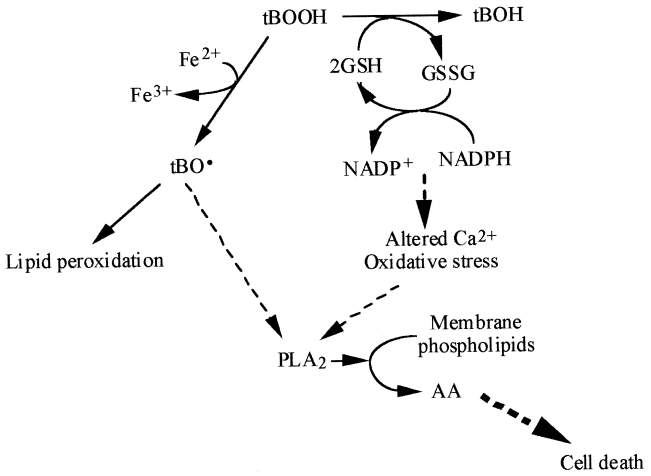


Fig. 6. Proposed mechanism of oxidative stress induced by *tert*-butyl hydroperoxide (tBOOH) in rat hepatocytes. tBOH, *tert*-butanol; tBO˙, *tert*-butoxyl radical.

manipulation of the antioxidant and radical scavenging capacity of the cells, as well as the removal of free iron by chelators, affected the response to TBHP to different extents. ROS formation obtained with different compounds correlated significantly with the levels of [^{14}C]-AA. Moreover, time-course experiments revealed that the DCF fluorescence was detected about 5.5 min after the TBHP addition to cell suspensions, whereas the accumulation of [^{14}C]-AA was never observed before 7.5 min. These results suggest a cause-effect relationship between both parameters. The iron chelator deferoxamine completely inhibited the TBHP-induced ROS generation, and also abolished the [^{14}C]-AA release (Fig. 1 and Table 2). These data indicate that the mobilization of AA depends on an iron supply, which is also necessary for generating *tert*-butoxyl radicals. Promethazine may have blocked TBHP-induced lipid peroxidation without altering the formation of *tert*-butoxyl radicals.

α-Tocopherol has been reported to be a typical peroxyl radical scavenger, but a poor inhibitor of lipid peroxidation induced by alkoxyl radicals [36]. Accordingly, under the conditions we have used, α-tocopherol inhibited lipid peroxidation in a dose-dependent manner, but did not decrease ROS efficiently (Table 2). However, α-tocopherol completely inhibited the [^{14}C]-AA mobilization stimulated by TBHP. The action of vitamin E as a non-specific PLA_2 inhibitor reported recently [37] could explain the observed effect on the AA mobilization.

In conclusion, it appears that TBHP triggers a series of events in rat hepatocytes that ultimately lead to a PLA_2-mediated release of AA from membrane phospholipids. Endogenous GSH seems to play a major role in this effect, while lipid peroxidation-related events are unlikely to be involved. We speculate that specific ROS generated in iron-dependent reactions and different from lipid peroxyl radicals are involved in PLA_2 activation, this process being important in TBHP-induced hepatocyte injury (see Fig. 6). The exact mechanism and target proteins involved in the oxidant-induced PLA_2 activation remain to be elucidated and are the objective of investigations in course.

Acknowledgements

We are grateful to Montserrat Busto for her skillful technical assistance. This work was supported by Scientific Research Grants UPV 081.327-EB005/96 and Gobierno Vasco PI-1999-118. C.M. and R.M. were awarded Predoctoral Training Grants from the Spanish Education Ministry and the Basque Government, respectively.

References

[1] Remacle J, Raes M, Toussaint O, Renard P, Rao G. Low levels of reactive oxygen species as modulators of cell function. Mutat Res 1996;316:103–22.
[2] Buttke TM, Sandstrom PA. Redox regulation of programmed cell death in lymphocytes. Free Radic Res 1995;22:389–97.
[3] Lander HM. An essential role for free radicals and derived species in signal transduction. FASEB J 1996;11:118–24.
[4] Palmer HJ, Paulson KE. Reactive oxygen species and antioxidants in signal transduction and gene expression. Nutr Rev 1997;55:353–61.
[5] Ginn-Pease ME, Whisler RL. Redox signals and NF-kappa B activation in T cells. Free Radic Biol Med 1998;25:346–61.
[6] Kondakova IV, Peiretti F, Nalbone G, Lafont H. Phospholipase A stimulation in tumor cells by subtoxic concentration of tert-butyl hydroperoxide. Biochim Biophys Acta 1995;1258:297–302.
[7] Rashba-Step J, Tatoyan A, Duncan R, Ann D, Pushpa-Rehka TR, Sevanian A. Phospholipid peroxidation induces cytosolic phospholipase A2 activity: membrane effects versus enzyme phosphorylation. Arch Biochem Biophys 1997;343:44–54.
[8] Albano E, Bellomo G, Parola M, Carini R, Dianzani MU. Stimulation of lipid peroxidation increases the intracellular calcium content of isolated hepatocytes. Biochim Biophys Acta 1991;1091:310–6.
[9] Mayer RJ, Marshall LA. New insights on mammalian phospholipase A_2(s); comparison of arachidonoyl-selective and -nonselective enzymes. FASEB J 1993;7:339–48.
[10] Chakraborti S, Michael JR, Gurtner GH, Ghosh SS, Dutta G, Merker A. Role of a membrane-associated serine esterase in the oxidant activation of phospholipase A_2 by *t*-butyl hydroperoxide. Biochem J 1993;292:585–9.
[11] Chen X, Gresham A, Morrison A, Pentland AP. Oxidative stress mediates synthesis of cytosolic phospholipase A_2 after UVB injury. Biochim Biophys Acta 1996;1299:23–33.
[12] Akiba S, Nagatomo R, Hayama M, Sato T. Lipid peroxide overcomes the inability of platelet secretory phospholipase A_2 to hydrolyze membrane phospholipids in rabbit platelets. J Biochem 1997;122:859–64.
[13] Kafoury RM, Pryor WA, Squadrito GL, Salgo MG, Zou X, Friedman M. Lipid ozonation products activate phospholipases A2, C, and D. Toxicol Appl Pharmacol 1998;150:338–49.
[14] Babenko N, Ruiz-Larrea MB, Martínez R, Martín C, Lacort M. Inhibition by estrogens of the oxidant-mediated mobilization of arachidonic acid in hepatocytes. J Physiol Biochem 1998;54:77–84.
[15] Ruiz-Larrea MB, Garrido MJ, Lacort M. Estradiol-induced effects on glutathione metabolism in rat hepatocytes. J Biochem 1993;113:563–7.
[16] Fee JA, Teitelbaum HD. Evidence that superoxide dismutase plays a role in protecting red blood cells against peroxidative hemolysis. Biochem Biophys Res Commun 1972;49:150–8.

[17] Leal AM, Ruiz-Larrea MB, Martínez R, Lacort M. Cytoprotective actions of estrogens against tert-butyl hydroperoxide-induced toxicity in hepatocytes. Biochem Pharmacol 1998;56:1463–9.
[18] Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968;25:192–205.
[19] Jaworek D, Gruber W, Bergmeyer HU. Adenosine-5′-triphosphate. Determination with 3-phosphoglycerate kinase. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis, Vol. 4. New York and London: Academic Press, 1974. p. 2097–2101.
[20] Peterson GL. A simplification of the protein assay method of Lowry *et al*. which is more generally applicable. Anal Biochem 1977;83: 346–56.
[21] Brigelius R, Muckel C, Akerboom TP, Sies H. Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. Biochem Pharmacol 1983;32: 2529–34.
[22] Folch J, Less M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497–509.
[23] Royall JA, Ischiropoulos H. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 1993;302:348–55.
[24] Rush GF, Gorski JR, Ripple MG, Sowinski J, Bugelski P, Hewitt WR. Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. Toxicol Appl Pharmacol 1985;78:473–83.
[25] Sies H, Summer KH. Hydroperoxide-metabolizing systems in rat liver. Eur J Biochem 1975;57:503–12.
[26] Bellomo G, Jewell SA, Thor H, Orrenius S. Regulation of intracellular calcium compartmentation: studies with isolated hepatocytes and t-butyl hydroperoxide. Proc Natl Acad Sci USA 1982;79:6842–6.
[27] Rush GF, Alberts D. *tert*-Butyl hydroperoxide metabolism and stimulation of the pentose phosphate pathway in isolated rat hepatocytes. Toxicol Appl Pharmacol 1986;85:324–31.
[28] Sen CK, Khanna S, Reznick AZ, Roy S, Packer L. Glutathione regulation of tumor necrosis factor-alpha-induced NF-kappa B activation in skeletal muscle-derived L6 cells. Biochem Biophys Res Commun 1997;237:645–9.
[29] Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. FASEB J 1996;10:709–20.
[30] Finkel T. Redox-dependent signal transduction. FEBS Lett 2000;476: 52–4.
[31] Behl C, Widmann M, Trapp T, Holsboer F. 17-beta estradiol protects neurons from oxidative stress-induced cell death in vitro. Biochem Biophys Res Commun 1995;216:473–82.
[32] Bonnefont AB, Munoz FJ, Inestrosa NC. Estrogen protects neuronal cells from the cytotoxicity induced by acetylcholinesterase-amyloid complexes. FEBS Lett 1998;441:220–4.
[33] Zaulyanov LL, Green PS, Simpkins JW. Glutamate receptor requirement for neuronal death from anoxia-reoxygenation: an *in vitro* model for assessment of the neuroprotective effects of estrogens. Cell Mol Neurobiol 1999;705–18.
[34] Poli G, Cheeseman K, Slater TF, Dianzani MU. The role of lipid peroxidation in CCl4-induced damage to liver microsomal enzymes: comparative studies *in vitro* using microsomes and isolated liver cells. Chem Biol Interact 1981;37:13–24.
[35] Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 1999;27:612–6.
[36] Maiorino M, Coassin M, Roveri A, Ursini F. Microsomal lipid peroxidation: effect of vitamin E and its functional interaction with phospholipid hydroperoxide glutathione peroxidase. Lipids 1989;24: 721–6.
[37] Farooqui AA, Litsky ML, Farooqui T, Horrocks LA. Inhibitors of intracellular phospholipase A_2 activity: their neurochemical effects and therapeutical importance for neurological disorders. Brain Res Bull 1999;49:139–53.