Oxidative DNA damage by *t*-butyl hydroperoxide causes DNA single strand breaks which is not linked to cell lysis. A mechanistic study in freshly isolated rat hepatocytes

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Abstract In rat hepatocytes, DNA damage by *t*-butyl hydroperoxide (tBOOH) was measured by using the fluorimetric analysis of alkaline DNA unwinding. The electrophoretic profile of genomic DNA suggests single rather than double DNA strand breaks formation. Oxidative DNA modifications, measured as increased 8-hydroxy-deoxyguanosine content, were not detected. Lysis of hepatocytes and DNA strand breaks induced by tBOOH did not correlate, indicating that both processes are not interconnected. Since *o*-phenanthroline prevents against tBOOH-mediated effects on both DNA and membrane integrity, we discussed about a putative role of iron.

Key words: DNA fragmentation; Oxidative stress; Organic hydroperoxide; Free radical; Rat hepatocyte

1. Introduction

Many physical, environmental toxicants and chemicals (including metabolic poisons and chemotherapeutic drugs) are harmful for cells by mechanisms involving reactive oxygen species overproduction [1]. Such reactive intermediates can react with and modify major macromolecules, i.e. the appearance of strand breaks in DNA [2,3].

The molecular mechanisms by which oxidants trigger the cascade leading to cell death are however not fully elucidated, but loss of calcium homeostasis may contribute in a major way to the onset of cytotoxicity. Indeed, a rapid increase in free cytosolic calcium concentration occurred in isolated rat hepatocytes injured by *t*-butyl hydroperoxide (tBOOH), an organic hydroperoxide widely used as model compound to induce an oxidative stress [4–7]. It has also been reported that the activation of an endonuclease, a process elicited by Ca^{2+} changes, plays a crucial role in DNA fragmentation [8–10].

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Abbreviations: BSA, bovine serum albumin; DPPD, *N,N'*-diphenyl-*p*-phenylendiamine; dsDNA, double-stranded DNA; FADU, fluorometric analysis of DNA unwinding; LDH, lactate dehydrogenase; oPT, *o*-phenanthroline; tBOOH, *t*-butyl hydroperoxide; 8-OH-dG, 8-hydroxy-deoxyguanosine.

This work was undertaken with the aim to study and characterize the DNA damage induced by tBOOH. We further analysed whether this putative DNA damage was linked to the tBOOH-mediated cell death. Oxidant injury was evaluated by following the formation of DNA strand breaks, the time course of LDH leakage and the formation of the DNA adduct 8-hydroxy-deoxyguanosine (8-OH-dG). The extent and the nature of DNA strand breakage were evaluated by the method of fluorometric analysis of the rate of alkaline DNA unwinding (FADU) and by the electrophoretic profile of genomic DNA run on agarose gel, respectively. The cytotoxicity of tBOOH was modulated by using the inhibitor of lipid peroxidation *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), the iron chelator *o*-phenanthroline (oPT) and zinc sulfate and aurintricarboxylic acid (ATCA) as an endonuclease inhibitor.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–280 g) were purchased from Iffa-Credo (Les Oncins, France) and housed in individual cages in a temperature- and light-controlled room. They received standard diet AO3 (UAR, France) and water ad libitum.

2.2. Chemicals

Bovine serum albumin (BSA, fraction V), *o*-phenanthroline (oPT), *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) and tBOOH were purchased from Sigma (St Louis, MO). Collagenase was from Boehringer (Mannheim, Germany). Ethidium bromide, zinc sulfate ($ZnSO_4$) and sodium dodecyl sulfate (SDS) were from Merck (Darmstadt, Germany). Agarose was from FMC BioProducts (Rockland, ME). Aurintricarboxylic acid (ATCA) was from Aldrich (Steinheim, Germany). Dulbecco's modified Eagle medium (DMEM) was purchased from Flow Laboratories (Irvine, UK). All other chemicals and reagents were of the purest grade available.

2.3. Preparation of isolated hepatocytes

Hepatocytes were isolated using the standard procedure described by Berry and Friend [11] and slightly modified by Krack et al. [12]. Briefly, animals were anaesthetized with an i.p. injection of pentobarbital (60 mg/kg) and cells were isolated by liver perfusion with Krebs solution containing collagenase. The yield of hepatocytes was usually in the range of $350–400 \times 10^6$ cells per liver, with a viability varying from 85 to 95% as estimated by dye cell exclusion of erythrosin B. Cells were then suspended in 40 ml of DMEM supplemented with 0.3% BSA, at a final concentration of 5×10^5 cells/ml. Cell suspensions were incubated at 37°C in a thermoregulated shaking water bath (100 oscillations/min) under a continuous flow of O_2/CO_2 (95%/5%).

2.4. Assays

Hepatocytes viability was estimated by measuring the activity of lactate dehydrogenase (LDH) according to the procedure of Wrobleski and Ladue [13] both in the culture medium and in the cell pellet obtained after centrifugation as described elsewhere [12]. The results are

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expressed as a ratio of released activity to the total activity. The release of LDH and erythrosine B staining gave essentially the same results.

The fluorimetric analysis of DNA unwinding (FADU) was performed according to the procedure of Birnboim [14]. Cells were washed and added into immobilized test tubes and cell lysis was performed for 15 min. The pH was increased by adding, successively and carefully, the alkaline solutions in order to allow DNA unwinding. After neutralization, the percentage of double-stranded DNA (dsDNA) formed was detected by measuring the fluorescence of samples after addition of ethidium bromide. Measurements were performed in a Perkin Elmer fluorimeter with 520 and 575 nm as excitation and emission wavelenghts, respectively. Values are expressed in percentage of dsDNA (D), which can be transformed in Qd units by applying the following equation [15]:

$$Qd = 100 \times \log \frac{D \text{ from untreated cells}}{D \text{ from treated cells}}$$

this relation is directly proportional to the number of strand breaks present in the genome of treated cells, thus allowing a quantitative analyse.

Detection of 8-OH-dG was performed by following the procedures first described by Floyd et al. [16] and further modified by Fiala et al. [17] and Berger et al. [18]. Briefly, cells were washed, homogenized and nuclei were isolated by centrifuging at $32 \times g$ for 15 min. They were further resuspended in ice-cold TE/SDS and DNA was extracted twice with isopropanol and precipitated at −20°C with isoamyl alcohol. Samples were dissolved in TE buffer and digested for 1 h with RNAse A and T1, 50 μg/ml and 45 U/ml, respectively. The detection of 8-OH-dG was performed by HPLC/EC after enzymic hydrolysis in the laboratory of Professor J. Cadet (Grenoble-France) as described in [18].

For analysis of genomic DNA, cells were washed with ice-cold PBS and lysed in TE buffer (10 mM Tris-HCl, 10 mM EDTA, pH 7.4) containing 1% SDS and 0.1 mg/ml proteinase K. After 4 h incubation at 37°C, DNA was extracted twice with phenol, once with phenol-chloroform, once with chloroform and precipitated by ethanol. Samples were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA) and digested for 2 h with 0.1 mg/ml RNAse A. The DNA fragmentation was analysed on a 1% agarose gel in the presence of 0.5 μg/ml ethidium bromide. A *Hin*dIII digest of λ phage DNA was used as molecular size standards.

Protein content was measured according to Lowry et al. [19] using BSA as standard.

2.5. Statistics

For statistical comparison of results at a given time point, data were analysed using Student's t test. A P value less than 0.05 was set as the minimum level of significance.

3. Results

3.1. Effects of tBOOH on DNA integrity and on cellular viability

The effect of varying concentrations of tBOOH on DNA integrity is shown in Table 1. The formation of strand breaks, leading to a decreased percentage of dsDNA as measured by the FADU procedure, was observed as soon as 15 min after the start of incubation for all tBOOH concentrations. This DNA damage did not result from enhanced cell death induced by tBOOH, since a significant increase of LDH leakage was only observed for the high concentration (0.5 mM) after 120 min of incubation. For lower concentrations of tBOOH, no significant LDH leakage was observed as compared with untreated cells. It was further shown that for all tBOOH concentrations, the amounts of 8-hydroxy-deoxyguanosine (8-OH-dG) were similar to that determined in untreated cells: at 0.25 mM tBOOH the production of 8-OH-dG ng/100 μg DNA was 1.2 and 1.4 after 15 and 120 min, respectively, while in untreated cells it was 1.2 and 1.1 for the same time intervals.

Since no DNA fragments lower than 20 kb appeared in DNA gel electrophoresis (Fig. 1), DNA damage induced by tBOOH seems to be the consequence of single strand breaks formation. Indeed, the electrophoretic profile of genomic DNA extracted from cells incubated in the presence of 0.1, 0.25 or 0.5 mM (lanes b–d, respectively) was similar to that from untreated cells (lane a).

3.2. Possible pathways of tBOOH metabolism involved in DNA damage and in cell death

We decided to investigate by which mechanisms tBOOH may lead to single strand breaks formation. Table 2 shows that neither zinc nor aurintricarboxylic acid were able to protect against the deleterious effect by tBOOH on both cellular survival and DNA integrity. On the contrary, aurintricarboxylic acid seems to increase the cytotoxicity of tBOOH. The addition of DPPD resulted in a non-significant decrease of tBOOH-mediated DNA damage, while a partial protection against tBOOH-mediated cell lysis was observed. When hepatocytes were incubated in the absence of tBOOH, DPPD was without effect on DNA integrity (data not shown). Only the addition of oPT was able to protect cells against the oxidant injury by tBOOH at the two levels of DNA and cellular integrity.

4. Discussion

This work was undertaken to evaluate how the oxidative DNA damage induced by tBOOH can influence its cytotoxicity. We observed that neither an increased 8-OH-dG content (the major oxidative end-product) nor the formation of DNA double strand breaks occur in cells exposed to tBOOH over a range of concentrations.

DNA can be damaged by different mechanisms including the

Table 1
Effects of tBOOH on both DNA and cellular integrity

tBOOH (mM)	D double stranded DNA (%)		LDH leakage (%)		DNA adducts[a] (μmol 8-OHdeoxyguanosine/mg DNA)	
	15 min	120 min	15 min	120 min	15 min	120 min
0	73 ± 2	70 ± 7	9.0 ± 0.8	18 ± 1.7	3.40	3.11
0.1	57 ± 5*	55 ± 6*	8.5 ± 0.7	22 ± 0.9	3.12	ND
0.25	46 ± 4*	49 ± 7*	10.7 ± 0.6	23 ± 1.5	3.39	3.96
0.5	24 ± 6**	19 ± 3**	9.9 ± 0.5	40 ± 1.2**	3.68	ND

Hepatocytes were incubated for 120 min at 37°C. At 15 and 120 min of incubation, aliquots of cell suspension were taken and parameters were evaluated as described under Section 2. Values are mean ± S.E.M. of at least three separate experiments.
[a]Values are means of two separate experiments. ND, not determined.
*$P < 0.05$ as compared with tBOOH 0 mM.
**$P < 0.05$ as compared with tBOOH 0, 0.1 and 0.25 mM.

activation of a calcium-dependent endonuclease [8,9], arylation of DNA [20], and/or direct oxidative modification to DNA bases [2,3]. Nevertheless, the influence of such a process in lethal cell injury still remains unclear [21–25]. In agreement with a previous report [26], we shown that DNA strand breaks did not correlate with LDH leakage induced by tBOOH (table 1).

Although not universally accepted [10], DNA fragmentation in both lymphocytes [8] and hepatocytes [9,27,28] has been reported to be mediated by the activation of a calcium-dependent endonuclease. Despite the fast increase of free cytosolic Ca^{2+} concentration by tBOOH [7], no internucleosomal DNA fragmentation presenting a 'ladder profile' was observed, indicating that an apoptotic-like DNA cleavage is unlikely to occur.

DNA damage by tBOOH (as measured by the FADU method) is likely to result from DNA single strand breaks and/or by formation of alkali labile sites [15,29]. It may be triggered by two major mechanisms (Fig. 2): in the first one, iron metabolism plays a key role thus leading to free radical formation and further radical-mediated processes (lipid peroxidation, covalent binding to DNA, etc.). The second mechanism is depending on thiol oxidation followed by the activation of a calcium-dependent endonuclease which can lead to DNA strand breaks [8,9]. This latter pathway can be prevented by using endonuclease inhibitors like aurintricarboxylic acid [30] and zinc [31]. However, these compounds were unable to inhibit the effects of tBOOH on both cell LDH leakage and DNA unwinding.

The results observed by using oPT suggests that an intracellular source of iron seems to be required in order to express the damaging effects of tBOOH on both cell and DNA integrity. Supporting this view, it has been reported that the oPT-ferrous iron complex (oPT_3Fe^{2+}) is unable to catalyse a Fenton reaction [32]. Such a reaction can also be catalysed by other transition metals than iron, e.g. copper [1]. The pK_d of the oPT-copper complex (oPT_3Cu^{2+}) is similar to that of divalent iron complex, 20 and 21, respectively [33], but paradoxically, the copper complex increased the DNA damage [34]. We concluded that copper is therefore unlikely involved and oPT by complexating

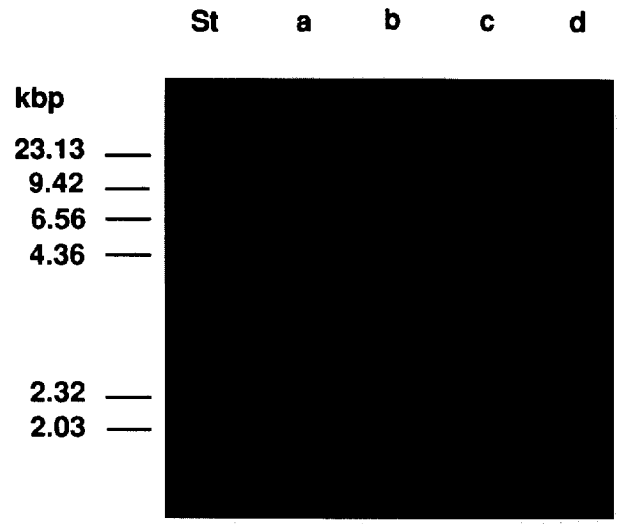

Fig. 1. Agarose gel electrophoretic pattern of ethidium bromide stained genomic DNA extracted from cells incubated in the absence and in the presence of tBOOH. Control untreated cells (lane a). *t*-butyl hydroperoxide at concentrations of 0.1 mM (lane b), 0.25 mM (lane c) and 0.5 mM (lane d). λ/*Hin*dIII molecular size standards (St) and arrows indicate their molecular weights in kbp.

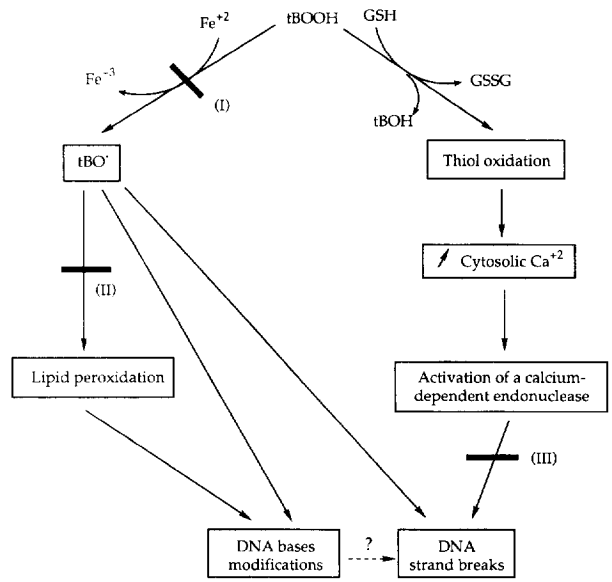

Fig. 2. Possible pathways leading to DNA strand breaks. Metabolism of tBOOH was modulated by using the iron chelator oPT at 0.1 mM (I); the antioxidant DPPD at 1 μM as inhibitor of lipid peroxidation (II); and the inhibitor of endonuclease aurintricarboxylic acid at 0.3 mM (III).

ferrous iron avoids the formation of secondary free radicals. Indeed, iron and organic hydroperoxides are closely related through a Fenton chemistry thus yielding highly deleterious free radicals:

$$(Fe^{2+} + tBOOH \rightarrow tBO^{\bullet} + Fe^{3+} + OH^{-}).$$

Alkoxyl radicals (tBO•) are unable to react with DNA in order to form free radical adducts, but they can either dismutate to yield methyl radicals [35,36] although DNA bases methylation is a less probable process [37], or initiate a lipid peroxidation process. Peroxidized lipids (or secondary oxidized by-products) might interact with nucleic acids as suggested by Fraga and Tappel [38] thus leading to DNA fragmentation. The

Table 2
Influence of compounds which modulate tBOOH-metabolism on both cell and DNA integrity impaired by tBOOH

Treatments	DNA damage (*Qd* units)	Cytolytic activity (%)	
		60 min	120 min
tBOOH	46.5 ± 7.6	100	100
+ Zn^{2+} (0.1 mM)	43.9 ± 5.9	103	99
+ ATCA (0.3 mM)	54.4 (n = 2)	129 (n = 2)	120 (n = 2)
+ DPPD (1 μM)	28.8 ± 6.3	45	75
+ oPT (0.1 mM)	10.9 ± 2.3*	21	30

Hepatocytes were incubated for 120 min at 37°C. To test cellular protection, cells were incubated in the presence of tBOOH (1 mM), while for DNA integrity tBOOH was 0.5 mM. At the indicated times, aliquots of cell suspension were taken and both DNA unwinding (to calculate *Qd*) and LDH leakage (to calculate cell protection) were measured as described under Section 2. Results of *Qd* are expressed as mean values ± S.E.M. of at least three separate experiments. For estimation of cytolytic activities, values of LDH leakage for untreated and tBOOH-treated cells were 9.4 ± 0.6 and 22.5 ± 1.7 and 90.1 ± 3.4 and 95.2 ± 2.1 at 60 min and 120 min, respectively.
*$P < 0.05$ as compared with tBOOH-treated cells.
n = 2, values are means of two separate experiments.

use of lipid peroxidation inhibitors like DPPD shows, however, that it is unlikely that oxidation of lipids is involved in DNA damage induced by tBOOH.

The results reported herein indicate that no direct relationship exists between DNA damage and cell death by tBOOH. They also rule out both the formation of oxidized DNA bases and the activation of a calcium-dependent endonuclease as mechanisms by which tBOOH induces DNA single strand breaks. Rather, they suggest that tBOOH-dependent DNA damage is mediated by some form of metal-catalysed Haber-Weiss-like reaction within the cell. The chemical nature and the exact source of such transition metal pool (iron being the best candidate) still remains unknown and needs to be further investigated.

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