Hepatotoxic effects of tert-butyl hydroperoxide (t-BHP) and protection by antioxidants

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Organic peroxides which are known to cause oxidative stress serve as a convenient model for studying the biochemical process involved. The prooxidant role of tert-butyl hydroperoxide (t-BHP) has been studied\textsuperscript{1,2} as a model for oxidative cellular injury. In liver, t-BHP is metabolized to t-butanol and then conjugated by glutathione\textsuperscript{3}. It may also lead to cytochrome P\textsubscript{580} mediated hemolytic scission capable of initiating lipid peroxidation.

The membrane damage by t-BHP involves the deregulation of Ca\textsuperscript{2+} homeostasis\textsuperscript{4}, transient to sustained increase in cytosolic calcium ion concentration.

To understand the mechanism of hepatotoxicity of t-BHP, freshly isolated cultured rat hepatocytes were used to assess the effect on their viability. Cultured rat hepatocytes have several important advantages as an experimental model as they can be readily isolated in high yield, have good viability and can be cultured for many hours in a medium devoid of Ca\textsuperscript{2+} and serum without any loss of viability.

Materials and Methods

Animals—Male albino Wistar strain rats weighing 100–150 g were used. They were maintained in the Department of Biochemistry, Dr. R.M.L. Avadh University, Faizabad at 25°±5°C and 65±5% RH under standard animal house. The rats were provided Lipon pellet diet and water ab libitum.

Chemicals—Trichloro acetic acid (TCA), HEPES, collagenase (Type IV, from Clostridium histoliticum), ethylene glycol bis tetra acetic acid (EGTA), heparin, deferoxamine (DFO), methylthiazioteletrazolium (MTT), sodium pyruvate, bovine serum albumin (BSA), thio barbituric acid (TBA), ascorbic acid, NADH, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and other chemicals and reagents used were purchased from BDH Ltd. Merck India Ltd., Ranbaxy India Ltd. and Qualigenes India Ltd. HPLC grade quartz double distilled water was employed throughout the studies.

Isolation of hepatocytes—Hepatocytes were prepared as per the method of Moldeus et al.\textsuperscript{6} using following reagents:

(i) Modified Hanks Buffer (136 mM NaCl, 5 mM KCl, 1 mM MgSO\textsubscript{4} and 0.3 mM NaHCO\textsubscript{3}) containing 0.5 mM EGTA; (ii) Heps (25 mM); BSA (2%); Collagenase (0.12%); CaCl\textsubscript{2} (4 mM); (iii) Krebs–Henseleit Buffer (118.1 mM NaCl, 48 mM KCl, 1 mM KH\textsubscript{2}PO\textsubscript{4}, 2.9 mM CaCl\textsubscript{2}, 23.8 mM NaHCO\textsubscript{3} and 25 mM Hepes); (iv) MEM–amino acids and vitamins; Calf serum; Glucose (1.7 mM).

The livers were isolated and the perfusion was initiated in situ at 122 ml/min flow rate. The liver was cut free, immersed in buffer at 37°C and the flow rate adjusted at 80 ml/min in rat. Liver was perfused for 5 min. with a modified Hanks buffer (pH 7.4) (136 mM
NaCl, 5 mM KCl, 1 mM MgSO₄, 0.3 mM NaHCO₃) containing 0.5 mM EGTA, 25 mM Hepes (free acid), and 2% BSA. Subsequently liver was perfused with a modified Hanks buffer containing 0.12% collagenase, 4mM CaCl₂ and 2% BSA for an additional 10 min. At the end of perfusion, the liver which was swollen and pale was detached from canula and cells were dispersed in a Krebs--Henseleit buffer.

Cells were then filtered through a nylon mesh (150 μm) as per Moldeus et al.°

The hepatocytes were incubated at a concentration of 1×10⁶ cells/ml in Erlenmeyer flask at 37°C and flushed with 95% O₂ and 5% CO₂. The viability of cells was checked by MTT reduction and LDH release.

(a) Cell Viability of isolated hepatocytes was checked in terms of Methylthiazoletetrazolium reduction: The cell viability of isolated hepatocytes was monitored according to the method described by Carmichael et al.° The conversion of soluble yellow dye to insoluble purple formazan by active mitochondrial dehydrogenases in living cell was employed to assay the cell viability. Hepatocytes were incubated with t-BHP for 30 minutes before MTT reduction was measured in terms of optical density at 570 nm with background subtraction of 690 nm.

(b) Cell viability of isolated hepatocytes in terms of lactate dehydrogenase release: The cell viability of isolated hepatocytes in terms of lactate dehydrogenase release was monitored according to the method of Moldeus et al.° The LDH activity was assayed in the supernatant, after centrifuging the cell suspension at 200 g for 5 min and absorbance was recorded spectrophotometrically at 340 nm. Total LDH activity of cell was determined after Triton X-100 treatment.

(c) Effect of t-BHP on malondialdehyde level in hepatocytes: Malondialdehyde (MDA) liberated during the course of lipid peroxidation was estimated by the thiobarbituric acid (TBA) method.° Liver homogenate (10% w/v) was centrifuged at 3000 rpm and the mitochondrial fraction and liver homogenate diluted with Tris--KCl. Specified concentrations of the reagents were added in different sets and reaction was stopped by the addition of cold TCA, after incubation. To the centrifuged supernatant, TBA was added and kept in boiling water for 20 min. The optical density was recorded at 535 nm spectrophotometrically, after cooling.

Statistical analysis—The data are the mean of three separate observations alongwith standard deviations employing Student’s t test.

Results

Effect of t-BHP on hepatocyte viability—Effect of t-BHP was observed in two ways: (i) in terms of MTT reduction, and (ii) in terms of LDH release as indicators of hepatocyte viability. In absence of t-BHP, the viability of hepatocytes in terms of MTT reduction was found to be 100%. The range of t-BHP concentrations varied from 0.05-1 mM. As the concentration of t-BHP increased the viability of hepatocytes decreased in a concentration dependant linear manner. At 0.05 mM concentration of t-BHP, 79% cell viability was observed, while at 1.0 mM the viability was 21% (Fig.1 a).

In the absence of t-BHP, no LDH release took place while at 0.05 mM concentration of t-BHP, the LDH release was found to be 31% and at 1 mM concentration the LDH release increased to 79% (Fig.1 b).

Effect of t-BHP on malondialdehyde levels in hepatocytes—In the absence of t-BHP, the lipid peroxidation was found to be 0.194 nmoles/mg of MDA/mg of protein whereas at 0.05 mM concentration of t-BHP, the MDA level was 0.321 nmoles/mg of protein and at 1 mM of t-BHP the MDA levels increased to 0.513 nmoles/mg of protein (Fig.1 c).

Protective effect by antioxidants on t-BHP induced cell death—Cells were incubated with antioxidants for 4 hr prior to incubation with t-BHP. In the control group (without t-BHP), the cell viability was found to be 100%. With addition of t-BHP at 0.1 mM concentration, the cell viability was reduced upto 61%. The data presented in (Fig. 2) show that α-tocopherol at 0.1 mM concentration accorded 10% protection against t-BHP. Addition of BHT at 0.1 mM concentration showed 36% protection against t-BHP; DMSO (0.1 mM) accorded 6% protection against t-BHP; GSH at concentration 1mM exhibited 21% protection against t-BHP while DFO at concentration 0.1 mM accorded 11% protection against t-BHP. BHT accorded maximum protection. Ascorbic acid at 0.1 mM concentration promoted loss of cell viability by 20% against t-BHP, indicating a prooxidant role. The prooxidant concentration of ascorbic acid did not cause any significant change.

Discussion

Kakkar et al.° have used organic hydroperoxides such as t-BHP as a convenient model for studying oxidative stress. Biological effects of t-BHP have been studied as a model for oxidative cellular injury.°
Fig.1—Effect of t-BHP on hepatocytes viability in term of (a) MTT reduction (b) LDH release and (c) malondialdehyde level in hepatocytes.

It is clear from the results that t-BHP is able to generate oxidative stress in hepatocytes responsible for decreased cell viability as it exerts its toxic effects by acting on a wide variety of sites. Masaki et al.\(^{11}\) and Nieminen et al.\(^{12}\) have reported that t-BHP reduced the inner mitochondrial membrane potential along with loss of ATP and metabolic acidosis. Castilho et al.\(^{13}\) have reported oxidative damage of mitochondria, induced by t-BHP in the presence of Ca\(^{2+}\). Kennedy et al.\(^{14}\) have characterized the formation of free radicals by t-BHP in rat liver mitochondria in vitro. Similarly cumene hydroperoxide and benzoyl peroxides cause peroxidative membrane changes\(^{15}\) including mitochondrial swelling\(^{16}\). The membrane damage by t-BHP was interpreted by Frei et al.\(^{14}\) as to involve deregulation of Ca\(^{2+}\) homeostasis. La\(^{15}\) and ruthenium red were found to reduce the membrane potential changes and Ca\(^{2+}\) alteration caused by the hydroperoxide. A variety of hepatotoxins, including bromobenzene, acetaminophen, t-BHP\(^{17}\) can deplete soluble and protein thiols and disrupt Ca\(^{2+}\) homeostasis. Treatment of hepatocytes with t-BHP led to a release of both mitochondrial and extramitochondrial Ca\(^{2+}\) stores\(^{18}\). The present results showed that loss of cell viability took place in a concentration dependant manner, when freshly isolated hepatocytes were incubated with t-BHP, such dose dependant responses have been found with t-BHP earlier on the oxidative swelling of mitochondria\(^{9}\).

It is evident from the results that oxidative stress is involved in hepatotoxic action of t-BHP. Masaki
et al.\textsuperscript{19} reported that lipid peroxidation occurs in the cultured hepatocytes as rapidly as the rise in cytosolic calcium. Such lipid peroxidation in suspended hepatocytes renders them readily permeable to extracellular calcium ions. It was observed that isolated rat hepatocytes incubated in absence of extracellular Ca\textsuperscript{2+} increased level of TBA reactive substances and had a marked loss of mitochondrial and cytosolic GSH occurring concomitant with GSSG formation and GSH efflux. These results were highly suggestive that extracellular Ca\textsuperscript{2+} omission generates oxidative stress and enhances the malondialdehyde production in hepatocyte subjected to Ca\textsuperscript{2+} deprivation.

The biphasic response of ascorbic acid on lipid peroxidation, especially in the presence of external Fe\textsuperscript{2+} has been encountered. It is interesting that in the mitochondrial swelling also ascorbate shows a biphasic response depending on the concentration used\textsuperscript{20}. Based on the findings it seems that BHT and reduced glutathione act as potential antioxidants against t-BHP induced cell death.

Thus t-BHP exerts its deleterious effects through the formation of free radicals as is evident from the protective effect of the antioxidants employed in the study.

References


4 Frei B, Winterhalter K H & Richter C, Quantitative and mechanistic aspects of the hydroperoxide induced Ca\textsuperscript{2+} release from rat liver mitochondria, Eur J Biochem., 149 (1985) 633.

5 Fisher R A, Statistical methods for research works, 11\textsuperscript{th} ed. (Oliver & Boyd Edinburgh, UK) 1950.