0026-895X/88/0304132-08$02.00/0

MOLECULAR PHARMACOLOGY, 34:413-420

Effect of Hypoxia on *tert*-Butylhydroperoxide-Induced Oxidative Injury in Hepatocytes

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Received March 16, 1988; Accepted June 16, 1988

SUMMARY

Toxicity of *t*-butylhydroperoxide (*t*-BuOOH) was studied at different steady state O_2 concentrations under conditions at which O_2 deficiency alone did not cause cell death. *t*-BuOOH-induced cell death was more rapid in hypoxic than normoxic cells; the maximal rate of cell death occurred in anoxic cells. *t*-BuOOH elimination was independent of O_2 concentration and was complete within 15 min; *t*-Butanol was produced at the same rate and was the only product detected by gas chromatography. Measurement of radical production by formation of adducts of the spin-trapping agent N-*tert*-butylphenylnitrone showed that the amount of radicals trapped was 0.02% of the amount of peroxide added and was the same under anoxic and oxygenated (214 μM O_2) conditions. These results show that the O_2 dependence of *t*-BuOOH-induced toxicity is not related to quantitative alterations in its metabolism. Lipid peroxidation was lowest in anoxic cells and increased as the O_2 concentration was increased to 1.07 mM O_2, showing that enhanced toxicity during hypoxia and anoxia was not due to enhanced lipid peroxidation. In contrast, O_2 deficiency impaired the ability of cells to maintain and recover GSH and NADPH pools after addition of *t*-BuOOH. GSH was decreased to a greater extent in anoxic cells than in normoxic cells, and the GSH content remained lower in these cells for up to 30 min. This decrease was due both to a decrease in the rate of synthesis and to decreased supply of the NADPH needed for the reduction of GSSG. Taken together, these results show that O_2 deficiency has little effect on metabolism of *t*-BuOOH but impairs the ability of cells to maintain cellular GSH and renders them more susceptible to injury from oxidizing agents. This suggests that oxidative injury under hypoxia or following ischemia may not require a marked stimulation in generation of oxidative species but may occur as a consequence of the impaired ability to tolerate or repair oxidative injury.

Tissue O_2 concentration directly affects cellular metabolism and thereby affects the function of many drug-metabolizing and detoxication systems (1, 2). In addition to limiting the activities of enzymes that utilize O_2 as substrate (e.g., cytochromes P-450 and monoamine oxidase), O_2 deficiency results in decreased ATP production and increased utilization of glucose by glycolysis. During hypoxia, decreased ATP concentration limits drug sulfation (3), and enhanced utilization of glucose limits the formation of the UDP-glucuronic acid that is needed for drug glucuronosylation (4). In principle, such processes could also limit the production of NADPH by the pentose phosphate pathway and the mitochondria. Metabolic changes such as these could be important in the susceptibility to oxidative injury because GSH, an important component of antioxidant defenses, is synthesized by an ATP-requiring pathway and is maintained in reduced form by the NADPH-requiring GSSG reductase reaction (5). Thus, the ability of cells to maintain GSH may be diminished under hypoxic conditions; this may impair catabolism of peroxides and render cells more susceptible to oxidative injury.

This research was supported by National Institutes of Health Grants GM-36538 and GM-29433.

Trudell and co-workers (6) have recently found that hypoxia exacerbates the toxicity of *t*-BuOOH in cultured hepatocytes. Their studies have important implications concerning hepatic necrosis following hypoxia or exposure to halogenated hydrocarbons and raise fundamental questions about the importance of reductive activation, impaired detoxication, and selective vulnerability of liver during hypoxia. To investigate the mechanism by which hypoxia potentiates oxidative injury, we examined the O_2 dependence of *t*-BuOOH-induced death of freshly isolated hepatocytes. The effects of O_2 concentration on metabolism of *t*-BuOOH were studied by measuring elimination of *t*-BuOOH, formation of *t*-butanol, and formation of *t*-BuOOH-associated free radicals, at different O_2 concentrations. Responses of the cellular GSH and NADPH pools to *t*-BuOOH under aerobic and anoxic conditions were measured to determine whether anoxic cells had an impaired ability to maintain and recover normal thiol and redox status after exposure to *t*-BuOOH. The results show that, as with cultured hepatocytes, *t*-BuOOH is more toxic to freshly isolated hepatocytes under hypoxic conditions than under normoxic conditions. This increased cytotoxicity was found to be due to the impaired ability of cells to tolerate and recover from oxidative

ABBREVIATIONS: *t*-BuOOH, *t*-butylhydroperoxide; PBN, *N-tert*-butylphenylnitrone.

stress rather than to O_2-dependent changes in the rate of peroxide metabolism, lipid peroxidation or free radical generation. These results show that injury due to the combined effects of hypoxia and oxidative stress, such as that caused by exposure to halothane, CCl_4, or post-ischemic reperfusion, may occur without a marked increase in production of oxidants due to an enhanced susceptibility of hypoxic cells to oxidative injury.

Experimental Procedures

Materials. *t*-BuOOH, GSH, GSSG, NADPH, 1,1-diphenyl-2-picrylhydrazyl (free radical), L-cysteine·HCl, L-methionine, digitonin, thiobarbituric acid, collagenase (Type IV), and GSSG reductase (Type III) were purchased from Sigma Chemical Company (St. Louis, MO). GSH peroxidase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Silicone oil (density 1.050, high temperature) and PBN were purchased from Aldrich Chemical Company (Milwaukee, WI). Light mineral oil (paraffin oil, Saybolt viscosity 125/135) was obtained from Fisher Scientific (FairLawn, NJ). HPLC-grade methanol and chloroform were purchased from American Burdick and Jackson (McGaw Park, IL). All other chemicals were of reagent grade and were purchased locally. Distilled deionized water was used for HPLC solutions and deionized water was used for other solutions.

Cell preparation and incubations. Hepatocytes were prepared by the collagenase perfusion method of Moldeus *et al.* (7) from livers of male Sprague-Dawley rats [Kng: (SD)BR; King Animal Laboratories, Oregon, WI] weighing 150–250 g. Rats were fed normal rat chow and water *ad libitum*. Hepatocytes prepared in this manner are metabolically intact by several criteria including GSH content (8) and pyridine nucleotide status (9). Cell viability, measured as the ability to exclude 0.2% trypan blue, was 90–99% immediately after preparation. Before experimentation, cells were maintained at room temperature in a shaking water bath for up to 4 hr without loss in viability. Hepatocytes (10^6 cells/ml) were incubated at 37° in modified Krebs-Henseleit buffer (in mM: NaCl, 140; KCl, 5; $MgSO_4$, 1.5; KH_2PO_4, 1.0; $CaCl_2$, 3.0) supplemented with 10 mM HEPES, pH 7.4. Because the cells were from fed animals that have an abundance of glycogen and because exogenous glucose does little to preserve the metabolic state in hepatocytes under these hypoxic conditions (4), we did not include glucose in the media. However, such an inclusion may improve recovery of NADPH levels after *t*-BuOOH treatment (see below). Steady state anoxic, hypoxic, normoxic, and hyperoxic conditions were maintained by incubating cells in rotating round bottom flasks under argon or calibrated O_2-containing gas mixtures, either obtained from Specialty Gases (Atlanta, GA) or mixed as previously described (3). *t*-BuOOH (0.6 mM final concentration) was added after exposing cells to the appropriate gas mixtures for 30 min.

Measurement of *t*-BuOOH and thiobarbituric acid-reactive substance. *t*-BuOOH concentrations were determined spectrophotometrically with an enzyme-coupled assay involving GSH peroxidase, GSSG reductase, NADPH, and excess GSH (10). The extent of lipid peroxidation in control and *t*-BuOOH-treated cells was measured as the amount of thiobarbituric acid-reactive material (11).

Measurement of *t*-butanol. Aliquots (0.5 ml) were transferred to 1.5-ml micro-centrifuge tubes containing 125 μl of saturated $Ba(OH)_2$. A total 125 μl of 20% $ZnSO_4$ containing a known amount of isopropanol (1:2000 dilution) was added. The contents were mixed thoroughly and centrifuged. Aliquots of the supernatant were analyzed on a Perkin-Elmer Series 8310 gas chromatograph equipped with a hydrogen flame ionization detector. *t*-Butanol (R_T 14.2 min) was completely separated from isopropanol (internal standard, R_T 10.4 min) by a 3.7 m × 2 mm i.d. sialized glass column packed with 80/100 mesh Porapak S (Supelco, Bellefonte, PA) at 185° using nitrogen, at a flow of 35 ml/min, as carrier gas.

EPR measurements of PBN-adduct formation. Five min before introduction of *t*-BuOOH, PBN was added to achieve a final concentration of 50 mM. This concentration did not decrease cell viability during the course of the experiments. Spin-trapped PBN-adducts were extracted into chloroform by the method of Folch *et al.* (12) at various times after addition of *t*-BuOOH to cells under argon (0 μM O_2) or air (214 μM O_2). Extracts from 5×10^6 cells were dried under N_2 or argon, resuspended in 200 μl of chloroform, and placed in 1-mm i.d. quartz capillary tubes. Although samples were dried under N_2 or argon, all other steps were aerobic so that free radical adducts that had been reduced by the cells to corresponding hydroxylamines would air-oxidize back to the free radicals (13). EPR spectral measurements were performed at ambient temperature with an IBM Bruker, model ER 200D spectrometer. Instrument settings were as follows: microwave power, 20.5 mW; modulation amplitude, 2.0 G; time constant, 0.1 sec; scan range, 100 G; and scan time, 4 min. Diphenylpicrylhydrazyl (free radical) in solution was used as a standard to quantify absolute spin concentrations by double integration of first derivative spectra.

Measurement of GSH and pyridine nucleotides. Cells were separated from the incubation mixture by centrifugation through a silicone-mineral oil mixture (14) into 1 M perchloric acid for measurement of GSH and $NADP^+$ or 0.5 M KOH containing 50% (v/v) ethanol and 35% (w/v) $CsCl_2$ for measurement of NADPH. Cellular thiols (consisting primarily of GSH) were determined by a spectrophotometric method (15); GSH and GSSG were determined by HPLC after derivatization with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene (16) and using an Altex Ultrasil NH_2 column (Beckman Instruments, San Ramon, CA). The pyridine nucleotides were determined by HPLC using a μBondapak C18 column (Waters Associates, Milford, MA) (9). For measurements of NADPH, cell extracts were stored on ice and analyzed within 2 hr.

Results

The hepatocyte preparation used for these experiments has been used extensively for studies of hypoxia (2–4) and *t*-BuOOH-induced toxicity (17). Although they do not provide information on the long-term survival of cells exposed to hypoxia or oxidative injury such as can be done with culture hepatocytes (6), they provide sufficient material to readily assess metabolic and detoxication systems. In addition, the oxygen concentrations in solution can be determined reliably and the viability can be directly assessed. Under the conditions of the current studies (modified Krebs-Henseleit buffer, 37°), the viability of control hepatocytes was largely retained for at least 5 hr under air (214 μM O_2) and 2.5 hr under argon (0 μM O_2). The concentration of *t*-BuOOH to which the cells were exposed (0.6 mM) was selected because it resulted in about 50% cell death in 1 hr under 1.07 mM O_2; this optimized the ability to measure O_2-dependent effects on *t*-BuOOH-induced toxicity.

O_2 Dependence of *t*-BuOOH-induced toxicity. Exclusion of trypan blue was measured as a function of time in cells maintained under various steady state O_2 concentrations (Fig. 1A). After introduction of *t*-BuOOH, cell death occurred more rapidly in anoxic than in oxygenated cells; by 2 hr, the anoxic cell population was almost completely nonviable but that at 20 μM O_2 was ≈50% viable. There was no significant loss in the viability of control cells within 2 hr at any O_2 concentration.

To provide a measure of the relative sensitivity of cells to *t*-BuOOH as a function of O_2 concentration, cell viability at 60 min was expressed as a function of O_2 concentration (Fig. 1B). Cells maintained at concentrations between 20 and 214 μM O_2 were most resistant to *t*-BuOOH-induced toxicity. This range is noteworthy because it includes the physiological range of O_2 concentrations in the liver (18). *t*-BuOOH-induced cell death was increased at O_2 concentrations below 20 μM and was maximal under anaerobic conditions. Hyperoxia also increased *t*-BuOOH toxicity although not as extensively as anoxia.

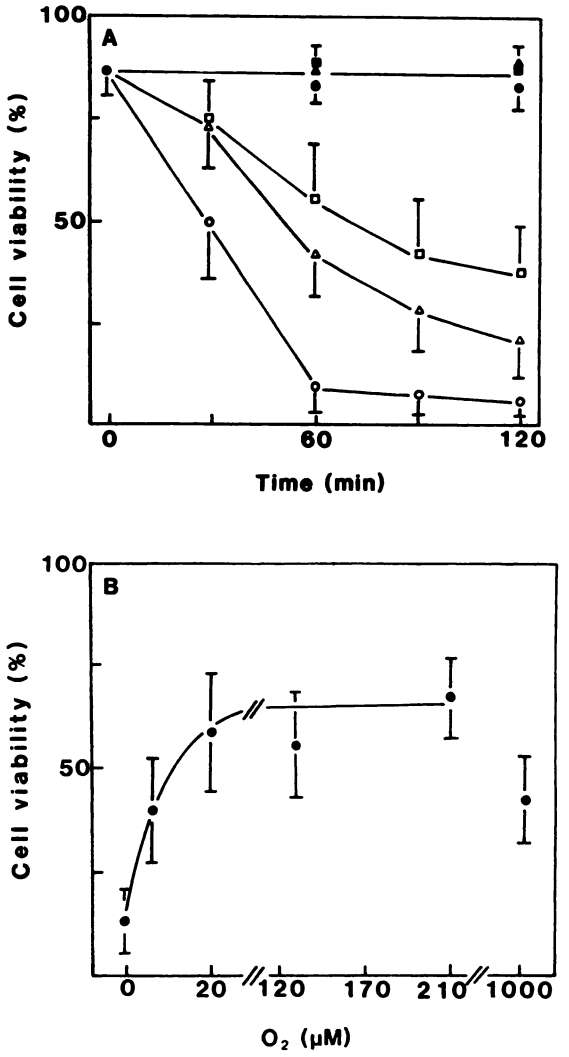

Fig. 1. O_2 dependence of *t*-BuOOH-induced toxicity. A, Time course of viability loss by hepatocytes as measured by trypan blue exclusion after introduction of 0.6 mM *t*-BuOOH. Cells (10^6/ml) were maintained under steady state O_2 concentrations of 0 μM (*circles*), 20 μM (*squares*), or 1.07 mM (*triangles*). *open symbols*, *t*-BuOOH-treated cells; *closed symbols*, control cells. B, Viability of cells 60 min after introduction of *t*-BuOOH for O_2 concentrations ranging from 0 μM to 1.07 mM. Values are the mean ± standard error of six cell preparations for all data points except 6, 125, and 214 μM O_2 (B), which represent three cell preparations. Values for 0 μM O_2 are significantly different ($p < 0.05$) from those for 20 μM and 1.07 mM O_2 at 60 min.

The O_2 dependence of *t*-BuOOH-induced toxicity is distinct from that for CCl_4 (19) and halothane (20), which are most toxic under hypoxic conditions, or paraquat (21), which is most toxic under hyperoxic conditions. In principle, three factors could contribute to the pattern of toxicity of *t*-BuOOH: a) deficient catabolism under hypoxic and anoxic conditions could result in prolonged exposure to the oxidant, b) generation of a toxic species could be enhanced under hypoxic and anoxic conditions, or c) there could be an increased susceptibility of hypoxic and anoxic cells due to alterations in protective or repair systems.

Metabolism of *t*-BuOOH. To investigate whether impaired *t*-BuOOH elimination could account for the observed O_2 dependence of *t*-BuOOH toxicity, we measured the rate of disappearance of *t*-BuOOH from cells at 0 μM, 20 μM, and 1.07 mM O_2. These three O_2 concentrations were selected as being representative of extremes in hepatocellular susceptibility to *t*-BuOOH (see Fig. 1B). As shown in Fig. 2, the O_2 concentration did not affect the rate of *t*-BuOOH elimination, indicating that the O_2 dependence of *t*-BuOOH toxicity is not due to variations in this rate or in the length of exposure of cells to *t*-BuOOH.

The metabolism of *t*-BuOOH by GSH peroxidase involves a 2-e^- reduction to *t*-butanol. As measured by gas chromatography, *t*-butanol was the only major volatile product from *t*-BuOOH in hepatocytes (data not shown). The rate of appearance of *t*-BuOOH was the same as that of *t*-BuOOH disappearance (35 nmol/10^6 cells/min) and there was no difference between anoxic and oxygenated (214 μM O_2) cells (Fig. 3). *t*-Butanol remained constant for up to 1 hr, indicating that its subsequent metabolism by hepatocytes is very slow. Addition of *t*-butanol (600 nmol/10^6 cells) to anoxic and oxygenated cells and measurement of the amount remaining at various times (Fig. 3, *inset*) confirmed that the cells do not catabolize *t*-butanol at an appreciable rate. These experiments further showed that at this concentration, *t*-butanol did not affect cell viability for up to 2 hr (results not shown). Thus, accumulation of *t*-butanol or a further metabolite of *t*-butanol metabolism is not responsible for the O_2 dependence of *t*-BuOOH-induced toxicity.

Measurement of *t*-BuOOH-dependent radical formation. In addition to the 2-e^- reduction of *t*-BuOOH to *t*-butanol, various systems present in cells are capable of catalyzing the formation of alkoxy and alkylperoxy radicals (22, 23). Both cytochrome P-450 and cytochrome *c* can catalyze homolytic scission of peroxides (see Ref. 24). Formation of *t*-butoxy and/or *t*-butylperoxy radical(s) at significantly elevated rates in the reductive environment of hypoxic and anoxic cells could result in enhanced toxicity. To examine whether *t*-BuOOH-induced free radical generation was enhanced under anoxic conditions, we compared the rates of spin-trapping of radicals by PBN in anoxic and oxygenated (214 μM O_2) cells.

Addition of *t*-BuOOH to cells preequilibrated with PBN

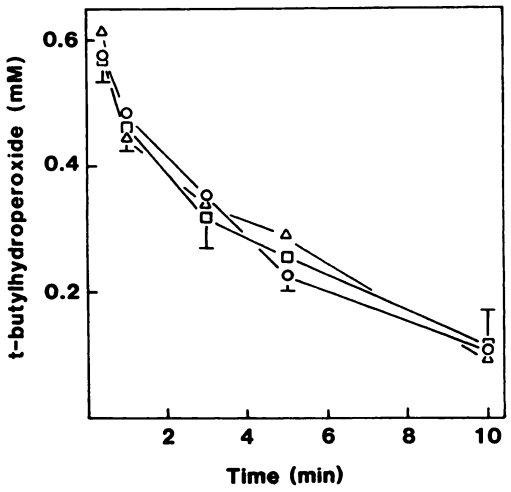

Fig. 2. Rate of *t*-BuOOH elimination by hepatocytes. The amount of peroxide remaining at various times after introduction of 0.6 mM *t*-BuOOH to hepatocytes maintained at 0 (○), 20 μM (□), or 1.07 mM O_2 (△) was measured by the GSH peroxidase, GSSG reductase recycling assay. Values represent means for five cell preparations. For clarity, standard error bars are given only for 20 μM O_2 values but were similar for all measurements. Values for different O_2 concentrations were not significantly different for any time points.

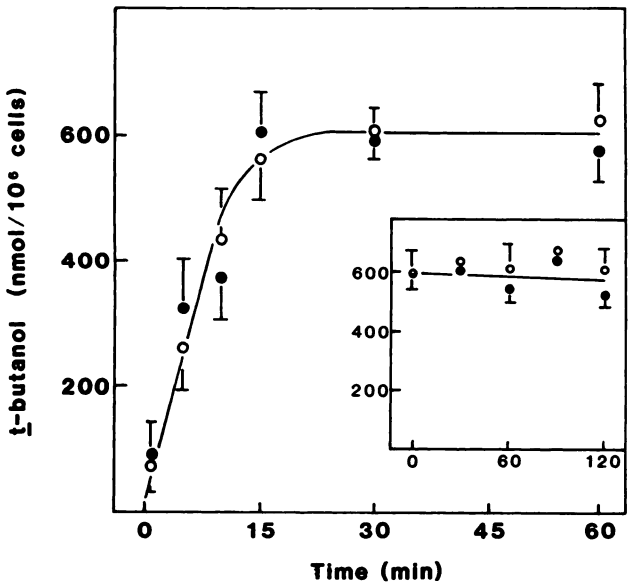

Fig. 3. Rate and extent of formation of *t*-butanol after introduction of *t*-BuOOH to isolated hepatocytes. At various times after introduction of 0.6 mM *t*-BuOOH to hepatocytes maintained at 0 μM (●) or 214 μM (○) O_2, 0.5-ml aliquots were removed for analysis of *t*-butanol content. Samples were prepared as described in Experimental Procedures and 2–4 μl were analyzed by gas chromatography. The *t*-butanol content of cells upon addition of *t*-butanol (0.6 mM) is shown in the *inset*. Values represent the mean ± standard error for three cell preparations and were not significantly different for cells at 0 or 214 μM O_2 for any time points.

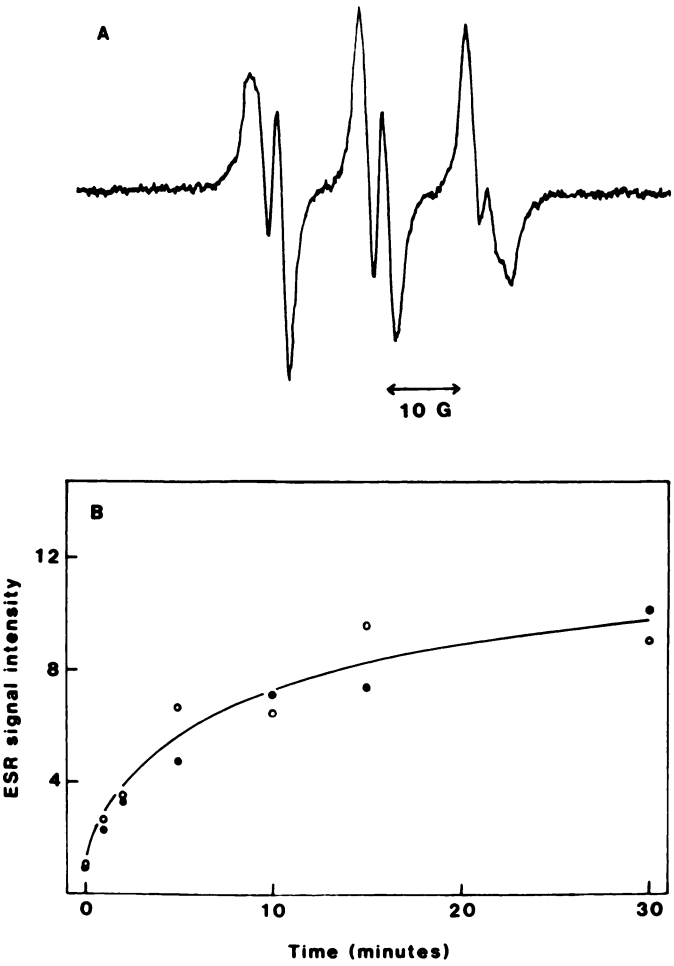

Fig. 4. Formation of PBN adducts after introduction of *t*-BuOOH to hepatocytes. PBN was added directly to cells incubated at 0 or 214 μM O_2 for 25 min to achieve a final concentration of 50 mM. After 5 min, 0.6 mM *t*-BuOOH was added to cells. PBN adducts were extracted and analyzed as described in Experimental Procedures. A, Spectrum obtained from aerobic cells at 10 min (gain, 10×10^6). B, Signal intensity of the PBN spectra with time after addition of *t*-BuOOH to cells at 0 μM (●) or 150 μM O_2 (○). At the early time points, values were corrected for the slight reaction of PBN with *t*-BuOOH during the extraction procedure; subtractions ranged from 5 to 22% of the signal intensity. Values shown are the average of five experiments. Standard errors ranged from 12 to 28% of the mean signal intensity.

resulted in the formation of a stable spin-adduct with a characteristic EPR spectrum (three doublets; see Fig. 4A) with the following hyperfine splitting constants: $a_N = 14.1$ G, $a_H^B = 3.1$ G. The PBN-adduct signal intensity increased rapidly after addition of *t*-BuOOH and approached a maximal value by 10 min (Fig. 4B), corresponding to the period of *t*-BuOOH elimination by cells. The mean signal intensity did not increase significantly after 10 min and in some experiments decreased by 10 to 15% between 10 and 30 min. Hepatocytes are able to reduce PBN-nitroxides, resulting in signal loss (13). However, as judged by the maintenance of the PBN-adduct signal for up to 30 min, the contribution of this effect appears to be minimal and is not likely to affect the interpretation of the present results. Addition of PBN to control hepatocytes did not cause time-dependent changes in signal intensity.

The rate and extent of PBN-adduct accumulation was similar in anoxic and oxygenated (214 μM) cells. Using diphenylpicrylhydrazyl as a standard, approximately 0.26 nmol of free radical spins per 10^6 cells were trapped by PBN during the active period of *t*-BuOOH metabolism with a $t_{1/2}$ of about 3 min. This accounted for about 0.02% of the *t*-BuOOH added to the cells. Thus, the rate and amount of radical formation due to *t*-BuOOH metabolism are independent of the O_2 concentration.

Because O_2-dependent differences in radical-initiated lipid peroxidation have been implicated in the toxicity of other hepatic agents (2, 19, 20), we examined the extent of *t*-BuOOH-induced lipid peroxidation in cells at 0 μM, 20 μM, and 1.07 mM O_2. As measured by the amount of thiobarbituric acid-reactive material, *t*-BuOOH-induced lipid peroxidation increased with increasing O_2 concentration (Fig. 5), consistent with the participation of O_2 in the reactions of peroxidation only at high O_2 concentrations. Thus, lipid peroxidation is not a primary factor in the increased toxicity of *t*-BuOOH under anoxic conditions but may be involved in the toxic process in hyperoxic cells. This is in contrast to studies of halothane- and CCl_4-induced toxicities (19, 20) in which reductive activation is coupled with O_2-dependent lipid peroxidation to create greatest toxicity under hypoxic but not anoxic conditions.

Alterations in GSH and pyridine nucleotide concentrations. *t*-BuOOH-induced depletion of GSH and NADPH in hepatocytes is well documented (see Ref. 25). To examine the effect of O_2 deficiency on *t*-BuOOH-dependent GSH loss, cellular GSH was measured as acid-soluble thiols at various times after addition of *t*-BuOOH under normoxic and anoxic conditions (Fig. 6A). Acid-soluble thiols were extensively decreased at 5 min but began to recover by 15 min. Thiol concentration at 5 min was significantly less by the paired *t* test ($p < 0.05$) in anoxic cells than in cells at 20 μM and remained significantly lower at 15 and 30 min ($p < 0.05$, $p < 0.01$, respectively). For comparison, data are also included for hyperoxic conditions (Fig. 6, *broken lines*). The thiol content for all

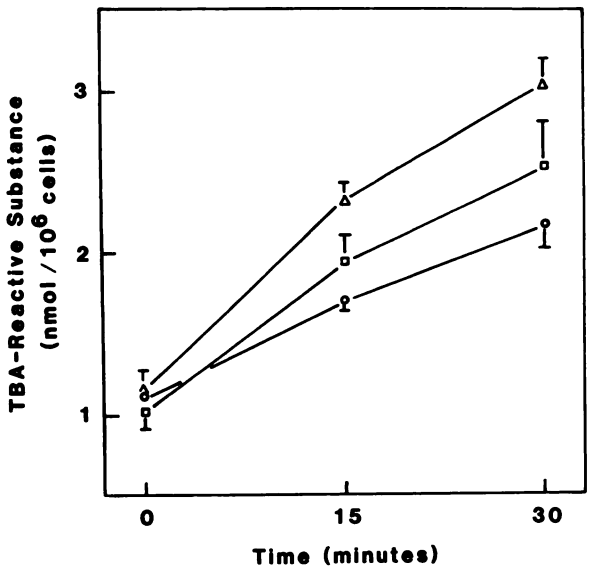

Fig. 5. Lipid peroxidation in hepatocytes exposed to *t*-BuOOH. The accumulation of thiobarbituric acid-reactive substance was measured as an indication of lipid peroxidation in cells at 0 μM (○), 20 μM (□), and 1.07 mM O_2 (△). Conditions of cell incubations were as described in the legend to Fig. 1. Values are mean ± standard error for three cell preparations.

hyperoxic incubations was between those of the anoxic and normoxic (20 μM) value (significantly different at $p < 0.05$ by paired analyses), indicating that the thiol recovery is optimal at the O_2 concentration that approximates the normal physiological value.

Because hepatocytes constantly release GSH and also release GSSG during oxidative stress, we examined whether loss of GSH could in part be due to impaired synthesis. GSH synthesis in isolated hepatocytes is limited by availability of precursor sulfur-containing amino acids (26) so cells were supplemented with either L-methionine (0.5 mM) or L-cysteine (0.25 mM). The acid-soluble thiol pool (Fig. 6A) was significantly higher when cysteine was included in the incubations at either 0 μM or 20 μM O_2 ($p < 0.05$); increase due to methionine was significant only at 30 min for the aerobic incubations. Measurement of GSH by HPLC at 30 min showed that the differences in the thiol content of amino acid-supplemented cells were due to differences in GSH (Table 1). For comparison, recovery under hyperoxic conditions are included in Fig. 6 and Table 1. The results show that recovery under hyperoxic conditions is also significantly greater than recovery under anoxic conditions. Thus, addition of GSH precursors to cell suspensions enhanced recovery of GSH, and anoxic cells showed an impaired ability to utilize these precursors for synthesis of GSH. Measurements of cell viability after 60 min showed that amino acid inclusions had little effect (increased viability, 0–10% of total cells) in normoxic and hyperoxic incubations, but viability (in percentage of total cells) in anoxic cells was 20% more than in their absence. Thus, in spite of the impaired synthesis under anoxic conditions, the small increase in synthesis was associated with a greater preservation of viability.

To determine whether the impaired recovery of GSH during anoxic could also be partially due to deficient reduction of GSSG back to GSH after exposure to the peroxide, we measured cellular GSH and GSSG by HPLC 30 min after addition of the hydroperoxide (Table 2). GSSG was slightly elevated in *t*-BuOOH-treated cells at all O_2 concentrations (statistically significant when analyzed relative to paired controls), and the GSH/GSSG ratio was decreased at all O_2 concentrations and was lowest under anoxic conditions. These changes could be due to impaired reduction of GSSG as a result of direct inactivation of GSSG reductase by the peroxide or due to limitations in supply of NADPH. To examine whether GSSG reductase was inactivated by the concentration of *t*-BuOOH used in these studies, purified GSSG reductase was incubated with 0.6 mM *t*-BuOOH at 37° for 15 min. Enzyme activity was not altered, suggesting that direct inhibition of the reductase was not the cause of the decreased GSH/GSSG ratio.

To examine whether the decreased GSH/GSSG ratio could be due to decreased availability of NADPH, we measured the effects of *t*-BuOOH on NADPH and $NADP^+$ pools in cells at 10 μM, 20 μM, and 1.07 mM O_2. The results showed that the extent of *t*-BuOOH-induced depletion and recovery of cellular NADPH varied with the O_2 concentration (Fig. 7). Ten min after addition of *t*-BuOOH, NADPH was decreased in all cells but was lower in anoxic and hyperoxic (1.07 mM O_2) cells than in cells maintained at 20 μM O_2 (Fig. 7). After 30 min, NADPH values remained decreased in anoxic cells but had essentially reestablished their original values at 20 μM O_2. However, analysis of $NADP^+$ showed that, even under normoxic conditions,

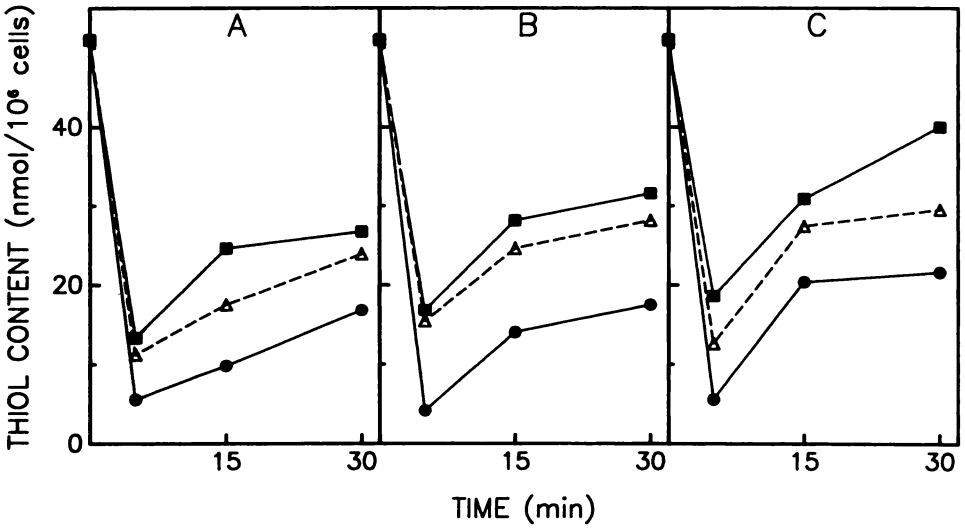

Fig. 6. Thiol loss and recovery in hepatocytes after addition of *t*-BuOOH. Cells were maintained at steady state O_2 concentrations of 0 μM (●), 20 μM (■), and 1.07 mM (△). Cell aliquots (0.5 ml) were removed at various times after addition of 0.6 mM *t*-BuOOH and thiol levels were determined by the Saville assay (15). Methionine, glycine, and glutamate (B) or cysteine, glycine, and glutamate (C) were added to the cell suspensions 30 min before introduction of *t*-BuOOH. Values represent nmol/10^6 viable cells and are given as means of five, four, and three cell preparations for A, B, and C. Standard errors (omitted for clarity) ranged from 5 to 23% of the mean value. Anaerobic cell values were significantly different from those for cells under 20 μM and 1.07 mM O_2 at 30 min with *p* values as follows: A, <0.01, <0.01; B, <0.01, <0.025; C, <0.05, <0.20.

TABLE 1

Effect of methionine or cysteine supplementation on the recovery of GSH in t-BuOOH-treated Cells

Incubations were performed as described in the legend to Fig. 6. Preparation of samples for HPLC analysis of GSH was performed as described in Experimental Procedures. Values are mean ± standard error for three cell preparations. For methionine-supplemented cells, anaerobic cell values are different from values for cells incubated at 20 μM and 1.07 mM O_2 at $p < 0.1$. For cysteine-supplemented cells, anaerobic cell values are significantly different from cells at 20 μM O_2 ($p < 0.05$).

O_2	GSH Content		
	−Amino acids	+Methionine	+Cysteine
μM	nmol/10^6 viable cells		
0	12.5 ± 3.2	23.7 ± 4.6	28.4 ± 4.2
20	18.2 ± 5.2	32.4 ± 7.9	35.8 ± 6.0
1070	19.7 ± 5.2	27.6 ± 1.3	35.1 ± 5.3

TABLE 2

Effect of t-BuOOH on cellular GSH and GSSG after 30 min

Cells were maintained at the designated O_2 concentrations for 30 min before introduction of t-BuOOH. Thirty minutes after addition of 0.6 mM t-BuOOH, cell aliquots (0.5 ml, 0.5 × 10^6 cells) were removed and derivatized for HPLC analysis of GSH and GSSG as described under Experimental Procedures. Control values were obtained from cells incubated for 60 min. Values are the mean ± standard error for four cell preparations and are expressed per 10^6 viable cells.

O_2	GSH	GSSG	GSH/GSSG
μM	nmol/10^6 cells		
Control			
0	29.0 ± 5.7	1.3 ± 0.7	22.4
20	32.0 ± 6.2	1.3 ± 0.8	24.6
1070	34.3 ± 8.3	1.0 ± 0.8	34.3
+0.6 mM t-BuOOH			
0	12.5 ± 3.2	1.6 ± 0.6	7.8
20	18.2 ± 5.2	1.9 ± 1.1	9.6
1070	19.7 ± 5.2	1.2 ± 1.0	16.4

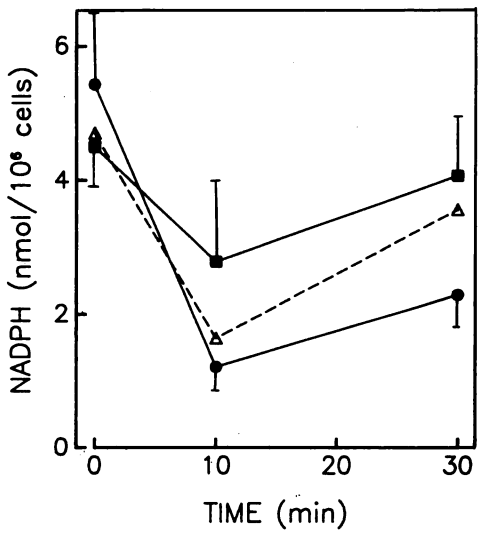

Fig. 7. NADPH loss and recovery in hepatocytes exposed to t-BuOOH. Cells were maintained at steady state O_2 concentrations of 0 μM (●), 20 μM (■), and 1.07 mM (△). At various times after addition of 0.6 mM t-BuOOH, cell aliquots (0.5 ml) were prepared for HPLC separation and analysis as described under Experimental Procedures. Values are means ± standard errors for four cell preparations expressed as nmol/10^6 viable cells. The anoxic cell value at 30 min is significantly different from the 20 μM value at $p < 0.01$. No significant differences occurred between control values.

the NADPH/$NADP^+$ ratio did not fully recover by 30 min (Table 3). t-BuOOH-induced loss of the reduced form was accompanied by a small but insignificant decrease in the total pool size (Table 3). Anoxic cells are therefore unable to maintain a normal supply of NADPH to maintain reduction of GSSG to GSH. Thus, both impaired GSH synthesis and GSSG reduction appear to contribute to poor recovery of the normal GSH homeostasis after oxidative stress under anoxic conditions.

Discussion

The toxicity of several compounds that cause oxidative injury varies with the O_2 concentration. Hepatotoxic halocarbons such as CCl_4 and halothane are more injurious to hypoxic than to normoxic tissue because the reduced state of the cell potentiates their reductive activation (19, 20). Toxicity is decreased at higher O_2 concentrations, which favor oxidative metabolism. Conversely, toxicity is limited as tissues become anoxic because O_2 is required for other deleterious reactions, such as lipid peroxidation (2). In contrast, the toxicity of some compounds increases monotonically with the O_2 concentration. Paraquat is more toxic to cells as the O_2 pressure increases up to 0.2 atm (21), apparently as a consequence of increasing production of O_2^- and H_2O_2. Thus, during exposure of cells to various chemical agents, hypoxia or hyperoxia may potentiate oxidative injury by increasing production of potent oxidants.

Because organic hydroperoxides directly cause oxidative cell death, they provide a convenient means to study the O_2 dependence of oxidative stress. Their use minimizes the possibility that the measured O_2 dependence is an O_2 dependence of generation of a potent oxidant and allows one to examine the importance of the O_2 dependence of metabolism and cellular protective mechanisms. In the current study with t-BuOOH, we found a direct pattern for the O_2 dependence of toxicity, i.e., toxicity was enhanced under hypoxic conditions and was maximal in anoxic cells. This pattern was consistent with the pattern of toxicity of t-BuOOH in cultured hepatocytes (6) and unlike the O_2 dependence of the toxicity of CCl_4, halothane, or paraquat, for which the O_2 dependence of toxicity is related to O_2-dependent alterations in the generation of potent oxidants.

t-BuOOH was converted almost completely to t-butanol in

TABLE 3

Effect of t-BuOOH on the cellular content of NADPH and $NADP^+$ after 30 min

Cell incubations and sample preparation were preformed as described in the legend to Fig. 7 and in Experimental Procedures. Values are per 10^6 viable cells given as the mean ± standard error for four cell preparations with addition of t-BuOOH or three cell preparations for control values of NADPH without the peroxide and two preparations for control values of $NADP^+$ without peroxide.Values are different from the corresponding 20 μM O_2 values as indicated in footnotes.

O_2	NADPH	$NADP^+$	NADPH + $NADP^+$	reduced/oxidized
μM	nmol/10^6 viable cells			
Control				
0	4.2 ± 0.7	0.52	4.7	8.0
20	4.4 ± 0.6	0.58	5.0	7.6
1070	4.4 ± 0.5	0.58	5.0	7.6
+0.6 mM t-BuOOH				
0	2.4 ± 0.4[a]	2.6 ± 0.5[b]	5.0	0.9[a]
20	4.1 ± 0.6	1.1 ± 0.4	5.2	3.7
1070	3.5 ± 1.1	1.2 ± 0.6	4.7	2.9

[a] $p < 0.01$.
[b] $p < 0.05$.

anoxic and oxygenated cells, and this metabolite was not toxic to cells at the concentrations used in these studies. The rate of peroxide conversion to *t*-butanol was independent of the O_2 concentration; thus, a variation in the length of exposure to the peroxide cannot account for the enhanced toxicity under anoxic conditions. The lack of an O_2-dependent effect on conversion to *t*-butanol in spite of a substantial decrease in GSH concentration is surprising given that the major GSH peroxidase has a ping-pong mechanism (27). This observation, as well as a comparison of rates of *t*-BuOOH loss (35 nmol/10^6 cells/min) and maximal rates of NADPH supply by hepatocytes under these conditions (8 nmol/10^6 cells/min),[1] indicate that a substantial portion of *t*-BuOOH is being metabolized by a pathway other than the well studied GSH peroxidase/GSSG reductase system.

As determined by the formation of spin adducts upon addition of PBN and *t*-BuOOH to cells, *t*-BuOOH-associated free radical production was not enhanced by hypoxia. Therefore, the rate of radical formation is not a factor in the hypoxic and anoxic potentiation of *t*-BuOOH-induced toxicity. The asymmetry of the EPR signal suggests that more than one radical was trapped by PBN. PBN can react with the alkylperoxy, alkoxy, and alkyl radicals of *t*-BuOOH as well as with HO· and HOO· liberated by homolytic scission of *t*-BuOOH (28, 29). Although no difference was noted in the degree of asymmetry of the signal obtained from anoxic and oxygenated cells, the type of radical formed could be different in anoxic and oxygenated cells and these radicals could have different reactivities. Characteristics of the EPR spectrum may not be indicative of the initial radical(s) trapped because alkylperoxy and alkoxy PBN-spin adducts are known to decompose to carbon-centered spin adducts at ambient temperatures (28, 30). Thus, additional studies with spin-trapping agents that gave characteristic adducts are needed to identify the radicals formed in anoxic and oxygenated cells, but the data presented show that the rates of radical trapping by PBN are the same and therefore indicate that the enhanced toxicity under anoxic conditions is not due to substantial differences in free radical production.

Measurement of thiobarbituric acid-reactive substance as an estimate of lipid peroxidation further indicates that radical-initiated processes are not responsible for the enhanced toxicity under anoxic conditions. Lipid peroxidation was minimal in anoxic cells, increased with O_2 concentration, and did not correlate with cell death. This is distinct from the O_2 dependence of lipid peroxidation that occurs with halothane and CCl_4, which are reductively activated and have maximal lipid peroxidation under hypoxic O_2 concentrations (19, 20). The lack of association between the O_2 dependence of toxicity of *t*-BuOOH and radical production suggests that the basis for the O_2 dependence of toxicity is distinct from those for other compounds (e.g., CCl_4, halothane).

The anoxic cell is metabolically and energetically distinct from normoxic cells; many of the associated metabolic and energetic changes have been summarized in terms of a neahypoxic state, wherein the functional capacities of metabolic systems in hypoxic and anoxic cells are suppressed to prolong survival and allow for recovery upon restoration of O_2 supply (31). Cellular ATP content is decreased, ionic distributions are perturbed, and metabolic pools, such as adenylates and pyridine nucleotides, are decreased (2–4, 31). The current study shows that these changes can limit the ability of cells to respond to oxidative injury. Both GSH and NADPH pools are decreased by exposure to *t*-BuOOH and these pools do not recover by 30 min. Decreased NADPH in anoxic cells may be related to a decreased energy-dependent transhydrogenation and/or to impaired function of the pentose phosphate pathway due to the limited supply of glucose 6-phosphate that is available in hypoxic cells (32). An inadequate supply of NADPH results in an impaired ability to reduce GSSG to GSH (26). However, it must be noted that, in the data shown in Table 2, there is little difference in the GSH/GSSG ratio for cells treated with *t*-BuOOH under anoxic conditions as compared with normoxic (20 μM) conditions. Although this indicates that GSH/GSSG may be a relatively unimportant determinant of toxicity under these conditions, it may also be a consequence of multiple pools of GSH within the cell, which are not equally affected by the *t*-BuOOH. In recent experiments, we have found that the mitochondrial fraction of hepatocytes, obtained by digitonin fractionation, is more dramatically affected under these conditions by *t*-BuOOH than is the whole cell contents as presented here.[2]

The rate of resynthesis of GSH from precursor amino acids is also impaired during anoxia and may be due to decreases in the ATP concentration required for synthesis from cysteine, glutamate, and glycine. The improved recovery of GSH under anoxia with cysteine as compared with methionine also suggests that the transsulfuration pathway may be limited by hypoxia. The decreased toxicity in cells supplied with the precursor amino acids shows that GSH synthesis is an important factor in recovery of cells from oxidant exposure.

t-Butylhydroperoxide is widely used for studies of pathological processes involving oxidative injury. Although the details of oxidative injury have not been definitively resolved, imbalances in oxidation and reduction are known to occur during injury by ionizing radiation (33), halogenated hydrocarbons (19, 20), redox-cycling compounds (24), hyperoxia (21), and reperfusion of ischemic tissues (34). When generation of oxidants exceeds the capacity of GSH-dependent reductant systems to maintain normal redox status, protein thiol oxidation can occur. Because Ca^{2+} transport systems are among the macromolecular targets of oxidation and are inactivated by oxidants, perturbation of Ca^{2+} homeostasis occurs and may be ultimately responsible for irreversible injury (17). Thus, in addition to the impaired ability of hypoxic cells to maintain GSH pools, a perturbation in the ability of hypoxic or anoxic cells to maintain Ca^{2+} homeostasis could also render these cells more susceptible to oxidative injury (2). In earlier studies of the effects of anoxia on mitochondrial Ca^{2+}, we found that this pool is decreased by 50 to 70% after 30 min of anoxia (35). Thus, the possibility remains that the enhanced susceptibility to oxidative injury during O_2 deficiency involves altered Ca^{2+} distribution as well as impaired recovery of normal thiol status.

Tissue hypoxia is commonly encountered in the clinical setting in association with obstructive lung diseases, anemias, and pathophysiological and iatrogenic vascular obstructions. Considerable research during recent years has been focused on the possibility that oxidative injury upon reperfusion after

[1] D. L. Tribble and D. P. Jones. Oxygen dependence of oxidative stress: rate of NADPH supply for maintaining the GSH pool during hypoxia. Manuscript submitted.

[2] D. L. Tribble, T. Y. Aw, and D. P. Jones, unpublished.

ischemia can account for much of the injury resulting from vascular obstructions and efforts have been focused on measures to limit generation and/or stimulate elimination of oxidants. The current results show that in combination with efforts to decrease the concentrations of oxidants to which cells are exposed during these pathological processes, efforts should be made to prevent the loss of detoxication and repair processes that are essential for preservation of the viable state.

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