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HYPOXIA AND OXYGEN DEPENDENCE OF CYTOTOXICITY IN RENAL PROXIMAL TUBULAR AND DISTAL TUBULAR CELLS

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Abstract—Ischemia and hypoxia are major causes of renal failure and altered oxygen supply may affect renal responses to toxic chemicals. *In vitro* experiments were designed to evaluate the susceptibility of isolated proximal tubular (PT) and distal tubular (DT) cells from rat kidney to brief periods of oxygen deprivation and to assess how variations in oxygen supply affect chemical-induced cytotoxicity. Isolated cells were incubated for 1 hr in either oxygen (95% O_2/5% CO_2), air (21% O_2), or nitrogen (95% N_2/5% CO_2) atmosphere. PT cells exhibited no injury due to brief oxygen deprivation whereas DT cells exhibited moderate, but significant injury, indicating that DT cells are more susceptible than PT cells to hypoxic injury. The cytotoxicity of chemicals that alter cellular redox status [i.e. *tert*-butyl hydroperoxide (tBH), menadione, methyl vinyl ketone] and the cytotoxicity of "chemical hypoxia" [i.e. KCN + iodoacetic acid] were greatest in air, intermediate in oxygen, and lowest in nitrogen. In contrast, the cytotoxicity of the alkylating agent *N*-dimethylnitrosamine was independent of oxygen concentration and the cytotoxicity of *p*-aminophenol was related directly to oxygen concentration. The mechanism of the oxygen dependence of chemical injury was investigated further, employing tBH as a model toxicant. tBH metabolism was oxygen independent in both PT and DT cells. Depletion of cellular protein sulfhydryl groups by tBH increased with increasing oxygen concentration and lipid peroxidation due to tBH was inhibited in nitrogen but was not different in air as compared with oxygen. Although these processes may contribute to the much lower toxicity in nitrogen as compared with oxygen, it does not explain the higher toxicity in air as compared with that in oxygen. Other processes that predominate at lower oxygen concentrations but that only produce injury if enough oxygen is present are likely to be responsible for the enhanced susceptibility of both PT and DT cells to oxidants in air as compared with oxygen.

Renal function is dependent on a constant supply of oxygen for ATP production and normal physiological function [1, 2]. Contributing factors to this dependence are the high rate of renal blood flow, the large number of active transport systems in renal plasma membranes, and the presence of energy-dependent biosynthetic pathways, such as gluconeogenesis, in certain renal cell populations. If the oxygen supply to the kidneys is disrupted, due to either decreased blood flow (i.e. ischemia) or decreased oxygen content of blood (i.e. simple hypoxia), renal function will be altered and injury may occur [1]. Supranormal oxygen concentrations (i.e. hyperoxia) may also lead to injury by increasing cellular oxidative stress. Individual renal cell populations, because of functional specialization and site-specific metabolic requirements, may exhibit characteristic susceptibility to altered oxygen supply. For example, the pars recta (S3) segment of proximal tubular (PT)† cells and the medullary thick ascending limb cells in isolated, perfused kidneys are particularly susceptible to injury from hypoxia [3–7].

While cellular injury may occur directly from altered oxygen supply, other mammalian cells, such as isolated rat hepatocytes, are resistant to limited periods of anoxia or hypoxia [8–11]. Although cells may not exhibit overt signs of cytotoxicity, their biochemical or physiological function may be altered so that their responses to exogenous stimuli, such as cytotoxic chemicals, may be modified. The oxygen dependence of processes such as bioactivation and detoxification reactions [12, 13] and the oxygen dependence of secondary cellular responses, such as lipid peroxidation, may be important in determining how cellular injury is affected by oxygen supply. The occurrence of many forms of cellular injury, not during an ischemic or hypoxic episode, but only during reflow or reoxygenation [13], and the involvement of reactive oxygen metabolites in cellular injury due to ischemia [14], suggest that oxygen-dependent mechanisms play an important role in the biochemical mechanism of injury.

We have developed an *in vitro* model to study cellular function in two distinct populations of cells from rat renal cortex, one derived from the PT region and one from the distal tubular (DT) region [15, 16]. With these two cell preparations, we

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† Abbreviations: PT, proximal tubular; DT, distal tubular; LDH, lactate dehydrogenase; GSH, glutathione; GSSG, glutathione disulfide; PrSH, protein sulfhydryl group; MDA, malondialdehyde; MVK, methyl vinyl ketone; NDA, *N*-dimethylnitrosamine; tBH, *tert*-butyl hydroperoxide; MD, menadione; IAA, iodoacetate; and PAP, *p*-aminophenol.

characterized and compared the effects of specific nephrotoxicants, such as cephaloridine [15], and of different classes of toxic chemicals, such as oxidants [17] and alkylating agents [18], on cellular viability and function under standard incubation conditions, which include an atmosphere of 95% O_2/5% CO_2. In these studies, we identified differences in the susceptibility of PT and DT cells to several types of injury.

In the current studies, three general questions were explored: (1) are renal PT and DT cells susceptible to injury from brief periods of hypoxia or hyperoxia? (2) is the injury that is produced by various chemicals oxygen dependent? and (3) what are some of the cellular processes responsible for the oxygen dependence of *tert*-butyl hydroperoxide (tBH)-induced cytotoxicity? PT and DT cells were incubated in either oxygen (95% O_2/5% CO_2), air (21% O_2), or nitrogen (95% N_2/5% CO_2) atmosphere in the presence and absence of selected chemical toxicants. Irreversible cellular injury [release of lactate dehydrogenase (LDH)] and cellular redox status [i.e. protein sulfhydryl (PrSH) concentrations, lipid peroxidation] were measured. We found that whereas PT cells were relatively resistant to injury from 60 min of hypoxia, DT cells exhibited significantly increased release of LDH after 60 min of incubation in the nitrogen atmosphere as compared with the air or oxygen atmosphere. Susceptibility of both cell populations to several classes of cytotoxic chemicals was markedly oxygen dependent. Oxidants and chemical hypoxia were most toxic in air, somewhat less toxic in oxygen, and much less toxic, if at all, in nitrogen. Detoxification of tBH was not affected by oxygen supply, indicating that metabolism has no role in oxygen dependence of cytotoxicity. In contrast, tBH-induced changes in lipid peroxidation and PrSH depletion were markedly oxygen dependent. This indicates that these pathological processes may determine the extent of injury in different oxygen-containing atmospheres. A preliminary report of this work has been presented [19].

MATERIALS AND METHODS

Materials. Percoll, collagenase (type I; EC 3.4.24.3), GSH peroxidase (glutathione:hydrogen-peroxide oxidoreductase; EC 1.11.1.9), GSSG reductase (NADPH:oxidized-glutathione oxido-reductase; EC 1.6.4.2), catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase; EC 1.11.1.6) from bovine liver, tBH, menadione (MD), methyl vinyl ketone (MVK), *N*-dimethylnitrosamine (NDA), *p*-aminophenol (PAP), 5,5'-dithio-bis-(2-nitrobenzoic acid), and 1,1,3,3-tetraethoxypropane were purchased from the Sigma Chemical Co. (St. Louis, MO). 4,6-Dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid) was purchased from the Aldrich Chemical Co. (Milwaukee, WI).

Isolation of rat renal PT and DT cells. Isolated renal cortical cells were obtained by collagenase perfusion [20] from male Fischer 344 rats (200–300 g; Charles River Laboratories, Wilmington, MA). Prior to surgery, rats were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg body weight). Animals were housed in the Wayne State University vivarium, were allowed access to food and water *ad lib.*, and were kept in a room on a 12-hr light–dark cycle. To obtain enriched populations of PT and DT cells, cortical cells were subjected to density-gradient centrifugation in Percoll, as previously described [15]. Briefly, cortical cells (5 mL, 5 to 8 × 10^6 cells/mL) were layered on 35 mL of 45% (v/v) isosmotic Percoll solution in 50-mL polycarbonate centrifuge tubes and were centrifuged at 4° for 30 min at 20,000 *g* in a Sorvall RC2B centrifuge in an SS34 rotor. The density gradient produced (1.016 to 1.120 g/mL) was continuous and concave with an inflection point at 1.057 g/mL. PT cells (1.02 to 1.05 g/mL) and DT cells (1.08 to 1.12 g/mL) were identified by measurement of marker enzyme activity distributions [15]. On this basis, purity of the PT cell preparation was estimated to be 97% and that of the DT cell preparation was estimated to be 88%. Qualitative assessments of cell-type identity, such as the response of cellular respiration rates to certain metabolites or metabolic inhibitors were also performed to confirm the identity of the two cell populations [15, 16].

Before incubations, cells were diluted 5-fold with Krebs–Henseleit buffer, pH 7.4, containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 2% (w/v) bovine serum albumin, and metabolic substrates (5 mM glucose, 5 mM glutamine), were washed to remove Percoll, and were resuspended in fresh buffer at concentrations of 1 to 4 × 10^6 cells/mL. Cell concentrations were determined in the presence of 0.2% (w/v) trypan blue in a hemacytometer, and cell viability was estimated by measuring the release of LDH activity from the cells in the presence and absence of detergent [21]. All buffers were equilibrated with 95% O_2/5% CO_2 and incubations were performed at 37° under an atmosphere of either 95% O_2/5% CO_2 (oxygen), air (21% O_2), or 95% N_2/5% CO_2 (nitrogen) as indicated, in a Dubnoff metabolic shaking incubator (60 cycles/min).

Experimental protocol. Before any experiments were performed, isolated PT and DT cells were placed in 25-mL plastic Erlenmeyer flasks and the flasks were sealed with serum bottle stoppers. The atmosphere was equilibrated with 95% O_2/5% CO_2 by gassing for 1 min, and the cells were preincubated for 15 min at 37° with either Krebs–Henseleit buffer or other additions as indicated. This preincubation period served to stabilize several parameters in the cell preparations, such as adenine nucleotides and GSH concentrations, and thus provided for better cells. The 15-min preincubation time was chosen based on previous studies with freshly isolated renal cortical cells and with the purified PT and DT cell preparations [15–18, 20, 21]. Additions to cell suspensions were made from concentrated stock solutions so that cells were minimally diluted. The 95% O_2/5% CO_2 (oxygen) atmosphere is the one that is typically used in isolated renal cell preparations to ensure adequate oxygen supply to all cells in suspension [21]. In subsequent experiments that were performed in either oxygen, air, or nitrogen atmosphere, isolated cells were placed in 25-mL Erlenmeyer flasks and the atmosphere was equilibrated with the appropriate gas mixture by

gassing for 1 min. Measurement of dissolved O_2 concentrations in cell suspensions on a Gilson 5/6H oxygraph with a Clark-type electrode at 37° showed that 1 min was sufficient to produce stable pO_2 values; gassing for longer periods of time resulted in little change in pO_2. In cell suspensions gassed with the nitrogen atmosphere, the final pO_2 was approximately 10 μM. Because of the configuration of the incubation flasks, the volume of cells added, and the endogenous respiration rates of the two cell populations, changes in oxygen concentration in the airspace above the cells in the incubation flasks during the 60-min incubations were not significant.* Thus, the isolated cells in suspension were exposed to essentially constant (<3% decrease) oxygen concentrations during incubations.

Assays. Intracellular concentrations of PrSH groups were measured by a spectrophotometric method based on that of Ellman and Lysko [22]. Aliquots of cells (0.5 mL) were layered on 1.0 mL of 20% (v/v) Percoll in saline and centrifuged in a microcentrifuge for 30 sec at 10,000 *g*; then the cells were resuspended in 0.5 mL of saline. Trichloroacetic acid (0.1 mL of 30%, w/v) was added to precipitate protein and samples were centrifuged in a microcentrifuge for 2 min at 10,000 *g*. Protein pellets were resolubilized in 0.5 mL of 0.1 M NaOH and pH was adjusted to between 6.0 and 8.0 with HCl. Aliquots of resolubilized protein pellets were mixed with 5,5'-dithio-bis-(2-nitrobenzoic acid) (1 mg/mL, final concentration), absorbance was measured at 410 nm (= *A*1), a small volume of a saturated aqueous solution of *N*-ethylmaleimide was then added, and absorbance was measured again at 410 nm (= *A*2). PrSH concentration was determined from the ΔA_{410} (= *A*1 − *A*2) using a molar extinction coefficient for *p*-nitrothiophenolate anion of 13,600 $M^{-1}\,cm^{-1}$.

Lipid peroxidation was estimated by measuring the formation of malondialdehyde (MDA) according to Stacey *et al.* [23]. Aliquots of cell suspensions (0.5 mL) were mixed with 0.5 mL of 10% (w/v) trichloroacetic acid and 1.0 mL of 0.76% (w/v) thiobarbituric acid and the mixtures were heated for 15 min. After cooling to room temperature, insoluble material was removed by centrifugation, and the absorbance at 532 nm was measured in supernatants. Acid hydrolysates of 1,1,3,3-tetraethoxypropane were used as MDA standards. Standard curves were linear (regression line: $y = 0.00280 + 0.05624x$; $r^2 = 1.000$) between 0.2 and 10 nmol MDA per mL.

Metabolism of tBH was measured by equating tBH content after various incubation times with NADPH oxidation rate, as measured by a GSH peroxidase/GSSG reductase coupled, spectrophotometric assay [24]. When measured in the presence of catalase, the assay measures tBH content only; in the absence of catalase, the assay measures all peroxides, including reactive oxygen metabolites generated in cells during exposure to tBH.

Statistics. All values are the means ± SEM of measurements made on the indicated number of separate cell preparations. Significant differences between means for data obtained in either PT or DT cells were first assessed with either a one-way or a two-way analysis of variance. When significant "F-values" were obtained with the analysis of variance, the Fisher's protected least significant difference *t*-test was performed to determine which means were significantly different from one another with two-tail probabilities of less than 0.05 considered significant.

RESULTS

Modulation of chemical toxicity by oxygen concentration. The oxygen dependence of many bioactivation and detoxification reactions in mammalian cells [12, 13] suggests that alterations in oxygen supply to cells will alter xenobiotic metabolism and disposition and, hence, will alter the susceptibility of cells to chemical-induced cytotoxicity. To investigate this hypothesis, isolated renal PT and DT cells were incubated for 1 hr in either oxygen, air, or nitrogen atmosphere and in the presence of either buffer or various test chemicals, all shown previously [15–18] to produce cytotoxicity in these cell populations (Figs. 1–3).

PT cells incubated with only buffer for 1 hr exhibited no significant increases in LDH release in either air or nitrogen atmosphere as compared with oxygen atmosphere (Fig. 1A). This indicates that PT cells are resistant to injury from brief periods of lowered oxygen concentration. In contrast, DT cells incubated with only buffer for 1 hr did exhibit significant increases in LDH release in both air and nitrogen atmosphere (34.0 and 39.5% LDH release, respectively) as compared with oxygen atmosphere (25.0% LDH release) (Fig. 1B). DT cell viability is thus moderately, but significantly diminished by brief periods of incubation in oxygen concentrations below that typically employed in isolated cell preparations (i.e. 95% O_2).

tBH and MD were used as model agents that produce toxicity by causing oxidative stress. Incubation of isolated PT cells with 1 mM tBH for 1 hr produced a significant increase in LDH release as compared with buffer-incubated cells in both oxygen and air (Fig. 1A). There was no significant difference in the response in the two atmospheres, although LDH release was slightly higher in air than in oxygen. Under nitrogen, however, tBH did not produce any increases in LDH release. Incubation of isolated PT cells with 1 mM MD for 1 hr produced

* The ideal gas law was used to calculate the amount of oxygen available in the 25-mL Erlenmeyer flasks used for incubations. When capped with a rubber serum bottle stopper, the total volume of the flasks is 31 mL. Since incubation volumes were generally 2 mL, the airspace above the cells was 29 mL. Using the gas law, $n = pV/RT$, where n is the number of moles of the gas, p is the pressure in atm, V is the volume in liters, R is the gas constant in atm · liter · °K^{-1} · mol^{-1}, and T is the temperature in °K, n = (1 atm)(0.029 liter)/(8.205 × 10^{-2} atm · liter · °K^{-1} · mol^{-1})(310°K) = 1.14 × 10^{-3} mol O_2 if the atmosphere is composed of 100% O_2. If the average basal rates of oxygen consumption for PT and DT cells are 15 and 25 nmol O_2/min per 10^6 cells, respectively [15], and each incubation flask contains 2 mL of cells at 2 × 10^6 cells/mL, total oxygen consumption in 1 hr will be at most 3.6 and 6.0 μmol in PT and DT cells, respectively. Even in an air atmosphere (i.e. 21% O_2), less than 3% of the total available oxygen will be consumed during the 1-hr incubation.

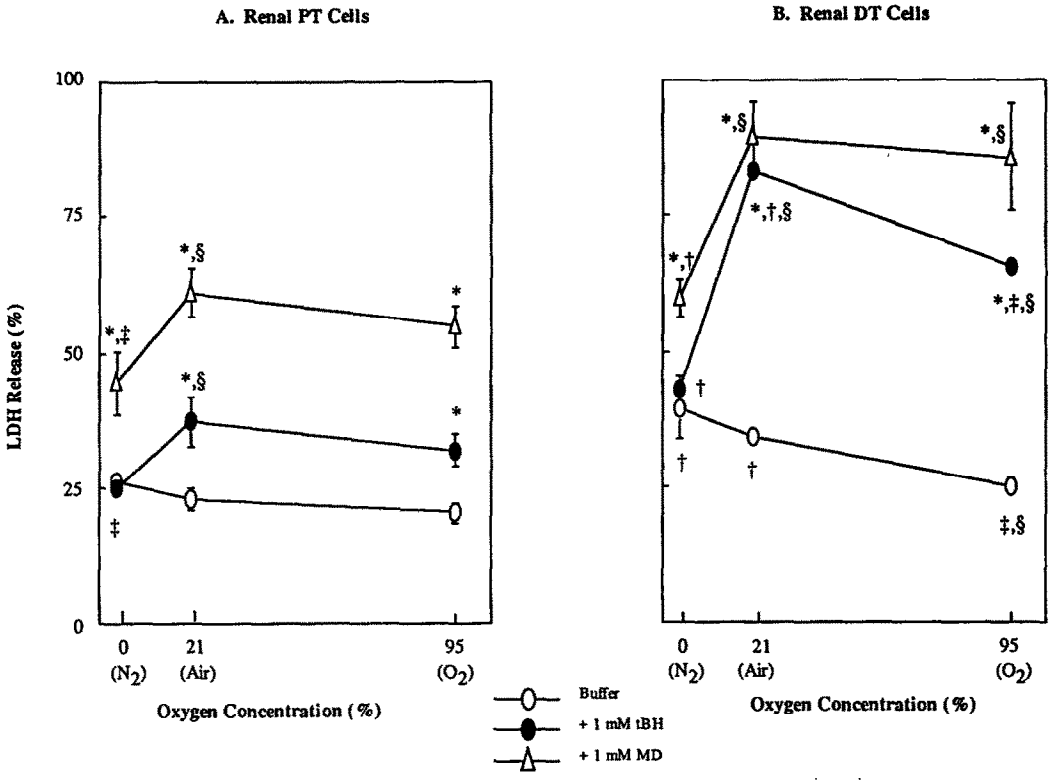

Fig. 1. Oxygen dependence of cytotoxicity caused by tBH and MD. Isolated PT (A) and DT (B) cells (2 to 3 × 10^6 cells/mL) were incubated at 37° in an atmosphere of either 95% O_2/5% CO_2 (= oxygen), air, or 95% N_2/5% CO_2 (= nitrogen) for 60 min. LDH release was measured after incubating cells for 60 min with either buffer, 1 mM tBH, or 1 mM MD. Results are the means ± SEM of incubations from 4 to 7 separate cell preparations. Key: (*) significantly different ($P < 0.05$) from the corresponding values for cells incubated with buffer in the same atmosphere; (†) significantly different ($P < 0.05$) from sample incubated with the same addition in oxygen; (‡) significantly different ($P < 0.05$) from sample incubated with the same addition in air; and (§) significantly different ($P < 0.05$) from sample incubated with the same addition in nitrogen.

a similar pattern of LDH release in different atmospheres; the only difference between tBH and MD was that under nitrogen, MD still produced a significant increase in LDH release versus cells incubated with buffer, although the increase was significantly lower than that produced in air. Qualitatively similar responses were observed with tBH and MD in isolated DT cells, although greater toxicity occurred as compared with PT cells, as shown previously [16, 17], and the difference in LDH release from DT cells due to tBH was significantly greater in air than in oxygen (Fig. 1B).

Alkylating agents such as MVK produce cytotoxicity in PT and DT cells by a mechanism that includes alterations in cellular thiol-disulfide status [18]. The oxygen dependence of cellular redox status suggests that MVK-induced cytotoxicity may vary with cellular oxygen supply. Other alkylating agents, such as NDA, produce cytotoxicity in PT and DT cells by a mechanism that is independent of cellular redox status [18], which suggests that NDA-induced cytotoxicity may not be affected by cellular oxygen supply. These hypotheses were tested by incubating PT and DT cells with either 1 mM MVK or 1 mM NDA for 1 hr in either oxygen, air, or nitrogen atmosphere and measuring LDH release (Fig. 2). NDA produced small increases in LDH release in both cell populations, and cytotoxicity exhibited no apparent dependence on oxygen concentration. MVK-induced cytotoxicity, in contrast, exhibited a small oxygen dependence in PT cells but a marked oxygen dependence in DT cells; in both cell populations, LDH release tended to be higher in air than in oxygen; in DT cells, MVK-induced cytotoxicity was completely eliminated in nitrogen atmosphere.

To determine if other mechanisms of chemical-induced cytotoxicity also exhibit an oxygen dependence similar to or different from that described above for oxidants and certain alkylating agents, LDH release induced by two other model toxicants was assessed in oxygen, air, and nitrogen atmospheres (Fig. 3). We used the model of "chemical hypoxia," which involves incubation with 1 mM KCN + 1 mM iodoacetate (IAA) to simultaneously inhibit cellular energy metabolism by oxidative phosphorylation and glycolysis, respectively. We found previously that DT cells were much more sensitive to injury

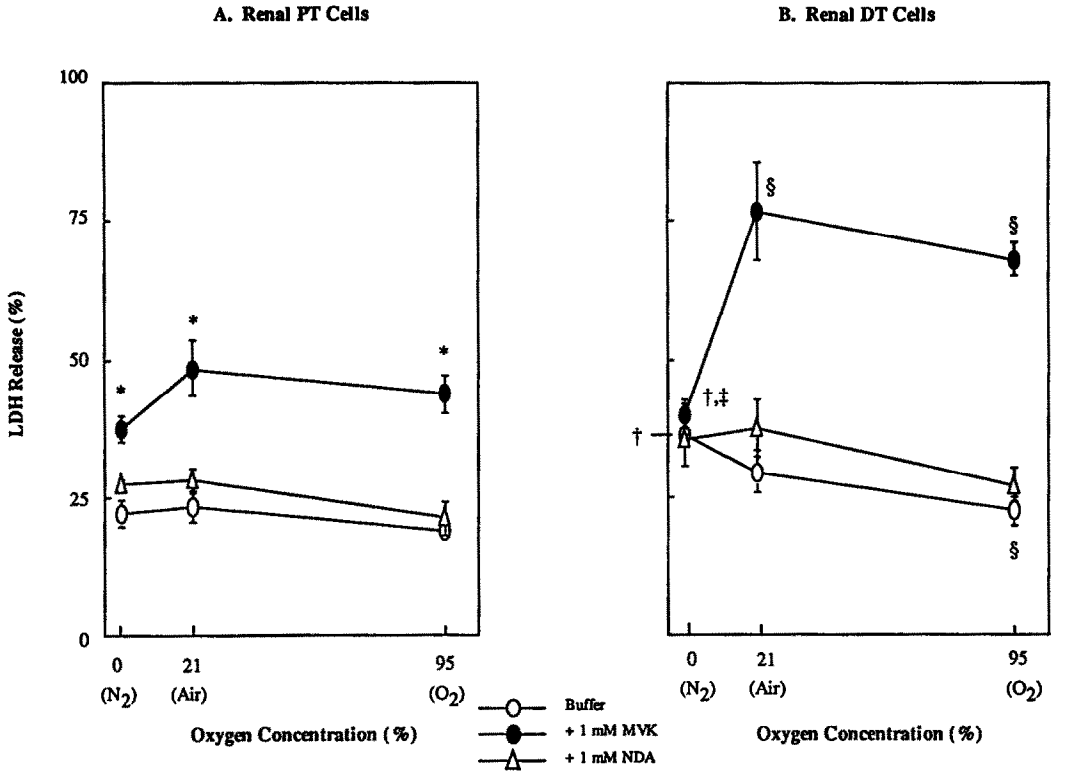

Fig. 2. Oxygen dependence of cytotoxicity caused by MVK and NDA. Isolated PT (A) and DT (B) cells (2 to 3×10^6 cells/mL) were incubated at 37° in an atmosphere of either 95% O_2/5% CO_2 (= oxygen), air, or 95% N_2/5% CO_2 (= nitrogen) for 60 min. LDH release was measured after incubating cells for 60 min with either buffer, 1 mM MVK, or 1 mM NDA. Results are the means ± SEM of incubations from 4 to 7 separate cell preparations. Key: (*) significantly different ($P < 0.05$) from the corresponding values for cells incubated with buffer in the same atmosphere; (†) significantly different ($P < 0.05$) from sample incubated with the same addition in oxygen; (‡) significantly different ($P < 0.05$) from sample incubated with the same addition in air; and (§) significantly different ($P < 0.05$) from sample incubated with the same addition in nitrogen.

from chemical hypoxia than PT cells in oxygen atmosphere [16, 25, 26]. These results were confirmed in the present study, where KCN + IAA produced a small increase in LDH release from PT cells that was not statistically significant (25.2 ± 3.2% for KCN + IAA versus 17.6 ± 1.7% for buffer) and a large, significant increase in LDH release from DT cells (61.0 ± 7.3% for KCN + IAA versus 19.0 ± 2.9% for buffer). In contrast, the cytotoxicity pattern in the air atmosphere was quite different: significantly greater LDH release from both PT and DT cells due to chemical hypoxia in air was observed (68.7 and 81.7%, respectively) as compared with LDH release from PT and DT cells due to chemical hypoxia in oxygen (25.2 and 61.0%, respectively). Furthermore, cytotoxicity in PT cells due to chemical hypoxia was comparable to that in DT cells in the air atmosphere. In the nitrogen atmosphere, LDH release due to chemical hypoxia was diminished substantially in PT cells as compared with LDH release in the air atmosphere; LDH release from PT cells in nitrogen due to chemical hypoxia was not significantly higher than that from PT cells incubated with buffer. A similar pattern was observed in DT cells, although LDH release due to chemical hypoxia was still significantly higher than that from DT cells incubated with buffer.

PAP is a potent nephrotoxicant that acts by arylating cellular nucleophiles, predominantly GSH and PrSH groups [27]. The oxygen dependence of PAP-induced cytotoxicity was different from any of the other model chemicals tested, but was similar in both PT and DT cells (Fig. 3). PAP-induced LDH release was directly dependent on oxygen, being greatest in the oxygen atmosphere, intermediate in the air atmosphere, and not significantly different from that due to buffer in the nitrogen atmosphere.

Oxygen dependence of tBH-induced cytotoxicity. To investigate the mechanism by which cellular oxygen supply alters chemical-induced cytotoxicity in isolated renal PT and DT cells, we employed tBH as a model chemical. tBH metabolism and effects of tBH on cellular lipid peroxidation and PrSH redox status were measured in PT and DT cells incubated in oxygen, air, and nitrogen atmosphere.

tBH metabolism to *tert*-butanol is the primary detoxification mechanism for tBH. Alterations in rates of tBH detoxification with pO_2 will directly

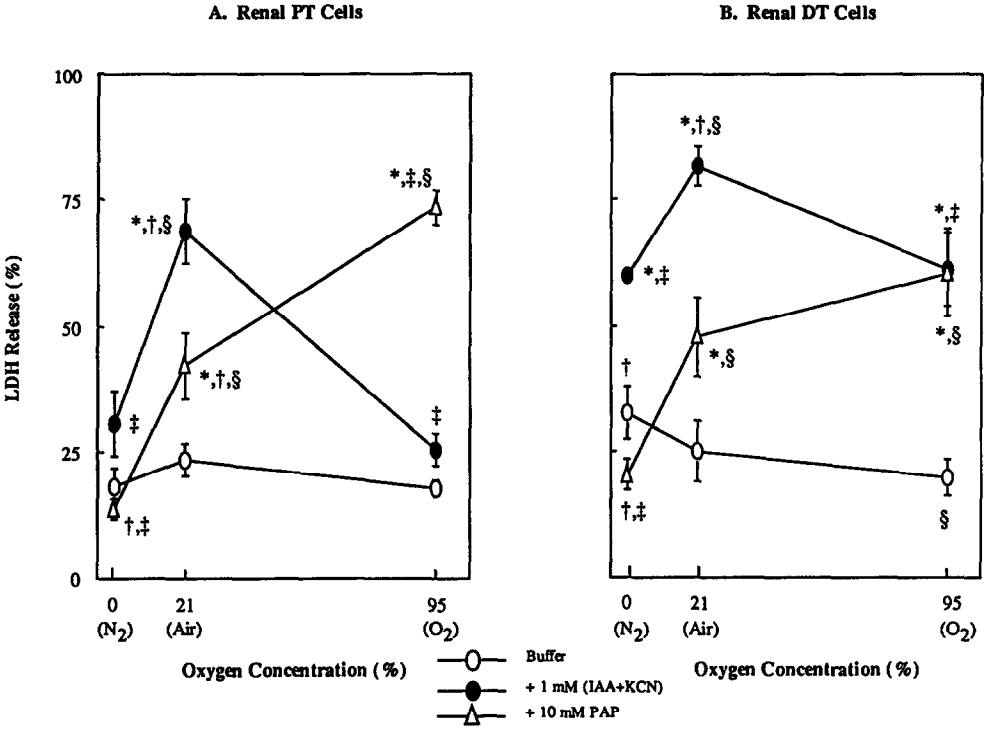

Fig. 3. Oxygen dependence of cytotoxicity caused by chemical hypoxia (IAA + KCN) and PAP. Isolated PT (A) and DT (B) cells (2 to 3 × 10^6 cells/mL) were incubated at 37° in an atmosphere of either 95% O_2/5% CO_2 (= oxygen), air, or 95% N_2/5% CO_2 (= nitrogen) for 60 min. LDH release was measured after incubating cells for 60 min with either buffer, 1 mM IAA + 1 mM KCN, or 10 mM PAP. Results are the means ± SEM of incubations from 4 to 7 separate cell preparations. Key: (*) significantly different ($P < 0.05$) from the corresponding values for cells incubated with buffer in the same atmosphere; (†) significantly different ($P < 0.05$) from sample incubated with the same addition in oxygen; (‡) significantly different ($P < 0.05$) from sample incubated with the same addition in air; and (§) significantly different ($P < 0.05$) from sample incubated with the same addition in nitrogen.

affect cellular susceptibility to tBH-induced injury. To determine if differences in tBH detoxification contribute to the observed oxygen dependence of tBH cytotoxicity, tBH metabolism was measured in renal PT and DT cells under either oxygen or nitrogen atmosphere (Table 1). tBH metabolism to *tert*-butanol was measured by coupling GSH peroxidase and GSSG reductase, thereby equating peroxide content at two time points with NADPH oxidation rate [24]. In the presence of catalase in the assay mixture, only organic peroxides (i.e. tBH) are measured. No effect of oxygen concentration on tBH metabolism was observed in either cell population, although the rate of tBH metabolism in DT cells was approximately 50% higher than that in PT cells. In the absence of catalase in the assay mixture, all peroxides, including hydrogen peroxide formed as a consequence of lipid peroxidation and oxidative stress, are measured. Under these conditions, total peroxide metabolism indeed exhibited a significant oxygen dependence; total peroxide content was reduced by 27 and 31% in PT and DT cells, respectively, in nitrogen as compared with oxygen atmosphere.

Because the results described above indicate that formation of reactive oxygen metabolites in PT and DT cells due to tBH exposure is oxygen dependent, we then determined if tBH-induced oxidative stress and redox changes are oxygen dependent. Lipid peroxidation, as determined by MDA formation, was measured in renal PT and DT cells incubated for up to 2 hr with either buffer or 1 mM tBH in either oxygen, air, or nitrogen atmosphere (Fig. 4). In either PT cells incubated with buffer (Fig. 4A) or in DT cells incubated with buffer (Fig. 4C), no significant time- or oxygen-dependent effects on the amounts of MDA measured were observed, although MDA contents in both cell types in air at both 1 and 2 hr were slightly higher than MDA contents in either oxygen or nitrogen. Incubation of either PT (Fig. 4B) or DT (Fig. 4D) cells with tBH in oxygen produced approximately 2-fold increases in MDA content after 1 or 2 hr as compared with cells incubated with buffer in oxygen. MDA formation due to tBH clearly required oxygen, as indicated by the complete absence of any increase in MDA content due to tBH in nitrogen atmosphere in either PT or DT cells. Although MDA formation due to tBH in air did not differ significantly from that due to tBH in oxygen atmosphere, MDA formation due to tBH in air atmosphere was not significantly different from that due to buffer at the 1-hr

Table 1. Oxygen dependence of tBH metabolism in isolated PT and DT cells

Atmosphere	tBH metabolism (nmol reduced/min per 10^6 cells) PT cells	DT cells
+Catalase		
Oxygen	38.9 ± 3.5	60.0 ± 5.6
Nitrogen	40.6 ± 4.8	65.9 ± 9.6
−Catalase		
Oxygen	44.0 ± 4.0	81.5 ± 10.1
Nitrogen	32.3 ± 3.9*	56.6 ± 6.7*

Isolated PT and DT cells (2 to 3 × 10^6 cells/mL) were incubated with 1 mM tBH at 37° under an atmosphere of either 95% O_2/5% CO_2 (= oxygen) or 95% N_2/5% CO_2 (= nitrogen) for 10 min. Peroxide content was measured in neutralized, perchloric acid extracts at 0 and 10 min by a pyridine nucleotide-linked enzymatic assay. Peroxide content in the presence of catalase is equivalent to organic peroxides only and that in the absence of catalase is equivalent to organic peroxides and hydrogen peroxide. Results are the rates of change of peroxide content per min over the 10-min incubation period and are the means ± SEM of measurements from 12 separate cell preparations.

* Significantly different ($P < 0.05$) from the corresponding rate in the presence of oxygen.

time point, suggesting diminished rates of lipid peroxidation relative to cells incubated only with buffer.

Intracellular PrSH concentrations in PT and DT cells incubated with buffer did not vary in a predictable manner with oxygen concentration (Fig. 5). In both cell populations, however, incubation with 1 mM tBH for 1 hr in either oxygen or air produced significant decreases in cellular PrSH concentration as compared with cells incubated with buffer in the same atmosphere. In oxygen, intracellular PrSH concentrations decreased by 35% in PT cells and by 40% in DT cells due to tBH. In air, the tBH-induced decreases in intracellular PrSH concentrations, while significant, were less than those in oxygen (28% decrease in PT cells and 27% decrease in DT cells). In nitrogen, however, PrSH concentrations did not change significantly due to tBH.

DISCUSSION

We have developed procedures to prepare highly enriched suspensions of rat renal PT and DT cells and to use these cells as *in vitro* models to study biochemical mechanisms of chemical and pathological injury in specific nephron regions [15–18]. With this system, toxicologic and pathologic responses can be studied at cellular and biochemical levels. In the present work, the effects of brief exposures of isolated renal PT and DT cells to atmospheres that contain suboptimal oxygen concentrations (i.e. functionally hypoxic) and the effects of varying oxygen concentration on patterns of chemical-induced cytotoxicity in these cells were studied. An understanding of how variations in oxygen supply affect renal cell function and susceptibility of renal cells to chemical injury is important clinically because of the prevalence of renal ischemia and hypoxia in many disease states.

PT cells incubated with buffer for 1 hr exhibited no significant changes in amounts of LDH released due to changes in oxygen supply (Figs. 1–3). In contrast, DT cells incubated with buffer for 1 hr exhibited significantly higher amounts of LDH release in both air and nitrogen as compared with those released in oxygen. These results indicate that brief exposure of DT cells to oxygen concentrations that are below the optimal 95% concentration generally used in isolated cell incubations will result in some cell injury. In contrast, PT cells are resistant to injury from brief exposure to hypoxia, similar to the resistance observed with isolated hepatocytes [8–10].

We next investigated the toxicological implications of the biochemical changes that occurred during hypoxia. Jones *et al.* [13] have recently reviewed the effects of hypoxia on drug metabolism and drug-induced toxicity in hepatocytes. The same considerations should apply to isolated renal cells, although the specific responses in renal PT and DT cells demonstrated some quantitative and qualitative differences from those observed in hepatocytes. Jones *et al.* [13] described three general patterns of oxygen dependence for these processes. The most common pattern is an increase in toxicity with increasing oxygen concentration, and is normally determined by the oxygen dependence of formation of a toxic product. The plot of toxicity versus pO_2 can be sigmoidal if oxidant production obeys Michaelis–Menten kinetics and has a physiologically relevant K_m, the plot can be linear if oxidant production is first-order, or the plot can be hyperbolic if multiple processes are involved in oxidant formation. A second pattern of toxicity is observed with halogenated hydrocarbons, such as carbon tetrachloride [28], in which toxicity is greater in hypoxic conditions than in normoxic or anoxic conditions. The mechanism for this pattern involves the balance between reductive and oxidative activation processes and the requirement for the presence of some O_2 to initiate toxicity. A third pattern of toxicity versus pO_2 is observed with tBH in freshly isolated hepatocytes [29] and in hepatocyte primary cultures [30]. In this case, injury is maximal in anoxia and is minimal in normoxia. The mechanism responsible is impaired cellular detoxification during oxygen deprivation, which causes the cells to have an enhanced susceptibility to injury [13, 29, 31].

To obtain a general characterization of the toxicologic response of renal PT and DT cells to varying oxygen concentration, cells were incubated in either oxygen (95% O_2/5% CO_2), air (21% O_2), or nitrogen (95% N_2/5% CO_2) atmosphere in the presence of two model classes of chemicals (oxidants, alkylating agents) or in the presence of two other model toxicants (chemical hypoxia, PAP). Three types of responses were observed in both PT and DT cells: First, oxidants (i.e. tBH, MD), alkylating agents that alter cellular thiol-disulfide status (MVK), and chemical hypoxia exhibited maximal toxicity in

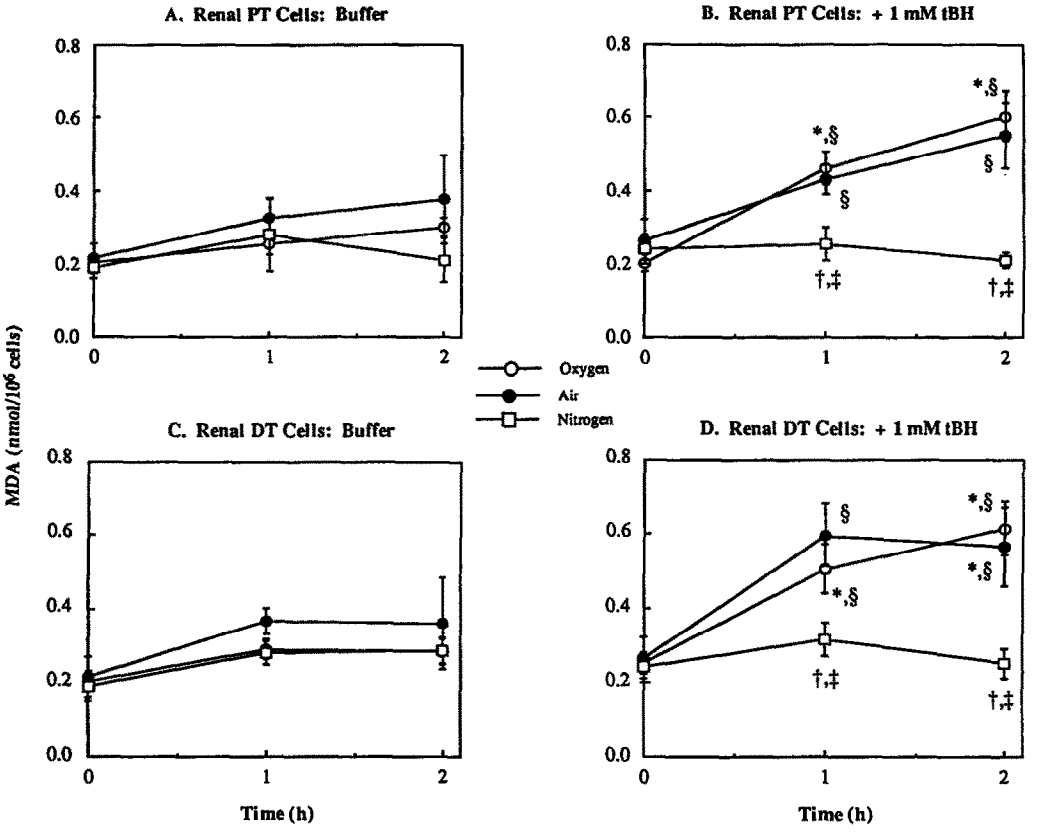

Fig. 4. Lipid peroxidation after tBH treatment under oxygen, air, or nitrogen atmosphere. Isolated PT (A, B) and DT (C, D) cells (2 to 3 × 10^6 cells/mL) were incubated at 37° under an atmosphere of either 95% O_2/5% CO_2 (= oxygen), air, or 95% N_2/5% CO_2 (= nitrogen) for 60 min with either buffer or 1 mM tBH. Lipid peroxidation was assessed by equating malondialdehyde (MDA) formation with thiobarbiturate reactive materials and measuring increases in absorbance at 532 nm. Results are the means ± SEM of measurements from 4 separate cell preparations. Key: (*) significantly different ($P < 0.05$) from the corresponding values for cells incubated with buffer in the same atmosphere; (†) significantly different ($P < 0.05$) from sample incubated with the same addition in oxygen; (‡) significantly different ($P < 0.05$) from sample incubated with the same addition in air; and (§) significantly different ($P < 0.05$) from sample incubated with the same addition in nitrogen.

air and minimal toxicity in nitrogen; second, PAP cytotoxicity was directly dependent on oxygen concentration, being greatest in oxygen, less in air, and nontoxic in nitrogen; and third, cytotoxicity of NDA, which alkylates hard nucleophiles, was independent of oxygen concentration. While responses were qualitatively the same in the two cell populations, their magnitude was greater in DT cells, as found previously [15–18].

tBH was chosen as a model for further investigation for two principal reasons: first, we have characterized extensively the cytotoxicity of tBH in these isolated cells under standard incubation conditions (i.e. an atmosphere of 95% O_2/5% CO_2); and second, previous studies in freshly isolated rat hepatocytes [29] and in primary hepatocyte cultures [30] have detailed the dependence of cellular toxicity on pO_2, showing that injury is maximal in anoxia and is minimal in normoxia. The oxygen dependence of tBH-induced cytotoxicity may result from several factors, including (1) oxygen-dependent tBH metabolism, (2) oxygen-dependent processes in the cell that are directly affected by tBH exposure, such as lipid peroxidation and cellular thiol-disulfide status, (3) oxygen-dependent cellular detoxification pathways that alter cellular susceptibility to oxidants, and (4) other oxygen-dependent processes or functions in the cell that modulate cellular susceptibility to exogenous chemicals.

Measurement of tBH metabolism revealed some potentially important differences between PT and DT cells. We showed previously [17] that PT cells contain slightly higher activities of GSH peroxidase and several-fold higher activities of catalase than DT cells. When tBH metabolism was measured in the current study (Table 1), however, we found that the rate of tBH metabolism with catalase in the assay mixture (a procedure that measures specifically organic peroxides) was 50% higher in DT cells than in PT cells and was not oxygen dependent. This demonstrates that a significant portion of tBH metabolism in DT cells must be catalyzed by

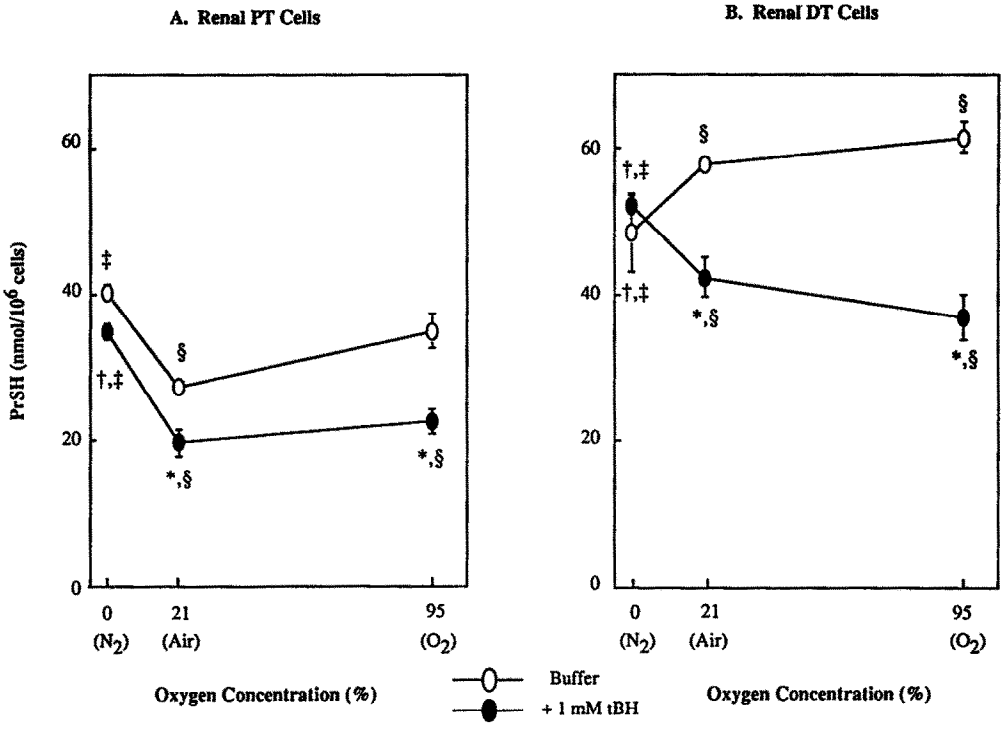

Fig. 5. Protein sulfhydryl (PrSH) status after tBH treatment under oxygen, air, or nitrogen atmosphere. Isolated PT (A) and DT (B) cells (2 to 3 × 10^6 cells/mL) were incubated at 37° under an atmosphere of either 95% O_2/5% CO_2 (= oxygen), air, or 95% N_2/5% CO_2 (= nitrogen) for 60 min with either buffer or 1 mM tBH. PrSH concentrations were measured by a spectrophotometric assay involving treatment of acid extracts with 5,5'-dithio-bis-(2-nitrobenzoic acid). Results are the means ± SEM of measurements from 3 to 6 separate cell preparations. Key: (*) significantly different ($P < 0.05$) from the corresponding values for cells incubated with buffer in the same atmosphere; (†) significantly different ($P < 0.05$) from sample incubated with the same addition in oxygen; (‡) significantly different ($P < 0.05$) from sample incubated with the same addition in air; and (§) significantly different ($P < 0.05$) from sample incubated with the same addition in nitrogen.

another enzyme besides GSH peroxidase. Moderate protection of DT cells from tBH-induced cytotoxicity by exogenous lactate (Lash LH and Pedrosi BM, unpublished data) suggests that this additional pathway for tBH metabolism may be NADH-linked rather than NADPH-linked, as suggested previously by Jones and colleagues [32] in isolated hepatocytes. These results indicate that the first possible mechanism suggested for the oxygen dependence of tBH-induced cytotoxicity, oxygen-dependent metabolism, is not correct.

When catalase was omitted from the assay mixture to measure tBH metabolism (a procedure that measures all peroxides) and activity was measured in the presence of oxygen or nitrogen atmosphere, two results were striking. First, DT cells exhibited a 2-fold higher rate of peroxide metabolism than PT cells. This is consistent with the higher amounts of basal and tBH-induced lipid peroxidation found in DT cells as compared with PT cells (Fig. 4). Second, the measurement of total peroxide metabolism showed a significant oxygen dependence, with the rate of peroxide metabolism decreasing by 27 and 31% in PT and DT cells, respectively, under nitrogen atmosphere as compared with oxygen atmosphere. The oxygen dependence of tBH-induced PrSH depletion (Fig. 5) is also consistent with these findings. Thus, the total peroxide metabolism data, the lipid peroxidation data, and the PrSH data support the second suggested mechanism for oxygen dependence of tBH-induced cytotoxicity, i.e. that oxygen-dependent processes in the cell that are directly affected by tBH exposure contribute to the cytotoxicity responses in different atmospheres.

For chemicals such as PAP, the first suggested mechanism, that of oxygen-dependent metabolism, may explain the cytotoxicity versus oxygen concentration pattern because PAP cytotoxicity increased directly with oxygen concentration. The most probable explanation is that formation of the reactive metabolite from PAP is oxygen dependent and it is this metabolite that is directly responsible for cytotoxicity.

For the other chemicals, including tBH, the oxygen dependence was more complex because toxicity was maximal in air and was minimal or absent in nitrogen. This pattern suggests that a combination of suggested mechanisms No. 2, No. 3, and possibly No. 4 may be responsible for the results. Thus, potential reasons for enhanced toxicity of chemicals such as tBH in

air as compared with oxygen are that: (1) oxygen concentration in air may be adequate for bioactivation reactions but may be insufficient for detoxification processes; (2) there may be reductive activation with oxygen-dependent enhancement of cytotoxicity, such as occurs with carbon tetrachloride and halothane in hepatocytes [28]; with tBH, the following reaction may occur:

$$ROOH + 1\,e^- \rightarrow RO^{\cdot} + OH^-,$$

where the alkoxy radical produces cell injury; and (3) renal PT and DT cells, especially the latter, may be inherently more susceptible to many types of chemical injury in air because there may be adequate oxygen supply for oxygen-dependent processes that lead directly to cell injury, such as lipid peroxidation, but there may not be adequate oxygen supply for other important functions in the cell, such as active transport and ATP synthesis.

In conclusion, this study characterized the susceptibility of renal PT and DT cells to brief incubations in suboptimal oxygen concentrations, investigated the oxygen dependence of cytotoxicity induced by various chemicals, and characterized further the biochemical mechanisms responsible for the oxygen dependence of chemical injury, employing tBH as a model toxicant. Brief periods (60 min) of oxygen deprivation caused moderate cytotoxicity in DT cells but not in PT cells. Qualitatively, PT and DT cells responded similarly to the model chemicals in the presence of varying oxygen concentrations. The oxygen dependence pattern exemplified by tBH may be due to the oxygen dependence of cell function in general and to the oxygen dependence of toxicologic processes that cause cell injury.

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REFERENCES

1. Jones DP, Renal metabolism during normoxia, hypoxia and ischemic injury. *Annu Rev Physiol* **48**: 33–50, 1986.
2. Soltoff DP, ATP and regulation of renal cell function. *Annu Rev Physiol* **48**: 9–31, 1986.
3. Brezis M, Rosen S, Silva P and Epstein FH, Selective vulnerability of the medullary thick ascending limb to anoxia in the isolated perfused rat kidney. *J Clin Invest* **73**: 182–190, 1984.
4. Brezis M, Shanley P, Silva K, Spokes K, Lear S, Epstein FH and Rosen S, Disparate mechanisms for hypoxic cell injury in different nephron segments: Studies in the isolated perfused rat kidney. *J Clin Invest* **76**: 1796–1806, 1985.
5. Ruegg CE and Mandel LJ, Bulk isolation of renal PCT and PST II. Differential responses to anoxia or hypoxia. *Am J Physiol* **259**: F176–F185, 1990.
6. Shanley PF, Brezis M, Spokes K, Silva P, Epstein FH and Rosen S, Hypoxic injury in the proximal tubule of the isolated perfused rat kidney. *Kidney Int* **29**: 1021–1032, 1986.
7. Shanley PF, Rosen MD, Brezis M, Silva P, Epstein FH and Rosen S, Topography of focal proximal tubular necrosis after ischemia with reflow in the rat kidney. *Am J Pathol* **122**: 462–468, 1986.
8. Andersson BS, Aw TY and Jones DP, Mitochondrial transmembrane potential and pH gradient during anoxia. *Am J Physiol* **252**: C349–C355, 1987.
9. Aw TY, Andersson BS and Jones DP, Mitochondrial transmembrane ion distribution during anoxia. *Am J Physiol* **252**: C356–C361, 1987.
10. Aw TY, Andersson BS and Jones DP, Suppression of mitochondrial respiratory function after short-term anoxia. *Am J Physiol* **252**: C362–C368, 1987.
11. Hochachka PW, Defense strategies against hypoxia and hypothermia. *Science* **231**: 234–241, 1986.
12. Jones DP, Hypoxia and drug metabolism. *Biochem Pharmacol* **30**: 1019–1023, 1981.
13. Jones DP, Aw TY and Shan X, Drug metabolism and toxicity during hypoxia. *Drug Metab Rev* **20**: 247–260, 1989.
14. Weinberg JM, The cell biology of ischemic renal injury. *Kidney Int* **39**: 476–500, 1991.
15. Lash LH and Tokarz JJ. Isolation of two distinct populations of cells from rat kidney cortex and their use in the study of chemical-induced toxicity. *Anal Biochem* **182**: 271–279, 1989.
16. Lash LH, Susceptibility to toxic injury in different nephron cell populations. *Toxicol Lett* **53**: 97–104, 1990.
17. Lash LH and Tokarz JJ, Oxidative stress in isolated rat renal proximal and distal tubular cels. *Am J Physiol* **259**: F338–F347, 1990.
18. Lash LH and Woods EB, Cytotoxicity of alkylating agents in isolated rat kidney proximal tubular and distal tubular cells. *Arch Biochem Biophys* **286**: 46–56, 1991.
19. Lash LH, Oxygen dependence of chemical injury in renal proximal tubular and distal tubular cells. *FASEB J* **6**: A938, 1992.
20. Jones DP, Sundby G-B, Ormstad K and Orrenius S, Use of isolated kidney cells for study of drug metabolism. *Biochem Pharmacol* **28**: 929–935, 1979.
21. Lash LH, Isolated kidney cells in the study of chemical toxicity. In: *In Vitro Toxicology: Model Systems and Methods* (Ed. McQueen CA), pp. 231–262. The Telford Press, Caldwell, NJ, 1989.
22. Ellman G and Lysko H, A precise method for the determination of whole blood and plasma sulfhydryl groups. *Anal Biochem* **93**: 98–102, 1979.
23. Stacey NH, Cantilena LR Jr and Klaassen CD, Cadmium toxicity and lipid peroxidation in isolated rat hepatocytes. *Toxic Appl Pharmacol* **53**: 470–480, 1980.
24. Heath RL and Tappel AL, A new sensitive assay for the measurement of hydroperoxides. *Anal Biochem* **76**: 184–191, 1976.
25. Lash LH, Mitochondrial function and toxicity in renal proximal tubular (PT) and distal tubular (DT) cells. *Toxicologist* **10**: 178, 1990.
26. Lash LH, Protection from chemical hypoxia in isolated renal cells. *Toxicologist* **11**: 140, 1991.
27. Newton JF, Kuo C-H, Gemborys MW, Mudge GH and Hook JB, Nephrotoxicity of *p*-aminophenol, a metabolite of acetaminophen, in the Fischer 344 rat. *Toxicol Appl Pharmacol* **65**: 336–344, 1982.
28. Shen ES, Garry VF and Anders MW, Effect of hypoxia on carbon tetrachloride hepatotoxicity. *Biochem Pharmacol* **31**: 3787–3793, 1982.
29. Tribble DL, Jones DP and Edmondson DE, Effect of hypoxia on *tert*-butylhydroperoxide-induced oxidative injury in hepatocytes. *Mol Pharmacol* **34**: 413–420, 1988.
30. Costa AK, Heffel DF, Schieble TM and Trudell JR, Toxicity of *t*-butylhydroperoxide in hepatocyte monolayers exposed to hypoxia and reoxygenation. *In Vitro Cell Dev Biol* **23**: 501–506, 1987.
31. Tribble DL and Jones DP, Oxygen dependence of oxidative stress: Rate of NADPH supply for maintaining the GSH pool during hypoxia. *Biochem Pharmacol* **39**: 729–736, 1990.
32. Kowalski DP, Aw TY, Park Y and Jones DP, Postanoxic oxidative injury in rat hepatocytes: Lactate-dependent protection against *tert*-butylhydroperoxide. *Free Radic Biol Med* **12**: 205–212, 1992.