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Oxidative Stress and 1-Methyl-2-nitroimidazole Cytotoxicity

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ABSTRACT. The 2-nitroimidazoles have been used clinically to radiosensitize resistant hypoxic cells, but a dose-limiting peripheral neuropathy has restricted their therapeutic effectiveness. A model compound, 1-methyl-2-nitroimidazole (INO_2), was used to investigate the possible role of oxidative stress in this normal tissue toxicity. Chinese hamster ovary (CHO) cells were 10–15 times more resistant to 20 mM INO_2 under aerobic than hypoxic conditions. In comparison, a pair of transformed rat embryo fibroblasts (ER17-1^{wtp53} and ER12L5^{mtp53}), differing in their p53 genotype, were approximately 3- to 4-fold more sensitive than Chinese hamster ovary cells to INO_2 under aerobic conditions, but had the same sensitivity as Chinese hamster ovary cells under hypoxic conditions. These results are consistent with an earlier hypothesis that the mechanism of aerobic toxicity is different from that of hypoxic toxicity (nitroreduction) and show that neither toxicity is dependent on cellular p53 status. There was an increase in the production of reactive oxygen intermediates and a decrease in the antioxidant glutathione following aerobic exposure to INO_2, which correlated with cell survival in all three cell lines. No evidence of reductive adducts of the 2-nitroimidazole 2-(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl)acetamide (EF5) was found by immunofluorescent techniques in aerobic cells. Differing activities of the antioxidant enzymes superoxide dismutase and catalase could be correlated with INO_2 aerobic cytotoxicity. DNA strand breaks, as measured by the comet assay, paralleled the appearance of INO_2 aerobic cytotoxicity in all three cell lines. Taken together, these results strongly support the conclusion that the aerobic toxicity of INO_2 is due to active oxygen species created by futile redox cycling of the parent compound. BIOCHEM PHARMACOL **56**;3:335–344, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 2-nitroimidazoles; oxidative stress; aerobic cytotoxicity

The 2-nitroimidazoles have been studied extensively for their use as radiosensitizers, hypoxic cytotoxins, and molecular markers of hypoxic regions in solid tumours [1–3]. Their selective hypoxic cytotoxicity and use as imaging agents for hypoxia are dependent on the bioreduction of these compounds to reactive intermediates and the binding of these reductive species to intracellular macromolecules. Bioreduction occurs only under extremely low oxygen tensions and, therefore, is selective for hypoxic regions [4]. In contrast, the use of 2-nitroimidazoles as radiosensitizers requires the intact compound to act as an oxygen mimic and potentiate the lethal effects of ionizing radiation in hypoxic but not aerobic cells [5]. Clinical trials with 2-nitroimidazoles as adjuncts to radiotherapy have been limited by a dose-dependent peripheral neuropathy [1, 6]. The peripheral neuropathy that was observed consisted of numbness, burning, tingling, and changes in sensation of the peripheral extremities and could be reversed if administration of the drug was terminated [1, 6]. A retrospective clinical analysis suggested that the onset of neurotoxicity could be avoided if plasma concentrations were maintained below a critical value [7]. By combining all clinical trials with nitroimidazoles as adjuncts to radiotherapy, positive results were observed in head and neck and bladder cancers, suggesting that therapeutic efficacy was achieved [1]. However, the dose-limiting peripheral neuropathy is still considered the major limitation in achieving loco-regional control of solid tumours with 2-nitroimidazoles.

In vivo testing with the 2-nitroimidazole misonidazole suggested that axonal degeneration of the Wallerian-type, resulting in secondary demyelination of peripheral nerves, was the cause for the observed transient peripheral neuropathy [8]. These authors also showed evidence of significant decreases in lactate/pyruvate ratios measured in the brain stem, suggesting inhibition of glycolysis as a factor mediating neurotoxicity. *In vitro* studies with 2-nitroimidazoles confirmed ultrastructural disruption and degradation of the neurofilament lattice, which appeared to be mediated through stimulation of Ca^{2+}-dependent proteases [9, 10]. Other *in vitro* analyses also indicated alterations in glycolysis, that were accompanied by reductions in intracellular nonprotein thiols, specifically GSH [11, 12]. The observed *in vitro* results therefore suggest that depletion of

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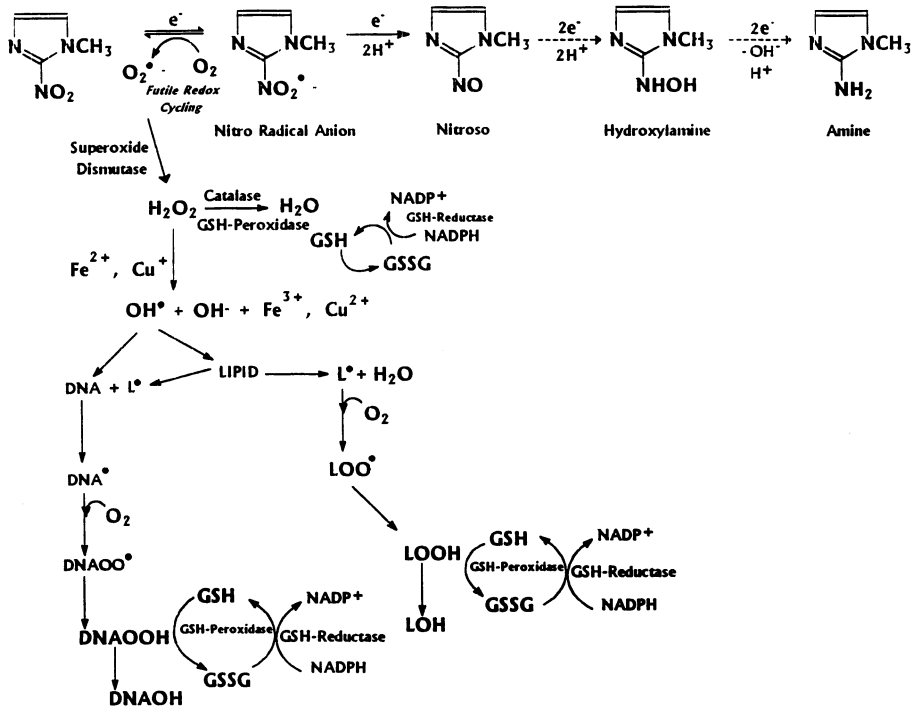

FIG. 1. Bioreductive pathway of INO_2 including both the proposed hypoxic and aerobic metabolism. Hypoxic cytotoxicity is thought to be mediated by the two-electron nitroso (INO) intermediate, whereas aerobic cytotoxicity is proposed to proceed through the generation of ROIs by futile cycling of the parent compound with the one-electron nitro radical anion in the presence of oxygen. Cellular targets of aerobic metabolism include DNA and lipids (L) that are oxidized by the production of $OH^{\cdot}$ from H_2O_2 via reactions with transition metals (Cu^{+} and Fe^{2+}) (based on Ref. 12).

key energy requirements and intracellular thiols may be the mechanism by which neuronal degradation and demyelination occur, which results in the peripheral neurotoxicity of 2-nitroimidazoles under normoxic conditions.

It has been hypothesized that the aerobic toxicity of 2-nitroimidazoles is caused by the production of intracellular ROIs[‖] generated by the futile cycling of the parent 2-nitroimidazole compound (Fig. 1) [12]. However, experimental data supporting this mechanism of aerobic toxicity for 2-nitroimidazoles are limited. Three rodent fibroblast lines have been identified that have the same sensitivity to a model 2-nitroimidazole, INO_2, under hypoxic exposure conditions but differ from one another by a factor of 3- to 4-fold in their sensitivity to INO_2 under aerobic conditions. The present work investigates the biochemical and biological effects of aerobic exposures to INO_2 in these cells in terms of ROI generation, reduction products of 2-nitroimidazoles, antioxidant enzyme activities, GSH depletion, and DNA damage. The results support the hypothesis that the mechanism of aerobic toxicity is due to ROI production and that cell sensitivity is modulated by the ability of the cell to detoxify these species.

MATERIALS AND METHODS

Cells and INO_2

Cells used in this study were: CHO cells, subclone AA8-4, that were originally obtained from Dr. L. H. Thompson (Lawrence Livermore Laboratories) and REF (ER17-1^{wtp53} and ER12L5^{mtp53} cells) that were transformed by activated EJ-H-*ras* and HPV-16 E7 oncoproteins and differ in their p53 genotype. ER17-1^{wtp53} cells are normal wildtype (wtp53) cells, whereas ER12L5^{mtp53} cells have a mutant p53 (mtp53) containing a C:T base change predicted to give rise to a serine to proline change [13]. The transformed REF clones were provided by Dr. S. Benchimol (Ontario Cancer Institute). CHO cells were grown routinely in monolayer or suspension cultures at 37° in growth medium consisting of α-MEM supplemented with 10% FBS (Sigma) and had a doubling time of 12 hr. REF clones were grown as monolayers under the same conditions as CHO cells and had a similar doubling time.

INO_2 was synthesized using a protocol described previously [14]. Cellular exposures to 20 mM INO_2 were performed following incubation of stirred suspension cultures (10 mL with a cell density of 10^6 cells/mL) in 40-mL polyshell vials (John's Scientific, Inc.) for varying lengths of time at 37° under either aerobic conditions (95% air:5% CO_2) or hypoxic conditions (N_2:5% CO_2 < 10 ppm O_2) (Gas Dynamics Inc.) in α-MEM plus 10% FBS as described previously [2].

Cell Survival Assay

Cells were treated with INO_2 under aerobic or hypoxic conditions, and cell survival was measured as previously described [2]. A concentration of 20 mM was chosen as a compromise that allowed significant aerobic toxicity to be obtained over reasonable exposure times (6–30 hr) in all three cell lines. For all of the following cellular analyses (ROI levels, GSH levels, comet assay), cells were treated to

[‖] *Abbreviations:* CHO, Chinese hamster ovary; EF5, 2-(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl)acetamide; FBS, fetal bovine serum; INO_2, 1-methyl-2-nitroimidazole; α-MEM, minimum essential medium; $O_2^{\cdot -}$, superoxide radical anion; $OH^{\cdot}$, hydroxyl radical anion; REF, rat embryo fibroblasts; ROIs, reactive oxygen intermediates; and SOD, superoxide dismutase.

an equitoxic survival of 1 to 0.1% following aerobic exposure to 20 mM INO_2. To achieve this level of equitoxicity, CHO cells were treated for 30 hr, and the ER17-1^{wtp53} and ER12L5^{mtp53} cells were treated for 6 hr. To obtain an equitoxic survival of 1 to 0.1% under hypoxic treatment to 20 mM INO_2, all three cell lines were treated for up to 2 hr.

Determination of ROIs

Levels of ROIs were measured at different times following a 20 mM INO_2 acute aerobic exposure of ER17-1^{wtp53}, ER12L5^{mtp53} or CHO cells by using the non-fluorescent dye dihydrorhodamine-123, which is converted to the fluorescent rhodamine-123 (Molecular Probes) in the presence of $O_2^{\cdot -}$ and H_2O_2. Aliquots of cells (10^6 cells/mL) were removed at timed intervals following 20 mM INO_2 aerobic treatment, washed, and pretreated with 3 μM cyclosporin A to inhibit P-glycoprotein [15]. Fluorescent staining with dihydrorhodamine-123 (1 μM) was then performed for 15 min following a procedure described previously [16]. Fluorescence was monitored using a Coulter flow cytometer, and the mean fluorescence intensity of 10,000 cells was measured.

Measurement of GSH Levels

Total intracellular glutathione levels were measured at different time points during a 20 mM INO_2 acute aerobic exposure of CHO, ER17-1^{wtp53}, and ER12L5^{mtp53} cells using the enzyme recycling assay [17] as modified by Bump and Brown [18].

Immunofluorescence of EF5

Intracellular binding of a reduction product of the pentafluorinated derivative of etanidazole (EF5) was measured in all three cell lines under hypoxic and aerobic conditions. Cells were treated with 100 μM EF5 and incubated for 6 hr in cell suspension at a cell density of 10^6 cells/mL with sampling occurring every 2 hr beginning at time 0. Cells were processed as previously described [3, 19]. Briefly, cells were washed in ice-cold PBS containing 4% paraformaldehyde, rinsed in PBS, and resuspended in blocking solution (nonspecific binding blocked with a protein blocking solution); the blocking solution was removed, the cells were rinsed in PBS, and Elk3-51 monoclonal antibody, which was conjugated to the Cy3 fluorochrome, was added. After 6 hr at 4°, cells were washed extensively with PBS and stored in PBS containing 1% paraformaldehyde. The intracellular binding of reduced EF5 intermediates was detected by immunofluorescent flow cytometry using a Coulter flow cytometer. The mean fluorescence intensity of Elk3-51 of 10,000 events was plotted for all six treatment conditions. The EF5 and Elk3-51 antibody were gifts from Dr. C. Koch (University of Pennsylvania).

Antioxidant Enzyme Assays

Intracellular Cu/Zn SOD activity and catalase activity were measured using the methods of Begleiter [20]. For the Cu/Zn SOD assay, KCN (33 μM) was added to block peroxidase activity and, following cell sonication, the cell lysates were centrifuged (250 *g*) and cell debris was pelleted and removed from the assay measurement. Total GSH-peroxidase activity, which measures both intracellular non-selenium-dependent GSH-peroxidase that belongs to the GSH-*S*-transferase α-subclass and selenium-dependent GSH-peroxidase, was measured using the protocol of Lawrence and Burke [21]. Total cellular protein was measured using the Lowry assay, and enzyme activity is presented as the mean of at least three independent experiments with calculated standard deviations.

Alkaline Comet Assay

DNA strand breaks were measured following a 20 mM INO_2 acute aerobic exposure of CHO, ER17-1^{wtp53} and ER12L5^{mtp53} cells at varying time periods by following the procedure of Olive *et al.* [22] as modified by Hu *et al.* [23].

RESULTS

Differential Hypoxic and Aerobic Cytotoxicity of INO_2

CHO, ER17-1^{wtp53}, and ER12L5^{mtp53} cells were treated with 20 mM INO_2 under aerobic and hypoxic conditions, and their clonogenic cell survival was assayed. Figure 2a shows that under aerobic conditions, the transformed REF clones were 3- to 4-fold more sensitive to the aerobic cytotoxicity of INO_2 than the CHO cells at the 10% survival level. A similar 3- to 4-fold differential in toxicity was observed following continuous exposure to INO_2 at 1–2 mM during the entire 8-day colony formation assay for survival (data not shown). In contrast, hypoxic treatment of 20 mM INO_2 produced only small differences (1.2 to 1.4) below 10% cell survival in the three cell lines studied (Fig. 2b). No significant differences in cell survival were observed in INO_2 toxicity towards the p53 expressing ER17-1^{wtp53} and ER12L5^{mtp53} cells, either under aerobic or hypoxic conditions (Fig. 2a and b), indicating that p53 status was not a factor determining toxicity. The differential response of the cells to INO_2 under aerobic but not hypoxic exposure conditions was consistent with the hypothesis that the mechanism of cell death was different under air versus hypoxia.

Production and Detoxification of ROIs Following Aerobic INO_2 Exposure

ROI production in these cell lines was assessed as a measure of the possible role redox cycling of INO_2 may play in aerobic drug toxicity. The production of $O_2^{\cdot -}$ occurs as a result of the one-electron reduction of the parent INO_2 compound to the nitro radical anion, which in the presence

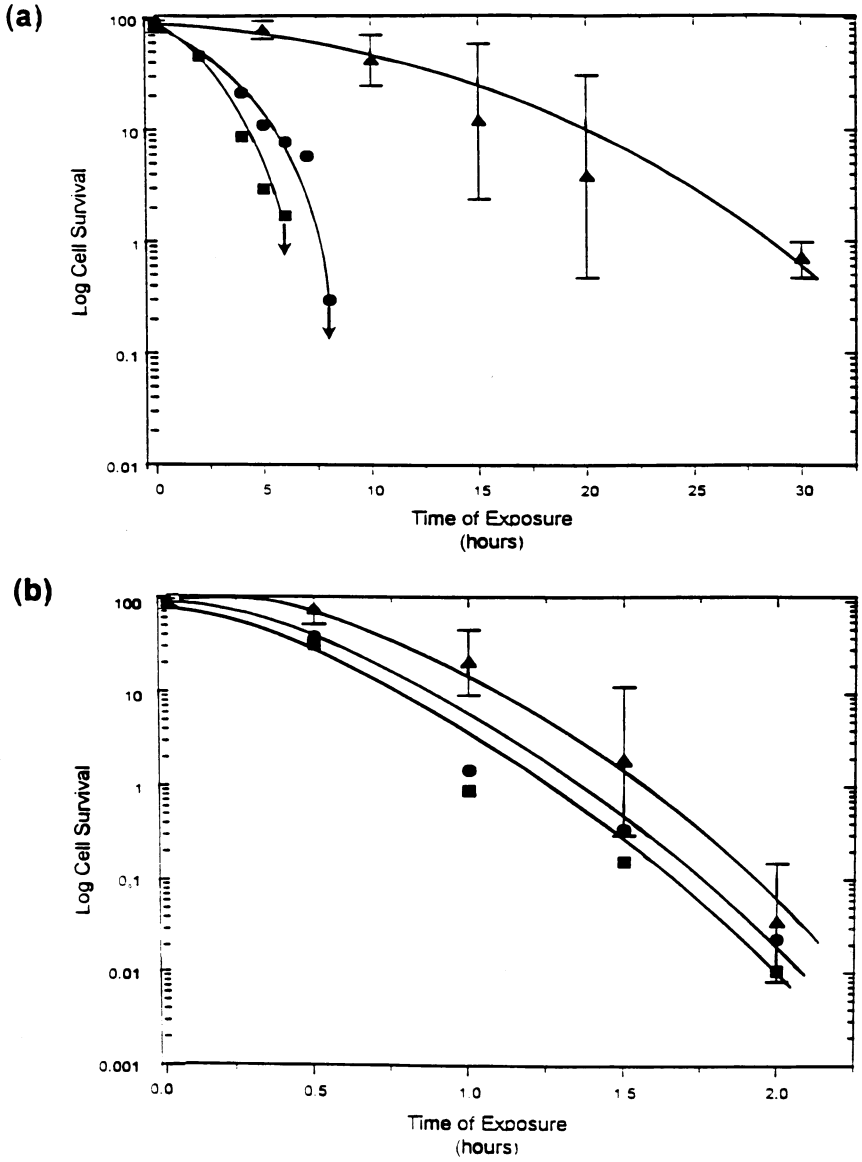

FIG. 2. Clonogenic cell survival following 20 mM INO_2 aerobic (a) and hypoxic (b) treatment. The plotted values for ER17-1^{wtp53} (■), ER12L5^{mtp53} (●), and CHO (▲) curves represent the means of at least three independent experiments. Arrows indicate that no colonies were observed, and survival was less than the data point. The representative error bars represent the SEM and are shown on the CHO survival curve for both plots.

of oxygen, is back-oxidized to the parent INO_2 generating $O_2^{\cdot -}$. This model of futile cycling of the parent INO_2 compound with the nitro radical anion and the concomitant generation of ROIs are shown in Fig. 1. In contrast, under low partial pressures of oxygen, further bioreduction past the nitro radical anion occurs, generating reactive nitroso and hydroxylamine reduction derivatives that react chemically with critical cellular molecules [2].

The mean fluorescence intensity of rhodamine-123 provides an indirect approach for measuring the presence of ROIs following INO_2 aerobic treatment. Cells were exposed to 20 mM INO_2 under aerobic conditions; aliquots were removed as a function of time, washed, and assayed for the presence of ROIs. Fluorescence of rhodamine-123 increased over controls in ER17-1^{wtp53} and to a somewhat lesser extent in ER12L5^{mtp53} cells over 1–6 hr of INO_2 exposure (Fig. 3a). This time period corresponded to changes in cell colony forming ability from 60% to less than 5% survival (Fig. 2a). In contrast, no evidence for ROI increase was seen in CHO cells over 5–30 hr when cell survival went from 80% to less than 1% (Fig. 3b).

Evidence of Nitroreduction

The hypothesis that the aerobic toxicity of 2-nitroimidazoles is mediated by the production of ROIs by futile cycling predicts the reduction of 2-nitroimidazoles, and their retention in aerobic cells should not occur. Due to the increased aerobic sensitivity of the transformed REFs to INO_2 compared with the CHO cells, the amount of nitroreduction was assayed under both aerobic and hypoxic conditions. Immunofluorescent analysis of the intracellular retention of the reduced pentafluorinated 2-nitroimidazole EF5 allows for specific recognition of intracellular adducts that are formed between reduced EF5 and cellular macromolecules [19]. Figure 4 represents the accumulation of the reduction product(s) of EF5 under hypoxic and aerobic conditions in all three cell lines. Hypoxic metabolic activation of EF5,

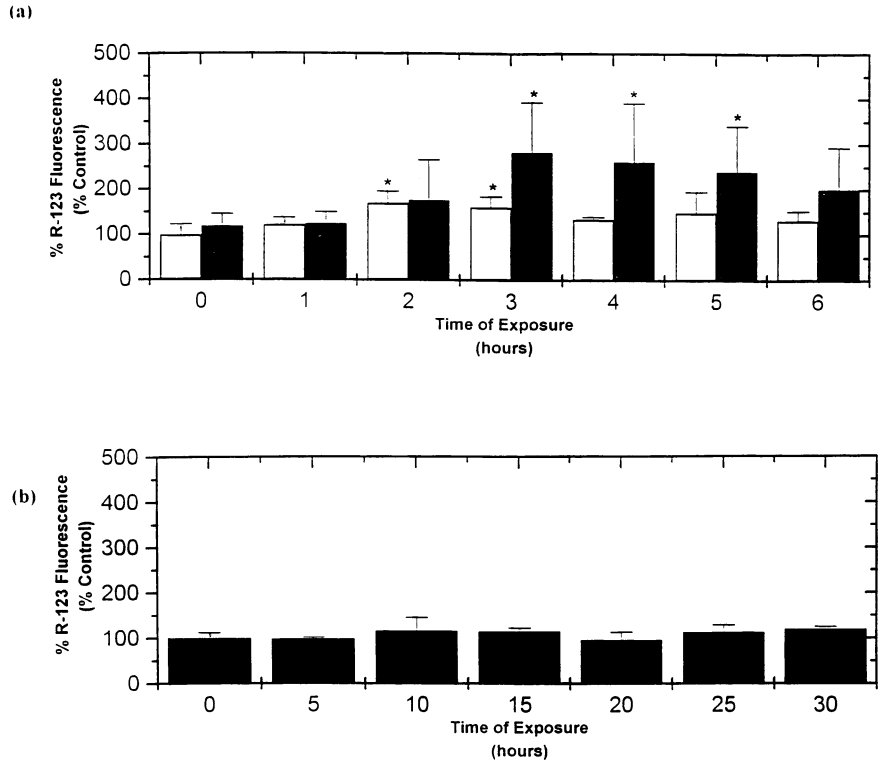

FIG. 3. Production of ROIs in ER17-1^{wtp53} (black bars) and ER12L5^{mtp53} (white bars) cells in (a) and in CHO cells (black bars) in (b) following 20 mM INO_2 treatment. ROI production was directly proportional to the fluorescence of rhodamine-123. Values are represented as percent-mean fluorescence intensity (as a percent of control for each time point) of at least three independent experiments. Control values did not change significantly during the incubation time. Error bars represent SDs of the mean. Asterisks represent significant differences ($P < 0.05$) from the zero time point.

measured as EF5 adducts, occurred in all three cell lines with increased adduct formation in the transformed REFs compared with the CHO cells. Reduction and binding of EF5 were not observed in any of these cell lines under aerobic exposure conditions (Fig. 4), even up to 6 hr when survival of transformed REFs was reduced by 2–3 orders of magnitude (Fig. 2a). These results are consistent with different mechanisms of action of INO_2 under hypoxic and aerobic conditions.

Intracellular Antioxidant Enzyme Activities and GSH Levels

To see if detoxification of ROIs may be a cause of increased resistance and/or sensitivity to INO_2, the levels of the antioxidant enzymes SOD, catalase, and GSH-peroxidase were measured in the three cell lines. The role that these antioxidant enzymes play in the detoxification of ROIs following INO_2 aerobic metabolism is shown in Fig. 1.

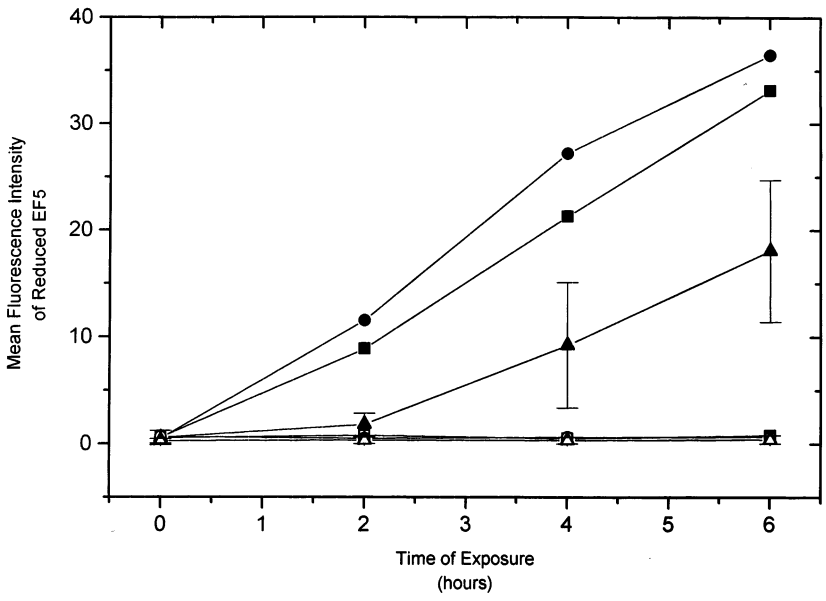

FIG. 4. Intracellular accumulation of reduced EF5 as measured by mean fluorescence intensity of reduced EF5 adducts under hypoxic conditions in ER17-1^{wtp53} (■), ER12L5^{mtp53} (●), and CHO (▲) cells and under aerobic conditions in ER17-1^{wtp53} (□), ER12L5^{mtp53} (○), and CHO (△) cells as a function of exposure time. Values plotted are the means of two independent experiments, and error bars represent the range. Data for all cells under aerobic conditions were indistinguishable from zero.

TABLE 1. Antioxidant enzyme profile and GSH levels

Cell line	SOD (units/mg protein)	GSH-peroxidase (nmol/min/mg protein)	Catalase (units/mg protein)	GSH levels (nmol/10^6 cells)
CHO	5.6 ± 1.1	5.18 ± 0.03	5.86 ± 0.32	39 ± 15
ER12L5^{mtp53}	30.5 ± 1.7	5.31 ± 0.13	2.92 ± 0.16	47 ± 13
ER17-1^{wtp53}	14.7 ± 1.9	6.09 ± 0.68	2.74 ± 0.15	36 ± 7

Endogenous enzyme activities of Cu/Zn SOD (units/mg of protein), catalase (units/mg of protein), non-selenium-dependent GSH-peroxidase (GST-α subclass) (nmol/min/mg of protein), and intracellular GSH levels (nmol/10^6 cells) were measured in ER17-1^{wtp53}, ER12L5^{mtp53}, and CHO cells, and the values presented are the means ± SD of at least three independent experiments.

Table 1 shows that differences were observed in the amount of cytosolic SOD (Cu/Zn SOD) activity in the three cell lines. ER12L5^{mtp53} cells had a two-fold higher activity than the ER17-1^{wtp53} cells, whereas the CHO cells had 2- to 3-fold less Cu/Zn SOD than the ER17-1^{wtp53} cells. No statistical difference was observed in the levels of activity of GSH-peroxidase among the three cell lines (Table 1). However, a two-fold significant increase ($P < 0.001$) in the catalase activity in the CHO cells compared with the sensitive transformed REF cells was observed (Table 1). These differences in SOD and/or catalase activities may contribute to detoxification of ROIs and modulate cell killing.

The ubiquitous and most abundant cellular nonprotein thiol responsible for intracellular detoxification of xenobiotics, including ROIs, is GSH. Several enzyme reactions are dependent on this tripeptide, specifically GSH-reductase and GSH-peroxidase (Fig. 1). Although endogenous GSH levels were similar between the three cell lines (Table 1), Fig. 5 shows that following 20 mM INO_2 treatment, GSH depletion was observed in all three cell lines but in a different time-dependent manner. At equitoxic (1%) cell survival, the kinetics of depletion to approximately 40% (of control) was observed at 30 hr for the CHO cells and 30 and 20% at 6 hr for the ER12L5^{mtp53} and ER17-1^{wtp53} cells, respectively (Fig. 5). The pattern of GSH depletion in all three cell lines paralleled loss of cell colony forming ability with time of aerobic exposure to INO_2 (Fig. 2a). Intracellular depletion of GSH also paralleled ROI production in the sensitive REF cells, suggesting that oxidative stress occurs following aerobic INO_2 treatment of these cells.

DNA Strand Breaks with INO_2 Aerobic Treatment

The generation of ROIs, specifically the ($OH^{\cdot}$) through Fenton chemistry, can induce single-strand breaks in DNA [24]. The alkaline comet assay measures the production of DNA strand breaks and, therefore, was utilized to assess DNA damage following 20 mM INO_2 aerobic exposure in the three cell lines. The kinetics of DNA damage (Fig. 6) paralleled those of cell survival (Fig. 2a) in all three cell lines following 20 mM aerobic treatment, with similar levels of DNA strand breaks observed at equitoxic survival, 1% to 0.1%.

DISCUSSION

The aerobic cytotoxicity of 2-nitroimidazoles has been suggested previously to be due to alterations in the pentose cycle and glycolysis, which would result in perturbations of

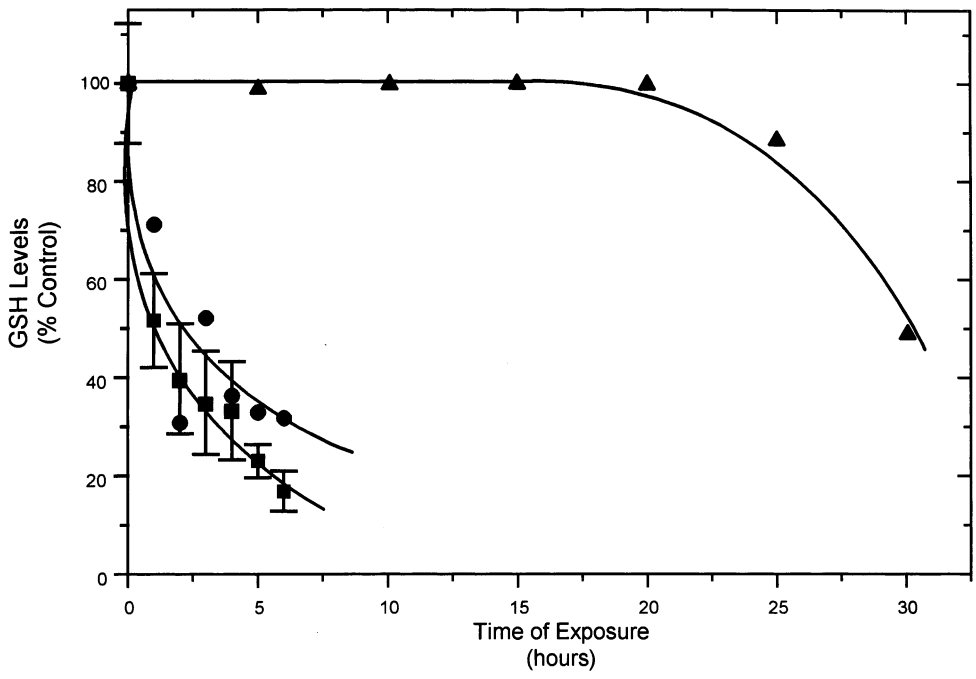

FIG. 5. Intracellular GSH levels following 20 mM INO_2 aerobic treatment as a function of exposure time. GSH levels are plotted as percent control for ER17-1^{wtp53} (■), ER12L5^{mtp53} (●), and CHO (▲) cells during 20 mM INO_2 aerobic exposure. Values represent the means of at least three independent experiments, and error bars represent the SDs of the mean and are shown on the ER17-1^{wtp53} curve. Absolute values for GSH levels for the cell lines at time zero are listed in Table 1.

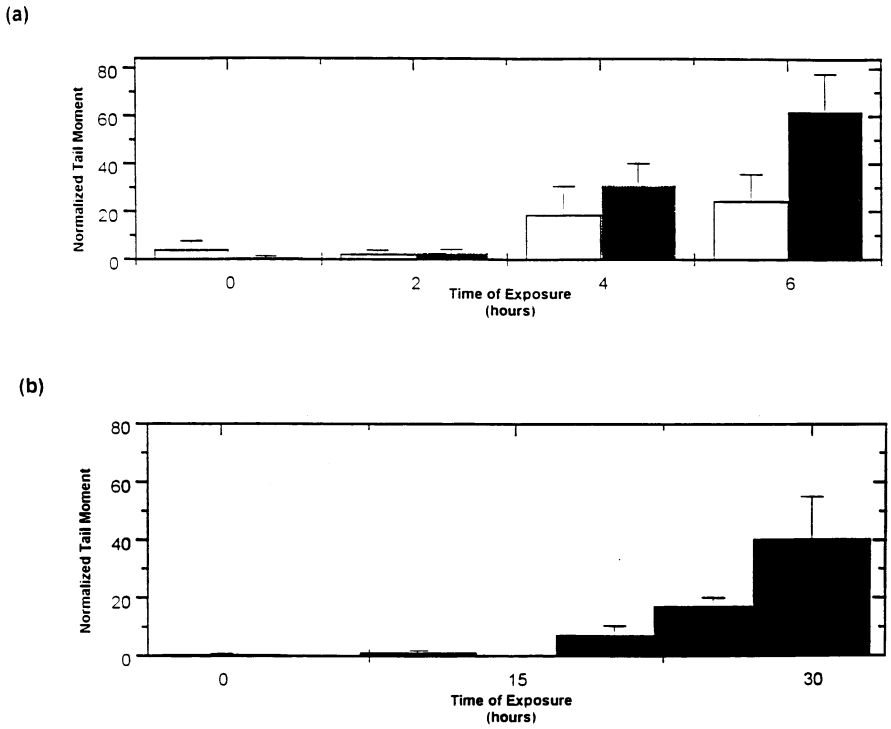

FIG. 6. DNA damage following 20 mM INO_2 aerobic treatment as a function of time. The alkaline comet assay measures the amount of total DNA strand breaks, and DNA damage is represented as the normalized tail moment. Values are plotted in graph (a) for ER17-1^{wtp53} (black bars) and ER12L5^{mtp53} (white bars) cells, and graph (b) CHO cells (black bars), and they represent the means of at least three independent experiments. Error bars represent the SEMs.

critical energy requirements such as ATP [12]. In addition to glycolytic alterations, depletion of critical intracellular nonprotein thiols was also observed following aerobic treatment to 2-nitroimidazoles [12]. The importance of investigating the aerobic cytotoxicity of 2-nitroimidazoles is to provide a mechanism for the understanding of the aerobic neurotoxicity that was observed in patients when 2-nitroimidazoles underwent clinical testing as radiosensitizers of radioresistant hypoxic tumours [1, 6, 7]. It is the focus of this paper, therefore, to further characterize the mechanism(s) involved in the aerobic cytotoxicity of 2-nitroimidazoles by using a simple N-1 substituted analogue, INO_2, as a model compound. This compound has been shown previously to be similar to misonidazole in its aerobic and hypoxic toxicity towards CHO cells [2].

Transformed REF, which differ in the genotype of the tumour suppressor p53, had similar aerobic sensitivity to INO_2, while CHO cells were 3–4 times more resistant (Fig. 2a). This aerobic cytotoxic differential between the REF and CHO cell lines was also maintained following continuous exposure to 1–2 mM INO_2 during the 8- to 10-day period of the colony assay (data not shown), showing that the difference between cell lines was maintained over a wide drug concentration range. This is important since blood levels of drug at which human peripheral neuropathies occur are of the order of 0.1 to 0.3 mM [6, 7]. In contrast, the selective hypoxic cytotoxicity to 20 mM INO_2 was similar for all three cell lines (Fig. 2b), consistent with different mechanisms for aerobic versus hypoxic cytotoxicity of 2-nitroimidazoles.

Figure 1 displays the proposed pathways involved in both aerobic and hypoxic metabolism of INO_2, such that under hypoxic conditions further enzymatic nitroreduction occurs, which produces the reactive toxic reductive nitroso (two-electron reduction product) and the hydroxylamine (four-electron reduction product) derivatives. Previous work reported that the hydroxylamine was responsible for the selective hypoxic cytotoxicity of 2-nitroimidazoles [25, 26]. However, more recent data suggest that the nitroso is a toxic electrophile that could also mediate the preferential hypoxic cytotoxicity observed with 2-nitroimidazoles [14, 27].

CHO [28] and REF (data not shown) cells die apoptotically after hypoxic exposure to INO_2. The similar sensitivities of the two REF cell lines, differing in their p53 status, suggest that this cell death occurs via a p53-independent pathway. Evidence of other investigators indicates that apoptosis induced by DNA damage requires p53, whereas p53-independent apoptosis has been described for stimuli that mimic physiological cell deletion signals [29].

The aerobic cytotoxicity is hypothesized to be due to the futile redox cycling between the parent compound and the one-electron reduced nitro radical anion (Fig. 1). This cycling allows for the generation of $O_2^{\cdot -}$, which may be enzymatically or spontaneously reduced to H_2O_2. The generation of these ROI may be further exacerbated by the production of the very reactive and damaging $OH^{\cdot}$ from H_2O_2 through Fenton chemistry [24, 30].

Following 20 mM INO_2 aerobic treatment, it was observed that the ER17-1^{wtp53} and ER12L5^{mtp53} cells produced ROIs, as measured by increased fluorescence of rhodamine-123 (Fig. 3, a and b). The CHO cells did not generate any detectable amounts of ROIs compared with the controls. The amount and rate of ROI production were

qualitatively associated with INO_2 sensitivity of the REF and CHO cell lines (Fig. 2a). The INO_2-sensitive ER17-1^{wtp53} and ER12L5^{mtp53} cells did not produce the same amounts of ROIs under aerobic conditions (Fig. 3a), though their survival (Fig. 2a) was statistically similar. Thus, ROI production does not quantitatively correlate with aerobic cell survival for the two sensitive REF cell lines, indicating that other modulating factors are present. No elevation in the levels of ROIs was observed in the CHO cells, even up to the time of INO_2-induced aerobic toxicity (Fig. 2a). This may be due to a limitation of sensitivity of the ROI detection protocol. In particular, the staining procedure with dihydrorhodamine-123 was for 15 min at 5-hr intervals for up to 30 hr during the aerobic exposure of CHO cells to INO_2. If the production of ROIs occurs at a rate that is slow relative to removal, the increase in rhodamine-123 fluorescence would be minimal. By this explanation, the rate of production of ROIs and/or ROI removal in CHO cells following INO_2 aerobic exposure may be different compared with the transformed REFs.

Using EF5 as a representative 2-nitroimidazole, retention of metabolically reduced drug was observed in all three cell lines under hypoxic conditions (Fig. 4). The initial rate of product accumulation was greater in both REF cell lines than in CHO cells but did not lead to statistically different rates of cell killing with hypoxic INO_2 treatment (Fig. 2b). However, aerobic metabolism and intracellular retention of EF5 was not evident in any of the three cell lines, suggesting that the generation of stable reductive products is not the mechanism of the aerobic cytotoxicity of 2-nitroimidazoles (Fig. 1). In fact, there was no increase in the mean fluorescence intensity of aerobic cells even at 6 hr versus zero time. At 6 hr, the mean fluorescence of hypoxic cells was approximately 50-fold greater than that of aerobic cells. It must be emphasized that a 200-fold higher concentration of INO_2 (20 mM) was added to cells compared with EF5 (0.1 mM), and this could contribute to the differences in the aerobic and hypoxic accumulation of EF5. However, previous work with the ^{14}C-labelled 2-nitroimidazole misonidazole at micromolar levels showed a two-fold increase in the rate of drug accumulation in human HeLa versus CHO cells, which correlated well with the two-fold difference in hypoxic cell killing at millimolar levels of misonidazole [31]. There is, of course, an implicit assumption in the use of EF5 as a marker of hypoxia, that the mechanism by which it is retained and/or accumulated in hypoxic versus aerobic cells is the same as for INO_2. At present, this appears to be a valid assumption [19].

Antioxidant enzymes that have been shown to play a key role in modifying the cellular response to oxidative stress in cells are SOD, catalase, and GSH-peroxidase [32]. Intracellular thiols, specifically the nonprotein thiol GSH, have also been pivotal in detoxifying ROIs, either directly or by conjugation with GSH-dependent enzymes. It was observed that the sensitive ER17-1^{wtp53} and ER12L5^{mtp53} cells had higher levels of cytosolic Cu/Zn SOD activity compared with the CHO cells, whereas catalase activity was almost two-fold higher in the CHO cells compared with the sensitive transformed REFs (Table 1). Both GSH-peroxidase activity and endogenous GSH levels were similar in all three cell lines (Table 1). Cerutti and collaborators have emphasized that no one antioxidant enzyme activity predicts the ability of a cell to withstand oxidative stress. It is the balance between individual components that is important, e.g. the ratio of Cu/Zn SOD and catalase activities has been demonstrated to be important for resistance to oxidative stress [33]. They have shown that 2- to 4-fold differences in SOD or catalase levels can cause significant changes in cell sensitivity to H_2O_2. One possible model for the present results with INO_2 is that the high levels of SOD in REF cells versus CHO cells convert the high levels of $O_2^{\cdot}$ – in these cells to higher H_2O_2 levels than occur in CHO cells. Lower levels of catalase in REF cells result in higher levels of $OH^{\cdot}$ produced by Haber–Weiss chemistry and the Fenton reaction [30, 34].

DNA strand breaks were observed in all three cell lines following 20 mM INO_2 treatment (Fig. 6). It must be acknowledged that such breaks are not a unique marker for oxidative damage because they are also observed under hypoxic drug exposure conditions [26]. The appearance of DNA strand breaks occurred with increasing depletion of the protective antioxidant GSH following 20 mM INO_2 aerobic exposure treatment in all three cell lines (Fig. 5). Furthermore, cell survival at 20 mM INO_2 aerobic treatment in all three cell lines was directly proportional to the degree of GSH depletion (Fig. 2a and 5). The loss of GSH (and GSSG) is likely due to its reaction with electrophiles followed by export from the cell and/or the export of excess GSSG from the cell. Therefore, the loss of GSH and the increased generation of ROIs with the appearance of DNA damage suggest that cytotoxic oxidative stress is induced following INO_2 treatment under normoxic conditions.

It was of interest to note that at equitoxicity similar amounts of DNA damage were observed in the CHO cells at 25–30 hr as were observed in the more sensitive REFs at 4–6 hr (Fig. 6), particularly because CHO cells have a lower capacity of generating ROIs and an increased antioxidant detoxifying capacity due to elevated catalase activity. However, other factors that modify the response of DNA to oxidative stress may be involved, such as the ability of cells to repair damage produced by an oxidative environment or intracellular levels of transition metals. Transition metals such as iron and copper increase the production of $OH^{\cdot}$ from H_2O_2 by Fenton chemistry, and their cellular metabolism has also been linked with increased sensitivity to oxidative stress [35]. Thus, the intracellular levels of these metals would also contribute to the differential observed in the production of ROIs following aerobic INO_2 treatment in all three cell lines.

In conclusion, this paper provides experimental evidence that oxidative stress occurs following INO_2 aerobic exposure in all three rodent cell lines studied. The kinetics of cell survival, generation of ROIs, GSH depletion, and DNA damage are dependent on the antioxidant enzyme

profile and on the rate of ROI generation. Understanding the biochemical and cellular differences between the sensitive REFs and the CHO cells helps to define factors that may be involved in the neuronal tissue toxicity induced by 2-nitroimidazole therapy. Certainly neuronal cells, specifically sensory neurons, need to be tested for 2-nitroimidazole-induced aerobic toxicity. In such studies, molecular differences, such as expression of the anti-apoptotic *bcl-2* oncogene, which has been shown to participate in the antioxidant pathway [36, 37], will need to be considered. Studies of INO_2 aerobic cytotoxicity with motor [38] and sensory [39] neuronal cells are currently in progress.

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