Mitochondrial Dysfunction after Aerobic Exposure to the Hypoxic Cytotoxin Tirapazamine

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ABSTRACT

Tirapazamine (TPZ) is a bioreductive drug that exhibits a high degree of selective toxicity toward hypoxic cells, and at doses that are used clinically, little or no cell killing is observed in aerobic cells. Nonetheless, the effects of TPZ on aerobic tissues are still responsible for the dose limitations on the clinical administration of this drug. Clinical side effects include fatigue, muscle cramping, and reversible ototoxicity. We have investigated TPZ-induced changes in the mitochondria in aerobically exposed cells as a potential mediator of these side effects. Our data show that aerobic administration of TPZ at clinically relevant doses results in a profound loss in the mitochondrial membrane potential (MMP). We have observed that MMP occurs in a variety of cell lines in vitro and also occurs in muscle tissues in vivo. The loss in MMP is temporary because recovery occurs within 2 h. TPZ is directly metabolized within mitochondria to a DNA-damaging form, and this metabolism leads to both the cell-killing effects of TPZ on aerobic cells at high doses and to the loss in MMP at clinically relevant doses. Using cell lines derived from genetically modified mice with a targeted deletion in manganese superoxide dismutase, we have further distinguished the phenotypic effects of TPZ in mitochondria at high toxic doses versus those at clinically relevant doses. We have investigated several potential mechanisms for this TPZ-induced loss in MMP. Our results indicate no change in the rate of cellular respiration in TPZ-treated cells. This implies that the loss in MMP results from an inability of the inner mitochondrial membrane to sustain a potential across the membrane after TPZ treatment. Incubation of cells with an inhibitor of the mitochondrial permeability transition suggests that the loss of MMP may result from the regulated opening of a large mitochondrial channel.

INTRODUCTION

Recognition of the adverse effects of tumor hypoxia on cancer therapy has led to the development of bioreductive drugs that are specifically toxic to hypoxic cells. TPZ (3-aminobenzotriazine-1,4-dinitride; WIN 59075; SR 4233; Tirazone) is the lead compound in a new class of bioreductive agents and has high selectivity for killing hypoxic cells in a range of different cell lines (1). For equivalent killing, aerobic cells require a TPZ dose that is 50–200 times greater than that for hypoxic cells. This allows the delivery of TPZ in the clinic at doses that are extremely toxic to hypoxic cells and essentially nontoxic (with respect to cell killing) to aerobic cells (2). TPZ is undergoing Phase II and Phase III clinical testing and published recently have shown significant clinical potential for this drug (3). Exposure to TPZ under hypoxic conditions leads to DNA single- and double-strand breaks, chromosome aberrations, and cell death (4). The toxic species has been inferred to be a radical that is produced upon a cofactor-dependent, 1-electron reduction of TPZ (5). This radical is highly reactive and in the absence of oxygen is converted to a stable 2-electron reduction product known as SR4317 by reacting with cellular constituents (5). The radical is believed to be the relevant toxic species because both the parent TPZ and the 2-electron stable reduction product SR4317 are nontoxic (6). The existence of this radical has been experimentally demonstrated in an in vitro system by electron paramagnetic resonance (7). Generation of the intermediate radical by exogenous enzymes in a cell-free in vitro assay is capable of causing DNA strand breaks in plasmid DNA (8, 9). In the absence of exogenously added enzymes or cellular protein fractions, there is no reduction of TPZ, and correspondingly no DNA damage is detected. In the presence of oxygen, the TPZ radical is rapidly back oxidized to the parent compound, concomitant with the production of superoxide (7). Under aerobic conditions, the 2-electron reduction product SR 4317 cannot be detected, even when high doses of TPZ are used, indicating a lack of any significant radical attack on cellular components.

Although TPZ is much less damaging under aerobic conditions, there are nevertheless toxicities observed in patients resulting from adverse effects of TPZ on presumably aerobic cells. These side effects include patient fatigue, muscle cramping, and reversible ototoxicity (10–13). Although muscle cramping itself is not dose limiting, this side effect has prevented the administration of TPZ with each daily fraction of radiation (11). These effects occur at TPZ doses that are far below those needed in tissue culture to kill aerobic cells, but they nonetheless limit the doses of TPZ that can be administered to patients. In this study, we have investigated damage to mitochondria in aerobic cells as a mechanism of toxicity that may limit the clinical efficacy of TPZ.

MATERIALS AND METHODS

Cell Culture. Details of the SCCVII cells used in these experiments have been described previously (14). HT1080, A549, and HeLa cells were obtained from the American Type Culture Collection. The CHO wild-type cells and a derivative engineered to overexpress NADPH P450 reductase were kindly provided by Dr. Sartorelli (Yale University School of Medicine, New Haven, CT; Ref. 15). The human osteoblast 143B cells and a derivative lacking mitochondrial DNA, 143B pΔy, were kindly provided by Dr. Michael King (Thomas Jefferson University, Philadelphia, Pennsylvania; Ref. 16). Cells were cultured in αMEM (CHO, HT1080, A549, and HeLa), Waymouth’s (SCCVII, MnSOD mouse embryo fibroblasts), or DMEM (143B cells) supplemented with 10% serum. The 143B osteoblast cells were grown in medium supplemented with uridine at a final concentration of 50 μg/ml.

Drugs and Chemicals. Rotenone, cyclosporin A, antimycin A, uridine, NADH, and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). Rh-123 and JC-1 were obtained from Molecular Probes (Eugene, OR). All tissue culture media and serum were from Life Technologies, Inc. TPZ was kindly supplied by Sanofi-Winthrop (Malvern, PA).
In Vitro Measurement of MMP. We used the fluorescent cationic dye Rh-123 to measure changes in the MMP essentially as described (17). Briefly, exponentially growing cells were loaded with 5 μmol/L Rh-123 for 20 min. Cells were then washed three times and exposed to TPZ at various concentrations and for various times. At the end of the incubation, cells were washed again, harvested by trypsinization, and then resuspended in PBS containing 0.5 mg/ml propidium iodide. The cell populations were then analyzed by flow cytometry (Becton Dickinson FACScan), and the mean fluorescence of viable cells (propidium iodide negative) was determined. Rotenone (10 μM) was administered routinely as a control and was administered over the same time period as TPZ.

In some cases, cells were loaded with Rh-123 after treatment with TPZ or various inhibitors and then analyzed as described. Levels of oxygenation were achieved as described previously (18).

We also measured MMP using the cationic dye JC-1 as described (19). This dye forms J-aggregates under high MMP, causing a shift in fluorescence from green to red. Cells were preloaded with JC-1 (5 μg/ml) for 30 min. Fluorescence was monitored in both green and red channels of the flow cytometer. Data using this indicator of MMP were represented by the level of the red/green ratio relative to controls (20).

In Vivo Measurement of MMP. To measure MMP in mouse muscle tissue in vivo, Balb/c mice received injections of 10 μg/g of Rh-123 either alone or with 0.3 mmol/kg TPZ administered 30 min or 1 h after the Rh-123. Mice were sacrificed 1 h after the TPZ injection. Muscle tissue (∼150 mg) was isolated and minced with scissors and then disaggregated at 37°C for 1 h in an enzyme mixture consisting of DNAse (0.02%), pronase (0.05%), and collagenase (0.02%). The resulting suspension was centrifuged at 1500 rpm for 10 min. The supernatant was discarded, and the pellet was taken up in 5 ml of butanol, homogenized, and centrifuged. The supernatants were then analyzed. For calibration purposes, standards containing 10−4 to 10−7 mg/ml Rh-123 in butanol were prepared. All samples and standards were read on a fluorescence spectrometer (excitation, 511 nm; emission, 549 nm).

In one experiment, muscle tissue was collected and dissociated with the enzyme mixture for 15 min. Single-cell suspensions were then collected and assayed for fluorescence on a cellular basis using the flow cytometer.

Clonogenic Assays. Exponentially growing cells were exposed to varying doses of TPZ for 1 h. After exposure, cells were washed three times with PBS, harvested by trypsinization, centrifuged, resuspended in growth medium, and plated at different densities for colony survival. After 14 days growth, Petri dishes were stained with crystal violet, and colonies containing >50 cells were scored as survivors. The surviving fraction at each dose was determined as the ratio of survivors in the treated cell populations relative to the untreated controls.

Isolation of Mitochondria. HeLa cells (∼2 × 106) were harvested and washed in ice-cold Ca2+-free PBS, followed by 250 mm sucrose. The cells were resuspended in 6 ml of 250 mm sucrose and disrupted by six strokes of a Dounce homogenizer (Kontes, Vineland, NJ; 0.05 mm clearance) on ice. Nuclei and debris were pelleted at 3000 × g for 5 min. The supernatant (∼4.5 ml) was decanted and loaded onto a hybrid Percoll/metrizamide discontinuous density gradient (21), centrifuged at 50,500 × g (average) for 20 min at 4°C, and the mitochondrial fraction was collected by Pasteur pipette. Mitochondria were washed in Ca2+-free PBS, pelleted at 17,000 × g, resuspended to 10 mg/ml protein in Ca2+-free PBS, and stored on ice. Protein concentration was determined using the bicinchoninic acid method (BCA Protein Assay; Pierce, Rockford, IL). Mitochondrial purity was assessed by enzyme activity of citrate synthase (22), and cytosolic contamination was assessed by assay of lactate dehydrogenase.

Measurement of MMP in Isolated Mitochondria. Mitochondria (250 μg) were diluted to 50 μl in Ca2+-free PBS with or without 1 mM NADH and a range of TPZ concentrations and were incubated at room temperature for 30 min. The mitochondria were then diluted to 500 μl in energization buffer [125 mM KCl, 20 mM 4-morpholinepropanesulfonic acid, 10 mM Tris-Cl (pH 7.4), 0.5 mM EGTA, 2 mM rotenone, and 10 μM safranin]. Energization was induced by the addition of 1 mM succinate. The MMP was determined as the difference in absorbance at 554 and 524 nm (sA554-sA524; Ref. 23) using a Beckman DU 640 spectrophotometer. The MMP was evaluated as the net absorbance 5 min after succinate addition minus the net absorbance immediately prior to succinate addition. Treated samples values were expressed as a percentage of control.

Measurement of TPZ Metabolism in Isolated Mitochondria. Metabolism of TPZ was determined by measuring the levels of TPZ and its 2-electron reduction metabolite (SR4317) using HPLC as described previously (6). To detect the 2-electron reduction product, incubations were carried out under hypoxic conditions.

Measurement of DNA Damage in Isolated Mitochondria by QPCR. Isolated HeLa mitochondria (500 μg of total protein) were resuspended in 60 μl of Ca2+-free PBS containing various concentrations of TPZ either with or without 1 mM NADH in microcentrifuge tubes on ice. The tubes were transferred to 37°C for 30 min to allow TPZ metabolism. The mitochondria were then harvested by diluting with 1 ml of ice-cold Ca2+-free PBS and pelleted by centrifugation at 14,000 × g for 10 min at 4°C. Mitochondrial DNA was extracted from the pellets using spin column methodology (Dnasey kit; Qia- gen, Hilden, Germany). DNA was quantitated against a DNA standard (A125; Sigma Chemical Co., St. Louis, MO). Mitochondrial DNA (125 pg) was used as a template in a 25-μl QPCR reaction using mitochondria-specific primers and conditions designed to amplify a 16.2-kb fragment (24). PCR was performed for 22 cycles, and the products were resolved on 0.8% agarose gels. The bands were visualized by staining with Vistra green (Amersham, Arlington Heights, IL), followed by scanning the gel with a fluorescence scanner (Storm; Molecular Dynamics, Sunnyvale, CA). Quantitation was performed with Imagequant software (Molecular Dynamics).

Generation of MnSOD Knockout Cells. Newborn mice from MnSOD heterozygous crosses were obtained from mice generated at University of California-San Francisco containing a targeted deletion of the MnSOD (SOD2) gene (25). These mice were kindly provided by Dr. Pak Chan (Stanford University). All mice were genotyped using PCR by the Chan laboratory. These mice are designated CD1-Sod2 (tm1Cje) in accordance with the nomenclature used by the Jackson Laboratory. Newborn mice littersmotes that were wild type, heterozygous, and homozygous for the MnSOD deletion were decapitated and prewashed. The skin from each mouse was removed aseptically, washed in 0.25% trypsin, and placed in a stirred flask of fresh trypsin on ice for 5 h. Cell suspensions were collected and plated into individual tissue culture flasks. As cells became confluent, they were passaged at a ratio of 3:1 (approximately every 3 days). This passaging was continued on a regular basis until cells entered crisis. Cells that escaped crisis and continued to proliferate were expanded into immortal cell lines and used for the experiments with TPZ.

Measurement of Respiration. Measurements of cellular respiration were conducted in the presence or absence of TPZ using a sensitive oxygen electrode in a sealed container with a known concentration of cells. Measurement of dissolved oxygen concentrations within the stirred cell suspensions were made as a function of time with a Clark-type oxygen electrode coupled to a sensitive amplifier from Instech Laboratories (Plymouth Meeting, PA). For these purposes, the polarographic oxygen probe was inserted through a ceramic port into a small 10-ml spinner flask. The flask was filled completely with cells suspended in complete growth medium at a concentration of ∼106/ml. Calibration of the probe was made by gassing with known concentrations of humidified oxygen at 37°C and allowing solutions without cells to reach equilibrium. The background current (low level detection limit) of the probe was found by an enzymatic depletion of oxygen using phosphate buffer (5 mM KH2PO4, 15 mM K2HPO4) containing 10 mM glucose to which catalase (0.5 mg) and glucose oxidase (0.05 mg) were added. After calibration, the oxygen concentration under experimental conditions could be monitored with a chart recorder. Addition of TPZ or rotenone to the stirred suspension was made through the small syringe port in the ceramic top. The port was subsequently sealed.

RESULTS

TPZ Produces a Loss in MMP. To study the effect of the anticancer agent TPZ on the MMP, we exposed cells to the fluorescent cationic dyes Rh-123 or JC-1 as described (17, 26–29). These dyes accumulate in energized mitochondria in proportion to the relative potential difference across the mitochondrial membrane. Fig. 1a shows that in SCCVII cells, the Rh-123 fluorescence decreased to ∼30% of control values after 30-min incubation with TPZ. The loss in MMP was dose dependent and reached a maximum at doses >250 μM. The overall reduction in MMP observed in these cells after aerobic TPZ exposure is almost as great as that observed after incubation with inhibitors of oxidative phosphorylation such as rotenone.
MMP recovers when assessed 2 h after TPZ treatment (followed by assessment of MMP with postloading of Rh-123. This allowed us to show that the concentration of 0.6%. In was measured at normal atmospheric oxygen of 20%, as well as at a reduced oxygen atmosphere.

In various concentrations of TPZ and assayed for MMP using the cationic dye Rh-123 (circles). Evaluating the remaining Rh-123 fluorescence relative to controls. In the loss in MMP was measured at normal atmospheric oxygen of 20%, as well as at a reduced oxygen concentration of 0.6%. In circles and triangles, the human squamous cell carcinoma line HT1080 (c, and the human cervical carcinoma line HeLa (d) were treated with various concentrations of TPZ and assayed for MMP using the cationic dye Rh-123.

Fig. 1. Aerobic administration of TPZ causes a dramatic decrease in the level of MMP. The murine squamous cell carcinoma cell line SCCVII (a and b), the human squamous cell carcinoma line HT1080 (c, and the human cervical carcinoma line HeLa (d) were treated with various concentrations of TPZ and assayed for MMP using the cationic dye Rh-123 (circles). In a and b, SCCVII cells were preloaded with Rh-123 and then exposed to TPZ. MMP was evaluated by the remaining Rh-123 fluorescence relative to controls. In b, the loss in MMP was measured at normal atmospheric oxygen of 20%, as well as at a reduced oxygen concentration of 0.6%. In c and d, HT1080 (c) and HeLa (d) cells were treated with TPZ first, followed by assessment of MMP with postloading of Rh-123. This allowed us to show that the MMP recovers when assessed 2 h after TPZ treatment (triangles). Bars, SD.

or antimycin A. The drop is steep and occurs over the range of concentrations (0–100 μM) that are achieved clinically in patients administered this drug (10–13). At these doses, TPZ has been shown to produce little, if any, cell kill when administered under aerobic conditions (5, 30, 31). We have observed this loss in MMP after aerobic TPZ exposure in a variety of rodent and human cell lines including mouse squamous carcinoma cells (SCCVII; Fig. 1, a and b), mouse embryo fibroblasts (Fig. 6), CHO cells (Fig. 3), human squamous cell carcinoma cells (HT1080; Fig. 1c), human cervical carcinoma cells (HeLa; Fig. 1d; Fig. 4), human osteoblast cells (143B; Fig. 8, below) and human lung carcinoma cells (A549; not shown).

The loss of MMP by TPZ under aerobic conditions is dependent upon oxygen concentration. We measured MMP loss under standard oxygen concentrations (20%) and also at reduced oxygen levels (0.6%). At the lower oxygen levels, TPZ is still relatively nontoxic, although at this concentration, some degree of cell kill can be measured by clonogenic assays (30). Fig. 1b shows that there is still a substantial loss in the MMP under these reduced oxygen concentrations, although the effect is substantially less than that at 20%. This result indicates that oxygen, and perhaps the oxygen stress associated with futile cycling of TPZ, participates in the loss in MMP.

Reduction in Membrane Potential is Transitory. Fig. 1, c and d, show that the drop in MMP is transitory and recovers within a period of 2 h. When HT1080 cells (Fig. 1c) or HeLa cells (Fig. 1d) were treated with TPZ and then analyzed immediately afterward with Rh-123, a substantial loss of MMP was observed. However, when these cells were treated with TPZ and then allowed to recover for 2 h before assaying MMP with Rh-123, no significant loss was observed. This recovery in membrane potential is consistent with the fact that at the low doses of TPZ that produce the loss in MMP, no direct cell killing is produced. All cells exposed to these doses under normal aerobic conditions are able to recover and continue unlimited clonogenic capacity.

The Loss in MMP Occurs in Vivo. To establish the potential clinical relevance of this effect, we determined whether TPZ elicits the same phenotypic responses in normal tissues in vivo. To do this, mice were injected with Rh-123 and 30 min or 1 h later administered TPZ (experiments 1 and 2, respectively). One h later, the mice were sacrificed, and skeletal muscle tissue was removed and assayed for Rh-123 fluorescence. Fig. 2 shows results measured on homogenized bulk muscle tissue from control or TPZ-treated mice. In two separate experiments, TPZ caused a significant loss in Rh-123 fluorescence, implying a drop in MMP. In a subsequent experiment (experiment 3), we isolated individual cells from muscle tissue and analyzed MMP on a cellular basis using a flow cytometer. In this case, muscle tissue was disaggregated into a single-cell suspension, and the mean Rh-123 fluorescence was measured from individual cells. Again, TPZ resulted in a drop of MMP equivalent to ~30%, consistent with the data from bulk muscle tissue. These data suggest that the loss in MMP observed after physiological TPZ treatment of tumor cells in vitro also occurs in normal tissues in vivo. The somewhat smaller loss in MMP compared with the effect seen in vitro is probably a result of the lower oxygen levels in muscle tissue than in cells in vitro.

Metabolism of TPZ by Mitochondria Is Responsible for the Loss in MMP. The loss of MMP after TPZ exposure prompted us to examine the intracellular metabolism of TPZ under aerobic conditions. As a first test, we utilized a pair of genetically matched cell lines, one of which had been engineered to overexpress the cytosolic enzyme P-450 reductase (15). This enzyme has been shown previously to metabolize TPZ and has been implicated in the toxicity of this drug (32, 33). The activity of this enzyme in the overexpressing cell line is ~20 times higher than in the parent cell line (data not shown). Fig. 3 shows that this overexpression results in a dramatic sensitization to the aerobic cell-killing effects of high TPZ doses. Because...
All had 40 nm of NADH. No change in MMP is observed without NADH (Bars). h c NADH. isolated mitochondria with TPZ results in DNA damage. DNA damage was assessed by QPCR after exposure of mitochondria to TPZ in the presence (n allowing a quantitative measurement of the overall reduction of TPZ SR4317. The SR4317 reduction product can be measured by HPLC, reduction of the radical to the stable 2-electron reduction product unstable radical. Under hypoxic conditions, this radical is implicated ability to metabolize TPZ. TPZ undergoes a 1-electron reduction to an mitochondrial enzyme, we isolated mitochondria and measured their metabolism relevant to the loss in MMP does not occur via P-450 reductase and likely does not occur in the cytoplasm.

To determine whether TPZ was being metabolized directly by a mitochondrial enzyme, we isolated mitochondria and measured their ability to metabolize TPZ. TPZ undergoes a 1-electron reduction to an unstable radical. Under hypoxic conditions, this radical is implicated in causing DNA damage and cell death coincident with the further reduction of the radical to the stable 2-electron reduction product SR4317. The SR4317 reduction product can be measured by HPLC, allowing a quantitative measurement of the overall reduction of TPZ (6). Under aerobic conditions, little or no SR4317 is produced, presumably because in the presence of oxygen the TPZ radical is rapidly back oxidized to the parent compound, and in so doing, produces the potentially toxic radical superoxide. We thus incubated mitochondrial fractions with TPZ under hypoxic conditions and looked for the formation of the reduction product SR4317. We found that mitochondria preparations metabolized TPZ effectively (Fig. 4a). The metabolism resulting from equal protein concentrations from mitochondria was similar to that of whole-cell lysates. Reduction of TPZ by mitochondria was strictly dependent on NADH, indicating that it was enzyme mediated. Unlike whole-cell lysates, NADPH could not serve as a cofactor. In the whole-cell lysates, metabolism occurred using either NADH (perhaps mitochondrial enzymes) or NADPH (likely p450 reductase mediated) as a cofactor. Using a PCR-based assay, we also showed that reduction of TPZ under aerobic conditions by isolated mitochondria resulted in mitochondrial DNA damage (Fig. 4b). TPZ-induced damage in the isolated mitochondria was highly dependent upon the presence of the NADH cofactor, and thus presumably metabolism of TPZ.

We next determined whether the reduction of TPZ by the mitochondria was directly responsible for the loss in the MMP, or if the loss in MMP was a secondary effect in response to a more generalized cellular response to TPZ. For example, induction of apoptosis is often associated with a loss in MMP, even though the signals for apoptosis may occur outside of the mitochondria (34, 35). Isolated mitochondria were exposed to TPZ under aerobic conditions in the absence of other cytoplasmic and nuclear factors. The ability of the mitochondria to become energized was then assessed by measuring the uptake of the cationic dye safranin after stimulation with succinate. Consistent with the cellular results, isolated mitochondria exposed to TPZ under aerobic conditions exhibited a substantial loss in MMP (Fig. 4c). The effect of TPZ on the MMP required the addition of the cofactor NADH during TPZ exposure. In the absence of NADH, no metabolism of TPZ took place and no loss in MMP was observed. This result implies that the loss in MMP by isolated mitochondria is strictly dependent on reduction of TPZ by mitochondria.

![Diagram](image-url)
Superoxide Generation in Mitochondria Is Responsible for Toxicity but not for Loss of MMP. Generation of superoxide results from back-oxidation of the TPZ radical when metabolism occurs under aerobic conditions. One of the ways that superoxide becomes toxic is by conversion of this radical into the highly damaging and toxic 17OH radical through a Fenton reaction. Because the Fenton reaction requires Fe(II) or other divalent cation, this allowed us to test the possibility that direct radical damage to the mitochondrial membrane was responsible for the loss in MMP. To do this, we investigated whether the iron chelator desferrioxamine (100 μM) could reduce the effect of TPZ on the MMP. This chelator has been shown previously to provide a 3-fold protection against the aerobic cell-killing effects of high TPZ doses when added 2 h prior to TPZ exposure (36). We measured the loss of MMP in SCCVII cells that were preincubated with desferrioxamine for 2 h. Fig. 5 shows that under these conditions desferrioxamine provides only a modest level of protection against MMP loss. These data suggest that direct radical damage to the membrane is, if anything, only a minor contributor to the loss in MMP. We also obtained similar results when cells were preincubated with desferrioxamine for 12 h prior to TPZ exposure (data not shown).

We also tested the possibility that the generation of superoxide was responsible for the loss in MMP using a genetic approach. Superoxide generation in the mitochondria has been implicated previously in the high-dose toxic effects of aerobic TPZ exposure. Cells that were adapted to grow in TPZ showed increased levels of the mitochondrial form of superoxide dismutase, MnSOD (37). To directly test the role of superoxide generation in the mitochondria, we established cell lines from MnSOD knockout mice and their wild-type littermates (25). We established wild-type, heterozygous, and knockout fibroblast cell lines from day 14 embryos obtained from MnSOD heterozygous crosses. After these cell lines had gone through crisis, we measured the effects of aerobic TPZ exposure with respect to both loss in MMP and toxicity. Consistent with the previous data implicating superoxide and MnSOD in the aerobic toxicity of TPZ, the MnSOD knockout cells were highly sensitive to cell killing by high doses of TPZ under aerobic conditions (Fig. 6a). This result directly implicates superoxide generation in the mitochondria as a mediator of the cellular toxicity that results after aerobic administration of high doses of TPZ and establishes the mitochondria as an important target for the cell-killing ability of this drug.

In contrast to the results using clonogenic survival as an endpoint, loss of MnSOD had no effect on the loss in MMP observed after exposure of these cells to clinically achievable concentrations of TPZ. Fig. 6b shows that the mouse embryo fibroblasts derived from wild-type, heterozygous, and homozygous MnSOD knockouts respond identically to TPZ with respect to the resulting loss in MMP. These data provide further evidence that the loss in MMP after aerobic TPZ exposure is not attributable to direct oxygen-radical damage to the mitochondrial membrane. This result also dissociates the mechanistic basis for cell killing observed at high doses of TPZ and the loss in MMP at clinically relevant doses, even though mitochondria participate in both aspects.

Requirement of Oxygen and the Electron Transport Chain. The MMP is maintained by pumping protons out of the inner mitochondria space. These pumps are driven by energy liberated through respiration as electrons pass down the electron transport chain, eventually reacting with oxygen. TPZ could effect respiration, and ultimately MMP, by two possible mechanisms. The first is an inhibition of respiration and thus oxygen consumption, analogous to the situation, when agents such as rotenone and antimycin A block the electron chain. These agents interfere with the enzymes responsible for transferring electrons down the chain, resulting in inhibition of oxygen consumption and a loss of MMP. The other possibility is an uncoupling of electron transport and MMP. Agents that interfere with the ability of the membrane to retain a potential across it (i.e., agents that dissipate the membrane potential) result in an accelerated rate of oxygen consumption. In this case, electron transport and respiration run at a maximum rate, whereas under normal conditions the MMP balances or couples oxygen consumption to membrane potential.

In order to test whether inhibition of respiration and oxygen consumption was responsible for the drop in the MMP in cells treated aerobically with TPZ, we used a sensitive oxygen electrode to measure cellular respiration. This was conducted by placing a known number of cells in a sealed, stirred suspension and measuring the loss of dissolved oxygen within the media resulting from cellular consumption. Fig. 7 shows the oxygen depletion curves measured with this electrode. In each case we allowed an equilibration period after starting, which allowed us to determine the initial steady-state oxygen consumption. We then added TPZ or various inhibitors through a small porthole that was subsequently sealed. Addition of this known

Fig. 5. The iron-chelating agent, desferrioxamine, fails to protect against MMP loss. SCCVII cells were incubated with (•) or without (○) the iron chelator desferrioxamine for 2 h prior to treatment with varying concentrations of TPZ. TPZ-mediated loss of MMP was assayed by flow cytometry using the fluorescent dye Rh-123.

Fig. 6. Loss of MnSOD increases cellular sensitivity to TPZ-induced killing but not to TPZ-induced loss of MMP. Cell lines were established from newborn mice after MnSOD heterozygous crosses. In a, the clonogenic survival of wild-type (○) and homozygous MnSOD knockout (●) cells treated with varying concentrations of TPZ as shown. In b, the wild-type (○), heterozygous (+), and homozygous (●) cell lines were assayed for loss of MMP after treatment with TPZ, using flow cytometry. Bars, SD.
volume of TPZ or inhibitor caused a corresponding loss of cells that could be accounted for. Analysis of the data in Fig. 7 indicates that addition of 100 or 500 μM TPZ resulted in no change in the cellular oxygen consumption. The small decreases shown in Fig. 7 can be entirely attributed to the small loss of cells that occurs after administration of the drug. In contrast, addition of rotenone caused a nearly complete block in respiration.

We have also looked directly at the requirement of a functional electron transport chain to elicit a loss in MMP after TPZ exposure. This was carried out using genetically matched human osteoblast cell lines, one of which lacked mitochondrial DNA (16). These so-called "ρ0" cells contain energized mitochondria but lack a functional respiratory chain as a result of depletion of mitochondrial DNA and the corresponding proteins encoded by the mitochondria that are required in the chain. These cells generate a MMP by a mechanism opposite of that of normal cells. Instead of pumping out protons to create the MMP and generate ATP from ADP, these cells exchange ATP for ADP through the adenine nucleotide carrier and use the resulting energy to pump out protons. We analyzed the effects of aerobic TPZ administration in this pair of cells. The 143B wild-type osteoblast cell line exhibited a dose-dependent decrease in MMP analogous to that observed in other cells (Fig. 8). However, the ρ0 cells exhibited a much smaller drop in MMP in comparison with the wild-type cells. These data imply that a functional respiratory chain is required to observe the loss in MMP. We speculate that the enzyme that metabolizes TPZ within the mitochondria may be a part of the electron transport chain. Alternatively, electrons being passed down the electron chain may serve as the reducing species used by another enzyme in the mitochondria.

Mitochondrial Permeability Transition. Loss in MMP can occur by the regulated opening of a large pore complex in the inner mitochondrial membrane. The opening of this pore results in the so-called PT, allowing solutes with a molecular weight of ~<1,500 to equilibrate across the inner membrane. Loss in MMP can lead to the PT, and conversely the PT can lead to loss in MMP. Pore opening can be prevented by submicromolar concentrations of cyclosporin A (CsA) and its analogues. This inhibition by CsA allowed us to test whether the PT was responsible for the loss in MMP after TPZ administration. Fig. 9 shows that when the PT was inhibited in SCCVII cells with 100 nM CsA, aerobic administration of TPZ failed to produce a loss in Rh-123 fluorescence. This result implies that the loss in MMP after TPZ may be attributable to opening of the pore complex and the resulting mitochondrial PT.

DISCUSSION

Many cellular enzymes are capable of metabolizing TPZ, and it is becoming increasingly clear that the intracellular location of these enzymes is an important determinant of the consequences of that metabolism. Under hypoxic conditions, the enzymes responsible for the reduction of TPZ that produces DNA damage and presumably toxicity are located within the nucleus (38). The identities of these enzymes remain unknown. Under aerobic conditions, several enzymes have been shown to be mediators of the cellular response to TPZ including the cytosolic enzyme NADPH P-450 reductase. Our data show that reduction of TPZ by the mitochondrial compartment is also an important determinant of the cellular response to TPZ under aerobic conditions.

The response of cells to TPZ under aerobic conditions can be classified into two distinct categories. At very high doses (millimolar concentrations), TPZ produces toxicity in the form of direct cell killing. In contrast, at low, clinically relevant TPZ doses, a significant loss in MMP is observed. We speculate that the dose-limiting side
effects that have been observed in patients are attributable to this second cellular response. Interestingly, mitochondria appear to be important in both the high-dose and low-dose cellular responses. At high doses, metabolism of TPZ by the mitochondria results in the generation of superoxide. Under the appropriate conditions, some of this superoxide will be converted to toxic hydroxyl radicals, and both of these radical species are toxic when produced at high enough levels. Using cell lines derived from MnSOD knockout mice, we have shown that generation of superoxide by the mitochondria is an important contributor to TPZ-induced cell killing under aerobic conditions. This is consistent with a previous report showing increased expression of this enzyme in cell lines adapted to grow in the presence of TPZ (37). However, toxicity produced by reduction of TPZ outside of the mitochondria is equally important. Increased P-450 reductase activity in cells overexpressing this cytosolic enzyme results in a corresponding increase in cell killing under aerobic conditions. Thus, the cell-killing observed under aerobic high-dose TPZ exposure results from reduction of TPZ by at least two different enzymes in two different cellular locations. Interestingly, in neither of these two cases where we have genetically manipulated the cells to become sensitive to TPZ-induced cell killing at high doses does the sensitivity to loss of MMP change when assessed at low TPZ doses.

Mitochondria have also been directly implicated in the second aerobic response of cells to TPZ. After low, clinically relevant doses, we have observed a profound loss in MMP. At these exposures, the MMP recovers within 2 h after treatment, and little or no direct cell killing is observed. The loss in MMP is dose dependent, observable in two different cellular locations. Interestingly, in neither of these two cases where we have genetically manipulated the cells to become sensitive to TPZ-induced cell killing at high doses does the sensitivity to loss of MMP change when assessed at low TPZ doses.

We have investigated several mechanisms to explain the loss in MMP. One possibility was that the oxygen species produced as a result of TPZ reduction and back-oxidation produced direct damage to the inner mitochondrial membrane. These toxic oxygen radicals are important mediators of the aerobic cellular toxicity at high TPZ doses (36). However, we found that the loss in MMP at low TPZ doses was insensitive to the levels of these radicals. Using a genetic approach, we created cell lines from MnSOD-deficient mice. MnSOD is located within the mitochondria, and the cell lines derived from these mice are unable to remove mitochondrial superoxide. Although these cells become dramatically more sensitive to the high-dose killing effect of TPZ, the MnSOD knockouts responded identically to the wild-type cells with respect to a loss in MMP at low TPZ doses. Similarly, the loss in MMP could not be significantly abrogated by administration of the iron-chelating agents desferrioxamine or ICRF 187. These agents prevent hydroxyl radical production by blocking the Fenton reaction and have been shown previously to protect against cell killing at high TPZ doses (36). These results further differentiate the aerobic cellular responses at low and high doses of TPZ.

We also investigated the possibility that TPZ reduction by the mitochondria somehow interfered with respiration, possibly through inhibition of electron transport. Our data indicate that there is no change in electron transport (monitored by oxygen consumption in a closed system) when TPZ was added to cells. This result implies that the reduction of MMP is not because of a failure to transfer electrons across the inner membrane space. Instead, these data implicate an effect of TPZ on the ability of the inner membrane to maintain a potential across it. A previous study showed that high doses of TPZ could result in an increased respiration rate by uncoupling respiration from oxidative phosphorylation (41). Our data showing a drop in MMP is consistent with this finding because the loss in MMP would allow oxidative phosphorylation to proceed at a maximum rate. However, we did not observe any significant increase in the rate of oxygen consumption in our system. Nonetheless, the fact that respiration was occurring without generation of a normal MMP implies that ATP production would be significantly impaired.

The lack of direct radical damage to the membrane or inhibition of oxidative phosphorylation prompted us to look at the mitochondrial PT as a possible mechanism for the loss in MMP. The pore opening that activates the PT abrogates MMP loss in cells treated with TPZ. SCCVII cells treated with CsA were assayed for loss of MMP by flow cytometry after treatment with various concentrations of TPZ. Bars, SD.

**Fig. 9.** Inhibition of the PT abrogates MMP loss in cells treated with TPZ. SCCVII cells treated with (C) or without (F) CsA were assayed for loss of MMP by flow cytometry after treatment with various concentrations of TPZ. Bars, SD.

**Fig. 10.** Intracellular localization of TPZ metabolism mediates the downstream biological consequences.
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stress, thiols, agents, and NADH oxidation (42). Because TPZ can be expected to effect each of these modulators of pore opening, we tested whether specific inhibition of the pore opening would prevent the TPZ-induced loss in MMP. Preincubation of cells for 30 min prior and during TPZ exposure with 100 nm CsA completely abrogated the TPZ-induced loss in MMP. Thus, the loss in MMP appears largely attributable to the PT. Recent reports suggest that enhanced pore opening can result from the increased binding of matrix cyclophilin to the adenine nucleotide translocase (42, 43). This binding appears to lead to a change in the conformation of the adenine nucleotide translocase that dramatically increases the sensitivity of mitochondria to the PT. Oxidative stress also has been shown to cause a recruitment of cyclophilin to the inner mitochondrial membrane (44). Our results suggest that a similar effect may occur after aerobic administration of TPZ. In this case, the oxidative stress produced by TPZ directly within the mitochondria would lead to increased cyclophilin binding and sensitization to pore opening.

In conclusion, although TPZ is selectively toxic to hypoxic cells, the dose-limiting toxicities in patients are likely attributable to its effects on aerobic cells. The data presented here suggest that the side effects observed in patients may not be attributable to TPZ-induced cell killing in normal cells. Instead, they offer the explanation that these toxicities result from the ability of TPZ to damage mitochondria, cell killing in normal cells. From these data, one can conclude that the data presented here suggest that the side effects observed in patients administered TPZ.

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