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Nitroxide TEMPOL Impairs Mitochondrial Function and Induces Apoptosis in HL60 Cells

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Abstract The piperidine nitroxide TEMPOL induces apoptosis in a number of tumor cell lines through free radical-dependent mechanisms. As mitochondria play a major role in apoptosis as both source and target for free radicals, the present study focuses on mitochondrial effects of TEMPOL in a human promyelocytic leukemic cell line (HL-60). On 24-h exposure to TEMPOL, the following alterations were observed: 1) decrease in both the intracellular and mitochondrial glutathione pools; 2) impairment of oxidative phosphorylation; and 3) decrease in mitochondrial membrane potential. In addition, TEMPOL was found to specifically target complex I of the respiratory chain, with minor effects on complexes II and IV, suggesting that mitochondrial effects might play a role in TEMPOL-induced oxidative stress and apoptosis, and that TEMPOL might sensitize tumor cells to the pro-apoptotic effects of cytotoxic agents. J. Cell. Biochem. 82: 271–276, 2001. © 2001 Wiley-Liss, Inc.

Key words: apoptosis; mitochondria; glutathione; oxidative stress; TEMPOL

The piperidine nitroxide TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; TLP) is widely used as a spin labelling agent for EPR studies in biological systems [Mehlhorn and Packer, 1983]. Following the demonstration of its antioxidant properties, the compound has been proposed as cytoprotectant in a number of experimental settings involving oxidative stress, including irradiation-induced cell damage, myocardial ischemia/reperfusion injury, closed head injury, and anthracycline cardiotoxicity [Samuni et al., 1991; Hahn et al., 1992; Monti et al., 1996; Zhang et al., 1998]. However, ongoing studies by our group have shown that TPL is also able to exert cytotoxic effects, which are mediated by oxidative stress and depend on induction of apoptosis [Gariboldi et al., 1998]. In this respect, TPL behaves similarly to other known antioxidants, such as pyrrolidine dithiocarbamate and N-acetylcysteine, which exhibit pro- or antioxidant activity, and exert cytoprotective or cytotoxic effects, depending on the specific microenvironment [Chinery et al., 1998; Della Ragione et al., 2000]. Interestingly, growth inhibition by TPL shows some degree of selectivity, as lower concentrations of the nitroxide are required to inhibit the growth of tumor cells in comparison with non-neoplastic cells of similar lineage, and does not exhibit cross resistance in cells with a multidrug resistant phenotype [Gariboldi et al., 1998]. Based on these properties, TPL might have a role as antiproliferative agent in cancer chemotherapy; however, the specific subcellular target(s) involved in its growth inhibitory effect remain(s) to be identified. Previous studies by our group showed that TPL tends to accumulate in the mitochondrion in myocardial cells [Monti et al., 1996]. As mitochondria play a pivotal role both in free radical-mediated events and in the apoptotic process, the present study investigates the possible link between mitochondrial events and TPL-induced cell death in the HL60

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human promyelocytic leukemic cell line. The effects of TPL have been examined, both on mitochondrial electron transport chain and on parameters which are directly related to the onset of apoptosis, such as disruption of the potential across the inner mitochondrial membrane ($\Delta\psi_m$) and depletion of the mitochondrial gluathione (GSH) pool.

MATERIALS AND METHODS

All standard chemical and cell culture reagents, unless otherwise indicated, were purchased from Sigma Aldrich srl.

Cell Culture, Growth Curves and Assessment of Apoptosis

HL60 cells were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 10% FBS (Mascia-Brunelli), and 1% antibiotic mixture. To obtain growth curves, cells were seeded at 3×10^5/ml and treated with different TPL concentrations (1.0, 2.5, and 5 mM). Aliquots of cell suspensions were withdrawn every 12 h and counted in the presence of Trypan blue to assess cell viability. Apoptosis was visualized in cells exposed to TPL for 24 h by fluorescence microscopy, using the In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim, Mannheim, Germany) according to the manufacturers' instructions.

Isolation of Mitochondria

Mitochondria were isolated from HL-60 cells by differential centrifugation, as previously described [Piedimonte et al., 1986]; the fraction obtained exhibited succinate dehydrogenase levels 3–4-fold higher than total homogenates.

HPLC Determination of Mitochondrial TPL Levels

Mitochondria were isolated according to standard fractionation procedures and TPL levels were determined in the organic phase obtained by octanol extraction (1:2) using a Jasco PU-980 HPLC system equipped with a Coulochem 5100A electrochemical detector connected to a 5011 (ESA) analytical cell. A LiChrospher 60 RP SELECT B (5 μm) column (Merck: length 12.5 cm, internal diameter 4 mm was used with a mobile phase consisting of Na-acetate buffer 0.1 mM: methanol (80:20). HPLC analysis was performed at room temperature under isocratic conditions, with a flow rate of 1 ml/min.

Determination of Total Cellular and Mitochondrial GSH

GSH concentration was determined on total cell extracts and on the mitochondrial fraction from cells exposed to TPL for 24 h by the fluorometric method described by Hissin and Hilf [1976], using o-phthaladehyde (OPT) as a fluorescent reagent.

Determination of $\Delta\psi_m$

The effect of TPL on $\Delta\psi_m$ was assessed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) by measuring the intracellular incorporation of $DiOC_6(3)$ (250 nM in PBS at 37°C for 30 min), a cationic fluorescent dye which accumulates into mitochondria as a function of $\Delta\psi_m$.

Mitochondrial Respiration and ATP Formation

Oxygen consumption rates were determined polarographically, in whole HL-60 cells and in isolated mitochondria, using a Clark-type oxygen electrode (Yellow Springs Instruments Co.) at 25°C in respiratory medium. Mitochondrial respiration was monitored with the use of a 3-ml reaction vessel and a Clark oxygen electrode in respiration buffer (10 mM Tris, pH 7.4, 1.5 mM phosphate, 10 mM KCl, 0.1 mM EDTA, 0.3 M mannitol, 13 mM glutamate). States 3 and 4 of respiration were determined in the presence or absence of 187 μM ADP, respectively, according to the method described by Chance and Williams [1956]. At the end of the measure, aliquots of the reaction mixtures were used for protein assay and for determinations of ATP, which were performed after protein precipitation with 50% TCA by the luciferin-luciferase reaction [Lyman and DeVincenzo, 1967].

Activities of Mitochondrial Complexes

The effect of TPL on the activities of mitochondrial complexes was assessed as previously described [Colleoni et al., 1996]. The effect of TPL on complex I (NADH-ubiquinone reductase) specific activity was assessed on freeze-thawed mitochondria from HL-60 cells by measuring the rate of NADH oxidation inhibitable by 2 mM rotenone in the presence of antimycin (2 μg/ml) and sodium azide (0.2 mg/ml) to block complex III and complex IV, respectively. The molar extinction coefficient (ε) for NADH was 6.81 $mM^{-1}\,cm^{-1}$. The effect of TPL on complexes II (succinate-ubiquinone

reductase) and III (succinate-ferricyanide reductase) was evaluated on freshly isolated mitochondria in the presence of rotenone block of complex I, measuring the TTFA (0.1 M)-inhibitable rate of reduction of 2,6-dichlorophenol-indophenol (DCIP; $\varepsilon = 21\ mM^{-1}\ cm^{-1}$) for Complex II or of $Fe_3(CN)_6$ ($\varepsilon = 1.2\ mM^{-1}cm^{-1}$) for Complex II + III. The activity of complex IV (cytochrome oxidase) was determined by measuring oxygen consumption (state 3 and 4) using tetramethyl-p-phenylenediamine/ascorbate (0.2 mM/1 mM) as an artificial electron donor, thereby shunting electron transfer through complexes I to III.

RESULTS

When HL60 are grown in the presence of different TPL concentrations, a clear antiproliferative effect is observed at 48 h with 1 mM TPL, whereas cytotoxicity develops for higher nitroxide concentrations, starting at 36 h (Fig. 1a). TUNEL analysis at 24 h shows a concentration-dependent increase in apoptotic cells (Fig. 1b).

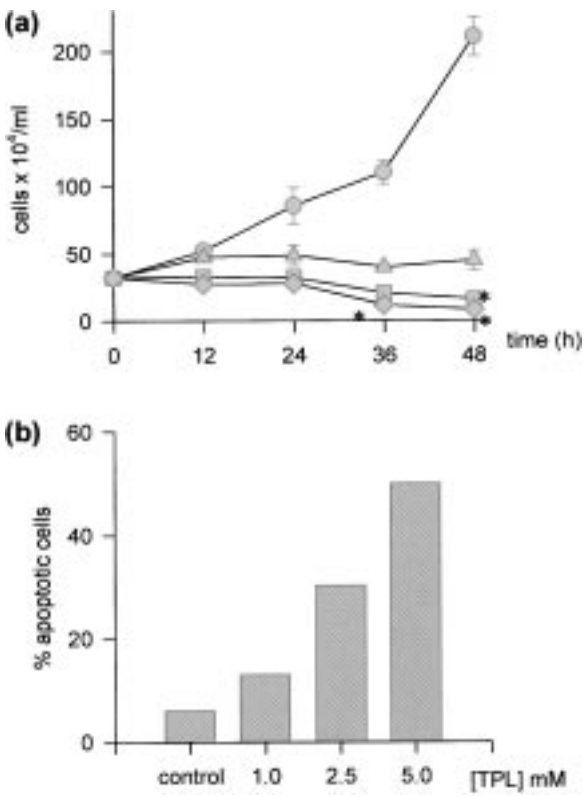

Fig. 1. Effect of TPL on the growth and survival of HL60 cells. **a:** Growth curves in control cells (●) and in cells trated with 1.0 (▲), 2.5 (■) or 5.0 mM (◆)TPL. Mean±SD of four independent experiments. **b:** percent of apopototic cells following 24 h exposure to TPL.

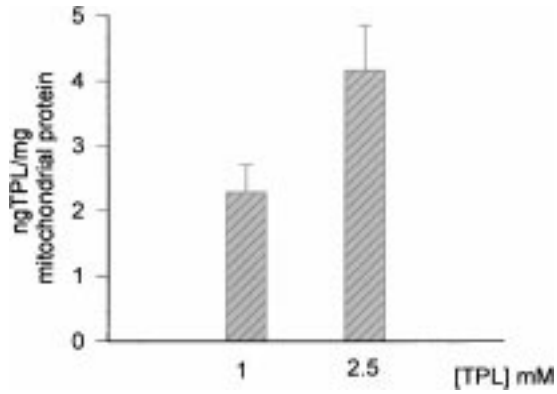

Fig. 2. TPL levels in mitochondria isolated form HL60 cells following 24 h exposure to TPL. Mean±SE of three determinations.

Figure 2 shows that mitochondria from TPL-treated HL60 cells accumulate detectable amounts of TPL, which are directly related to the nitroxide concentration in the culture medium.

Figure 3 shows the results of the fluorometric determination of the total cellular and mito-

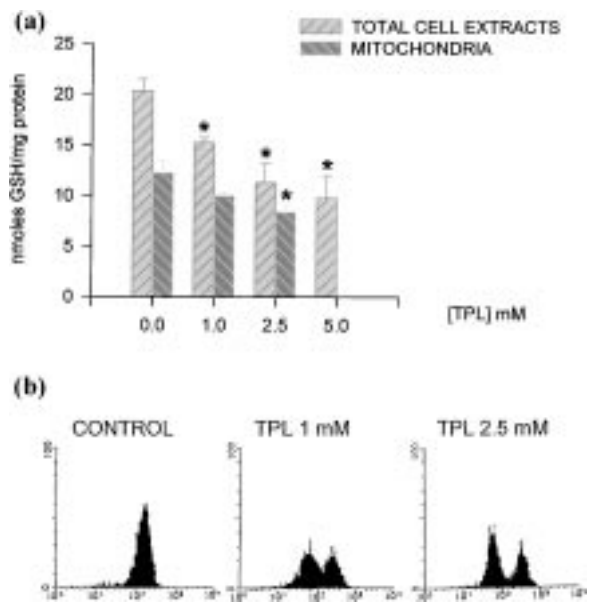

Fig. 3. **a:** GSH content in total cell extracts and in mitochondria obtained from HL60 cells exposed to TPL for 24 h. Mean±SE of three independent experiments. $^*p < 0.05$ vs. control. **b:** Flow cytometric determination of $\Delta\psi_m$ in HL60 cells treated with TPL for 24 h. Cell numbers are reported on the *y*-axis as a function of fluorescence intensity, expressed in arbitrary units on the abscissa. A typical histogram is shown. Similar data were obtained in four independent experiments.

chondrial GSH pools. A concentration-dependent decrease can be observed for both pools following exposure to TPL for 24 h (Fig. 3a). This effect is accompanied by the appearance of a sub-population of HL60 cells exhibiting $\Delta\psi_m$ values lower than control cells, as assessed by flow cytometry using $DiOC_6(3)$ as fluorescent probe (Fig. 3b).

Figure 4a shows that 24 h exposure of HL60 cells to TPL induces a concentration-dependent decrease both in oxygen consumption and in ATP formation. Similar results are obtained when these two parameters are measured in mitochondria isolated from TPL-treated HL60 cells. Oxygen consumption, both in the presence (state 3) and in the absence (state 4) of added ADP, and ATP formation exhibit a parallel decline following exposure of HL60 cells to increasing TPL concentrations.

Figure 5 shows the effect of TPL on the function of complexes I through IV in the mitochondrial electron transport chain. A significant, concentration-dependent decrease can be observed in the activity of complex I (NADH-ubiquinone reductase) following exposure of HL60 cells to TPL for 24 h (a). A significant inhibition is also apparent for the activities of complexes II (succinate-ubiquinone reductase, b) and IV (cytochrome oxidase, d) in mitochondria from HL60 cells exposed to the higher TPL concentration (2.5 mM) for 24 h. Comparison of the effect on complex II with the TPL-induced decrease in succinate-ferricyanide reductase activity (corresponding to Complex II + III activity, c) indicates that the contribution of Complex III inhibition to the overall effect of TPL on the mitochondrial electron transport chain is negligible.

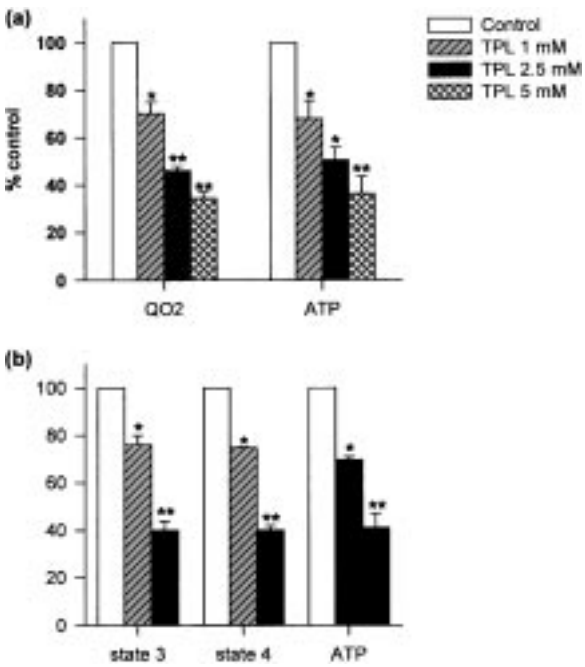

Fig. 4. Effect of 24 h exposure to TPL on oxygen consumption and ATP formation in intact HL60 cells (**a**) and in mitochondria isolated from treated cells (**b**). State 3 and state 4 refer to oxygen consumption in the presence and in the absence of 187 μM ADP, respectively. $^*p < 0.05$ vs. control; $^{**}p < 0.05$ vs. control and vs. 1 mM TPL. Mean ± SE of three independent experiments.

DISCUSSION

Previous studies by our group indicate that the piperidine nitroxide TPL is able to exert cytoprotective or cytotoxic effects. Such dual behavior is not uncommon among antioxidants, and compounds such as PDTC have indeed been shown to induce cell death in different human tumor cell lines depending on the specific microenvironmental conditions [Komarov et al., 1997; Chinery et al., 1998; Della Ragione et al., 2000]. TPL has been found to exert antiproliferative effects on a number of cell lines derived from human solid and hematological malignancies [Gariboldi et al., 1998]. Data obtained in some of these cell lines indicate that cell death depends largely upon triggering of apoptosis due to induction of cellular oxidative stress. However, the intracellular target for TPL action remains elusive. Studies performed on isolated rat hearts perfused with TPL-containing saline demonstrated that intracellular TPL could be recovered mainly from the cytosolic and mitochondrial fractions. These data have been confirmed by the results obtained in the present study (Fig. 2), leading to the hypothesis that TPL might directly target mitochondrial structures. Such a mechanism of action would be particularly appealing in view of two considerations; 1) mitochondrial events have been assigned a crucial role in the apoptotic process; and 2) TPL exerts its cytotoxic effect also in cell lines lacking p53 expression (such as the HL-60 cell line used for the present study). Induction of p53 and downstream genes is probably the major pathway in DNA damage-induced apoptosis; the requirement for activation of this pathway would be bypassed if TPL were to act primarily on mitochondrial structures.

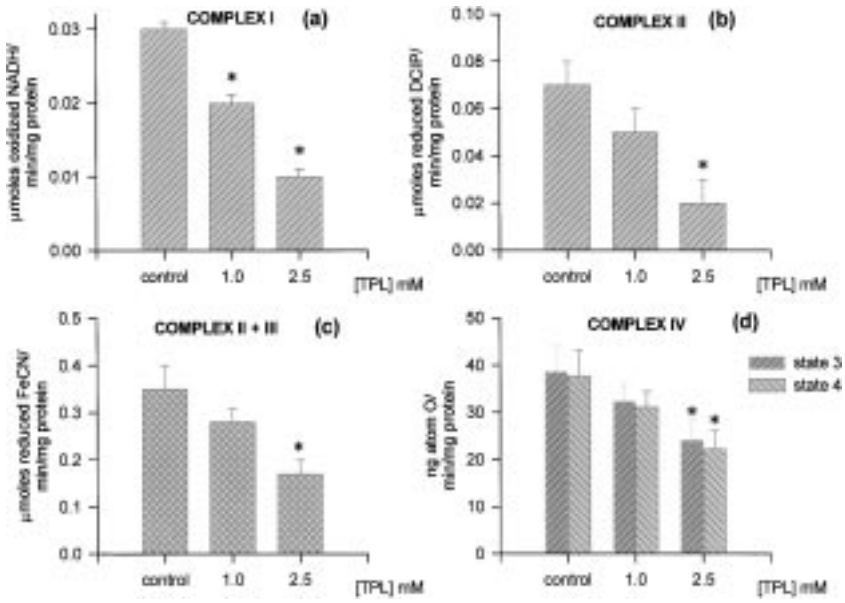

Fig. 5. Effect of TPL on the activity of the respiratory complexes in mitochondria isolated from HL60 cells exposed to TPL for 24 h; (**a**) NADH-ubiquinone reductase; (**b**) Succinate-ubiquinone reductase; (**c**) Succinate-ferricyanide reductase; (**d**) cytochrome oxidase. Mean ± SD of three independent experiments. $^*p < 0.05$ vs control.

The role played by mitochondria in the apoptotic process has recently been re-evaluated, following the observation that collapse of the electrochemical gradient of the inner membrane ($\Delta\psi_m$) and release of apoptogenic factors through the outer mitochondrial membrane are general features of this mode of cell death [Kroemer et al., 1998]. Dissipation of the $\Delta\psi_m$ occurs irrespective of the cell type and of the apoptosis inducer and constitutes an early event as compared to the other hallmarks of apoptosis at the levels of the nucleus, cytoplasm, and plasma membrane. Current hypotheses on apoptosis predict that various damage pathways and proapoptotic signal transduction cascades converge at the level of the mitochondrion, in line with the fact that a number of physiological effectors can induce an irreversible mitochondrial permeability transition (PT). Several of the metabolic consequences of mitochondrial PT are well-known byproducts of apoptosis: uncoupling of the respiratory chain with hyperproduction of reactive oxygen species, disruption of mitochondrial (and later extra-mitochondrial) Ca^2+ homeostasis, and liberation of protease activators with loss of cytochrome c (which blocks the respiratory chain). All these consequences of PT themselves favor PT. This suggests a dual role for certain molecules, including reactive oxygen species, in apoptosis: as facultative constituents of signal transduction pathways initiating the vicious cycle of PT and as constant by-products of the death process.

In this general scenario, the data obtained in this study suggest two possible mechanisms for the apoptotic effect of TPL. 1) TPL directly targets the mitochondrion with a subsequent impairment of the electron transport chain, as indicated by its effect on complex I, and, to a lesser extent, on complexes II and IV, which would induce electron leakage and oxygen-derived free radical formation. This is supported by observations by our group, as well as by other authors, indicating that TPL and its non-hydroxylated analog TEMPO elicit H_2O_2 formation by a number of human tumor cells [Voest et al., 1992]. Although complex III, which is unaffected following TPL exposure, is considered as the major site for H_2O_2 generation, inhibition of complex I has been shown to contribute to ROS formation in the mitochondrion [Turrens and Boveris, 1980]. ROS generation and the subsequent depletion of mitochondrial GSH would lead to the $\Delta\psi_m$ alterations which characterize the point of no-return in apoptosis. 2) Alternatively, the earliest effect of TPL treatment could consist in GSH depletion, thereby inducing intracellular oxidative stress, which in turn would trigger

apoptosis. TPL has been demonstrated to cause a parallel depletion of both the total cellular and the mitochondrial GSH pools, in contrast with other GSH-depleting agents (e.g., buthionine sulfoximine) which are much more effective on the cytosolic than on the mitochondrial pool. Mitochondria, as the site of aerobic respiration and of production of toxic ROS, are highly dependent on GSH for the prevention of oxidative damage but are incapable of de novo synthesis of the tripeptide [Griffith and Meister, 1985]. Under physiological conditions mitochondrial GSH levels are tightly controlled by the cell; depletion of this critical thiol pool by TPL might then result in mitochondrial impairment and inhibition of the electron transport chain. A sequence of events similar to the one observed in TPL-treated HL-60 cells (GSH depletion and impairment of complex I, II and IV activities) has been described in neuronal cells as playing a relevant role in Parkinson's disease, in which apoptosis of dopaminergic neurons due to oxidative stress has been proposed as a possible etiologic factor [Merad-Boudia et al., 1998]. Studies aimed at elucidating the time-frame within which all the described events occur following TPL treatment will determine which of the two alternative models is actually involved in HL60 cell killing by TPL. Meanwhile, the present study confirms that TPL is a potentially useful agent in anticancer therapy.The evidence that modulation of the intracellular and/or intramitochondrial redox state by low-molecular weight compounds may influence the apoptotic threshold offers the exciting prospect that it may be possible to alter the sensitivity of cancer cells to cytotoxic drugs and thereby reduce the problem of drug resistance in the future.

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