2,3,5-tris(Glutathion-S-yl)hydroquinone (TGHQ), a metabolite of benzene, induces apoptosis in human promyelocytic leukemia (HL-60) cells. However, the mechanisms by which TGHQ induces apoptosis are unclear, and they were the focus of the present investigation. TGHQ stimulated the rapid formation (30 min) of reactive oxygen species (ROS) in HL-60 cells, and co-treatment with catalase or the antioxidant N-acetylcysteine (NAC) completely blocked TGHQ-induced apoptosis, implicating a causative role for ROS in HL-60 cell death. Western blot analysis revealed the complete disappearance of pro-caspase 9 between 1 and 2 hours after exposure of HL-60 cells to TGHQ, concomitant with the appearance of cleaved caspase 9 and increases in caspase 9 activity. The appearance of two cleaved forms of caspase 3 occurred subsequent to increases in caspase 9 activity. Levels of the anti-apoptotic Bcl-2 protein remained constant during TGHQ-induced apoptosis of HL-60 cells, but Bcl-2 S70 phosphorylation decreased. In contrast, changes in the subcellular localization of the pro-apoptotic molecule Bax were observed, with a rapid (15 – 60 min) increase in the ratio of cytosolic to mitochondrial Bax. Cytochrome c release from mitochondria to the cytosol occurred after Bax translocation and the dephosphorylation of pS70 Bcl-2. However, the mitochondrial inner transmembrane potential (ΔΨm) was maintained, even after cytochrome c was released from the mitochondria. Cyclosporin A, an inhibitor of the mitochondrial membrane permeability transition pore (PTP), did not completely rescue HL-60 cells from apoptosis. Taken together, we conclude that TGHQ facilitates ROS production, alters the post-translational modification of Bcl-2 and subcellular localization of Bax, culminating in the release of cytochrome c and caspase activation.

INTRODUCTION

The balance between cell death and cell proliferation is key to maintaining normal biological tissue function, especially in proliferating tissues, such as bone marrow. When cell proliferation becomes inappropriately dominant, it contributes to the development of neoplastic diseases, whereas excessive apoptosis can cause degenerative diseases and hematological disorders (Bellamy et al., 1995; Thompson, 1995). Apoptosis, programmed cell death, is triggered by two major cell death signaling pathways, death receptor–mediated (extrinsic) and stress/chemical–facilitated (intrinsic) signaling pathways (Wang, 2001). The death receptor–mediated pathway is triggered by the Fas/Apo-1 death ligand and is characterized by formation of the death-inducing signaling complex (DISC), leading to caspase 8 activation. In contrast, various stresses, including chemical toxicants, induce cell death via the mitochondrial-mediated pathway. Cytochrome c, released from mitochondria, assists in the assembly of the apoposome, in combination with the recruitment of pro-caspase 9 and Apaf-1, culminating in caspase 9 activation. These two signaling pathways activate effector caspases, such as caspase 3 and caspase 7, both of which cleave biologically important proteins, leading to cell death.

By regulating mitochondrial function, the Bcl-2 family members are key players in stress/toxicant–induced apoptosis. The Bcl-2 family members are divided into two subgroups, based on their ability to either promote or antagonize apoptosis (Chao and Korsmeyer, 1998; Gross et al., 1999; Wei et al., 2001). In general, the anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-xL, and Bcl-w, are integrated into the mitochondrial membrane, whereas the pro-apoptotic Bcl-2 family members, such as Bad and Bax, are localized in the cytosol in the absence of a death signal. The pro-apoptotic proteins are upregulated in response to death signals, and they translocate into mitochondrial membranes, where they interact with the anti-apoptotic members, contributing to apoptosis.

Abbreviations: GSH, glutathione; HPK1, hematopoietic progenitor kinase 1; ΔΨm, mitochondrial inner transmembrane potential; NF-xB, nuclear factor xB; PBS, phosphate-buffered saline; PTP, mitochondrial membrane permeability transition pore; ROS, reactive oxygen species; TBS-T, tris-buffered saline containing 0.1% Tween 20; TGHQ, 2,3,5 tris(glutathion-S-yl)hydroquinone.

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Cell survival signals can also influence the Bcl-2 family of proteins. For example, IL-3 activates the Akt survival signaling pathway, resulting in phosphorylation of pro-apoptotic Bad, which subsequently binds to and is sequestered by 14–3–3 (Datta et al., 2000; Zha et al., 1996; Zhou et al., 2000). Thus, the combination of post-translational modifications and the upregulation or downregulation of pro-apoptotic and anti-apoptotic protein expression determines the anti-apoptotic or pro-apoptotic function of the Bcl-2 family members.

Benzene is an industrial solvent used to produce plastics, nylon and other synthetic fibers, lubricants, and dyes; it is also a natural component of gasoline and cigarette smoke (Imbriani et al., 1995). Benzene causes bone marrow suppression in rodents and is both hematotoxic and leukemogenic in humans, leading to hematological disorders, such as aplastic anemia and acute myelogenous leukemia (Golding and Watson, 1999; Rinsky et al., 1981; Tunek et al., 1981). Benzene must be metabolized and bioactivated to mediate its toxic effects. A number of hydroquinone-thioether metabolites have been identified in the bone marrow of rats and mice exposed to a combination of hydroquinone and phenol, or benzene (Bratton et al., 1997). In particular, 2,3,5-tris(glutathion-S-yl)hydroquinone (TGHQ) causes hematotoxicity in rats, and it induces apoptosis in human promyelocytic leukemia (HL-60) cells (Bratton et al., 1997, 2000). TGHQ likely induces toxicity either by the generation of reactive oxygen species (ROS) or via the covalent binding of reactive metabolites to critical tissue macromolecules, or both (Bratton et al., 1997). Prior to the onset of apoptosis, TGHQ depletes cellular glutathione (GSH) levels and stimulates sphingomyelin turnover (Bratton et al., 2000). However, the mechanisms by which TGHQ induces apoptosis have not been fully elucidated.

In the present study we demonstrate that TGHQ facilitates ROS production, an essential element for inducing apoptosis, and stimulates cytochrome c release from mitochondria, leading to caspase activation, in the absence of a disruption in the mitochondrial membrane potential. Mitochondrial cytochrome c release therefore appears to be mediated by the ability of TGHQ to stimulate the subcellular relocation of pro-apoptotic Bax protein, which translocates to mitochondria, and in combination with the dephosphorylation of Bcl-2 protein, promotes cytochrome c release, contributing to TGHQ-induced apoptosis of HL-60 cells.

MATERIALS AND METHODS

Materials. TGHQ was synthesized and purified as previously described (Lau et al., 1988). Annexin V FITC kits were purchased from Immunotech (Marseille Cedex, France). Anti-Bax, anti-caspase 8, anti-cleaved caspase 9, and anti-caspase 3 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-caspase 9 antibody was obtained from Chemicon International (Temecula, CA). Anti-HPK 1, anti-lamin B, and anti-Bcl-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cytochrome c and anti-actin antibodies were purchased from PharMingen (Palo Alto, CA) and Oncogene (San Diego, CA), respectively. Caspase 9 activity assay kits were obtained from Oncogene. Protease inhibitor cocktail tablets were purchased from Roche (Mannheim, Germany). Tetramethylrhodamine methyl ester (TMRM) was purchased from Molecular Probes (Eugene, OR). Cyclosporin A was obtained from Calbiochem (San Diego, CA). Catalase, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and all other compounds were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents, including ethanol, were obtained from Fischer Scientific (Houston, TX).

Cell lines and culture conditions. HL-60 cells were maintained in Roswell Park Memorial Institute medium (RPMI 1640; Gibco BRL, Grand Island, NY) containing 20% fetal bovine serum (FBS) in a 37°C, 5% CO2-regulated incubator. Cells were routinely cultured at a density of 1.0 × 106 cells/ml. Immediately prior to all experiments, cells were washed and resuspended in RPMI 1640 containing 25 mM HEPES and 10% fetal bovine serum (FBS).

Annexin V FITC/propidium iodide apoptosis assay. The percentage of apoptotic cells was determined according to the manufacturer’s protocol using an annexin V FITC kit and an EPICS XL-MCL (Coulter, Miami, FL) flow cytometer.

ROS measurement using carboxy-H2DCFDA. Cells were loaded with 20 μM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA) (Molecular Probes) for 30 min. After TGHQ (200 μM) treatment, cells were pelleted, washed, and resuspended in PBS. Then, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein (carboxy-DCF) fluorescence was determined on an EPICS XL-MCL flow cytometer, with excitation at 495 nm and emission at 525 nm.

Measurement of mitochondrial inner transmembrane potential (ΔΨm). Cells were treated with TGHQ (200 μM) and collected at each time point. Cells were resuspended in 500 μl of PBS and labeled with TMRM (final concentration 150 nM). As a positive control, aliquots of cells were stained in the presence of 100 μM CCCP. Cells were subsequently incubated at 37°C for 30 min and returned to ice. Fluorescence was determined on an EPICS XL-MCL flow cytometer, with excitation at 495 nm and emission at 575 nm.

Preparation of nuclear and cytosolic extracts. After treatment of cells with TGHQ, nuclear and cytosolic extracts were prepared as described (Read et al., 1994), with slight modifications. Cells were harvested, washed with cold PBS, and resuspended in 100 μl of buffer A (10 mM HEPES [pH 8.0], 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 200 mM sucrose, 0.5 mM phenylmethylsulfonl fluoride [PMSF], 1 mM Na2VO4, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate and 0.5% Nonidet P-40) with protease inhibitors. Suspended cells were incubated for 5 min at 4°C. The lysed cells were microcentrifuged at 16,000 × g for 15 s at 4°C. The supernatants (cytosolic fraction) were saved, and the pellet (nuclear fraction) was resuspended in 40 μl of buffer B (20 mM HEPES [pH 7.9], 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 1 mM Na2VO4, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate) with protease inhibitors. The nuclear extracts were incubated at 4°C for 30 min and then microcentrifuged at 16,000 × g for 10 min. The resulting supernatants were diluted 1:1 with buffer C (20 mM HEPES [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT and 0.5 mM PMSF) with protease inhibitors. The cytosolic extracts were clarified by microcentrifugation at 14,000 × g for 30 min. Protein concentration was determined by the modified Lowry method (Biorad protein assay, Bio-Rad).

Preparation of mitochondria-enriched and cytosolic fractions. The mitochondria-enriched and cytosolic extracts were obtained as described elsewhere (Ganjoo and Eastman, 2002), with slight modifications. Briefly, 6 × 106 cells were incubated in ice-cold lysis buffer (75 mM NaCl, 1 mM Na2HPO4, 8 mM Na2HPO4, 250 mM sucrose, 1 mM Na2VO4, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate and protease...
inhibitors) with 26.25 μg digitonin in 100 μl on ice for 20 min, followed by centrifugation at 12,000 × g for 1 min. The supernatants were saved as the cytosolic fraction, and the pellet (mitochondria-enriched fraction) was resuspended in the same volume of lysis buffer without digitonin. The pellet was solubilized by sonication for 10 s at 4°C and stored as the mitochondria-enriched fraction.

**Preparation of total cell extracts.** RIPA buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid [EDTA], 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 1% Nonidet P-40 containing 0.5 mM PMSF, 1 mM Na3VO4, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate and protease inhibitors) was used to obtain total cell extracts. The cells were incubated for 30 min at 4°C in RIPA buffer followed by centrifugation for 20 min at 14,000 × g. The supernatants were used as total cell extracts.

**Western blotting.** Samples were mixed with sample buffer (Laemmili sample buffer, Bio-Rad) and then heated for 5 min at 100°C and loaded onto 10% or 12% SDS-polyacrylamide gels. After they were electrophoresed onto PVDF membranes, the sample blots were blocked for 1 h with 5% nonfat milk powder in TBS-T solution (25 mM Tris-HCl [pH 7.6], 0.2 M NaCl, and 0.1% v/v Tween 20) at room temperature. Membranes were incubated with the antibodies of interest at 4°C, unless stated otherwise. After washing three times in TBS-T for 5 min, membranes were incubated with horseradish-conjugated secondary antibodies. Bound antibodies were detected by enhanced chemiluminescence (ECL kit, Amersham, Arlington Heights, IL).

**Caspase 9 activity.** HL-60 cells were treated with 200 μM TGHQ for each time period (0, 0.25, 0.5, 1, and 2 h) and collected by centrifugation at 1000 × g for 3 min. Cells were washed with PBS twice. Cells were resuspended with extraction buffer (50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, and 0.5% Nonidet P-40) and incubated on ice for 10 min. The cell lysates were obtained by centrifugation at 1000 × g for 5 min. Caspase 9 activity was measured according to the manufacturer’s instructions provided in the fluorometric assay kit from Oncogene (San Diego, CA).

**Statistical analysis.** Data are presented as the mean ± SD. Statistical significance was determined by analysis of variance (ANOVA), followed by Tukey’s post hoc comparison.

**RESULTS**

**TGHQ Catalyzes ROS Generation in HL-60 Cells**

TGHQ induces apoptosis in HL-60 cells in a time-dependent manner (Fig. 1A) (Bratton et al., 2000). Although cellular GSH levels decrease significantly during TGHQ-induced apoptosis of HL-60 cells, that decrease occurs independent of ROS generation (Bratton et al., 2000). To determine whether TGHQ does in fact redox cycle and facilitate ROS formation in HL-60 cells, we determined ROS production with carboxy-H2DCFDA, which is converted to green fluorescent carboxy-DCF via oxidation. The rapid production of ROS occurred immediately after exposure of HL-60 cells to TGHQ, reaching maximal levels 30 min thereafter (Fig. 1B). The generation of ROS continued for at least 1 h, followed by a slight decline. Pretreatment of HL-60 cells with N-acetylcysteine (NAC), a precursor of GSH and an antioxidant, blocked TGHQ-induced apoptosis as well as ROS generation (Fig. 1C). Consistent with previous findings, catalase prevented TGHQ-induced apoptosis (Bratton et al., 2000), and it also reduced TGHQ-induced ROS formation (Fig. 1D).

**Initiator Caspase 9 and Effector Caspase 3 Are Cleaved into Their Active Forms and Activated Prior to the Onset of Apoptosis**

We subsequently examined the activation of caspases in HL-60 cells undergoing TGHQ-induced apoptosis. Western blot analysis of HL-60 cell cytosol revealed the complete disappearance of pro-caspase 9 (46 kDa) (Fig. 2A), together with the appearance of cleaved caspase 9 (35 kDa) (Fig. 2B), cytochrome c release from mitochondria (Fig. 2C), and two cleaved forms of caspase 3 (20, 17 kDa) (Fig. 3A). Concomitant with the appearance of the cleaved active form of caspase 9, increases in caspase 9 activity were detected at 1.5 h (Fig. 2D). The appearance of two cleaved forms of caspase 3 is consistent with the increased caspase 3 activity reported earlier (Bratton et al., 2000). The nuclear structural protein lamin B, and hematopoietic progenitor kinase 1 (HPK 1) are cleaved by active caspase 3 at 2 h (Fig. 3B and 3C). In addition, caspase 8, an initiator caspase in the death receptor–mediated signaling pathway, appears to be a substrate of active effector caspases during TGHQ-induced apoptosis (Fig. 4). The intermediate cleaved forms (41 and 43 kDa) of pro-caspase 8 appear at 2 h and reach maximum levels by 4 h, followed by the appearance of the final cleaved product (18 kDa) at 4 h.

**The Mitochondrial Inner Transmembrane Potential (Δψ_m) Is Maintained Subsequent to Caspase 9 Activation**

Disruption of the Δψ_m may contribute to the release of cytochrome c from mitochondria, initiating the cascade of events that result in caspase activation during stress-induced apoptosis (Halestrap et al., 2002; Wang, 2001). However, cytochrome c release may occur independent of a disruption in Δψ_m, suggesting that other pathways can regulate mitochondrial cytochrome c release (Bossy-Wetzel et al., 1998; Bratton and Cohen, 2001; Ly et al., 2003; Marzo et al., 1998). To examine whether a disruption of Δψ_m is required for the release of cytochrome c in TGHQ-induced apoptosis of HL-60 cells, we measured Δψ_m with the cationic fluorochrome, TMRM, which accumulates in the mitochondrial matrix driven by the membrane potential of mitochondria. CCCP was used as a positive control for disruption of Δψ_m. The Δψ_m remained intact for at least 2 h after exposure of HL-60 cells to TGHQ and was only marginally affected at 4 h (Fig. 5). To further examine the role of the Δψ_m and the mitochondrial membrane permeability transition pore (PTP) in TGHQ-induced apoptosis of HL-60 cells, cells were treated with cyclosporine A, which binds to cyclophilin D and inhibits the opening of the PTP. Cyclosporin A had only a modest effect on TGHQ-induced apoptosis (Fig. 6), suggesting that disruption of the Δψ_m and the PTP plays a minor role in TGHQ-induced apoptosis.
FIG. 1. TGHQ-induced apoptosis is ROS dependent. A. TGHQ induces apoptosis in HL-60 cells (■ early apoptotic/Annexin V+ and propidium iodide- □ late apoptotic/Annexin V+ and propidium iodide+). The percentage of apoptotic cells was determined as described in Materials and Methods. B. After 200 uM TGHQ treatment, the cells were collected and resuspended with PBS at each time point. Carboxy-DCF fluorescence was measured in an EPICS XL-MCL flow cytometer with excitation (495 nm) and emission (525 nm). Blue curves represent control cells and orange curves represent TGHQ-treated cells. C. (i) HL-60 cells were pretreated with 5 mM NAC for 2 h and then exposed to 200 uM TGHQ for 8 h. The percentage of apoptotic cells was determined as described in Materials and Methods. The white bar represents the control group and the black bar represents the TGHQ treated group. The bar with vertical lines and the bar with horizontal lines represent cells treated with NAC alone or cells co-treated with NAC and TGHQ respectively. (ii) After pretreatment with NAC for 2 h, cells were exposed to TGHQ for 30 min and carboxy-DCF fluorescence was measured as described in Materials and Methods. The blue and red lines represent control and TGHQ-treated cells, respectively. The pink and green lines represent cells treated with NAC alone or co-treated with NAC and TGHQ respectively. D. (i) HL-60 cells were co-treated with catalase 100 U and TGHQ (200 uM) for 8 h. White and black bars represent control and TGHQ-treated groups, respectively. The bar with horizontal lines represents TGHQ and catalase co-treated groups. (ii) After co-treatment with catalase and TGHQ, carboxy-DCF fluorescence was measured as described in Materials and Methods. The blue and red lines represent control and TGHQ treated cells, respectively. The pink line represents cells co-treated with TGHQ and catalase. Each value represents mean ± SD (**represent p < 0.01, Tukey’s post hoc). All data presented represent at least three separate experiments.
TGHQ Treatment Induces Dephosphorylation of the Anti-apoptotic Bcl-2 Protein

Because TGHQ-induced cytochrome c release from mitochondria occurs in the absence of a change in ΔΨmt, alternative pathways for mitochondrial cytochrome c release were investigated. The Bcl-2 family proteins are key players in stress/toxicant–mediated apoptosis via their ability to regulate mitochondrial function. Because the functions of Bcl-2 proteins are regulated at both the transcriptional and post-translational levels (Gross et al., 1999), we examined anti-apoptotic Bcl-2 protein from this perspective. In response to TGHQ treatment, total Bcl-2 protein levels remained constant. In contrast, phosphorylated S70 Bcl-2 decreased by 1h after TGHQ treatment, suggesting that TGHQ inhibits the anti-apoptotic function of Bcl-2 at the post-translational level and not at the transcriptional level (Fig. 7).

Pro-apoptotic Bax Is Upregulated and Translocates to Mitochondria During TGHQ-Induced Apoptosis

The pro-apoptotic Bax molecule translocates and homodimerizes in the mitochondrial membrane, thereby promoting apoptosis by disrupting mitochondrial function (Gross et al., 1999). Western blot analysis revealed that cytosolic Bax protein increases and reaches maximum levels 30 min after exposure of HL-60 cells to TGHQ, followed by a decrease in cytosolic Bax, concomitant with increases in mitochondrial Bax (Fig. 8). Mitochondrial Bax translocation and inhibition of anti-apoptotic Bcl-2 function by dephosphorylation at Ser 70 likely combine to facilitate cytochrome c release from mitochondria by 2 h.

FIG. 2. Cytochrome c release and initiator caspase 9 activation occurs between 1 and 2 h after TGHQ treatment. HL-60 cells were treated with 200 μM TGHQ and collected at each time point. The cytosolic and mitochondria-enriched fractions were prepared and western blot analyses were performed as described in Materials and Methods. Panels A and B show pro-caspase 9 and cleaved caspase 9 protein levels in cytosolic extract, respectively, and panel C represents mitochondrial and cytosolic cytochrome c. The primary antibody against cytochrome c was incubated at 4°C for 1.5 h. Panel D represents the activity of caspase 9, which was measured according to the manufacturer’s protocol (**represents statistically significant difference at p < 0.01 with Tukey’s post hoc). The western blot is illustrative of experiments repeated on at least three separate occasions.

FIG. 3. Caspase 3 is activated in TGHQ-treated HL-60 cells and cleaves its downstream substrates, lamin B and HPK 1. HL-60 cells were treated with 200 μM TGHQ and collected at each time point. The cytosolic extracts (A), nuclear extracts (B), and total extracts (C) were prepared, and western blot analyses were performed as described in Materials and Methods. A. Pro-caspase 3 and cleaved caspase 3 were identified. Panels B and C illustrate the intact and caspase-cleaved forms of lamin B and HPK 1. The western blot is illustrative of data obtained from at least two independent experiments.

FIG. 4. Cleaved caspase 8 is present in TGHQ-treated HL-60 cells. HL-60 cells were treated with 200 μM TGHQ and collected at each time point. The cytosolic extracts were prepared, and western blot analysis was performed as described in Materials and Methods. The western blot is illustrative of data obtained from at least two independent experiments.
DISCUSSION

TGHQ, a putative metabolite of benzene, generates ROS and induces apoptosis in HL-60 cells (Fig. 1). Both catalase and NAC block ROS generation and prevent TGHQ-induced apoptosis, indicating that ROS play an essential role in TGHQ-mediated toxicity. However, the mechanism by which TGHQ-catalyzed ROS generation engages apoptosis in HL-60 cells is unclear.

Caspases 9 and 3 are both activated in HL-60 cells exposed to TGHQ (Figs. 2 and 3). Caspase activation represents the “irreversible” or execution stage of apoptosis, because caspase-mediated proteolysis is irreversible (Thornberry and Lazebnik, 1998). Many proteins required for the maintenance of cell structure and function are substrates of active caspase 3. During TGHQ-induced apoptosis of HL-60 cells, lamin B, a nuclear structural protein, is cleaved by active effector caspases (Fig. 3B), leading to the disruption of nuclear architecture. Because lamin B is reported to be cleaved predominantly by caspase 6,
we can assume that caspase 6 is activated in TGHQ-treated HL-60 cells. In addition, HPK 1, which is predominantly expressed in hematopoietic tissue, is also a substrate for active caspase 3 (Fig. 3C). This finding is consistent with the report that intact HPK 1 and its caspase 3–cleaved form may support apoptosis of T lymphocytes (Schulze-Leuhrmann et al., 2002). In particular, the caspase 3–cleaved C terminal of HPK 1 blocks IxBα degradation, inhibiting the anti-apoptotic function of NF-κB. Caspase 8, an initiator caspase in the death receptor–mediated signaling pathway, is also a substrate of active effector caspases during TGHQ-induced apoptosis (Fig. 4); the significance of this remains unclear, however. We assume that caspase 6 is activated during TGHQ-induced apoptosis, because lamin B cleavage is observed. In addition, caspase 6 can cleave caspase 3 directly (Cowling and Downward, 2002). Thus, it is possible that activated caspase 6 cleaves caspase 8 directly during TGHQ-induced apoptosis of HL-60 cells. Another possibility is that active caspase 3 acts upstream of caspase 8. It has recently been suggested that caspase 3 and caspase 8 are associated with the Fas-associated death domain in lipid rafts, and that caspase 3 is required for complete caspase 8 activation during Fas-mediated cell death (Aouad et al., 2004).

Mitochondria play an important role during stress/toxicant–induced apoptosis (Green and Reed, 1998; Wang, 2001), because mitochondria contain many apoptosis-stimulating elements, including cytochrome c and Smac. In most cases of toxicant-induced apoptosis, cytochrome c is required for caspase 9 activation. Cytochrome c may be released from mitochondria into cytosol in several ways (Bratton and Cohen, 2001; Hengartner, 2000; Ly et al., 2003). One possible mechanism involves changes in the PTP, in which a loss in Δψm occurs. The PTP is believed to consist of the mitochondrial outer membrane voltage-dependent anion channel, the inner membrane adenine nucleotide translocase, and the mitochondrial benzodiapepine receptor. The adenine nucleotide translocase is associated with cyclophilin D. According to this mechanism, the apoptosis-inducing agent causes an opening of the PTP, the dissipation of Δψm, and the release of cytochrome c. However, in TGHQ-treated HL-60 cells, cytochrome c is released from mitochondria 2 h after TGHQ treatment (Fig 2C), a time at which the Δψm is still maintained (Fig. 5). In addition, pretreatment of HL-60 cells with cyclosporine A had little effect on TGHQ-induced apoptosis (Fig. 6). Alternatively, Bcl-2 family members may form a channel through which cytochrome c and other molecules can escape from mitochondria. Recently, Bax was found to translocate, oligomerize, and form a protein-permeable pore (Antonsson et al., 2000; De Giorgi et al., 2002; Gross et al., 1998). In addition, Bax may form a Bax/voltage–dependent anion channel hybrid channel to release cytochrome c (Shimizu et al., 1999). HL-60 cells exposed to TGHQ upregulate the pro-apoptotic Bax protein, which is subsequently translocated to mitochondria (Fig. 8). Moreover, TGHQ promotes the dephosphorylation of the anti-apoptotic S70 Bcl-2 protein (Fig. 7), suggesting that the redistribution of cytochrome c from the mitochondria to the cytosol during TGHQ-induced apoptosis of HL-60 cells may result not from the opening of PTP but via the formation of a conducting channel.

Debate continues over whether phosphorylation of Bcl-2 enhances or inhibits its anti-apoptotic function (Deng et al., 2004; Haldar et al., 1998; Ruvolo et al., 2001; Yamamoto et al., 1999). Phosphorylation of Bcl-2 at Ser70 correlates with increases in cell survival in chemotherapeutic drug–induced apoptosis (Ito et al., 1997; May et al., 1994). In contrast, paclitaxel induces Bcl-2 phosphorylation at Ser70 and causes cell death (Haldar et al., 1996, 1998). Korsmeyer’s group identified the phosphorylation sites of Bcl-2 at Ser70, Ser87 and Thr69 and suggested that phosphorylation inactivates Bcl-2 (Yamamoto et al., 1999). However, even in experiments in which a series of serine/threonine (S/T) → glutamate/alanine (E/A) mutants were created to mimic or abrogate phosphorylation, the phosphorylation of Bcl-2 had different effects on paclitaxel-induced apoptosis in different cell types. Such differences in the role of Bcl-2 phosphorylation on apoptosis are thus likely context specific and dependent on the initiating apoptotic insult. The role of the phosphorylation status of Bcl-2 must therefore be assessed in a context-specific fashion. In TGHQ treated HL-60 cells, dephosphorylation of S70 Bcl-2 may block the anti-apoptotic function of Bcl-2 and contribute to the apoptotic process. In support of this view, in TGHQ-treated HL-60 cells, GSH depletion induces sphingomyelin turnover and increases ceramide concentrations (Bratton et al., 2004).
Ceramide activates mitochondrial phosphatase 2A, which dephosphorylates pS70 Bcl-2, attenuating its anti-apoptotic activity. In addition, Bax translocates to mitochondria where it oligomerizes and forms a channel facilitating cytochrome c release. Cytosolic cytochrome c subsequently assists in initiating the activation of the caspase cascade and the cleavage of biologically important molecules, leading to cell death (VDAC, voltage-dependent anion channel; ANT, adenine nucleotide translocase).

In conclusion, TGHQ-induced apoptosis of HL-60 cells requires the generation of ROS, and decreases in GSH concentrations concomitant with an increase in ceramide. Ceramide-mediated activation of phosphatase 2A may then promote the dephosphorylation of Bcl-2, which, in combination with Bax, can then support mitochondrial cytochrome c release and activation of the caspase cascade (Fig. 9).

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REFERENCES


FIG. 9. Schematic illustration of the model of TGHQ-induced apoptosis in HL-60 cells. TGHQ facilitates ROS generation and causes a ROS-independent decrease in GSH. Subsequent increases in ceramide concentrations activate phosphatase 2A, which dephosphorylates pS70 Bcl-2, attenuating its anti-apoptotic activity. In addition, Bax translocates to mitochondria where it oligomerizes and forms a channel facilitating cytochrome c release. Cytosolic cytochrome c subsequently assists in initiating the activation of the caspase cascade and the cleavage of biologically important molecules, leading to cell death (VDAC, voltage-dependent anion channel; ANT, adenine nucleotide translocase).


