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Glutathione Peroxidase-1 Overexpression Prevents Ceramide Production and Partially Inhibits Apoptosis in Doxorubicin-Treated Human Breast Carcinoma Cells

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ABSTRACT

Reduced glutathione and *N*-acetylcysteine can inhibit both apoptosis and necrosis of several cell types, suggesting a critical role for reactive oxygen species (ROS) in cell death. However, how the cellular defense against oxidative stress is connected with other cell death mediators remains unclear. We selectively investigated the interaction of seleno-glutathione peroxidase-1 (GPx-1), the major enzyme responsible for peroxide detoxification in mammalian cells, with the cytotoxic response of T47D human breast cancer cells to doxorubicin, an anticancer drug known to promote production of ROS and apoptotic mediator ceramide. The sensitivity to doxorubicin-mediated cell death was compared in T47D/H3 containing low levels of endogenous GPx and T47D/GPx2 transfectant cells, which overexpress GPx-1. We show that T47D/GPx2 cells were significantly more resistant than T47D/H3 cells to doxorubicin (1 μM). The glutathione precursor, *N*-acetylcysteine also partially protected T47D/H3 cells from the lethal effect of doxorubicin, whereas L-buthionine-(*S*,*R*)-sulfoximine, an inhibitor of glutathione biosynthesis, sensitized both GPx-1–deficient and -proficient cells. Interestingly, in addition to a decrease in ROS production, the activation of neutral sphingomyelinase, sphingomyelin hydrolysis, and ceramide generation in response to doxorubicin was impaired in T47D/GPx2 cells compared with control cells. In contrast, GPx overexpression did not protect breast cancer cells from cell death induced by exogenous cell-permeant ceramide. Moreover, the basal activity of neutral sphingomyelinase was considerably lower in T47D/GPx2. Taken together, these results indicate that GPx-1 can regulate doxorubicin-induced cell death signaling at least in part by interfering with the activation of the sphingomyelin-ceramide pathway.

Reactive oxygen species (ROS) have been implicated in cell death regulation (Buttke and Sandstrom, 1994). Not only can apoptosis be induced by exposing cells to exogenous oxidants (Goldkorn et al., 1998), but also many chemical and physical agents capable of inducing cell death are also known to generate ROS. For example, anticancer drugs such as the anthracyclines daunorubicin or doxorubicin, which induce apoptosis in various tumor cells, elicit ROS formation (Mansat-de Mas et al., 1999). Finally, additional evidence that ROS are key molecules in cell death is provided by reports showing that apoptosis can be blocked by antioxidants (Liu et al., 1998; Mansat-de Mas et al., 1999). The proto-oncogene Bcl-2, a well-known antiapoptotic protein, can also function as an antioxidant (Hockenbery et al., 1993).

Alternatively, oxidative stress and apoptosis can be induced by decreasing the ability of a cell to detoxify ROS. Indeed, normal cellular homeostasis is a delicate balance between ROS formation and antioxidant defenses. Some studies have demonstrated that depletion of cellular glutathione (GSH) is an early event in damage-induced apoptosis (van den Dobbelsteen et al., 1996). Moreover, compounds such as buthionine sulfoximine (BSO) known to deplete intracellular stores of GSH render cells more susceptible to oxidative stress-induced apoptosis (Liu et al., 1998).

Several intracellular ROS detoxifying enzymes seem to be involved in cell protection. Superoxide dismutases convert the superoxide radical ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and molecular oxygen. In human breast cancer MCF-7 cells, resistance to TNFα is correlated with increased activity of

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ABBREVIATIONS: ROS, reactive oxygen species; GSH, glutathione; BSO, L-buthionine-(*S*,*R*)-sulfoximine; TNF, tumor necrosis factor; GPx, glutathione peroxidase; SMase, sphingomyelinase; SM, sphingomyelin; NAC, *N*-acetylcysteine; DAPI, 4′,6-diamidino-2-phenylindole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCFH-DA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

manganese-dependent superoxide dismutase (Manna et al., 1998). Catalase, which converts H_2O_2 to H_2O, can inhibit apoptosis induced by TNFα and interleukin-1α as well as lipid peroxidation (Bohler et al., 2000). A third group of antioxidant enzymes make up a family of glutathione peroxidases, including the classical cytosolic/mitochondrial selenium-dependent glutathione peroxidase-1 (GPx-1), which can reduce H_2O_2 and a variety of fatty acid hydroperoxides with the required participation of GSH as cofactor.

Overexpression of GPx has been reported to block ROS-induced apoptosis in several cell types, suggesting that inhibition of this enzyme is closely related to apoptotic cell death (Hockenbery et al., 1993). However, how GPx is connected with cell death signaling pathways remains to be elucidated.

Ceramide is a sphingolipid signaling molecule that regulates cellular differentiation, proliferation, and apoptosis. This second messenger is generally produced by sphingomyelinase (SMase)-catalyzed hydrolysis of sphingomyelin (SM), an abundant sphingolipid species in cell membranes. Ceramide can be generated by at least two distinct SMases: the acid lysosomal SMase and the neutral, magnesium-dependent SMase, probably located in the plasma membrane. Both enzymes could be activated in response to various stress stimuli, including anticancer drugs (Kolesnick and Krönke, 1998). GSH has been reported to inhibit the activation of the neutral, magnesium-dependent SMase and ceramide generation induced by TNFα in human mammary carcinoma cells (Liu et al., 1998). In addition, a link between ceramide and GSH has been proposed based on the use of *N*-acetylcysteine (NAC), a thiol antioxidant and GSH precursor. Pretreatment of leukemic (Mansat-de Mas et al., 1999) or cancer (Liu et al., 1998) cells with NAC resulted in inhibition of both ceramide production and cell death. Thus, because ceramide and oxidative stress seem to be intimately connected in cell death, it was of particular interest to examine the relationship between GPx, one of the major enzymes responsible for ROS detoxification in mammalian cells, and the ceramide pathway in response to exogenous insults.

To this end, human breast carcinoma T47D cells, which were stably transfected with a cDNA encoding human GPx-1 (Mirault et al., 1991; Legault et al., 2000), were used. Their susceptibility to doxorubicin, in terms of toxicity and cell signaling, was compared with that of parental cells, which are characterized by low endogenous GPx activity. Here, we report that overexpression of GPx-1 abolished doxorubicin-induced sphingolipid signaling. This phenomenon was accompanied by an inhibition of ROS formation and a partial protection against doxorubicin-induced apoptosis. Our data suggest that GPx plays a critical role in cell death signaling by regulating the SM-ceramide pathway.

Materials and Methods

Lipids and Reagents. [methyl-^{3}H]Choline chloride (81.0 Ci/mmol), [γ-^{32}P]ATP (6000 Ci/mmol) and [choline-methyl-^{14}C]SM (52 mCi/mmol) were purchased from PerkinElmer Life Sciences (Paris, France). *N*-Acetylcysteine, *N*-acetyl-D-sphingosine, L-buthionine-(*S*,*R*)-sulfoximine, 4′,6-diamidino-2-phenylindole (DAPI), GSH reductase, Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and sodium selenite were supplied from Sigma (Lisle d'Abeau, France). Octyl-β-glucoside (Ultrol grade) was from Calbiochem (Meudon, France); silica gel 60 thin-layer chromatography plates (Art. 5721) were from Merck (Darmstadt, Germany). Syto 16, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester (DCFH-DA) and monobromobimane were from Molecular Probes (Leiden, The Netherlands). Doxorubicin (Adriblastine) was from Pharmacia (St. Quentin Yvelines, France). All solvents and other reagents obtained from Merck or SDS (Peypin, France) were of analytical grade. RPMI 1640, Glutamax, hygromycin B, and antibiotics were from Invitrogen (Cergy-Pontoise, France); fetal calf serum was from BioMedia (Boussens, France).

Cell Culture. The human breast cancer T47D cell line, a differentiated epithelial substrain of ductal carcinoma origin, was transfected with plasmids pML-Hygro or pML-Hygro-HCMV-GPx, which contains part of a cDNA clone encoding human GPx-1 (Mirault et al., 1991). Empty vector and HCMV-GPx transfected cells are designated T47D/H3 and T47D/GPx2, respectively. All cells were grown in a humidified 5% CO_2 atmosphere at 37°C in RPMI 1640 medium containing 2 mM Glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin, 150 μg/ml hygromycin B, 0.1 μM sodium selenite, and 10% heat-inactivated fetal calf serum.

Determination of Cytotoxicity and Indices of Apoptosis. The whole cytotoxic effect was evaluated by using the tetrazolium-based MTT assay (Denizot and Lang, 1986). Cell viability was also estimated directly on six-well culture plates by staining with propidium iodide (4 μM) and Syto 16 (1 μM). Sensitivity of T47D cells to doxorubicin was also evaluated by a clonogenic assay: 250,000 cells were plated in a 30-mm-diameter dish, and treatment with 1 μM doxorubicin for 72 h. Then, the drug was removed and the cells were further incubated for 7 days in drug-free medium. Cells were fixed with 3% paraformaldehyde, stained with hematoxylin-eosin, and clones of more than 50 cells were counted.

Flow Cytometry Analyses. The percentage of cells in the different phases of the cell cycle and in the subdiploid apoptotic fraction was determined by flow cytometry, as described previously (Bilodeau et al., 2000). The cells were harvested, fixed in 70% cold ethanol, washed, stained with Hoechst 33342 (1 μg/ml) for 30 min on ice, and analyzed in a flow cytometer (Epics Elite ESP; Beckman Coulter, Inc., Fullerton, CA) using an Argon ion laser at 350 nm.

DNA Fragmentation Assay. After treatment with doxorubicin or ceramide, T47D cells were washed twice in phosphate-buffered saline, allowed to undergo lysis for 20 min at 4°C in 0.5 ml of lysis buffer [0.5% Triton X-100 (v/v), 20 mM EDTA, and 5 mM Tris-HCl, pH 8.0] and then centrifuged for 20 min at 27,000*g* to separate the DNA fragments from the chromatin pellet. The DNA content of pellet (resuspended in 1 ml of 1 mM EDTA in 10 mM Tris-HCl, pH 8.0 buffer) and supernatant was determined by the fluorometric DAPI procedure (Andrieu-Abadie et al., 1999).

Determination of Glutathione Peroxidase Activity. GPx activity was determined using a modification of the method of Steinbrecher (1988). Cultured cells were washed with phosphate-buffered saline, scraped, centrifuged, and sonically disrupted (Soniprep MSE sonicator). In a thermostat-equipped cuvette, 25 or 50 μg/ml of protein was added to the assay mixture containing 0.24 mM NADPH, 0.2 mM reduced GSH, 56 mU/ml GSH reductase, and 0.2 mM sodium azide in 50 mM Tris/0.1 mM EDTA, pH 7.6. The reaction was initiated by the addition of 20 μl of a 0.1% solution of cumene hydroperoxide, and the absorbance at 340 nm was monitored. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm that provides a spectrophotometric means of monitoring GPx activity. The rate of decrease in the A_{340} is directly proportional to the GPx activity in sample. One unit of enzyme activity was defined as the amount of enzyme that resulted in oxidation of 1 μmol of NADPH per minute.

Glutathione Determination. The intracellular content of GSH was determined with monobromobimane, a thiol-reactive probe. Exponentially growing cells were labeled with 100 μM monobromobimane for 30 min at 37°C. Cell pellets were suspended in 1 ml of phenol red-free culture medium. Then the fluorescence of cells was quantified at excitation and emission wavelengths of 395 nm and 470 nm, respectively, using a Jobin-Yvon JY3D fluorometer. A standard

curve was generated with known amounts of reduced GSH. Alternatively, GSH content was measured using a commercial kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions.

Determination of ROS. Production of ROS was assessed with DCFH-DA probe. This probe is an uncharged cell-permeant molecule that, once inside the cell, is cleaved by nonspecific esterases and releases carboxydichlorofluorescein, which is oxidized in the presence of ROS. Exponentially growing cells were labeled with 10 μM DCFH-DA for 30 min at 37°C before the reaction was stopped. Cells were washed three times with phosphate-buffered saline. Cell pellets were suspended in 1 ml of distilled water and sonicated at 4°C. The cell-associated fluorescence was recorded at excitation and emission wavelengths of 495 nm and 525 nm, respectively.

Sphingolipid Extraction and Analyses. Total intracellular SM levels were determined on cells metabolically labeled for 48 h with [methyl-^{3}H]choline (1 μCi/ml) using a previously described procedure (Andrieu et al., 1994). Cells pellets were suspended in 0.6 ml distilled water and sonicated for 2 × 15 s. After an aliquot was taken for protein determination (Smith et al., 1985), the lipids were extracted. Ceramide levels were quantified in the lipid extracts essentially as reported (van Veldhoven et al., 1995), using *Escherichia coli* diacylglycerol kinase and [γ-^{32}P]ATP. The *E. coli* strain expressing diacylglycerol kinase was a gift from Drs. D. Perry and Y.A. Hannun (Medical University of South Carolina, Charleston, SC). Radioactive ceramide-1-phosphate was isolated by thin-layer chromatography using chloroform/acetone/methanol/acetic acid/water [50:20:15:10:5 (v/v)] as the developing solvent and counted by liquid scintillation.

Sphingomyelinase Assay. Neutral SMase activity was determined on freshly isolated cell pellets essentially as described (Andrieu-Abadie et al., 1999), using [choline-methyl-^{14}C]SM (100,000 dpm/assay) as substrate.

Fluorogenic DEVD Cleavage Enzyme Assay. After incubation with doxorubicin, cells were sedimented. Cell pellets were homogenized in 10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 0.5% CHAPS, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin. Reaction mixtures contained 100 μl of cell lysates and 100 μl of 40 μM Ac-DEVD-AMC (Bachem, Voisins, France). After 30 min of incubation at room temperature, the amount of the released fluorescent product aminomethylcoumarin was determined at 351 and 430 nm (excitation and emission wavelengths, respectively).

Western Blot Analysis of Procaspase-3 and -7 Processing. Analysis of caspase-3 or -7 cleavage was assessed by Western blot using the cell lysates prepared for DEVD cleavage enzyme assay. Samples were loaded onto a 15% SDS-polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane. Caspase-3 and its cleaved fragments were detected by using a rabbit polyclonal antiserum (Tebu, Le Perray-en-Yvelines, France); caspase-7 was detected with a rabbit polyclonal antiserum (France Biochem, Meudon, France) and a goat anti-rabbit secondary antibody (Bio-Rad, Ivry-sur-Seine, France).

Statistical Analyses. Student's *t* test was used for statistical analysis.

Results

GPx Inhibits Doxorubicin-Induced Cell Death. Because doxorubicin is known to promote an oxidative stress and toxicity in mammalian cells, we investigated the effect of GPx overexpression on cell death induced by this agent. This was examined in cells overexpressing GPx (T47D/GPx2) compared with cells transfected with an empty vector (T47D/H3). The increase in GPx enzymatic activity over parental cells, as estimated using cumene hydroperoxide as substrate, was approximately 14-fold (Fig. 1A). In addition, their intracellular GSH content was found to be significantly higher, here almost doubled (Fig. 1B), in agreement with previous measurements (Mirault et al., 1991). GPx-1 activity was predominantly found in the membrane fraction (data not shown), consistent with a recent report demonstrating that GPx-1 is most concentrated within the mitochondria (Legault et al., 2000).

As illustrated in Fig. 2, A and B, the cytotoxic effect of doxorubicin on T47D/H3 cells was dose- and time-dependent, reducing cell viability by ~30% after 48 h exposure and by ~50% after 72 h exposure to 1 μM doxorubicin. At this concentration but not at higher concentrations, T47D/GPx2 cells were significantly less sensitive to anthracycline-induced cell death. In addition, a 7-day clonogenic assay performed on cells pretreated for 72 h with 1 μM doxorubicin indicated resistance of GPx-1 expressing cells (while in untreated wells, 110 to 150 clones were seen; 44 were found in T47D/GPx2 versus 5 in T47/H3 cells).

The effects of doxorubicin on cell cycle progression and the nature of cell death caused by the drug were investigated by monitoring the morphological changes (after cell staining with Syto 16/propidium iodide; Fig. 2C) with the use of flow cytometry (using the fluorescent probe Hoechst 33342 to stain DNA; Fig. 3), by quantifying DNA fragmentation (Fig. 4A), and by evaluating the ability of the drug to activate caspases (Fig. 4, B and C). When T47D/H3 cells were treated with 1 μM doxorubicin, nuclear condensation and cell shrinkage were observed, suggestive of apoptosis (Fig. 2C). Cell counts indicated 3- to 6-fold fewer apoptotic cells in T47D/GPx2 than in T47D/H3 cells. Accordingly, Fig. 3B shows that doxorubicin induced an S-phase cell cycle arrest, with features very similar to those described previously in T47D/H3 cells exposed to oxidative stress mediated by hyperoxia or menadione (Bilodeau et al., 2000). In addition to the inhibition of cell cycle progression, doxorubicin caused the appearance of a subdiploid cell fraction characteristic of apoptotic cell death. The presence of subdiploid cells and cell cycle block were both significantly reduced in the GPx-proficient cells. In contrast, the GPx status had no effect on cell cycle block and apoptosis mediated by staurosporine, a nonspecific protein kinase inhibitor and typical inducer of apoptosis (Fig. 3C). Two additional endpoints were used to further confirm the differential resistance of these cells to doxorubicin-mediated apoptosis: DNA fragmentation and caspase activation. DNA analysis revealed that 48-h exposure to 1 μM doxoru-

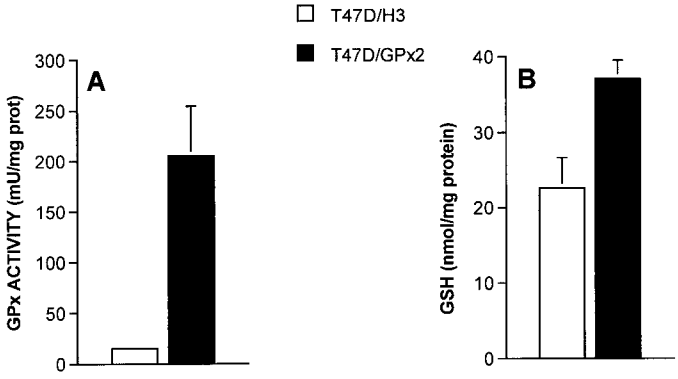

Fig. 1. Intracellular levels of glutathione and GPx activity in T47D cells. A, Intracellular GPx activity assayed with cumene hydroperoxide was measured in total homogenates as described under *Materials and Methods*. B, GSH levels were quantified in total cell extracts. Data correspond to means ± S.E. from at least three independent determinations of GSH and GPx.

bicin led to a significant increase in DNA fragmentation (Fig. 4A). On the other hand, activation of DEVDase started 24 h after cell treatment and reached a maximum at 48 h (Fig. 4B). Moreover, Western blot analysis revealed that a 48-h exposure of T47D/H3 cells to doxorubicin resulted in the proteolytic processing of caspase-3 and -7, two effector caspases (Fig. 4C).

Of interest was the finding that all these anthracycline induced-apoptotic events were partially blocked in T47D/GPx2 cells (Figs. 2, 3, and 4). These data suggest that GPx attenuates some but not all doxorubicin-activated apoptotic pathways. GPx overexpression did not influence the cellular uptake of doxorubicin (data not shown).

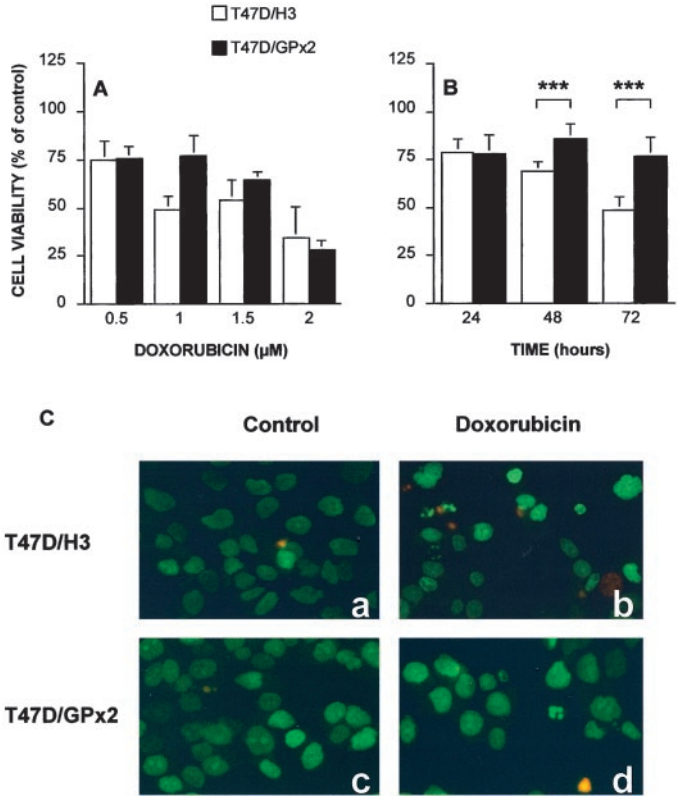

Fig. 2. GPx overexpression partially protects T47D cells from doxorubicin-induced cell death. T47D/H3 and T47D/GPx2 cells were incubated under serum-free conditions for 72 h at the indicated concentrations of doxorubicin (A) or for the indicated times in the presence of 1 μM doxorubicin (B). Cell viability was assessed by MTT assay. Results are means ± S.E. of five independent experiments (***, $p < 0.001$). Alternatively, morphological alterations of nuclei were evaluated by Syto 16/propidium iodide (1 and 4 μM, respectively) staining of T47D/H3 (a, b) and T47D/GPx2 (c, d) cells; in the absence (a and c) or presence (b and d) of 1 μM doxorubicin for 72 h (C).

GPx Inhibits Doxorubicin-Induced ROS Production. We next investigated the effect of GPx overexpression on intracellular ROS levels measured in doxorubicin-treated cells. As seen in Fig. 5, treatment of T47D/H3 cells with the chemotherapeutic drug stimulated a rapid and transient increase in ROS level (probably mostly peroxide) as measured by dichlorofluorescein fluorescence. This phenomenon was detectable and maximal within the first 5 min of incubation. By contrast, in T47D/GPx2 cells, doxorubicin failed to produce any detectable ROS increase in fluorescence, suggesting that in these cells, GPx-1 overexpression strongly reduced DCF-detected ROS accumulation consequent to superoxide formation via anthracycline redox-cycling.

GPx Inhibits Doxorubicin-Induced Ceramide Pathway. Because ceramide generation has been implicated in anthracycline-induced apoptosis (Bose et al., 1995; Andrieu-Abadie et al., 1999) but also in oxidant-stimulated cell death (Goldkorn et al., 1998), we measured ceramide levels in T47D cells treated with doxorubicin. In T47D/H3 cells, the amount of ceramide rapidly increased to reach maximal levels between 5 and 10 min after doxorubicin addition (Fig. 6A). No ceramide production was observed after 3, 6, or 24 h of treatment (data not shown). GPx overexpression completely abolished ceramide production induced by the anthracycline. In addition, GPx overexpression did not affect cell death induced by treatment with C_2-ceramide, an exogenous cell-permeant ceramide (Fig. 4A), further suggesting that GPx acted upstream from ceramide generation in cell death signaling.

Ceramide can be generated either through de novo synthesis by activation of ceramide synthase (Bose et al., 1995) or through SM hydrolysis catalyzed by SMase (Andrieu-Abadie et al., 1999). To determine the source of the ceramide produced by doxorubicin in T47D cells, we investigated the effect of this drug on SM levels. T47D/H3 cell stimulation with doxorubicin resulted in a SM decrease at 10 min (Fig. 6B, inset). Consistent with the results obtained on ceramide production, GPx overexpression blocked doxorubicin-induced SM hydrolysis.

Finally, because SM hydrolysis and concomitant ceramide generation suggested that doxorubicin activated a SMase, SMase activity was determined in doxorubicin-treated T47D cells. Of interest, the basal specific activity of neutral magnesium-dependent SMase was considerably lower in T47D/GPx2 compared with T47D/H3 cells (0.14 ± 0.03 versus. 1.00 ± 0.43 nmol/h/mg of protein, respectively). As shown in Fig. 6B, incubation of T47D/H3 cells with doxorubicin led to an increased activity of a SMase active at neutral pH in the presence of magnesium. No significant changes were ob-

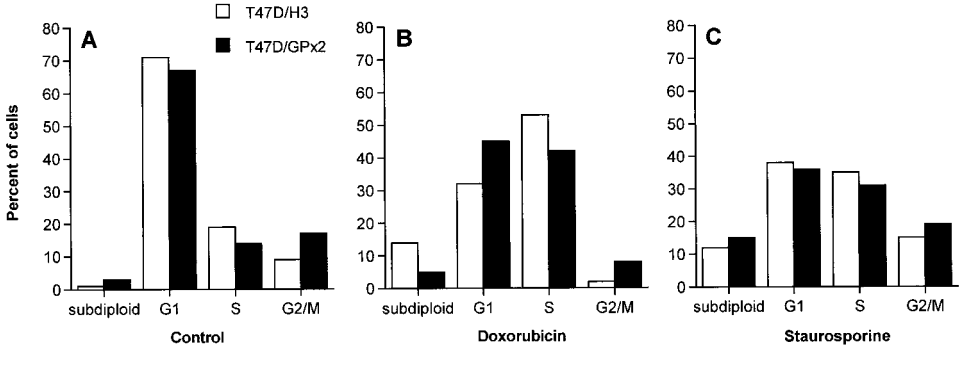

Fig. 3. GPx overexpression attenuates doxorubicin-induced cell cycle block and apoptosis. Representative cell cycle distributions and detection of apoptotic subdiploid cells by flow cytometry, after 48 h incubation in the absence (A) or presence (B) of 1 μM doxorubicin or 24-h exposure to 250 nM staurosporine (C).

served in the acidic SMase activity in the presence of doxorubicin (data not shown). In GPx overexpressing cells, the doxorubicin-induced increase in neutral SMase activity was completely abolished.

NAC and BSO Affects Doxorubicin-Induced Cell Death and Ceramide Production. To elucidate the protective mechanism of GPx on doxorubicin-induced cell death, we preincubated T47D cells for 2 h with NAC, a thiol antioxidant and GSH precursor, or with BSO, an inhibitor of GSH biosynthesis (Goldkorn et al., 1998), before doxorubicin treatment. As expected, intracellular GSH levels were increased by NAC (10 mM) and decreased by BSO (250 μM) (Fig. 7A). Although addition of exogenous NAC protected T47D/H3 cells from doxorubicin-induced cell death, BSO pretreatment sensitized both T47D/H3 and T47D/GPx2 cells to the lethal effects of the drug (Fig. 7C). The effects of these compounds on cell viability were correlated to ROS production (Fig. 7B). All these results further indicate that the protective effect of GPx is related to GSH, its cofactor.

Because the inhibitory effect of GPx overexpression on doxorubicin-induced ceramide generation suggested that GPx functioned upstream of SMase activation, doxorubicin-induced ceramide production was monitored after pretreatment of T47D/H3 cells with 10 mM NAC. Figure 7D shows that addition of NAC before doxorubicin treatment inhibited drug-induced ceramide accumulation, in agreement with similar effects produced by GPx overexpression (Fig. 6A).

Discussion

Anthracyclines and taxanes are the two most potent classes of chemotherapeutic drugs used for the treatment of advanced breast cancer. However, the signaling pathways triggered by these agents in breast cancer cells are not completely understood. The present study shows that GPx is an important regulator of doxorubicin-induced human breast cancer cell death signaling. Specifically, we show that overexpression of GPx inhibits ROS production, SM hydrolysis, and ceramide generation and leads to a partial protection from doxorubicin-induced apoptosis. These results demonstrate that the ceramide pathway is connected with oxidative stress via GPx in anthracycline-induced cell death.

Several lines of evidence suggest that ceramide and oxidative stress are intimately related: 1) exogenous hydrogen peroxide acts on cellular membranes to generate ceramide in epithelial cells (Goldkorn et al., 1998); 2) hypoxia, which is known to hyperproduce ROS, induces ceramide generation in cardiac (Bielawska et al., 1997) or neuronal (Yoshimura et al., 1998) cells; 3) cellular antioxidant systems and GSH regulate ceramide accumulation (Barroso et al., 1997; Liu et

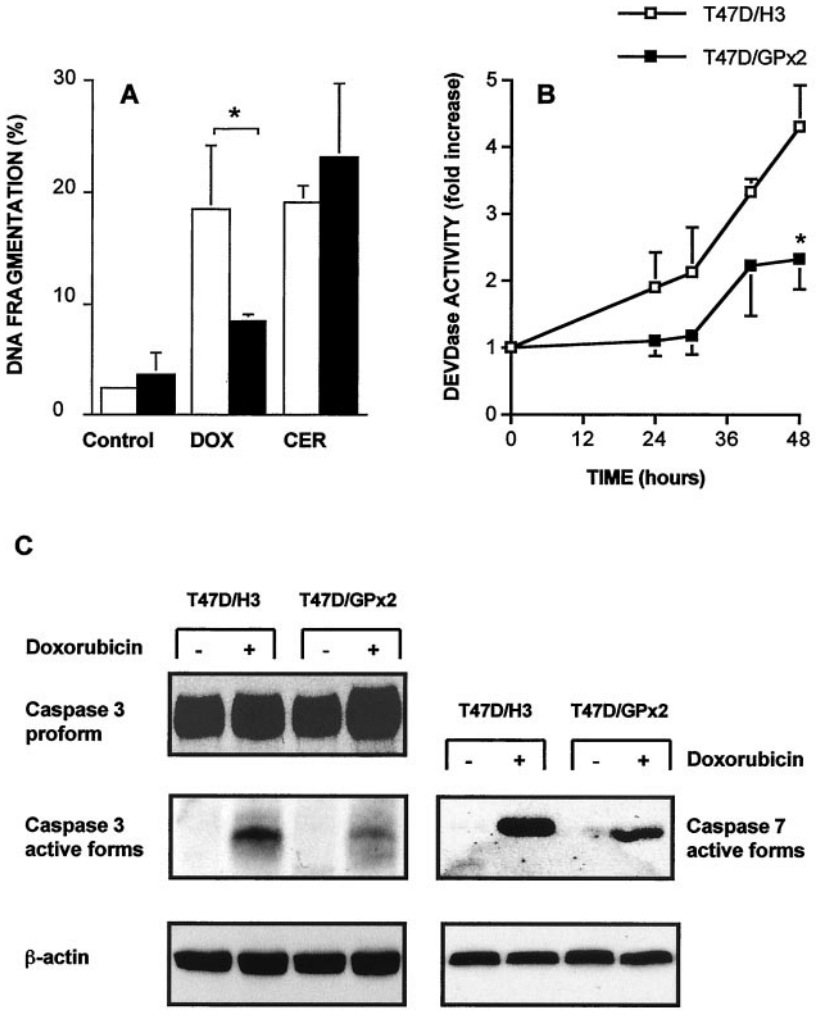

Fig. 4. Doxorubicin-induced apoptosis in T47D/H3 and T47D/GPx2 cells. T47D/H3 and T47D/GPx2 cells were incubated under serum-free conditions with 1 μM doxorubicin (DOX) for 72 h or with 20 μM C_2-ceramide (CER) for 24 h. Quantitative DNA fragmentation was determined by the spectrofluorometric DAPI procedure (A). Results are means ± S.E. of three independent determinations (*$p < 0.05$). B, cells treated without or with 1 μM doxorubicin for the indicated times were harvested and DEVDase activity was determined as described under *Materials and Methods*. Data are means ± S.E. of three independent experiments (*$p < 0.05$). C, extracts from cells treated as above for 48 h were subjected to 15% SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-caspase-3 or caspase-7.

al., 1998); 4) ceramide influences cellular oxidative stress via regulation of manganese-dependent superoxide dismutase (Pahan et al., 1999); 5) ceramide stimulates ROS production in leukemic cells (Quillet-Mary et al., 1997) but also in isolated rat liver mitochondria (Garcia-Ruiz et al., 1997); and 6) ceramide-induced apoptosis can be blocked by thioredoxin peroxidase overexpression in leukemic cells (Zhang et al., 1997). All these observations indicate that a positive feedback may exist between ROS and ceramide productions in the activation phase of programmed cell death.

Here we show that in T47D cells oxidative stress operates upstream from ceramide generation in doxorubicin-induced toxicity. Indeed, GPx overexpression or cell treatment with exogenous NAC, a thiol antioxidant and GSH precursor, prevented doxorubicin-induced ROS production, neutral SMase activation, SM hydrolysis, ceramide generation, and offered partial protection against drug-induced cell death. In accordance with these observations, cell-permeant ceramide promoted apoptosis in T47D, bypassing the doxorubicin-induced SMase activation. Neither GPx overexpression nor treatment with exogenous NAC blocked this event, indicating that GPx targets an event located upstream from ceramide.

Our data also demonstrate that doxorubicin-induced ceramide generation in T47D cells did originate from the breakdown of SM by a SMase assayed under neutral conditions and that this SMase activation could be inhibited by GPx overexpression. These observations are consistent with previous reports on the inhibitory effect of GSH on neutral SMase (Liu et al., 1998). In the same line, it is of particular interest to note that in the GPx-1-overexpressing cells, which exhibited higher GSH levels and lower basal ROS levels (Mehlen et al., 1995) compared with parental cells, neutral magnesium-dependent SMase activity was decreased. This strongly indicates that in the living cell this ceramide-generating enzyme is regulated by the intracellular redox state including GSH, further supporting previous data obtained in vitro (Liu and Hannun, 1997). Moreover, down-regulation of GSH levels by BSO, a specific inhibitor of GSH synthesis, increased doxorubicin-induced apoptosis in cells overexpressing GPx.

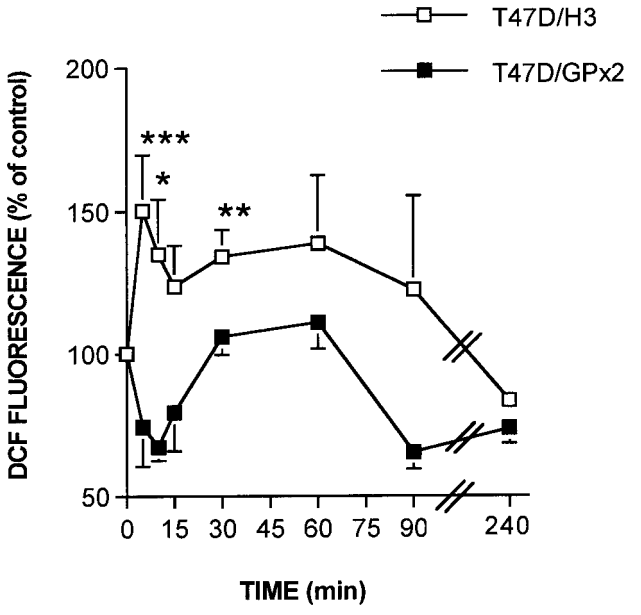

Fig. 5. Doxorubicin-induced ROS production is reduced in T47D/GPx2 cells. T47D/H3 and T47D/GPx2 cells were treated for the indicated times with doxorubicin (1 μM) under serum-free conditions. The DCFH-DA probe was added to the cells 30 min before each reaction was stopped. Cells were washed and cell fluorescence was quantified as described under *Materials and Methods*. Data are means ± S.E. of four independent experiments (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

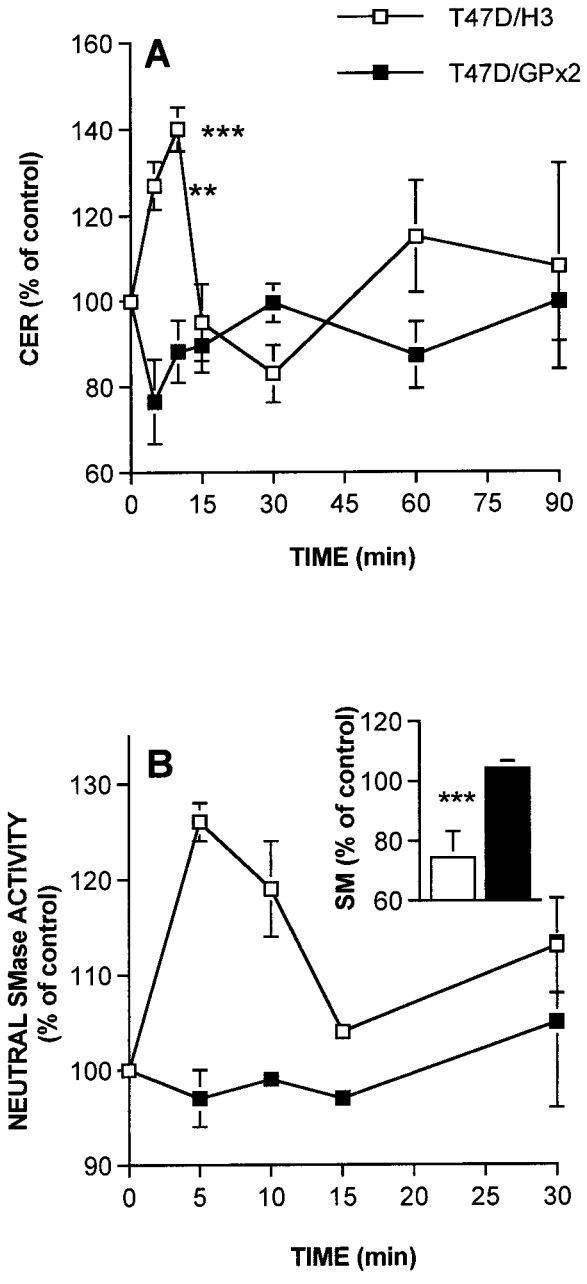

Fig. 6. Effect of GPx on doxorubicin-triggered activation of the sphingomyelin-ceramide pathway. T47D/H3 and T47D/GPx2 cells were incubated with doxorubicin (1 μM) under serum-free conditions. At the indicated times, cells were harvested and lipids were extracted. Ceramide was quantified using the diacylglycerol kinase assay (A). Results (pmoles per milligram of protein) are expressed as percentage of values found in cells incubated for the same time without the drug (***$p < 0.001$; **$p < 0.01$). Basal ceramide levels were 1485 ± 220 and 1965 ± 370 pmol/mg in T47D/H3 and T47D/GPx2 cells, respectively. Alternatively, neutral sphingomyelinase activity was determined on cell extracts (B). SM levels were determined on [^{3}H]choline-labeled cells that were incubated for 10 min with doxorubicin (***$p < 0.001$) (B, inset). Basal [^{3}H]choline-labeled SM levels were 244,500 ± 88,000 and 263,000 ± 73,000 dpm/mg in T47D/H3 and T47D/GPx2 cells, respectively. All data correspond to means ± S.E. of three independent experiments.

Taken together, our data clearly demonstrate that GPx and neutral SMase are closely related in cell death signaling. However, additional work is needed to clarify the exact molecular interactions between these two enzymes. These results also raise a number of important, still-debated questions, such as the subcellular localization (mitochondria or plasma membrane) of these events. Second, the contribution of phospholipid hydroperoxide GPx, another mitochondrial GPx known to prevent apoptosis induced by several inducers (Nomura et al., 1999), to ceramide production remains to be assessed.

Even though GPx overexpression blocked ROS and ceramide formation, it did not completely abolish doxorubicin-induced apoptosis in T47D cells. Indeed, we showed by monitoring cell viability, DNA fragmentation, caspase activation and morphological changes that doxorubicin also induced apoptosis, albeit to a lesser extent, in cells overexpressing GPx-1. One could speculate that doxorubicin triggers cell death via multiple pathways, one of them involving ceramide and ROS. As a matter of fact, it is known that the anthracycline drugs, including doxorubicin, kill cancer cells by various mechanisms, including free radical generation, cell membrane damage, and DNA cleavage via interaction with topoisomerase II (Cummings et al., 1991). Similar to a number of intercalative antitumor drugs and nonintercalative epipodophyllotoxins (VP-16 and VM-26), doxorubicin has been shown to induce single- and double-strand breaks in DNA via formation of topoisomerase-II-DNA cleavage complexes (see Liu, 1989). It is possible, if unlikely, that this mechanism of action cannot be prevented by GPx1. Regarding cell death signaling, some reports have revealed that doxorubicin may implicate the stimulation of the CD95(Fas) receptor-ligand system (Friesen et al., 1996). It was also demonstrated that induction of Fas ligand and apoptosis by doxorubicin is modulated by the redox state in chemosensitive- and drug-resistant tumor cells (Friesen et al., 1999). Generally, breast cancer cell lines are known to resist Fas-mediated apoptosis. but T47D cells may represent an exception because these cells express high levels of Fas (Keane et al., 1996). Whether GPx also interferes with CD95 signaling and prevents CD95-induced apoptosis in T47D cells is under current investigation.

Generation of ROS is viewed as one of the main mechanisms of anthracycline cytotoxicity (Muller et al., 1998). To counteract the harmful consequences of ROS generation, mitochondria are equipped with manganese-dependent superoxide dismutase and a redox cycle using reduced GSH and

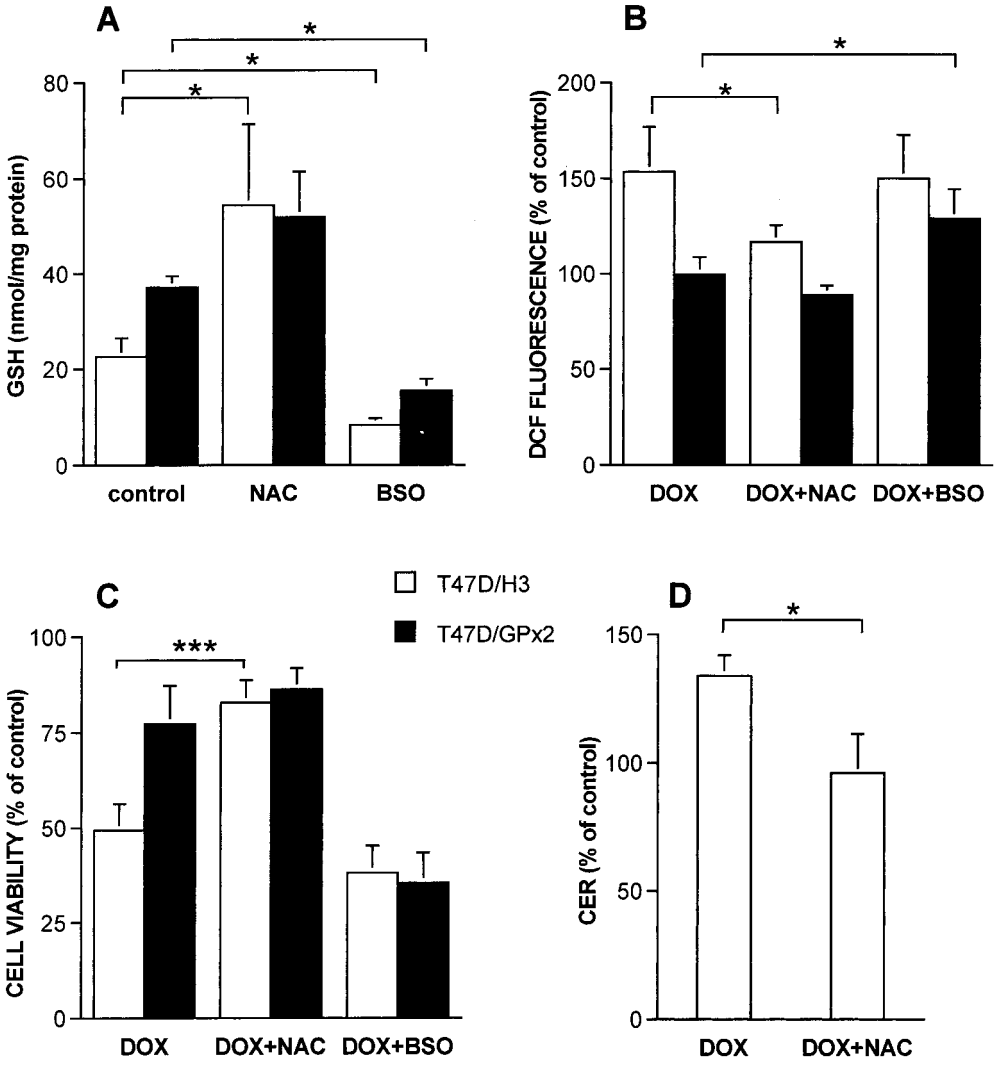

Fig. 7. Effect of NAC and BSO on GSH content and doxorubicin-triggered cell death signaling. T47D/H3 and T47D/GPx2 cells were incubated for 2 h in the absence or presence of NAC (10 mM) or BSO (250 μM). Then, intracellular GSH content was determined (A). After pretreatment for 2 h in the absence or presence of NAC or BSO, cells were incubated with 1 μM doxorubicin (DOX) for 10 min for ROS (B) and ceramide (D) determinations or 72 h for cell viability (C). Results are means ± S.E. of three independent experiments (***$p < 0.001$; *$p < 0.05$).

GPx (Richter et al., 1995). Four forms of human GPx have been described, including the classical GPx1, the gastrointestinal GPx2, the plasma enzyme GPX3, and the phospholipid hydroperoxide GPx4. Nomura et al. (1999) have reported that the latter GPx could suppress apoptotic cell death induced by several agents like etoposide and UV irradiation through the inhibition of cytochrome *c* release and caspase-3 activation. For GPx-1, although it might participate in human breast cancer MCF-7 cells resistance against doxorubicin treatment (Doroshow, 1995), little is known about the biological significance of this enzyme in apoptosis induced by chemotherapeutic agents. However, the protective effect of GPx-1 against ischemia-induced apoptosis has recently been demonstrated in experiments using GPx-1 knockout mice or transgenic mice overexpressing GPx-1 (Maulik et al., 1999). In the latter study, GPx-1 knockout mice showed early signs of apoptotic cardiac cell death after reperfusion. A significant number of apoptotic cells were found in GPx-1-deficient cardiomyocytes compared with nontransgenic control animals. On the contrary, very few apoptotic cells were detected in the hearts of the transgenic mice overexpressing GPx-1 (Maulik et al., 1999). Moreover, transgenic GPx-1 expression in mouse brain (Mirault et al., 1994) was shown to provide protection of neuronal functions such as synaptic transmission and long-term potentiation in hippocampal slices following transient hypoxic exposure (Furling et al., 2000).

Our data further substantiate the critical importance of GPx-1 in apoptosis because overexpression of this GPx form in human breast T47D cells attenuates doxorubicin-induced cell death. Whether other mitochondrial or membrane forms of GPx contribute to the cell defense against doxorubicin toxicity remains to be investigated. The recently described preferential localization of GPx-1 in mitochondria (Legault et al., 2000) makes GPx-1 a likely candidate for counteracting the effect of doxorubicin on ROS formation and ceramide generation, supporting the idea that this pathway may be at least partly mitochondria-dependent.

In summary, the results obtained in the present study led us to propose that in human breast cancer cells, apoptosis induced by doxorubicin is associated with the activation of a neutral SMase leading to SM hydrolysis and a concomitant ceramide generation. This event is preceded by ROS production, which can be counteracted by GPx overexpression, resulting in inhibition of ceramide accumulation and subsequent attenuation of the doxorubicin-induced apoptosis. Altogether, these results emphasize the notion that the cellular antioxidant defense influences the clinical efficacy of anthracyclines by modulating lipid-mediated intracellular signaling.

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References

Andrieu N, Salvayre R and Levade T (1994) Evidence against involvement of the acid lysosomal sphingomyelinase in the tumor-necrosis-factor- and interleukin-1-induced sphingomyelin cycle and cell proliferation in human fibroblasts. *Biochem J* **303:**341–345.

Andrieu-Abadie N, Jaffrézou JP, Hatem S, Laurent G, Levade T and Mercadier JJ (1999) L-carnitine prevents doxorubicin-induced apoptosis of cardiac myocytes: role of inhibition of ceramide generation. *FASEB J* **13:**1501–1510.

Barroso MP, Gomez-Diaz C, Villalba JM, Buron MI, Lopez-Lluch G and Navas P (1997) Plasma membrane ubiquinone controls ceramide production and prevents cell death induced by serum withdrawal. *J Bioenerg Biomembr* **29:**259–267.

Bielawska AE, Shapiro JP, Jiang L, Melkonyan HS, Piot C, Wolfe CL, Tomei LD, Hannun YA and Umansky SR (1997) Ceramide is involved in triggering of cardiomyocyte apoptosis induced by ischemia and reperfusion. *Am J Pathol* **151:**1257–1263.

Bilodeau JF, Faure R, Piedboeuf B and Mirault ME (2000) Hyperoxia induces S-phase cell-cycle arrest and p21(Cip1/Waf1)-independent Cdk2 inhibition in human carcinoma T47D-H3 cells. *Exp Cell Res* **256:**347–357.

Bohler T, Waiser J, Hepburn H, Gaedeke J, Lehmann C, Hambach P, Budde K and Neumayer HH (2000) TNFα and IL-1α induce apoptosis in subconfluent rat mesangial cells. Evidence for the involvement of hydrogen peroxide and lipid peroxidation as second messengers. *Cytokine* **12:**986–991.

Bose R, Verheij M, Haimovitz-Friedman A, Scotto K, Fuks Z and Kolesnick R (1995) Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. *Cell* **82:**405–414.

Buttke TM and Sandstrom PA (1994) Oxidative stress as a mediator of apoptosis. *Immunol Today* **15:**7–10.

Cummings J, Anderson L, Willmott N and Smyth JF (1991) The molecular pharmacology of doxorubicin in vivo. *Eur J Cancer* **27:**532- 535.

Denizot F and Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* **89:**271–277.

Doroshow JH (1995) Glutathione peroxidase and oxidative stress. *Toxicol Lett* **82–83:**395–398 .

Friesen C, Fulda S and Debatin KM (1999) Induction of CD95 ligand and apoptosis by doxorubicin is modulated by the redox state in chemosensitive- and drug-resistant tumor cells. *Cell Death Differ* **6:**471–480.

Furling D, Ghribi O, Lahsaini A, Mirault M-E and Massicotte G (2000) Impairment of synaptic transmission by transient hypoxia in hippocampal slices: improved recovery in glutathione peroxidase transgenic mice. *Proc Natl Acad Sci USA* **97:**4351–4356.

Friesen C, Herr I, Krammer PH and Debatin KM (1996) Involvement of the CD95 (APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nat Med* **2:**574–577.

Garcia-Ruiz C, Colell A, Mari M, Morales A and Fernandez-Checa JC (1997) Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J Biol Chem* **272:**11369–11377.

Goldkorn T, Balaban N, Shannon M, Chea V, Matsukuma K, Gilchrist D, Wang H and Chan C (1998) H_2O_2 acts on cellular membranes to generate ceramide signaling and initiate apoptosis in tracheobronchial epithelial cells. *J Cell Sci* **111:**3209–3220.

Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL and Korsmeyer SJ (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75:**241–251.

Keane MM, Ettenberg SA, Lowrey GA, Russell EK and Lipkowitz S (1996) Fas expression and function in normal and malignant breast cell lines. *Cancer Res* **56:**4791–4798.

Kolesnick RN and Krönke M (1998) Regulation of ceramide production and apoptosis. *Annu Rev Physiol* **60:**643–665.

Legault J, Carrier C, Petrov P, Renard P, Remacle J and Mirault M-E (2000) Mitochondrial GPx1 decreases induced but not basal oxidative damage to mtDNA in T47D cells. *Biochem Biophys Res Commun* **272:**416–422.

Liu LF (1989) DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* **58:**351–375.

Liu B, Andrieu-Abadie N, Levade T, Zhang P, Obeid LM and Hannun YA (1998) Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor α-induced cell death. *J Biol Chem* **273:**11313–11320.

Liu B and Hannun YA (1997) Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione. *J Biol Chem* **272:**16281–16287.

Manna SK, Zhang HJ, Yan T, Oberley LW and Aggarwal BB (1998) Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-κB and activated protein-1. *J Biol Chem* **273:**13245–13254.

Mansat-de Mas V, Bezombes C, Quillet-Mary A, Bettaieb A, de Thonel d'Orgeix A, Laurent G and Jaffrézou JP (1999) Implication of radical oxygen species in ceramide generation, c-Jun N-terminal kinase activation and apoptosis induced by daunorubicin. *Mol Pharmacol* **56:**867–874.

Maulik N, Yoshida T and Das DK (1999) Regulation of cardiomyocyte apoptosis in ischemic reperfused mouse heart by glutathione peroxidase. *Mol Cell Biochem* **196:**13–21.

Mehlen P, Kretz-Remy C, Briolay J, Fostan P, Mirault ME and Arrigo AP (1995) Intracellular reactive oxygen species as apparent modulators of heat-shock protein 27 (hsp 27) structural organization and phosphorylation in basal and tumor necrosis factor α-treated T47D human carcinoma cells. *Biochem J* **312:**367–375.

Mirault ME, Tremblay A, Furling D, Trépanier G, Dugré F, Puymirat J and Pothier F (1994) Transgenic glutathione peroxidase mouse models for neuroprotection studies. *Ann NY Acad Sci* **738:**104–115.

Mirault ME, Tremblay A, Beaudoin N and Tremblay M (1991) Overexpression of seleno-glutathione peroxidase by gene transfer enhances the resistance of T47D human breast cells to clastogenic oxidants. *J Biol Chem* **266:**20752–20760.

Muller I, Niethammer D and Bruchelt G (1998) Anthracycline-derived chemotherapeutics in apoptosis and free radical cytotoxicity. *Int J Mol Med* **1:**491–494.

Nomura K, Imai H, Koumura T, Arai M and Nakagawa Y (1999) Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. *J Biol Chem* **274:**29294–29302.

Pahan K, Dobashi K, Ghosh B and Inderjit S (1999) Induction of the manganese superoxide dismutase gene by sphingomyelinase and ceramide. *J Neurochem* **73:**513–520.

Quillet-Mary A, Jaffrézou J-P, Mansat V, Bordier C, Naval J and Laurent G (1997)

Implication of mitochondrial hydrogen peroxide generation in ceramide-induced apoptosis. *J Biol Chem* **272:**21388–21395.
Richter C, Gogvadze V, Laffranchi R, Schlapbach R, Schweizer M, Suter M, Walter P and Yaffee M (1995) Oxidants in mitochondria: from physiology to diseases. *Biochim Biophys Acta* **1271:**67–74 .
Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:**76–85.
Steinbrecher UP (1988) Role of superoxide in endothelial-cell modification of low-density lipoproteins. *Biochim Biophys Acta* **959:**20–30.
van den Dobbelsteen DJ, Nobel CSI, Schlegel J, Cotgreave IA, Orrenius S and Slater AF (1996) Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1 antibody. *J Biol Chem* **271:**15420–15427.
van Veldhoven PP, Bishop WR, Yurivich DA and Bell RM (1995) Ceramide quantitation: evaluation of a mixed micellar assay using E. coli diacylglycerol kinase. *Biochem Mol Biol Int* **36:**21–30.
Yoshimura S, Banno Y, Nakashima S, Takenaka K, Sakai H, Nishimura Y, Sakai N, Shimizu S, Eguchi Y, Tsujimoto Y et al. (1998) Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death. Inhibitory effects of Bcl-2 on ceramide formation and caspase-3 activation. *J Biol Chem* **273:**6921–6927.
Zhang P, Liu B, Kang SW, Seo MS, Rhee SG and Obeid LM (1997) Thioredoxin peroxidase is a novel inhibitor of apoptosis with a mechanism distinct from that of Bcl-2. *J Biol Chem* **272:**30615–30618.

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