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Molecular Iodine Induces Caspase-independent Apoptosis in Human Breast Carcinoma Cells Involving the Mitochondria-mediated Pathway*

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Molecular iodine (I_2) is known to inhibit the induction and promotion of *N*-methyl-*n*-nitrosourea-induced mammary carcinogenesis, to regress 7,12-dimethylbenz(a)anthracene-induced breast tumors in rat, and has also been shown to have beneficial effects in fibrocystic human breast disease. Cytotoxicity of iodine on cultured human breast cancer cell lines, namely MCF-7, MDA-MB-231, MDA-MB-453, ZR-75–1, and T-47D, is reported in this communication. Iodine induced apoptosis in all of the cell lines tested, except MDA-MB-231, shown by sub-G_1 peak analysis using flow cytometry. Iodine inhibited proliferation of normal human peripheral blood mononuclear cells; however, it did not induce apoptosis in these cells. The iodine-induced apoptotic mechanism was studied in MCF-7 cells. DNA fragmentation analysis confirmed internucleosomal DNA degradation. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling established that iodine induced apoptosis in a time- and dose-dependent manner in MCF-7 cells. Iodine-induced apoptosis was independent of caspases. Iodine dissipated mitochondrial membrane potential, exhibited antioxidant activity, and caused depletion in total cellular thiol content. Western blot results showed a decrease in Bcl-2 and up-regulation of Bax. Immunofluorescence studies confirmed the activation and mitochondrial membrane localization of Bax. Ectopic Bcl-2 overexpression did not rescue iodine-induced cell death. Iodine treatment induces the translocation of apoptosis-inducing factor from mitochondria to the nucleus, and treatment of *N*-acetyl-L-cysteine prior to iodine exposure restored basal thiol content, ROS levels, and completely inhibited nuclear translocation of apoptosis-inducing factor and subsequently cell death, indicating that thiol depletion may play an important role in iodine-induced cell death. These results demonstrate that iodine treatment activates a caspase-independent and mitochondria-mediated apoptotic pathway.

Iodine is essential to maintaining the normalcy of the thyroid and the breast. An iodine-deficient state renders the rat thyroid and the breast susceptible to physiological changes and leads to atypia, dysplasia, and hyperplasia (1). The results of iodine replacement therapy in the iodine-deficient rat model shows that different forms of iodine have different tissue responses; iodide (I^-) is found to restore the normal morphology and physiology of the thyroid gland, whereas molecular iodine (I_2) results in a decrease of rat breast hyperplasia and perilobular/ductal fibrosis (2). The beneficial effect of molecular iodine has also been documented in the human fibrocystic breast condition and in cyclic mastalgia (3, 4). Iodine, in conjunction with medroxy progesterone acetate (5), and an iodine-rich seaweed "wakame" diet (6) are shown to regress 7,12-dimethylbenz(a)anthracene-induced rat breast tumors, and this effect has been corroborated by high tumor tissue iodine content (5, 6) and induction of apoptosis at the tumor site (6). Iodide excess is known to induce apoptosis in the thyroid cells *in vitro* (7) and also in sodium iodide symporter and thyroperoxidase stably transfected non-small cell lung carcinoma cells (8). Earlier studies show that sodium iodide symporter facilitates iodide transport, and thyroperoxidase oxidizes iodide (I^-) to iodine (I_2), which is important for its organification (9). Propyl-thiouracil, an inhibitor of peroxidase, completely abolishes the cell death-inducing effect of iodide in thyroid cells, establishing I_2 as the mediator of apoptosis (7). The enhanced expression of sodium iodide symporter in human breast cancer tissue has been reported; however, its significance is unknown (9, 10). In addition to this, non-lactating breast tissue is known to be peroxidase-poor (11) and does not provide milieu conducive for iodide organification. On the other hand, molecular iodine is a highly reactive species and can be utilized without involvement of sodium iodide symporter and peroxidase activity (12).

Studies performed in the cell-free system show that iodine exposure to mitochondria isolated from breast tumor tissue causes swelling, organification of the mitochondrial proteins, and release of apoptogenic effectors from mitochondria that cause nuclear fragmentation (13). The mechanism of iodine action in breast cancer cells has not been studied to date. This led us to investigate the anti-proliferative and cytotoxic effects of iodine on breast cancer cells, which can be mediated through apoptosis.

Apoptosis is a physiological cell suicide program critical to development and tissue homeostasis. The caspases, a family of

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intracellular cysteine proteases, are the central executioners of apoptosis. Effector caspases, such as caspase-3 and -7, are activated by initiator caspases, such as caspase-9, through proteolytic cleavage. Once activated, effector caspases are responsible for the digestion of a diverse array of structural and regulatory proteins, resulting in an apoptotic phenotype (14). During apoptosis, divergent cellular stresses, such as DNA damage, heat shock, oxidative stress, withdrawal of growth factor, etc., also converge on mitochondria. Decrease in the mitochondrial transmembrane potential and altered cellular redox state are the early changes in mitochondria-mediated apoptosis (15). Mitochondrial intermembrane space contains several proteins that can either induce apoptosis involving caspases (*e.g.* cytochrome *c*), the secondary mitochondrial activator of caspases (Smac) and HtrA2/Omi or execute a caspase-independent apoptotic death program through apoptosis-inducing factor (AIF)[3] and endonuclease G (16–22). The Bcl-2 family of proteins, with both anti-apoptotic as well as pro-apoptotic members, is implicated in the regulation of mitochondria-mediated apoptosis. Two of the anti-apoptotic members, namely Bcl-2 and Bcl-x_L, confer resistance to apoptosis induced by a number of stimuli, whereas the other homologues Bid, Bax, Bak, and BH-3 domain-only proteins promote apoptosis (23, 24).

This study elucidates the detailed mechanism of molecular iodine-induced apoptosis in human breast cancer cells. Iodine treatment induces changes in members of Bcl-2 family proteins and leads to the activation and translocation of Bax to mitochondria. The release of AIF from mitochondria executes nuclear fragmentation in a caspase-independent manner. The results show that iodine exhibits strong antioxidant activity, and thiol depletion seems to play an important role in iodine-induced apoptosis.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Phenylmethylsulfonyl fluoride, protease inhibitor mixture, iodine, potassium iodide, DCF-DA, DTNB, *N*-acetyl-L-cysteine, propidium iodide (PI), JC-1, and RNase A were procured from Sigma. All cell culture reagents were purchased from Invitrogen. Antibodies against caspase-3, Bcl-2, Bcl-X_L, Bax, AIF, phosphorylated c-Jun, and β-actin, and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for p53, caspase-7, and cytochrome *c* (7H8.2C12) were kindly gifted by Drs. A. Sparks, Xiao-Ming Sun, and R. Jemmerson, respectively. Inhibitors specific to JNK (SP600015) and p38 (SB203580) were purchased from Alexis (San Diego, CA).

Cell Culture and Transfection—Human breast cancer cell lines, namely MCF-7, MDA-MB-231, MDA-MB-453, ZR-75-1, and T-47D, were procured from the National Centre of Cell Sciences (Pune, India). Human peripheral blood lymphocytes (PBMCs) were isolated from healthy volunteers using Lymphoprep lymphocyte separation medium (Eurobio). Cells were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified environment of 5% CO_2. pCAGGS-hbcl-2 carrying cDNA for the human *bcl-2* gene was a kind gift from Dr. Tsujimoto (Osaka University Graduate School of Medicine). To establish the Bcl-2-overexpressing MCF-7 cell line, 0.1 million MCF-7 cells were plated in a 33-mm^2 culture dish for 24 h before transfection and then co-transfected with either 1 μg of pCAGGS-hbcl-2 or control vector pCAGGS and 0.1 μg of pCDNA3 (for neomycin selection) using Cytofectene (Bio-Rad). The transfected cells were selected with geneticin (500 μg/ml) for 24 h followed by 100 μg/ml for 2 weeks. Stably transfected clones were analyzed for Bcl-2 expression levels by Western blotting.

Cytotoxicity Assay—The cytotoxic effect of iodine on breast cancer cell lines was analyzed using the trypan blue dye exclusion method. Human PBMCs were used as a non-cancerous cell control. The iodine solution was prepared by dissolving 1 g of iodine and 2 g of potassium iodide in 100 ml of water. The amount of molecular iodine was estimated using the sodium thiosulfate titration method (25). Accordingly, stock solution of 75 μM iodine was prepared. Cells (2×10^4) were plated in a 24-well plate and treated with potassium iodide (5 mM), different concentrations of iodine (1–4 μM), or left untreated for 48 h. Cells were collected by trypsinization and counted using trypan blue staining. Experiments were performed in triplicates, and the mean values were calculated. IC_{50} values for each cell line were interpolated from the dose-response curve.

Detection of Apoptosis—Flow cytometric analysis was performed to detect and quantify apoptosis. Cells were fixed in 70% ethanol and stained with 50 μg/ml PI in hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X-100) containing DNase-free RNase A for 30 min. Acquisition and analysis was performed by FACScan using Cell Quest Alias software (BD Biosciences). Cells with their DNA content less than that of G_1 phase cells (sub-G_1) were assumed to be apoptotic.

For DNA fragmentation analysis, MCF-7 cells were grown in 25-cm^2 culture flasks and were left untreated or treated with iodine (1–4 μM). Both attached and detached cells were collected and resuspended in 0.5 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% Triton X-100) for 30 min. Supernatant was treated with proteinase K (20 μg/ml) and RNase A (10 μg/ml), and DNA was extracted with phenol/chloroform and precipitated with absolute ethanol. DNA samples were electrophoresed on 1.8% agarose gel and visualized by ethidium bromide staining.

For TUNEL assay, MCF-7 cells on the coverslips, left untreated or treated (1–4 μM) with iodine for different time points, were fixed with freshly prepared 4% paraformaldehyde. Subsequently, the assay was performed using the ApoAlert® DNA fragmentation assay kit strictly according to the manufacturer's instructions (Clontech, Palo Alto, CA). Nuclei were counterstained with PI, and the TUNEL-positive cells were visualized using a fluorescence microscope (Nikon Microphot 4T).

[3] The abbreviations used are: AIF, apoptosis-inducing factor; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid; PI, propidium iodide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; PBMC, peripheral blood mononuclear cell; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; ROS, reactive oxygen species, $\Delta\psi_m$, mitochondrial membrane potential; JNK, c-Jun NH_2-terminal kinase; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

Caspase Inhibition Assay—To study the involvement of caspases in iodine-induced apoptosis, MCF-7 cells were pretreated for 1 h with 50 μM caspase inhibitors (R & D systems, Minneapolis, MN), general caspase inhibitor (Z-VAD-fmk), caspase-3 inhibitor (Z-DEVD-fmk), caspase-8 inhibitor (Z-IETD-fmk), and caspase-9 inhibitor (Z-LEHD-fmk) followed by iodine treatment at an IC_{50} iodine concentration of 2.7 μM for 48 h. Cell viability was determined using trypan blue staining.

Measurement of $\Delta\psi_m$, ROS, and Total GSH Content—The effect of iodine on the $\Delta\psi_m$ was determined using JC-1. This fluorescent probe exists as a green fluorescent monomer (emission 527 nM) at low mitochondrial membrane potential. Mitochondrial depolarization is indicated by an increase in green fluorescence (FL-1). MCF-7 cells (0.2 million) in a 6-well plate were treated with 3 μM iodine for different time points. The cells were incubated with JC-1 (1 μM) in serum-free medium for 30 min before termination of the experiments, and $\Delta\psi_m$ was estimated in a minimum of 10,000 cells by FACScan. ROS generation was analyzed by flow cytometry using DCF-DA. MCF-7 cells were treated with iodine (3 μM) alone or pretreated with L-NAC (2 mM) or L-NAC alone for 24 h and incubated with 10 μM DCF-DA in serum-free medium for 30 min, and levels of ROS were estimated by FACScan. Total cellular thiol content was determined by a method described earlier (26). In brief, the cell lysates were prepared as described above and stained with 1 ml of 20 mM DTNB. Prechilled ethanol (200 μl) was added to precipitate the macromolecules, and the absorbance of the supernatant was measured with a Dynetech MR5000 (Chantilly, VA) microplate reader at 410 nM.

Western Blotting—MCF-7 cells were cultured in 75-cm^2 culture flasks and treated with 3 μM iodine for different time points. Attached and detached cells were disrupted in 50 mM phosphate buffer containing phenylmethylsulfonyl fluoride (1 mM) and protease inhibitor mixture by rapid freeze-thaw cycles (three times). To prepare mitochondrial and cytosolic fractions, the cells were homogenized using a Teflon homogenizer in ice-cold mitochondria buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture, pH 7.4). The homogenate was centrifuged at 800 $\times$ *g* to remove nuclei. The supernatant was again centrifuged at 10,000 $\times$ *g* for 30 min. The supernatant from this centrifugation represents the cytosolic fraction and was analyzed for the release of proteins from mitochondria. The purity of the cytosolic and membrane fraction was confirmed using biochemical assay for lactate dehydrogenase and cytochrome oxidase, respectively. The protein concentration was determined using the Bradford protein assay kit (Bio-Rad). A 50-μg protein sample in each lane was separated on a 12% SDS-polyacrylamide gel. Proteins were electrotransferred to nitrocellulose membrane, and nonspecific sites on the membrane were blocked with 5% nonfat dry milk (w/v) overnight at 4 °C. The membrane was incubated with primary antibody and subsequently with appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were detected by Supersignal® West Pico chemiluminescent substrate (Pierce). Membranes were stripped and reprobed with β-actin as a protein loading control.

Immunofluorescence—MCF-7 cells were grown on sterile coverslips in a 6-well plate and treated with 3 μM iodine for 36 h. The cells were fixed with fresh 4% formaldehyde/PBS at 4 °C for 20 min and permeabilized with prechilled 0.2% Triton X-100/PBS. The cells were then incubated with goat anti-AIF antibody (1:100) in 3% bovine serum albumin/PBS overnight at 4 °C followed by incubation with fluorescein isothiocyanate-conjugated secondary antibodies. After PBS washes, nuclei were counterstained with PI. For analysis of the activation of Bax, cells were labeled with Mitotracker Red CM-H_2Xros (Molecular Probes) at 200 nM for 45 min to localize the mitochondria. Cells were permeabilized for 5 min with 0.0125% CHAPS/PBS to prevent artificial activation of Bax (27). The cells were then incubated with monoclonal anti-Bax YTH-6A7 antibody (Trevigen, Gaithersburg, MD) at the dilution of 1:100, which recognizes only the activated form of Bax, followed by incubation with fluorescein isothiocyanate-conjugated secondary antibodies. Images were captured on a confocal microscope (Radiance 200100, Bio-Rad).

RESULTS

Cytotoxicity of Iodine in Breast Cancer Cell Lines—For determining the effect of iodine on cell survival, five human breast cancer cell lines and PBMCs were incubated with increasing concentrations of iodine (1–4 μM) for 48 h or left untreated and analyzed for their viability using the trypan blue dye exclusion method. Potassium iodide (5 mM) did not show any cytotoxicity (data not shown), and iodine treatment significantly decreased the cell viability of all of the cell lines and PBMCs in a dose-dependent manner (Fig. 1*A*). The cell number of all of the breast cancer cell lines at 4 μM iodine concentration decreased below the initial number after 48 h, indicating that iodine is cytotoxic to cells. Cells treated with iodine rounded up and detached from the substratum, indicating cell death (Fig. 1*B*). Nuclear staining using fluorescent DNA binding dye (Hoechst-33258) displayed the typical morphological features of apoptosis with nuclear fragmentation in the iodine (4 μM)-treated cell lines, except in MDA-MB-231 where nuclei were sickle-shaped but not fragmented (Fig. 1*C*). In addition to this, iodine exhibited a anti-proliferative effect on human PBMCs; however, nuclear fragmentation was not observed (Fig. 1). These results indicate that iodine not only inhibits cell growth but also induces cell death.

Iodine Induces Apoptosis in Breast Cancer Cell Lines—To determine whether iodine-induced cytotoxicity is mediated through apoptosis, several assays were performed. Iodine-treated breast cancer cell lines were analyzed for their DNA content after staining with PI using FACScan. We found a dose-dependent increase of the cells in the sub-G_1 population and >80% of the cells to be hypodiploid after a 4 μM iodine treatment for 48 h; however, hypodiploidy was not observed in iodine-treated MDA-MB-231 cells and human PBMCs (Fig. 2, *A* and *B*), consistent with the absence of nuclear fragmentation (Fig. 1*B*). DNA fragmentation analysis of iodine-treated MCF-7 cells showed a laddering pattern characteristic of apoptosis, indicating internucleosomal DNA degradation (Fig. 2*C*).

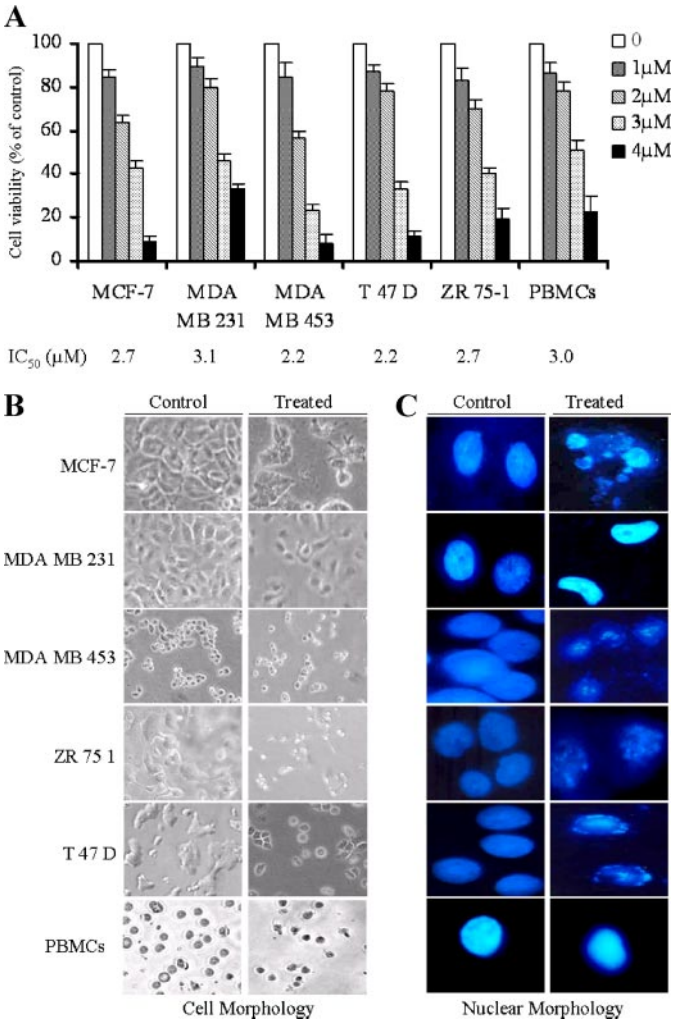


FIGURE 1. **Iodine decreases cell viability and induces cellular and nuclear morphology changes in human breast cancer cells.** *A*, cells were plated at the density of 4×10^4 cells/ml in a 24-well plate and were treated with different concentrations of iodine for 48 h in triplicates. Cell viability was analyzed using the trypan blue dye exclusion method. The negatively stained cells were counted in a hemocytometer. The percent of viable cells is expressed as mean ± S.D. IC_{50} values of iodine for each cell line is shown in the *lower panel*. PBMCs were used as the non-cancer cell control. *B* and *C*, cells were treated with iodine (4 μM) for 48 h, and morphological changes (*B*) were examined by phase contrast microscopy (10× objective). *Round-shaped* and floated cells are indicative of cell death. Nuclear morphology (*C*) was observed by Hoechst-33258 staining (60× objective). Nuclei exhibited condensation, margination, and fragmentation after iodine treatment. No nuclear condensation is observed in the nuclei of treated MDA-MB-231 and PBMCs.

MCF-7 cells were analyzed for TUNEL assay, a quantitative technique that reveals DNA strand breaks (Fig. 2*D*), and a dose- and time-dependent increase in the number of apoptotic cells was observed (Fig. 2*E*).

Iodine-induced Apoptosis Is Independent of Caspases—To test whether caspases are involved in iodine-induced apoptosis, the expression of effector caspase-3 and -7 was analyzed using Western blotting in MCF-7 cells. MCF-7 cells are known not to express a functional caspase-3 (28). In agreement, we also did not find its expression, but observed a corresponding 32-kDa band in the MDA-MB-231 cell line used as a positive control (Fig. 3*A*). Caspase-7 is another effector caspase that can take over functions of caspase-3 under certain conditions (29). Although increase in the expression of procaspase-7 was observed, its cleaved forms were not detected (Fig. 3*B*). Additionally, caspase inhibition assay using a general caspase inhibitor Z-VAD-fmk, as well as inhibitors specific to caspase-3, -8, and -9 (50 μM), did not affect cell viability of the untreated or iodine-treated MCF-7 cells (Fig. 3*C*). These results indicate that iodine-induced apoptosis is independent of caspase activation.

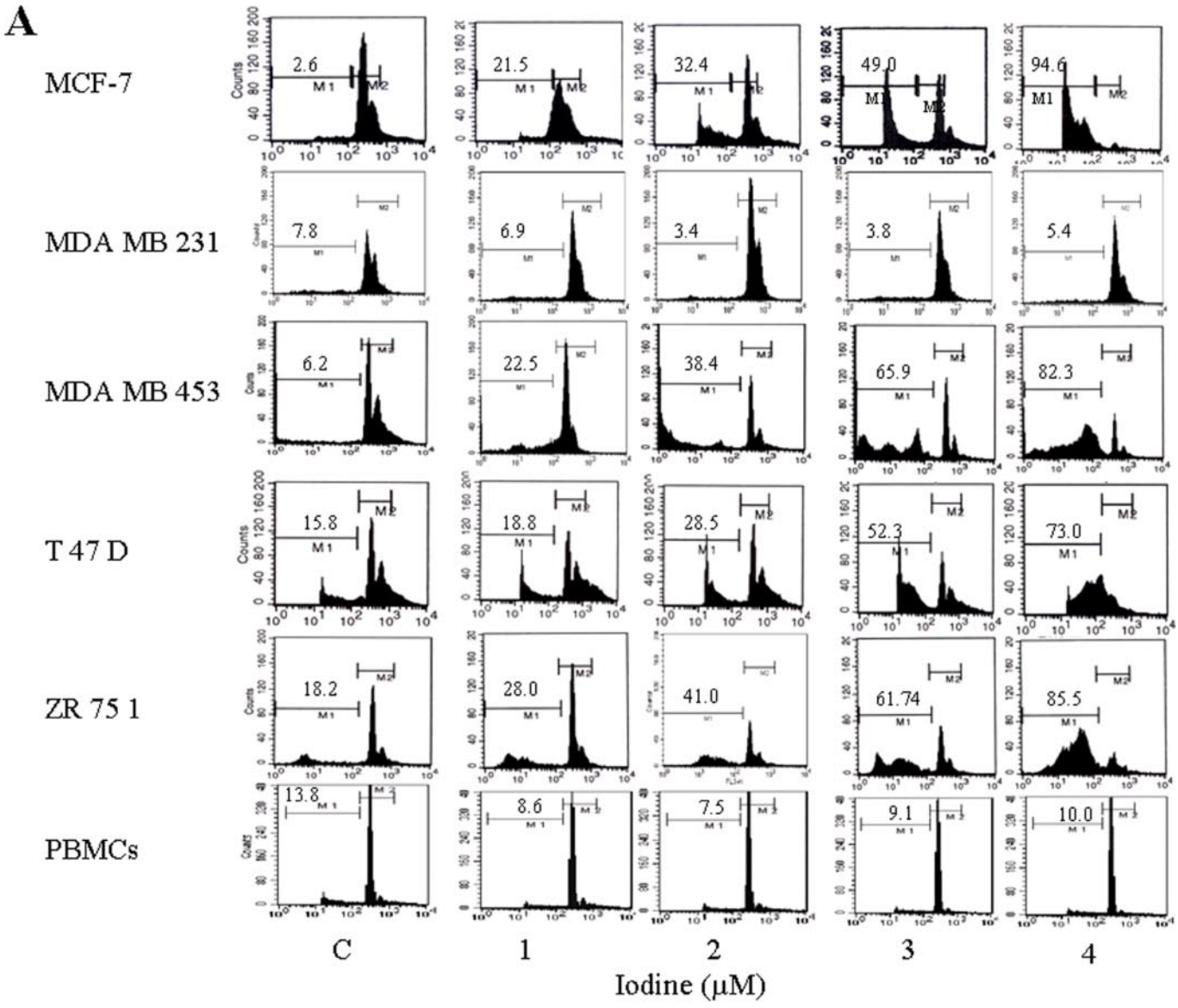
Iodine Induces Loss of $\Delta\psi_m$ and Decreases ROS Generation and Total Thiol Content—To determine the involvement of the mitochondria-mediated pathway in iodine-induced apoptotic cell death, we measured changes in $\Delta\psi_m$. Iodine treatment of MCF-7 cells resulted in a rapid dissipation of $\Delta\psi_m$ in a time-dependent manner detected by an increase in green fluorescence emission (Fig. 4*A*).

We analyzed levels of ROS in iodine-treated MCF-7 cells and found a 4-fold decrease in cellular ROS after 24 h of iodine treatment (Fig. 4*B*). Iodine treatment was also found to lower total thiol content (Fig. 4*C*). Pretreatment of L-NAC (2 mM) not only restored basal thiol content (Fig. 4*C*) but also the ROS levels comparable with control cells (Fig. 4*B*).

Role of Members of Bcl-2 Family Protein in Iodine-induced Apoptosis—To investigate whether iodine-induced apoptosis is a consequence of the altered expression of the members of Bcl-2 family proteins, Western blotting for Bcl-2, Bcl-x_L and Bax was performed (Fig. 5*A*). There was a decrease in the expression of anti-apoptotic Bcl-2 in a time-dependent manner. The decrease was significant after 24 h, and after 48 h, expression decreased up to 5-fold of the control (Fig. 5*B*). There was no alteration in the expression of Bcl-x_L (Fig. 5*B*). On the other hand, expression of Bax increased significantly after 12 h and was ~7-fold higher in comparison to the control after 48 h of iodine treatment (Fig. 5*B*). Bax is known to be a transcriptional target of p53 (30), and Western blot analysis of p53 showed a time-dependent increase in its expression (Fig. 5, *A* and *B*).

Bax has been shown to redistribute from cytosol to the mitochondria during the apoptotic process (31). Immunofluorescence analysis of untreated and iodine-treated cells stained with a conformation-specific antibody against the N-terminal sequence of Bax (green fluorescence) revealed its mitochondrial localization. Mitotracker Red was used to localize mitochondria. No green fluorescence was visible in control cells after 36 h of iodine treatment, whereas such fluorescence was detected in iodine-treated cells and was co-localized with red fluorescence of Mitotracker and resulted in the yellow color in the overlaid figure (Fig. 5*C*).

Release of Apoptogenic Molecules from Mitochondria—Decrease in $\Delta\psi_m$ and in the ratio of Bcl-2/Bax results in the loss of selective permeability of mitochondrial membrane that leads to the release of apoptogenic proteins normally confined to mitochondrial intermembrane space. Results of Western blotting showed the presence of cytochrome *c* and AIF in the cytosolic fraction of iodine-treated MCF-7 cells (Fig. 6*A*). AIF is synthesized as a 67-kDa preprotein and localizes in mitochondrial intermembrane space. Upon induction of apoptosis, AIF is processed to a 57-kDa form and released into the cytoplasm (32). The Western blot result shows the presence of 67-kDa AIF in the control as well as the treated sample; however, the 57-kDa form of AIF was observed only in the 24-h-treated sample (Fig.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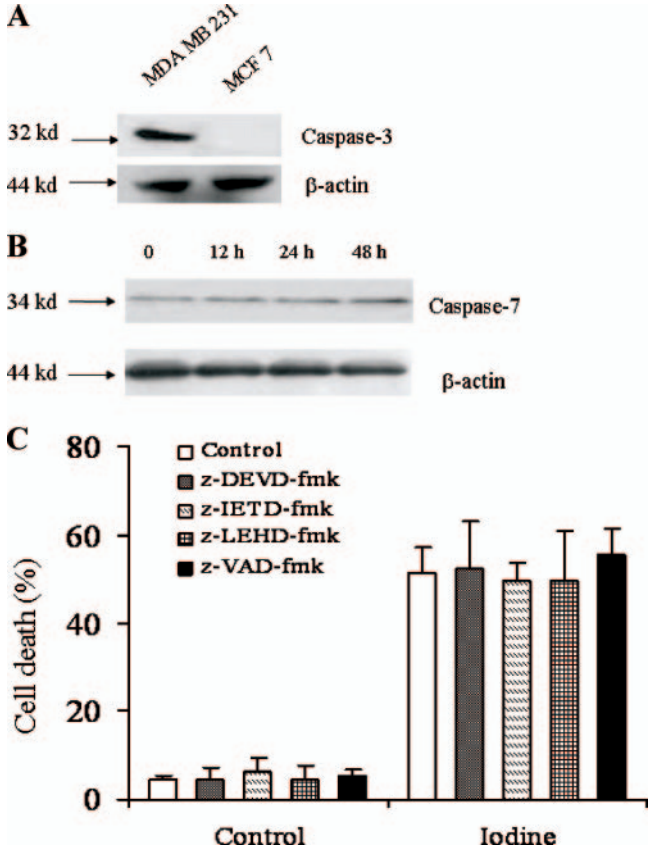

FIGURE 3. **Iodine induces caspase-independent cell death in breast cancer cells.** *A*, whole cell lysate was prepared from MCF-7 and MDA-MB-231 cells. A total 50 μg of cell lysate was separated on a 12% SDS-polyacrylamide gel and probed with caspase-3 antibody. Caspase-3 was not detected in MCF-7 cells. *B*, analysis of caspase-7 activation by Western blotting. MCF-7 cells, untreated or treated with iodine at 3 μM concentration for 12, 24, and 48 h and whole cell lysate were prepared. A total 50 μg of cell lysate from each time point was separated on a 12% SDS-polyacrylamide gel and probed with caspase-7 antibody. A band corresponding to 34-kDa procaspase-7 is observed only in treated samples, and no activated form was detected. The blot is representative of three independent experiments. *C*, caspase inhibitors do not have any effect on iodine-induced cell death in MCF-7 cells. Cells were treated with 2.7 μM iodine for 48 h in the presence or absence of caspase inhibitors (50 μM). Thereafter, cell viability was determined using the trypan blue dye exclusion method. Data represent mean ± S.D. from triplicate experiments.

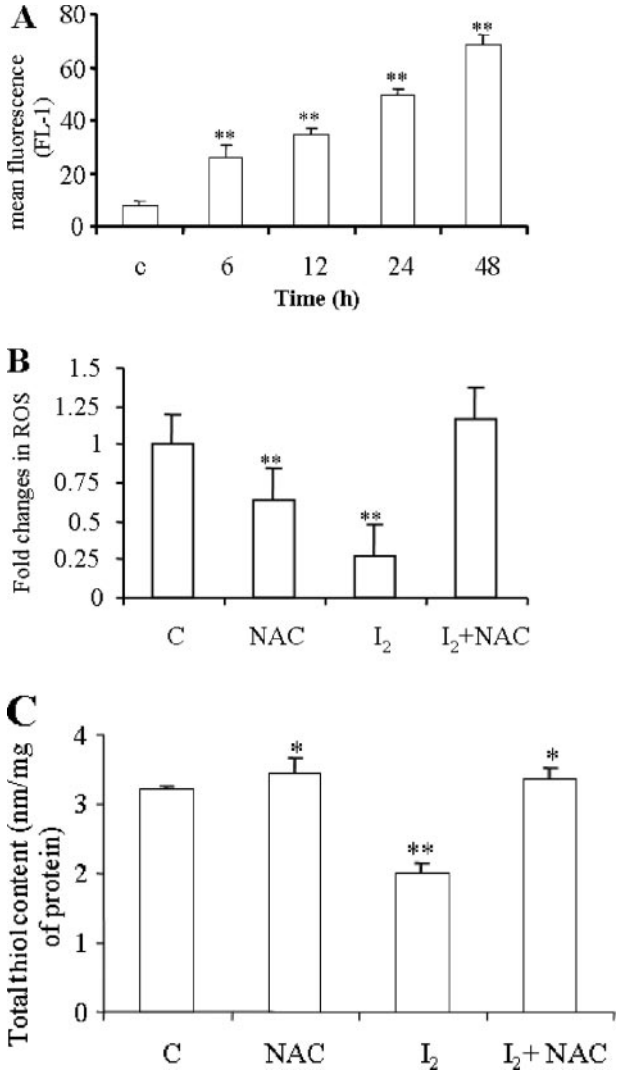

FIGURE 4. **Iodine treatment induces loss in $\Delta\psi_m$, decreases ROS production, and causes thiol depletion in MCF-7 cells.** *A*, MCF-7 cells were treated with 3 μM iodine for the indicated time periods. The loss in $\Delta\psi_m$ was measured by flow cytometry using JC-1. Increase in green fluorescence (*FL-1*) reflects loss in $\Delta\psi_m$. *B* and *C*, MCF-7 cells were treated with iodine (3 μM), L-NAC (2 mM), L-NAC (2 mM) + iodine (3 μM), or left untreated for 24 h, and ROS levels (*B*) were measured by flow cytometry using DCF-DA. Cell lysate was prepared from the above-treated cells, and total thiol content (*C*) was determined using the biochemical method. Data represent mean ± S.D. from triplicate experiments. *, $p < 0.05$; **, $p < 0.01$ (compared with control cells).

6*A*). Immunofluorescence studies confirmed translocation of AIF to the nucleus after 36 h of iodine treatment (Fig. 6*B*). Pretreatment of L-NAC (2 mM) for 1 h completely inhibited the nuclear translocation of AIF and completely inhibited iodine-induced cell death; however, pretreatment with the general caspase inhibitor (50 μM) did not prevent the same (Fig. 6*B*).

DISCUSSION

We propose a detailed mechanism of the molecular iodine (I_2)-induced apoptosis in human breast cancer cells that may explain iodine-induced breast cancer regression in experimental rat models as well as beneficial effects observed in human fibrocystic breast subjects (3–6). Iodine showed cytotoxic effects in the cultured human breast cancer cells and, in flow cytometry analysis using PI staining, confirmed apoptosis as a cell death mechanism in all of the cell lines tested, except in

FIGURE 2. **Iodine induces apoptotic cell death in human breast cancer cells.** *A*, flow cytometric analysis of iodine-induced apoptosis. Cells were treated with different concentrations of iodine (1–4 μM) or left untreated for 48 h and then quantified for their DNA content after PI staining. *Values* shown in *boxes* are the percent of cells with hypodiploid DNA. *B*, histogram represents the sub-G_1 fraction (%), the proportion of apoptotic cells. *Bars*, ± S.D. *C*, MCF-7 cells were treated with different concentrations of iodine for 48 h. DNA was isolated and the ladder formation was examined on 1.8% agarose gel electrophoresis. *Lane M*, 1-kb DNA ladder; *lane 1*, untreated; *lanes 2–5*, iodine (1–4 μM treated). The experiment was repeated three times independently. *D*, iodine induces apoptosis in MCF-7 cells in a time- and dose-dependent manner. Cells were grown on coverslips and treated with the indicated concentrations of iodine for 12, 24, and 48 h, and apoptosis was analyzed by TUNEL assay. Representative fluorescence micrograph of TUNEL (*bottom*) and PI (*top*) staining of untreated (*left*) and iodine (4 μM)-treated MCF-7 cells for 48 h is shown. *E*, the TUNEL-positive cells were counted in five different views, and results were expressed as mean ± S.D.

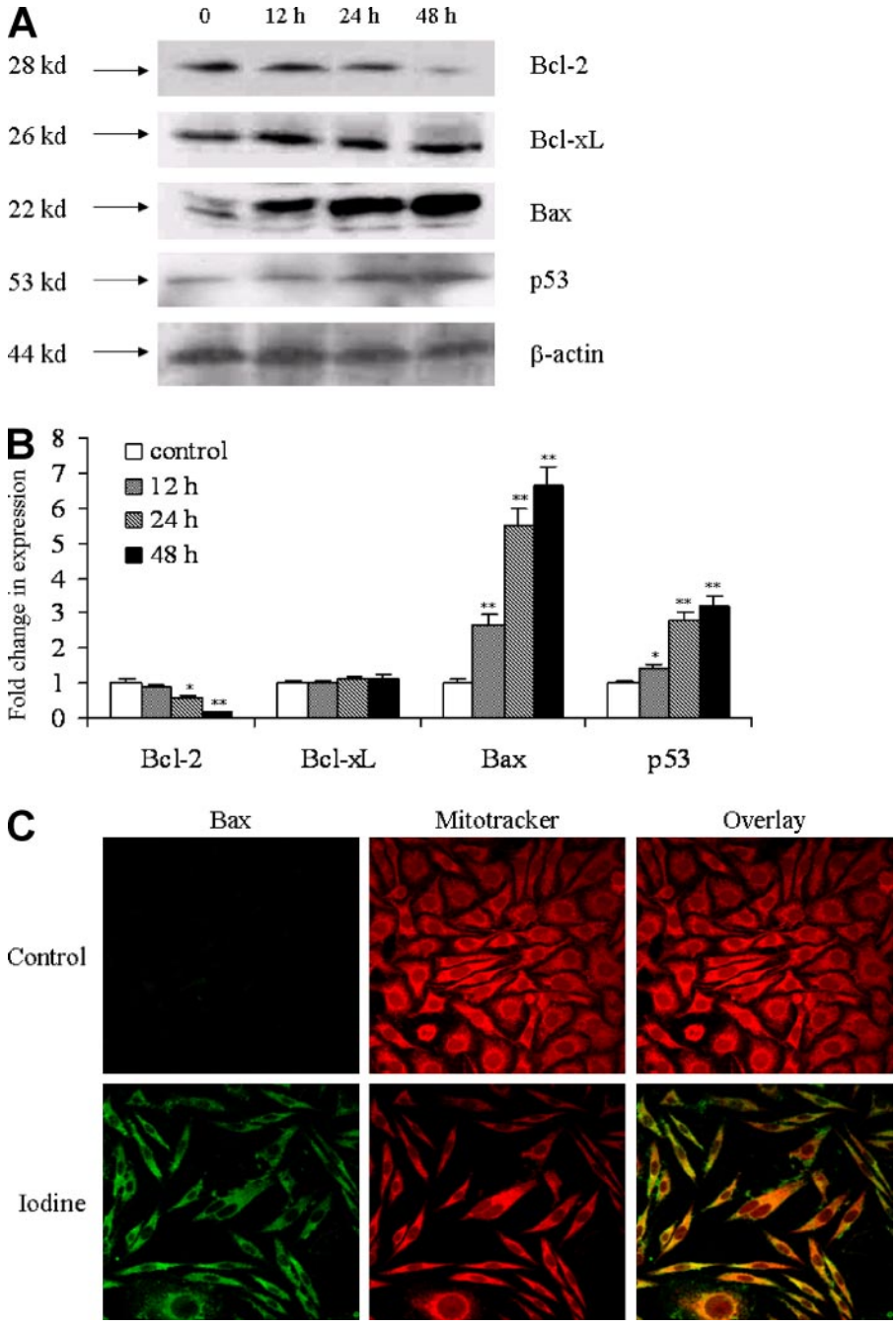

FIGURE 5. **Western blot analysis of Bcl-2, Bcl-x_L, Bax, and p53.** *A*, MCF-7 cells were treated with 3 μM iodine for 12, 24, and 48 h or left untreated. The protein extracts (50 μg) were separated on a 12% SDS-polyacrylamide gel and immunoblotted as described under "Experimental Procedures." β-actin was used as a control for loading of the proteins. *B*, densitometric quantitation of protein expression levels are shown as fold changes. *, $p < 0.05$; **, $p < 0.01$. *C*, iodine treatment leads to Bax activation and its mitochondrial membrane localization. Confocal microscopy examination of the activation status of Bax using conformation-specific antibody. Bax immunostaining colocalizes with the Mitotracker stain in iodine-treated MCF-7 cells, depicted in *yellow-orange* in the merged figure (*Overlay*).

MDA-MB-231. The inability of this cell line to undergo apoptosis in response to anti-cancer stimuli has been reported recently (33). The DNA laddering characteristic of apoptosis and the presence of TUNEL-positive MCF-7 cells in a dose- and time-dependent manner reconfirmed iodine-induced apoptosis. The cytotoxic effect of iodine on the five different breast cancer cell lines indicates its action is mostly independent of the p53 status (wild type in MCF-7, MDA-MB-231, and ZR-75–1 and mutant in MDA-MB-453 and T-47D) and estrogen receptor (wild type in MCF-7, T-47D, and ZR-75–1 and mutant in MDA-MB-231 and MDA-MB-453). In this study, human PBMCs were used to analyze the effect of iodine on cells of a non-cancerous origin. Iodine is anti-proliferative to PBMCs; however, the inability of iodine to induce apoptosis in PBMCs as well as in MDA-MB-231 cells suggests that iodine is selective in inducing apoptosis, depending upon the cellular context.

The MCF-7 cell line was used to delineate the detailed mechanism of iodine-induced apoptosis. Our results demonstrate iodine-induced apoptosis to be independent of caspases (Fig. 3). However, loss of $\Delta\psi_m$ on iodine treatment as early as 3 h (Fig. 4*A*) and the ability of iodine to cause significant reduction in free radical generation and glutathione levels supports mitochondria-mediated apoptotic cell death (Fig. 4, *B* and *C*). Earlier studies have shown that iodide-induced apoptosis is mediated by elevation of ROS (7, 8); however, molecular iodine has been hypothesized to have an antioxidant function (34). Recently, iodine treatment has been shown to lower lipid peroxidation in the *N*-methyl-*n*-nitrosourea-induced rat breast cancer model (12). The present data provide the first direct evidence that iodine acts as an antioxidant. Cells pretreated with the glutathione precursor L-NAC restored total cellular thiol content and ROS levels comparable with untreated cells, and cell viability was not compromised. These results suggest that thiol depletion is a major event during iodine-induced apoptosis. Interestingly, a recent report about tamoxifen-induced rapid cell death in MCF-7 cells suggests following a caspase-independent mitochondria-mediated cell death mechanism involving increase in ROS (35).

During apoptosis, the intracytosolic balance of members of the Bcl-2 family of proteins is critical to maintaining the integrity of mitochondrial membrane. A ~4-fold decrease in the levels of Bcl-2 accompanied with ~7-fold Bax up-regulation was found. Bcl-2 heterodimerizes *in vivo* with a conserved homologue of Bax and is also known to prevent Bax oligomer-

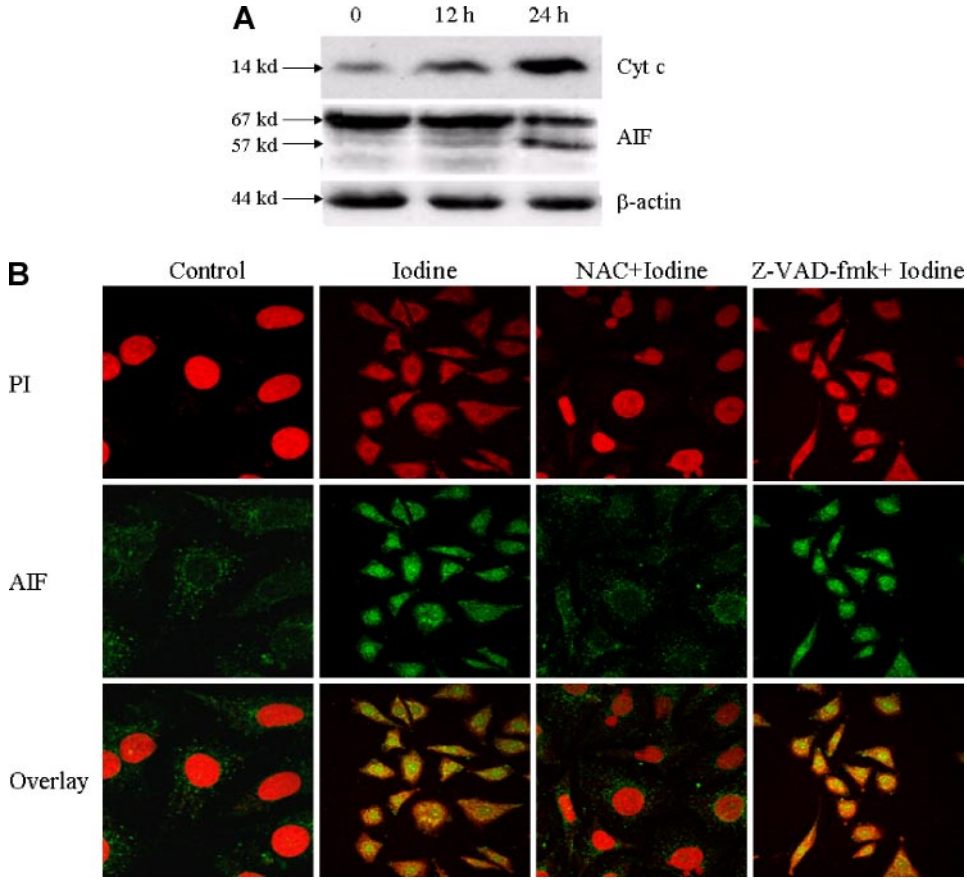

FIGURE 6. **Cytochrome *c* and AIF are released from the mitochondria during iodine-induced apoptosis.** *A*, MCF-7 cells were treated with 3 μM iodine for 12 and 24 h, and the cytosolic fraction was analyzed for the presence of cytochrome *c* and AIF by Western blotting on a 12% SDS-polyacrylamide gel. The processed form of AIF (57-kDa) is released only after 24 h of iodine treatment. *B*, representative confocal images showing iodine-induced AIF nuclear translocation and condensation. MCF-7 cells were grown on coverslips for 36 h after iodine treatment (3 μM) or left untreated and probed with AIF antibody (*green*). Nuclei were counterstained with PI (*red*). Pretreatment of L-NAC (2 mM) prior to iodine exposure prevents AIF nuclear translocation and subsequent nuclear fragmentation, and Z-VAD-fmk (50 μM) was unable to prevent AIF nuclear translocation and subsequent nuclear fragmentation. The concentrations of L-NAC and Z-VAD-fmk used are nontoxic to MCF-7-cultured cells (not shown).

ization and its insertion into the mitochondrial membrane (36, 37). A 3-fold increase in p53 expression suggests that increased expression of Bax may be p53-dependent, but this needs further investigation. However, pretreatment of the protein synthesis inhibitor cycloheximide has no effect on iodine-induced cell death, establishing that this process is independent of *de novo* protein synthesis (data not shown). The results of members of the Bcl-2 family of proteins and p53 are in contrast to the observations in iodide-induced apoptosis in thyroid and lung carcinoma cells, where levels of these proteins remain unaltered (7, 8).

Anti-apoptotic Bcl-2 protein has been shown to extend cell survival of resting B-cells and constitutes a common resistance mechanism against many anti-cancer stimuli (38, 39). The effect of iodine on the MCF-7 stably overexpressing Bcl-2 clone was analyzed. Bcl-2 overexpression had no effect on iodine-induced MPT or the prevention of cell death (data not shown). The inability of Bcl-2 overexpression in attenuating iodine-induced cells may be due to many fold increases of Bax accompanied with endogenous Bcl-2 degradation in response to iodine.

Our results suggest activation and insertion of Bax into the mitochondrial membrane subsequent to its increased expression. The exact mechanism of the activation of Bax, the minimum set of proteins required to permeabilize mitochondrial membrane, and whether mitochondrial proteins take part in membrane permeabilization still remain to be characterized. BH3 domain-only members of Bcl-2 family proteins have shown to induce conformational changes and activate Bax (40, 41). p53 can act analogously to BH-3-only proteins in directly activating Bax, which mediates membrane permeabilization (42). In addition to that, Bax-Bak oligomerization-mediated membrane perforation upstream to the release of AIF and endonuclease G is also reported (43). In the present study, we have not studied the involvement of BH3 domain-only protein and the cooperation of other Bcl-2 members in concert to Bax. Nevertheless, Bax is known to form large lipidic pores through which high molecular weight molecules can be passed (44). As a further step, we detected the presence of cytochrome *c* and the 57-kDa-processed form of AIF in the cytosolic fraction of iodine-treated MCF-7 cells. Release of cytochrome *c* leads to caspase-dependent cell death (17). The results show iodine-induced apoptosis to be independent of caspases and that AIF can execute a caspase-independent death program (21). Physiological functions of AIF are still not entirely clear; however, it possesses an NADPH-oxidase activity inseparable from apoptogenic activity (45). Immunofluorescence analysis confirmed the translocation of AIF in the nucleus and nuclear fragmentation. There are reports suggesting the requirement of caspase activation upstream to AIF release (43, 46, and 47). The inability of the general caspase inhibitor to block its nuclear translocation reconfirmed iodine-induced apoptosisto be caspase-independent. L-NAC pretreatment completely blocked AIF nuclear translocation and also cell death, suggesting thiol depletion may play an important role in the nuclear translocation of AIF. Studies performed in a cell-free system have also shown that thiols can prevent AIF-induced nuclear fragmentation (48).

Events leading to cellular stress activate various signaling cascades. The mitogen-activated protein kinase (MAPK) superfamily consists of three main protein kinase families, the extracellular signal-regulated protein kinases (ERKs), the JNKs, and the p38 family of kinases, and has been implicated in both apoptosis and survival signaling (49–51). We investigated the

possible involvement of the JNK and p38 MAP kinase signaling pathways using their respective inhibitors SP600125 (10 μM) and SB203580 (1 μM). These inhibitors had no effect on iodine-induced apoptosis in MCF-7 cells analyzed by fluorescence microscopy after PI staining (data not shown). Immunofluorescence analysis using antibodies showed no significant difference in the number of nuclei, with localization of activated c-Jun and p38 after 12 h of 3 μM iodine treatment (data not shown), suggesting the non-involvement of these two pathways. However, at present, the involvement of other signaling cascades cannot be ruled out.

Thyroid cells are able to iodinate polyunsaturated fatty acids to form iodolactones. α-Iodohexadecanal (alpha-IHDA) is a major iodolipid that has multiple inhibitory effects on adenylate cyclase, NADPH-oxidase, and thyroid peroxidase (52–54). Another iodolipid, 6-iodo-5-hydroxy-8,11,14-eicosatrienoic acid Δ-lactone (Δ-iodolactone) is known to specifically inhibit signal transduction pathways induced by local growth factors, such as epidermal growth factor and basic fibroblast growth factor (55). These iodocompounds seem to act as mediators of iodine function as an antioxidant and autoregulation of cAMP-independent thyroid cell proliferation, and the possibility of formation of such iodocompounds in iodine-treated breast cancer cells needs to be investigated as hypothesized earlier (56).

Taken together, the experimental evidences suggest the involvement of the activation of Bax, redistribution of AIF from mitochondria to the nucleus, and the important role of thiol depletion in molecular iodine-induced apoptosis. These results provide better insight into the action of iodine and establish mitochondria as the major mediator of its effect in breast cancer cells. Additional studies on the action of molecular iodine in animal tumor models are essential to assessing its therapeutic potential in breast neoplasia. Further investigations will be necessary to elucidate the mechanistic pathways involved in iodine transport, metabolism, and downstream signaling pathways.

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