Signaling pathways involved in the antiproliferative effect of molecular iodine in normal and tumoral breast cells: evidence that 6-iodolactone mediates apoptotic effects

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Abstract

Previous reports have documented the antiproliferative properties of I\(_2\) and the arachidonic acid (AA) derivative 6-iodolactone (6-IL) in both thyroid and mammary glands. In this study, we characterized the cellular pathways activated by these molecules and their effects on cell cycle arrest and apoptosis in normal (MCF-12F) and cancerous (MCF-7) breast cells. Low-to-moderate concentrations of I\(_2\) (10–20 \(\mu\)M) cause G1 and G2/M phase arrest in MCF-12F and caspase-dependent apoptosis in MCF-7 cells. In normal cells, only high doses of I\(_2\) (40 \(\mu\)M) induced apoptosis, and this effect was mediated by poly (ADP-ribose) polymerase-1 (PARP1) and the apoptosis-induced factor, suggesting an oxidative influence of iodine at high concentrations. Our data indicate that both I\(_2\) and 6-IL trigger the same intracellular pathways and suggest that the antineoplastic effect of I\(_2\) in mammary cancer involves the intracellular formation of 6-IL. Mammary cancer cells are known to contain high concentrations of AA, which might explain why I\(_2\) exerts apoptotic effects at lower concentrations only in tumoral cells.

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Introduction

Several studies have shown the beneficial potential of molecular iodine (I\(_2\)) in mammary pathologies either in humans or in animals (Ghent et al. 1993, Eskin et al. 1995, Funahashi et al. 1996, Kessler 2004, Garcia-Solis et al. 2005). It has been suggested that in thyroid gland the apoptotic iodide (I\(^{-}\)) effect is mediated by the derivatives of arachidonic acid (AA) or eicosapentaenoic acid such as 6-iodolactone (6-IL) or \(\omega\)-iodohexadecanal respectively (Dugrillon et al. 1994, Pisarev et al. 1994, Langer et al. 2003). The formation of these iodosides requires, in addition to iodide uptake by specific transporters, its oxidation by thyroperoxidase (Shah et al. 1986, Dohan et al. 2003). In mammary gland, I\(_2\) but not I\(^{-}\) supplementation alleviates human mastalgia (Ghent et al. 1993, Kessler 2004) and exerts a potent antineoplastic effect on pharma-co-induced mammary tumoral progression in rats (Funahashi et al. 1996, Garcia-Solis et al. 2005). The lack of an I\(^{-}\) effect has been explained by the fact that lactoperoxidase, the enzyme that oxidizes I\(^{-}\) and covalently binds it to the milk protein casein, is expressed in normal lactating but not in tumoral mammary gland (Shah et al. 1986, Anguiano et al. 2007). Recently, we also showed that methylnitro-sourea (MNU)-induced tumors contain fourfold more AA (the 6-IL precursor) than normal mammary glands, and that I\(_2\) supplementation is accompanied by a tenfold higher 6-IL content in these tumors (Arroyo-Helguera et al. 2006a). Our studies in the human tumoral mammary cell line MCF-7 show that I\(_2\) is taken up by a facilitated diffusion system and is then incorporated into iodosides similar to the 6-IL standard. Our data also show that both I\(_2\) and 6-IL exert antiproliferative and apoptotic properties (Arroyo-Helguera et al. 2006b); however, little is known about the molecular mechanisms involved in these effects.
Apoptosis is a form of cell death triggered during a variety of physiological conditions and is tightly regulated by a number of gene products that promote or block cell death at different stages. The most extensively studied are BCL2 family proteins (Reed 1997). In mammals, BCL2 has at least 20 relatives, including antiapoptotic proteins (BCL2, A1, and Mc11) and proteins that promote cell death (BAX, BAD, and BOK). Disequilibrium in the expression of these proteins releases factors from mitochondria and initiates the apoptotic cascade mediated by caspases (Oltvai et al. 1993, Zamzami & Kroemer 2001). Moreover, it was reported recently (Li & Osborne 2008) that elevated intracellular free radicals like reactive oxygen species (ROS) can trigger other death pathways that are independent of caspases and involve the activation of poly (ADP-ribose) polymerase-1 (PARP1) and apoptosis-inducing factor (AIF). PARP1 is a nuclear enzyme whose activity is rapidly stimulated by single- and double-stranded breaks in DNA, and it facilitates DNA repair in response to DNA damage caused by ROS. The overactivation of PARP1 depletes cellular reserves of NAD+ and ATP, leading to necrotic cell death via energy failure; it also has been reported that cleavage of PARP1 by caspases prevents excessive activation of the enzyme and that the cells can maintain levels of ATP and NAD+ sufficient to induce oligonucleosomal DNA fragmentation (Yu et al. 2002, Virag 2005). It is well established that iodine is an antioxidant at low or moderate levels, but it can act as a potent free radical at high concentrations (Yu et al. 2002, Virag 2005). In the present study, we analyzed the cell cycle arrest and the signaling pathways involved in the apoptotic effect of I2 and 6-IL in normal and tumoral mammary cell lines. Our data show that 1) tumoral cells are more sensitive than normal cells to apoptotic induction with I2, but the response of both cell types to 6-IL is similar, 2) in tumoral cells (MCF-7), low and moderate concentrations of I2 (10–20 μM) or 6-IL (0.5 μM) induce apoptosis, which is triggered by BAX caspases and AIF-PARP1 pathways, and 3) in normal cells (MCF-12F), only high I2 concentrations (40 μM) induce apoptosis, and this effect is dependent on AIF-PARP1.

Materials and methods
Materials
DMEM, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin–EDTA solutions were supplied by GIBCO-BRL. AA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and silica gel were purchased from Sigma Chemical Co. Iodine sublimed was obtained from Baker (Estado de Mexico, Mexico), and the proteinase inhibitor cocktail Mini Complete from Roche Applied Science. Antibodies specific for p53, p21, BAX, BCL2, cyclin D1, caspase-7, PARP1, and AIF were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the enhanced chemiluminescence (ECL) detection system was from Amersham.

Cell culture
Human breast epithelial cells MCF-12F were purchased from American Type Culture Collection (CRL-10783, ATCC, Marassas, VA, USA). The human breast cancer cell line MCF-7 (HTB-22, ATCC) was kindly supplied by Instituto Nacional de Cancerología de México (INCAN, Mexico, DF). All the cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (basal medium) at 37 °C and 5% CO2 for 24 h before treatment.

Synthesis of 6-IL
6-IL was synthesized according to the procedure described previously (Arroyo-Helguera et al. 2006b), where its purity was analyzed by reverse-phase HPLC and by ascending thin layer chromatography (TLC) using chloroform:methanol (97.5:2.5%, v/v). The spots were detected using iodine vapors. The 6-IL stock solution was stored at −80 °C and was diluted in ethanol to a concentration of 1 mM for working solution. Ethanol was 0.01% of the total cell culture media.

Cell cycle analysis
The cells were treated with or without 10–40 μM I2 or 0.1–1.0 μM 6-IL for 24 h, floating and adherent cells released by trypsinization where collected, washed with cold PBS (PBS), and fixed using NaCl (0.9%) in ice-cold 90% ethanol. The fixed cells were then incubated with 1 mg/ml DAPI stain. The DNA content from 10 000 cells was analyzed on a Partec 2.0 Flow Cytometer (Partec, Germany), and cell cycle parameters based upon DNA content were calculated using the Flomax Cytometer software. We also used cytometer analysis to detect cellular death. The cells were treated and fixed as described above, and the DNA content less than that of G1 phase cells (sub-G1) was assumed to indicate death.

Apoptosis assays
To quantify apoptosis, we use the APOPercentage apoptosis assay (Biocolor Ltd, Belfast, Ireland). This assay employs a dye that stains red the apoptotic cells

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undergoing the membrane flip-flop event by which phosphatidylserine is translocated to outer leaflet. Briefly, after 24 h of treatment with 10–40 μM I₂ or 0.1–1.0 μM 6-IL, the cells were incubated with APOPercentage dye for 30 min at 37 °C and 5% CO₂. After removing the culture medium and washing with PBS, we added the dye release reagent, and cell-bound dye was recovered in solution and measured at a wavelength of 550 nm, using a Microplate Reader model 680 (Bio-Rad). Apoptosis was also evaluated by DAPI staining. For this purpose, the cells were fixed with 4% paraformaldehyde, then stained for 30 min with DAPI (1 μg/ml), and analyzed by fluorescence microscopy to assess chromatin condensation.

DNA fragmentation

After induction of apoptosis with I₂ (10–40 μM) or 6-IL (0.1–1.0 μM), the cells (1 × 10⁶ cells per sample) were lysed with 500 μl lysis buffer (0.5% Triton X-100, 2 mM EDTA, 5 mM Tris–HCl (pH 8.0), and 200 μg/ml proteinase K) at 50 °C for 2 h. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The DNA was precipitated with 0.1 volume of 0.3 M sodium acetate and two volumes of ethanol. After recovery by centrifugation, the DNA pellet was dried and suspended in water with RNase A (7000 U/ml) and incubated for 2 h. DNA samples were subjected to electrophoresis on 2% agarose gels at 60 V and visualized by ethidium bromide staining under u.v. light.

Immunoblot analyses

The cells were pretreated with 20 μM I₂ or 1.0 μM 6-IL for 24 h, and then were lysed in an ice-cold lysis buffer (5 mM Tris (pH 7.4), 100 mM NaCl, 0.5% NP-40). The protein concentration was estimated using the Bradford assay (Bio-Rad), and 50–70 μg of cellular protein from each sample was separated by standard 12% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Life Science). Dilution of both primary and secondary antibodies was made in PBS containing 5% non-fat dry milk and 0.1% Tween 20. After 1-h incubation with the corresponding primary antibody against p53, p21, CD1, BAX, or BCL2 at 1–200 or 1–1000 dilutions, blots were washed three times, and the appropriate peroxidase-conjugated secondary antibody was added for another hour. The immunoblots were normalized for protein loaded by incubating with β-actin antibody. Immunoblots were visualized using ECL reagent (Amersham Pharmacia Biotech) and exposed to Biomax X-ray film (Kodak, Fisher Scientific). The films were scanned and the bands quantified with ImageQuant V2005 densitometry software (Amersham Biosciences).

PARP1 fragmentation and AIF redistribution

The cells were treated with 20 μM I₂ or 1.0 μM 6-IL. Nuclei-free cell lysates were prepared in NP-40 lysis buffer (30 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1.5 mM MgCl₂, 0.5% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonfyl fluoride, and protease inhibitor cocktail) followed by centrifugation at 800 g to remove nuclei. The supernatant contains mitochondrial proteins. Cytosolic or nuclear lysates containing 20 μg protein were resolved by 12% SDS-PAGE under non-reducing conditions. The proteins in the subcellular fractions were detected by immunoblots as described above.

Statistical analysis

One-way ANOVA was performed to determine the significance of differences between groups, followed by Tukey’s test for the significance of differences among multiple experimental groups. P < 0.05 were considered statistically significant.

Results

Analysis of cell cycle arrest and death in normal and cancer breast cells

The cell cycle analyses show that MCF-12F cells required more than 20 μM I₂ for arrest in G1 phase. By contrast, MCF-7 cells began arrest in G1 with as low as 10 μM and G1 and G2/M with 20 μM I₂ (Fig. 1A). In the case of 6-IL treatment, both cell lines started to show arrest and death with 0.5 μM 6-IL (Fig. 1B). A significant increase in dead cells (sub-G1) with all concentrations of I₂ is a further evidence that MCF-7 cells are more susceptible to this halogen than normal cells (Fig. 2).

Involvement of p53 and p21 in cell cycle arrest in I₂- and 6-IL-treated breast cells

I₂ treatment induced the time-dependent accumulation of p53 and p21 in MCF-7 but not in MCF-12F cells. By contrast, both MCF-7 and MCF-12F cells increased the expression of these proteins in response to 6-IL treatment (Fig. 3).

Low doses of I₂ and 6-IL induce apoptosis only in breast cancer cells

To determine whether the MCF-7 cell death induced by I₂ or 6-IL treatment is an apoptotic process, we used several assay methods. Results with the APOPercentage assay showed that doses of 10–40 μM I₂ or 0.5–1.0 μM 6-IL for 24 h induced apoptosis in a dose-dependent manner in MCF-7 cells but not in normal MCF-12F cells. Higher
doses of I$_2$ (40 μM) or 6-IL (0.5 μM) are necessary to induce apoptosis in the normal breast cells (Fig. 4A). Similar results were found using DAPI staining of nuclei, which revealed typical apoptotic nuclei with highly fluorescent, condensed chromatin (data not shown). These observations were confirmed by agarose gel electrophoresis, which indicated DNA ladder formation in MCF-7 cells treated with I$_2$ (10–40 μM) or 6-IL (0.1–1.0 μM) but not in normal cells (Fig. 4B).

I$_2$ and 6-IL modulate the expression of BCL2 family proteins only in MCF-7 cells

I$_2$ (20 μM) and 6-IL (0.5 μM) significantly induced the expression of proapoptotic BAX and caused down-regulation of antiapoptotic BCL2 proteins at 24 h, as measured by immunoblot in MCF-7 cells, in which the BAX/BCL2 ratio was increased with both I$_2$ and 6-IL treatments. By contrast, BAX and BCL2 show no change with I$_2$ treatment in MCF-12F cells (Fig. 5A).

The executioner caspase-7 is activated with I$_2$ and 6-IL only in MCF-7 cells

Immunoblot analysis from MCF-7 cells treated with I$_2$ (20 μM) or 6-IL (0.5 μM) revealed the presence of the activated, 17 kDa caspase fragment. Densitometric analysis showed significant increases in the caspase fragment, which became significant after 12 h of I$_2$ treatment, and with 6-IL they were significant beginning within the first 6 h of treatment (Fig. 5B).

Effect of I$_2$ and 6-IL on PARP1 cleavage and AIF redistribution in MCF-7 cells

To analyze the oxidative effect of I$_2$ and 6-IL, cleavage fragments of PARP1 and AIF were quantified. Figure 6 shows that control cells contained only the intact, 112-kDa PARP1 polypeptide, and it was present exclusively in the nuclear fraction. By contrast, in the cells treated with I$_2$ there was proteolytic cleavage of PARP1, resulting in the accumulation by 12 h of the
85 kDa PARP1 fragment in the nucleus and later in the cytosolic fraction. In the cells treated with 6-IL, this PARP1 cleavage fragment was detected in both compartments within the first 6 h of treatment. Accumulation of a 57 kDa cleavage fragment of AIF was detected after 12 h of I2 treatment in the cytosol and after 24 h in the nucleus of both normal and tumoral cells (Fig. 7). A similar AIF pattern was found when the cells were treated with 6-IL (data not shown).

**Discussion**

In this study, we demonstrate that I2 and 6-IL can induce cell cycle arrest in G1 or G2/M phases in normal and cancerous breast cells. These data agree with previous results showing that, in thyroid FRTL-5 cells, iodide induces cell cycle arrest in both G1 and G2/M phases (Smerdely et al. 1993). The p53 protein can regulate the G1 or G2/M transition by inducing p21, an inhibitor of cyclin/cyclin-dependent kinase (CDK) complexes (Roninson 2002). Using immunoblot analysis, we showed that cell cycle arrest induced by I2 in MCF-7 cells, and by 6-IL in both MCF-7 and MCF-12F cells, was dependent on the increased expression of p53 and p21 proteins. In normal cells, I2 treatment induces G1 cell cycle arrest;
however, it does not change the expression of p53 and p21 proteins.

Previous studies have established that at higher concentrations, I\(_2\) can act as a potent free radical; I\(_2\) treatment also causes depletion of thiol levels (Vitale et al. 2000, Joanta et al. 2006, Shrivastava et al. 2006). A shift in the redox state induces retinoblastoma gene (RB) dysfunction and results in a reduced release of E2F-binding factor and G1 cell cycle arrest. Regulators of cell cycle progression such as CDKs are enzymes that contain cysteine, tyrosine, and threonine residues that are important in regulating the phosphorylation of RB (Nevins et al. 1991, Yamauchi & Bloom 1997). Previous reports have demonstrated that I\(_2\) treatment induces the iodination of tyrosine, cysteine, and histidine residues (Venturi 2001), which could explain why normal breast MCF-12F cells treated with moderate levels of I\(_2\) were arrested in G1 phase.

In the present work, we demonstrated that a low dose of I\(_2\) induces apoptosis only in MCF-7, but not in MCF-12F cells. These findings agree with our previous data showing a reduction in mammary tumors without changes in normal tissues treated in vivo with a moderate I\(_2\) concentration (Garcia-Solis et al. 2005). In thyroid gland, the apoptotic iodine effect is mediated by iodinated AA derivatives such as 6-IL (Boeynaems & Hubbard 1980, Langer et al. 2003). It has been demonstrated that tumoral tissues contain high concentration of AA (Rillema & Mulder 1978). In agreement, our laboratory has shown that MNU-induced rat

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**Figure 5** Apoptotic BAX/caspases pathway activation. (A) Expression of BCL2 family proteins. Cells were incubated with 20 \(\mu\)M I\(_2\) and 0.5 \(\mu\)M 6-IL for 24 h. Proteins were resolved by 12% PAGE and analyzed by immunoblot as described in the Materials and methods section. (B) Induction of active caspase-7. The cells were incubated with similar treatments for the indicated times. The antibody against caspase-7 recognizes only the active, cleaved (17 kDa) form. The immunoblots are representative of three independent experiments with similar results. The proteins bands were scanned and quantified with a densitometer and normalized to the \(\beta\)-actin content of the same sample. The data are shown as mean \(\pm\) s.d. *\(P<0.05\) versus control cells.
mammary tumors also contain higher quantities of AA, and 6-IL was detected only in the lipids from MNU tumors from rats chronically I2 supplemented (Arroyo-Helguera et al. 2006a). In the present work, we show that 20- to 40-fold lower concentrations of 6-IL than I2 were sufficient to induce similar apoptosis effects in both normal and tumoral cells, which is the same ratio observed between iodide and 6-IL in the apoptosis-induced death in thyroid follicular cells (Langer et al. 2003). All of these data support the hypothesis that the apoptotic I2 effect is mediated by the formation of 6-IL in tumoral cells. Although the cellular mechanism involved in the 6-IL apoptotic effect has not yet been elucidated, we have proposed that the peroxisome proliferator-activated receptors (PPAR) could be the mediator. These ligand-dependent transcription factors are involved in the regulation of cell cycle control and apoptosis of several cancer cells, including MCF-7 (Berger & Moller 2002, Claria 2006). Recently, our laboratory showed that 6-IL is a specific ligand of PPARs with almost sixfold higher affinity than AA, which is one of the endogenous ligands of these receptors (Nunez-Anita et al. 2007).

Previous studies demonstrated that in MCF-7 cells, DNA fragmentation induced by I2 was accompanied by an increase in BAX, the release of cytochrome c, and nuclear translocation of AIF. These authors also showed that a caspase inhibitor did not block the apoptotic effect of I2, suggesting that caspase pathways were not involved (Shrivastava et al. 2006). In the present work, we confirmed the increase in BAX and the nuclear translocation of AIF, but we also detected the activated caspase-7 subunit of 17 kDa, as well as PARP1 cleavage, indicating the possibility that more than one pathway was activated by I2. These data agreed with a previous report showing that during iodine-induced goiter involution, the triggering of apoptosis is also mediated by caspases (Mutaku et al. 2002). Thus, we propose that in tumoral cells, at low concentrations I2 binds to AA, which is present at an elevated concentration, and forms 6-IL, which in turn activates PPAR, triggering BAX/caspase-dependent apoptosis. Although this pathway is not completely documented, it is known that p53, p21, and BAX proteins are up-regulated by PPARγ agonists (Elstner et al. 1998). By contrast, when present at higher concentrations either in normal or tumoral cells, I2 could be operating as an oxidizing agent that switches on apoptosis by the AIF/PARP1 pathway. Indeed, our data in MCF-12F cells demonstrated that only high concentrations of I2 induce apoptosis, and this event is not accompanied by changes in BAX or caspase 7, but by activation of AIF and PARP1 cleavage. This notion is consistent with a variety of other studies where it has been demonstrated that elevated intracellular free radicals can trigger death pathways that involve the activation of both PARP1 and AIF, without the participation of caspases (Yu et al. 2002, Virag 2005, Li & Osborne 2008).

As mentioned previously, iodine is an antioxidant at low or moderate concentrations but can act as a potent free radical at high concentrations (Vitale et al. 2000, Smyth 2003). Elevated concentrations of oxidized iodine (I2) could activate PARP1 and AIF. One view is that iodine could act within the nucleus and cause DNA or protein damage by iodinating double bonds, which then...
produce the overactivation of nuclear-located PARP1, generating the nuclear translocation of AIF and promoting DNA fragmentation and nuclear condensation. Another pathway could be through mitochondria, where the iodination of several components prompts the release of apoptotic species like AIF. These proposals are supported by our data and that of other authors demonstrating the presence of radiolabeled components, not related to thyroid hormones, in the nucleus and mitochondria after the $^{125}$I treatments (Upadhyay et al. 2002, Aceves et al. 2005). Indeed, the study in mitochondria showed that the apoptotic effect of high concentrations of iodide is more evident in normal than in tumoral cells, and that this effect is accompanied by an increase in mitochondrial membrane permeability (Upadhyay et al. 2002).

In conclusion, iodine and 6-IL induce cell arrest and/or apoptosis in normal and tumoral mammary cells by triggering the BAX-caspase and/or AIF/PARP1 pathways. This study also supports the view that the antineoplastic effect of I$_2$ in mammary cancer involves the intracellular formation of 6-IL.

**Declaration of interest**

We fully declare any financial or other potential conflict if interest.

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