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A complex between 6-iodolactone and the peroxisome proliferator-activated receptor type gamma may mediate the antineoplasic effect of iodine in mammary cancer

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ABSTRACT

Recently we and other groups have shown that molecular iodine (I_2) exhibits potent antiproliferative and apoptotic effects in mammary cancer models. In the human breast cancer cell line MCF-7, I_2 treatment generates iodine-containing lipids similar to 6-iodo-5-hydroxy-eicosatrienoic acid and the 6-iodolactone (6-IL) derivative of arachidonic acid (AA), and it significantly decreases cellular proliferation and induces caspase-dependent apoptosis. Several studies have shown that AA is a natural ligand of the peroxisome proliferator-activated receptors (PPARs), which are nuclear transcription factors thought to participate in regulating cancer cell proliferation. Our results show that in MCF-7 cells: (1) 6-IL binds specifically and with high affinity to PPAR proteins (EMSA assays), (2) 6-IL activates both transfected (by transactivation assays) and endogenous (by lipid accumulation) peroxisome proliferator response elements, and (3) 6-IL supplementation increases PPARγ and decreases PPARα expression. These results implicate PPARs in a molecular mechanism by which I_2, through formation of 6-IL, inhibits the growth of human breast cancer cells.

1. Introduction

Molecular iodine (I_2), but not potassium iodide (I^-) or thyroid hormones (TH), exhibits potent antiproliferative and apoptotic effects in mammary cancer models both *in vivo* [1,2] and *in vitro* [3,4]. These effects may be mediated by the activation of a complex signaling cascade that includes p53, bax/bcl2, caspases, as well as the AIF-PARP-1 pathways [4,5]. Moreover, in the mammary tumor cell line MCF-7, administration of I_2 but not I^- is accompanied by iodination of proteins and lipids [3], suggesting that the antitumoral effects require an oxidized iodine species such as I_2 and the formation of iodinated components. These suggestions agree with reports showing that I^- could exert cytotoxic effects in several cell types only if iodide is oxidized by thyroperoxidase (TPO) [6,7]. In thyroid the apoptotic effect is mediated by at least two lipids: an iodinated arachidonic acid (AA) derivative known as 6-iodo-5-hydroxy-eicosatrienoic acid or 6-iodolactone (6-IL) and by 2-iodohexadecanal [8–10]. Our group showed that MCF-7 cells treated with I_2 generate iodolipids with a migration similar to 6-IL, indicating that this iodolactone may also be produced after I_2 treatment of breast cancer cells [5]. Both I_2 and 6-IL activate the same signaling cascades, but 6-IL had an antiproliferative effect on MCF-7 cells that was 4-fold more potent than that of I_2 [3]. Polyunsaturated fatty acids such as eicosanoids and AA are natural ligands of peroxisome proliferator-activated receptors (PPARs) [11,12], which belong to the nuclear receptor superfamily of ligand-dependent transcriptional factors. Transactivation of these receptors requires ligand binding, heterodimerization with retinoic X receptors (RXRs), and binding of this complex to a PPAR-specific response element (PPRE) in the promoter region of target genes [13,14]. Three PPAR isoforms have been identified PPARα, PPARβ/δ and PPARγ, and all of them have been detected in human mammary tumoral cell lines including MCF-7 [15]. In general, PPARα is associated with the regulation of genes involved in proliferation [16,17], although some of its synthetic agonists can induce antiproliferation and apoptosis [18,19]. PPARβ/δ is expressed ubiquitously; its function in mammary cancer is uncertain, but it may enhance proliferation in certain sex hormone-sensitive cell lines such as breast and prostate [20,21]. PPARγ, which is expressed primarily in adipose tissue, promotes adipocyte and mammary gland cell differentiation [22], and it has anti-tumorigenic effects in many cancer cell types including mammary gland [21,23–25]. Here we show that 6-IL binds and activates PPAR, and that it exerts opposite effects

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on PPARα and PPARγ expression in MCF-7 cells. Together, these data support our hypothesis that the antiproliferative effect of I_2 is mediated by PPARs.

2. Experimental procedures

2.1. Materials

MCF-7 human breast cancer cells were purchased from INCAN (Instituto Nacional de Cancerología, México). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY). Arachidonic acid (purity > 99%) was from Calbiochem (La Jolla, CA), ^{125}I (17 Ci/mg) and γ^{32}P-ATP (12.5 μCi/μl) were purchased from Perkin Elmer Life Science (Boston, MA), and oligonucleotides were from Sigma (St. Louis, MO). The polyclonal anti-PPARα, anti-PPARβ/δ, and anti-PPARγ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the highest grade of purity commercially available.

2.2. Cell culture

The human breast cancer cell line MCF-7 was cultured routinely in DMEM supplemented with 10% (v/v) FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Chemical synthesis of ^{125}I-6-iodolactone (6-^{125}IL) and 6-IL

6-^{125}IL and 6-IL were synthesized and purified as described previously [3,26]. The purity was checked by thin layer chromatography on silica gel (TLC) using the solvent system CH_2Cl_2/MeOH (97.5:2.5). AA and 6-IL standards were visualized by iodine vapors, and the radiolabeled 6-^{125}IL was detected by autoradiography.

2.4. Protein extraction

Preparation of crude cytoplasmic and nuclear extracts was basically as described [27]. Briefly, adherent cells (10^7 cells per well) were scraped into 1.5 ml of phosphate buffered saline (PBS). The cell suspension was transferred to a microfuge tube and centrifuged at 4 °C for 5 min at 500 × *g*. The PBS was aspirated, and the cells were resuspended in 100 μl buffer A (0.32 M sucrose; 10 mM Tris HCl, pH 8; 3 mM $CaCl_2$; 2 mM $Mg(OAc)_2$; 0.1 mM EDTA; 0.5% NP-40; 1 mM dithiothreitol; 0.5 mM PMSF). The cellular lysate was centrifuged at 4 °C for 5 min at 500 × *g*, and the supernatant was transferred to a new tube (cytoplasmic fraction). The nuclear pellet was washed with 1 ml buffer B (buffer A without NP-40). The nuclei were centrifuged at 4 °C for 5 min at 500 × *g*, and the supernatant was removed. The pellet was resuspended in 30 μl buffer C (20 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 20 mM KCl; 0.2 mM EDTA; 25% glycerol; 0.5 mM DTT; 0.5 mM PMSF), and 30 μl buffer D (20 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 1% NP-40; 0.5 mM DTT; 0.5 mM PMSF) was added. The samples were incubated at 4 °C for 45 min, then centrifuged at 4 °C for 15 min at 14,000 × *g*, and aliquots of the supernatant (nuclear extract) were stored at −70 °C. Protein was determined using the Bradford method (Bio-Rad protein assay; Hercules, CA).

2.5. Electrophoretic mobility shift assays (EMSAs)

An established EMSA method was used [28]. Cytoplasmic or nuclear protein extracts (5–10 μg) were incubated for 1 h at room temperature with 1 μg poly(dI-dC) (Sigma), 6 μl of buffer (25 mM HEPES, pH 7.9; 2 mM EDTA; 1 mM DTT; 0.1 M KCl, 0.5 mM PMSF), and 20 ng of a [^{32}P]-labeled oligonucleotide in a final volume of 20 μl. AA, 6-IL, and 6-^{125}IL were added as indicated. For competition analysis, non-labeled *wt*PPRE at 25×, 50×, or 100×, or *mut*PPRE at 25× or 50× molar excess was added 15 min before ^{32}P-*wt*PPRE. DNA-protein or putative DNA–protein–ligand complexes were resolved at 120 V for 2–3 h in 0.5× TBE buffer on a native 6% polyacrylamide gel (5% for supershift), dried, and visualized using an intensifying screen and a Storm 860 phosphoimager scanner (Molecular Dynamics Inc., CA). For preparing ^{32}P-*wt*PPRE, both the sense and antisense oligonucleotides were labeled in a total volume of 20 μl containing 20 ng of oligonucleotide, 0.5 μl of T4 polynucleotide kinase (PNK), 2 μl of 10× PNK reaction buffer and 1.5 μl of gamma-^{32}P-ATP. A double-stranded oligonucleotide with the 3X-PPRE site was used (underlined) for shift assays and had the following sequence: *wt*PPRE (5′-GATCC-AGGGAAAAGGTCA TCAGGGAAAAGGTCACTAGGGAAAAGGTCAC-3′); this PPRE has been identified in the human gene, muscle-type carnitine palmitoyltransferase MCPT I [29]. As control, an oligonucleotide with a mutated 3X-PPRE site was used (mutated sites in boldface letters): sense *mut*PPRE (5′-AG**T**GAA**C**AGGTCATCAG**T**GAA-**C**AGGTCACTAG**T**GAA**C**AGGTCA-3′). For the supershift assay, antibodies (1.2 μg) were added to the reaction mixture, which was then incubated for 0.5 min at room temperature, and resolved in a native 5% polyacrylamide gel.

2.6. Gene transfer and transactivation experiments

Transient expression assays were performed with the established calcium phosphate precipitation method (Ca–PO_4) [30]. Briefly, MCF-7 cells were grown in six-well plates to a density of 2×10^5 cells/well in DMEM. One day later, the medium was changed, and cells were transfected by Ca–PO_4 with 6 μg/well of the following plasmids: PPRE3-TK-LUC [containing 3 copies of PPRE coupled to a minimal thymidine kinase (TK) promoter], pTK-LUC, or pCMX-βgal (β-galactosidase) (Promega, Madison, WI). Cells were incubated in the presence of a mixture of plasmid and Ca–PO_4 for 7 h, washed, and incubated in the presence of different concentrations (0.1–10 μM) of 6-IL, and rosiglitazone (RZ, a synthetic and specific PPARγ agonist) as well as 30–100 μM AA or vehicle alone (0.1% ethanol) for 24 h. Cell extracts were prepared and assayed for luciferase according to the manufacturer's instructions (Promega) and for β-galactosidase activity as described [31].

Table 1
Real-time PCR primer sequences.

Sense/antisense (5′ at 3′)		Aliening temp (°C)	Reference
PPARα	CATTTTAGTGTACACTGTGGTTTCC CAGCATTCAGGAAAACGGTT	62	GenBank acc. 624622R10075
PPARβ/δ	GTCGCACAACGCTATCC CTCCGGGCCTTCTTTTTGGTCA	62	Reference no. [50]
PPARγ	TCTCTCCGTAATGGAAGACC GCATTATGAGACATCCCCAC	62	Reference no. [51]
β-Actin	CCATCATGAAGTGTGACGTTG ACAGAGTACTTGCGCTCAGGA	55	Reference no. [32]