Antineoplastic effect of iodine and iodide in dimethylbenz[a]anthracene-induced mammary tumors: association between lactoperoxidase and estrogen-adduct production

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Abstract

Several groups, including ours, have reported that iodine exhibited antiproliferative and apoptotic effects in various cancer cells only if this element is supplemented as molecular iodine, or as iodide, to cells that are able to oxidize it with the enzyme thyroperoxidase. In this study, we analyzed the effect of various concentrations of iodine and/or iodide in the dimethylbenz[a] anthracene (DMBA) mammary cancer model in rats. The results show that 0.1% iodine or iodide increases the expression of peroxisome proliferator-activated receptor type γ (PPARγ), triggering caspase-mediated apoptosis pathways in damaged mammary tissue (DMBA-treated mammary gland) as well as in frank mammary tumors, but not in normal mammary gland. DMBA treatment induces the expression of lactoperoxidase, which participates in the antineoplastic effect of iodide and could be involved in the pro-neoplastic effect of estrogens, increasing the formation of DNA adducts. In conclusion, our results show that a supplement of 0.1% molecular iodine/potassium iodide (0.05/0.05%) exert antineoplastic effects, preventing estrogen-induced DNA adducts and inducing apoptosis through PPARγ/caspases in pre-cancer and cancerous cells. Since this iodine concentration does not modify the cytology (histology, apoptosis rate) or physiology (triiodothyronine and thyrotropin) of the thyroid gland, we propose that it be considered as an adjuvant treatment for premenopausal mammary cancer.

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Introduction

A robust body of information supports the notion that moderately high concentrations of iodine may reduce pathologies in several iodine-uptake tissues such as thyroid, mammary gland, intestine, and prostate (Venturi *et al*. 2000, Aceves & Anguiano 2009). It has been shown that iodide (I^-) exerts antiproliferative and apoptotic effects in thyrocytes and that the rate-limiting step for this effect is the conversion of I^- to a more reactive iodine species such as molecular iodine (I_2) catalyzed by thyroperoxidase (TPO). Indeed,

Vitale *et al*. (2000) showed that an excess of potassium iodide (KI) induces apoptosis in cultured thyroid cells, but if TPO activity is blocked with propylthiouracil, the apoptotic effect of KI is eliminated. Moreover, using lung cancer cells (without natural iodine uptake) transfected with the iodide/sodium symporter (NIS) or NIS/TPO, Zhang *et al*. (2003) observed that only in NIS/TPO-transfected cells did excess KI induce apoptosis, indicating that I^- from KI needs to be oxidized to have a cytotoxic effect. Data obtained in either *in vivo* (*N*-methylnitrosourea (MNU)) or *in vitro*

(the human mammary cancer cell line MCF-7) models of mammary cancer show that I_2 but not I^- (KI or NaI) exert an antineoplastic effect by increasing the expression of peroxisome proliferator-activated receptor γ (PPARγ), which triggers apoptotic mechanisms that include the Bax–caspase and apoptosis-inducing-factor (AIF) pathways (García-Solís *et al*. 2005, Shrivastava *et al*. 2006, Aceves *et al*. 2009). Moreover, we recently demonstrated that 6-iodolactone (6-IL), which is generated by the iodination of arachidonic acid (AA), is a specific ligand of PPARγ (Nuñez-Anita *et al*. 2009). The finding that I^- has no effect in these models has been explained by the absence of the same components required in thyrocytes: NIS for uptake of iodide and/or lactoperoxidase (LPO) for its oxidation. NIS is necessary to internalize I^- in several tissues (Dohan *et al*. 2003), whereas LPO, a protein homolog of TPO, oxidizes iodide and binds it to the lactoprotein casein, which is secreted together with free iodide into the milk (Kussendrager & Van Hooijdonk 2000). LPO is generally present in mammary glands only during pregnancy and lactation (Dohan *et al*. 2003), but it has been detected in some mammary cancer models (Anderson *et al*. 1984). It has been proposed that LPO is involved in mammary carcinogenesis, because of its ability to react *in vivo* and *in vitro* with estrogen molecules, oxidizing them to catechol estrogens that bind to DNA and induce mutations (Cavalieri *et al*. 1997, Lovstad 2006).

The dimethylbenz[a]anthracene (DMBA) model is considered a good model of human mammary cancer, because DMBA is a procarcinogen that is metabolically oxidized to a polycyclic aromatic hydrocarbon (PAH), a well-known class of environmental factors that contribute to the etiology of breast cancer in humans (Thompson 2000). Some reports have shown that the DMBA-induced mammary cancer cells express NIS and LPO (Anderson *et al*. 1984), and that co-administration of Lugol's solution – a mixture of I_2 and KI – with medroxyprogesterone acetate significantly decreases the growth of mammary tumors (Funahashi *et al*. 1996). However, the different chemical forms and doses of iodine have not been analyzed in this model. In this study, we investigate the effects of different combinations of iodine and/or iodide on mammary glands (non-tumoral and tumoral) from control and DMBA-treated rats and the participation of PPARγ and LPO in these effects. Our results confirmed that in this model, the iodide transporters NIS and pendrin (PEN) are present as well as LPO, and that iodine treatment overstimulates all of these components in the tumors. It was also shown that

iodine, at concentrations of 0.1% or more and as either I_2 or I^-, is necessary to induce the antineoplastic effect and that this effect is accompanied by significant increases in PPARγ expression and caspase-3 activity. Other important findings are that LPO is involved in protection by iodide and also in the pro-neoplastic 17β-estradiol (E_2) stimulation (increasing the level of DNA adducts), since its inhibition with methimazole (MMI) reduced both effects.

Materials and methods

Animals

Virgin female Sprague–Dawley rats were born in a colony maintained at the Instituto de Neurobiología, Universidad Nacional Autónoma de México (UNAM). Animals were kept under regulated temperature conditions ($22 \pm 1\,^{\circ}\text{C}$) at 50% humidity on a 12 h light:12 h darkness cycle and permitted *ad libitum* access to food (Purina Certified Rodent Chow) and water. Animals were housed and handled in accordance with UNAM Animal Use Committee Guidelines.

Iodine supplement

Virgin 5-week-old rats were supplemented with the following preparations: 0.05% I_2, 0.05% potassium iodide (0.05% KI), 0.07% I_2, 0.05% I_2 + 0.05% KI (MIX), 0.1% KI, 0.05% I_2 + 0.1% KI (Lugol), 3 µg/ml thyroxine (T_4), 0.05% MMI + 3 µg/ml triiodothyronine (T_3), and MMI/T_3 + MIX in drinking water for 22 weeks. Distilled water was used for drinking water and for all solutions. The concentration of the iodine preparations was confirmed by titration with sodium thiosulfate (Kenkel 1994).

Carcinogen administration and tumor incidence

Two weeks after the iodine treatments began, all rats were anesthetized with a mixture of ketamine and xylazine (Cheminova, Mexico City, DF, Mexico, 30 and 6 mg, respectively, per kilogram body weight), and a single intragastric dose of 20 mg/ml DMBA (Sigma) dissolved in 1 ml of sunflower oil was administered. The control group received 1 ml of sunflower oil alone. Tumor incidence and growth were analyzed for 20 weeks after DMBA administration. Endpoints for data analysis were: time to tumor appearance (tumor latency), percentage of animals that developed tumors (tumor incidence), number of tumors per rat (tumorigenesis), and combined size of tumors at the end of treatment (tumor volume). At the end of the experiment the rats from each group were killed by decapitation. Tumor sizes were measured using a

caliper, and volumes were calculated by the ellipsoid formula (Russo & Russo 2000). If the tumors reached the ethically maximal size of 3 cm^3, rats were anesthetized with the ketamine/xylazine mixture, and the tumors were surgically removed even before the end of the experiment. Normal or tumoral mammary glands were fixed in 10% neutral buffered formalin or frozen in dry ice and kept at $-70 \,^{\circ}\text{C}$. Blood was collected to determine circulating levels of total iodine, T_3, and TSH.

Thyroid status

Iodine, T_3, and TSH circulating levels

Total iodine was measured by HPLC and T_3 levels by the homologous RIA methods described previously (García-Solís *et al*. 2005). TSH levels were quantified by the ELISA method (Biosource, Camarillo, CA, USA).

Thyroid gland integrity

After 20 weeks of DMBA and/or iodine treatments, formalin-fixed, paraffin-embedded thyroid sections (5 µm) were dyed with the hematoxylin and eosin method, and frozen thyroids were used for the apoptosis assay (see below).

RT-PCR

PPARγ, NIS, PEN, and LPO expression were analyzed by quantitative real-time PCR (qPCR). Total RNA was obtained using the TRIzol reagent (Life Technologies, Inc., Carlsbad, CA, USA) dissolved in RNAase-free water (50 µl), and stored at $-70 \,^{\circ}\text{C}$. The extracted RNA (2 µg) was reverse transcribed using oligo-deoxythymidine. To eliminate genomic DNA contamination, we carried out the RT assay for each individual sample and as control, we used one tube that contained a sample from a pool of all samples but no transcriptase enzyme ($-$RT). We ran a standard PCR for each pair of oligos with two individual samples (random) and the $-$RT control. The sequence detector system Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) was used to perform qPCR with SYBRgreen

as a marker for DNA amplification. The reaction was performed with 1 µl of cDNA template and the qPCR supermix-UDG Kit (Invitrogen), using 40 cycles of three-step amplification (94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 30 s) and the gene-specific primers listed in Table 1. PCR generated only the expected specific amplicon, which was demonstrated in each case by the melting temperature profile (dissociation curve) and by electrophoresis of 5 µl of the PCR product through a 2% agarose gel containing ethidium bromide in TAE buffer. No PCR products were observed in the absence of template. Gene expression was calculated using the D cycle threshold method and normalized to the content of actin, a non-regulated housekeeping gene. The coefficient of variation for this gene was 15% in all RT-PCR assays, indicating that the significant changes observed in the different groups correspond to changes in the experimental genes.

Caspase-3 activity

Caspase-3 activity was measured using a standard colorimetric kit (Sigma). Briefly, frozen mammary glands, tumors, or thyroids were homogenized (1:2) with lysis buffer (50 mM HEPES, pH 7.4, 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), 4 °C). Samples were centrifuged at 16 000 ***g*** for 20 min at 4 °C, and supernatants were collected and stored at $-70 \,^{\circ}\text{C}$. Protein concentrations were determined by the Bradford method (Bio-Rad protein assay), and 100 µg protein was assayed for caspase-3 activity. The substrate was 200 µM Asp–Glu–Val–Asp (DEVD)-chromophore *p*-nitroanilide (pNa), which contains the pNa linked to a synthetic tetrapeptide DEVD. The final cocktail (300 µl) was incubated in assay buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.01% CHAPS, 5 mM DTT) for 3 h at 37 °C with continuous agitation and protected from light in a 96-well plate. Samples were read at 405 nm in an ELISA reader, and caspase-3 activity was expressed as nanomoles of pNa released/hour per milligram protein.

Table 1 Primer sequences

Gene	GenBank accession	Sense/antisense sequence primer	Size (bp)	Alignment temperature (°C)
PPARγ	AF156665	TCAAACCCTTTACCACGGTT/CAGGCTCTACTTTGATCGCA	147	55
LPO	BF404752	CCTAGGAAGGGACAGTCGGATA/CCTCCTGTCCTCCCAGACTAGA	120	55
NIS	U60282	CCGGATCAACCTGATGGACT/CCTGAGGGTGCCACTGTAAG	377	60
PEN	AF167412	CATTCTGGGGCTGGACCTC/CCTTCGGGCATTCACTTTCA	487	55
Cyc	M19533	AGACGCCGCTGTCTCTTTTCG/CCACACAGTCGGAGATGGTGATC	519	56

PPARγ, peroxisome proliferator-activated receptor γ; LPO, lactoperoxidase; NIS, sodium/iodide symporter; PEN, pendrin; Cyc, cyclophilin.

Quantification of *in situ* cell death

Cell death was detected in formalin-fixed, paraffin-embedded mammary gland or tumor sections using the *in situ* fluorescein/POD cell detection kit (Roche Molecular Biochemicals), which is based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method. Sections (5 μm) were prepared and treated according to the manufacturer's instructions. Diaminobenzidine was used as the chromogen, and sections were counter-stained with hematoxylin. TUNEL-positive cells were identified by a brown stain over the nucleus. Five regions, chosen at random, were analyzed, and labeling indices were expressed as the percentage of labeled cells among at least 500 cells per region.

Estrogen and LPO participation

In a second round of experiments, rats with frank tumors (~ 2 cm^2) were treated for 2 weeks with daily injections of E_2 (0.5 μg/day) and given drinking water with and without the LPO inhibitor MMI/T_3 only or together with MIX (MMI/T_3 + MIX). T_3 was used to maintain euthyroidism (Alva-Sanchez *et al.* 2009). The pro-neoplastic effect of E_2 and the possible protective effect of iodine (MIX) as well as how these effects were influenced by LPO inhibition (MMI/T_3) were analyzed by the number of DNA adducts, as well as by the change in the tumor growth (size of tumor) after 14 days. E_2, T_3, and MMI were obtained from Sigma.

DNA isolation and ^{32}P-postlabeling analysis of DNA adducts

Normal and tumoral mammary glands were pulverized in liquid nitrogen and homogenized in a solution of 20 mM Tris–HCl, pH 8.0, 250 mM NaCl, 100 mM EDTA, and 0.5% SDS. After RNase and proteinase K treatment, DNA was isolated by phenol–chloroform extraction. The DNA concentration was determined spectrophotometrically at 260 nm. DNA adducts were analyzed by the nuclease P1 enrichment version of the ^{32}P-postlabeling assay according to the protocol by Phillips & Arlt (2007). The labeled DNA adducts were separated by thin layer chromatography on PEI-cellulose plates (Macherey-Nagel, Düren, Germany) using the following solvents: D1, 1 M sodium phosphate, pH 6.0; D2, 3.6 M lithium formate, 8.5 M urea, pH 3.5; and D3, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. The plates were autoradiographed, and the diagonal radioactive zone was cut out for Cerenkov counting. All samples were run in triplicate.

Statistical analysis

The effects of dietary treatments on mammary cancer incidence were analyzed by 2×2 contingency tables and a χ^2 test. The effects of treatments on the other variables were analyzed by one-way ANOVA and Tukey's honest significant difference tests. Values with $P < 0.05$ were considered statistically significant.

Results

Figures 1 and 2 and Tables 2 and 3 summarize the chronic effect (22 weeks) of low or moderately high concentrations of iodine, iodide, or both, as well as LPO inhibition (MMI/T_3 treatment) on iodine and thyroid hormone status and on the incidence and progression (tumorigenesis and tumor volume) of DMBA-induced tumors. Circulating levels of total iodide were consistent with the low or moderate ingestion of iodine, and no significant modifications in circulating levels of T_3 or TSH were observed (Fig. 1).
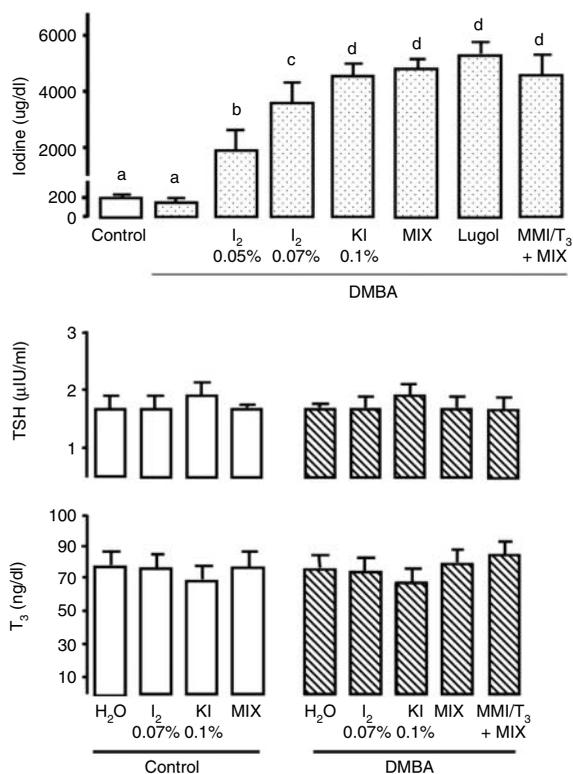

Figure 1 Thyroid status in supplemented animals with different chemical and combinations of iodine and/or methimazole. Five-week-old rats were supplemented for 22 weeks with 0.05 or 0.07% molecular iodine (I_2), 0.1% potassium iodide (KI), 0.05/0.05% I_2/KI (MIX), or 0.05/0.1% I_2/KI (Lugol) with or without 0.05% methimazole + 3 μg/ml triiodothyronine (MMI/T_3) in the drinking water. Different superscripts indicate significant differences ($P < 0.05$).
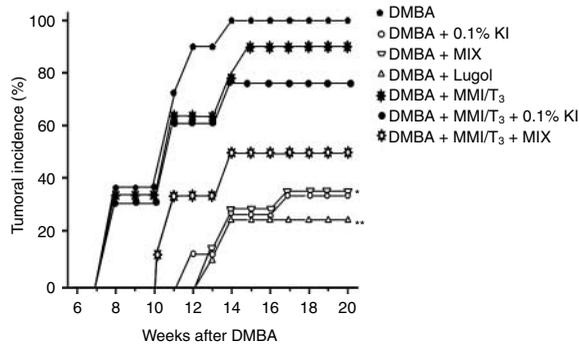
Figure 2 Effect of moderately high concentrations of iodine/iodide and/or methimazole/T_3 on tumoral incidence. Five-week-old rats were treated for 22 weeks with 0.1% potassium iodide (KI), 0.05/0.05% I_2/KI (MIX), or 0.05/0.1% I_2/KI (Lugol) with or without 0.05% methimazole + 3 μg/ml triiodothyronine (MMI/T_3) in the drinking water. At 7 weeks of age, rats were treated with a single dose (intragastric) of 20 mg DMBA. Data are expressed as the mean ± S.D. ($n = 8$). *$P < 0.05$ and **$P < 0.01$ compared with DMBA.

Incidence analyses showed that concentrations of 0.1% or higher of either chemical form of iodine were necessary to exert a significant antineoplastic effect (Fig. 2). The data also showed that inhibition of LPO activity impaired the antineoplastic effect of 0.1% KI and MIX (by 80 and 50% respectively). The tumorigenesis and tumor size were significantly lower in all iodine-treated animals in comparison with the DMBA control group (Table 3).

To analyze the cellular/molecular mechanisms involved in the antineoplastic effect of moderately high concentrations of iodine, we used samples of the MIX group. In Fig. 3, MIX treatment induced significant increases in PPARγ expression and caspase-3 activity in both damaged (DMBA-treated) mammary gland and frank tumors. These increases correlated with the significant rise in DNA rupture (TUNEL-positive cells) found in these tissues (Fig. 4).

To explore the effect of iodine supplement on the proteins needed for the uptake and metabolism of iodine, we measured the expression of NIS, PEN, and LPO in normal and tumoral mammary gland using lactating mammary tissue (10 days *post partum*, eight pups) as a positive control. The results showed that normal mammary gland contains low concentrations of NIS, PEN, and LPO. On the contrary, in both mammary gland and tumors from DMBA-treated animals all these proteins are overexpressed, and when tumors are treated with MIX this overexpression increases even more (Fig. 5).

Analyses of thyroid glands showed that only supplements containing the highest concentrations of iodide (0.1% KI and Lugol) exhibited flat thyroid epithelium, but no change in caspase-3 values was observed at any dose (Fig. 6).

To analyze the possible participation of LPO in the oxidation of iodide and/or in the transformation of E_2 into electrophilic metabolites, a second experiment was performed in animals with frank tumors. In this experiment (Fig. 7), we analyzed the number of DNA adducts in mammary gland and tumors as well as the change in tumor size after 2 weeks of treatment with E_2, MIX, and/or MMI/T_3. E_2 treatment increased the number of DNA adducts and decreased tumor growth. MIX treatments significantly reduced the number of DNA adducts in both DMBA-damaged mammary tissue and tumors, and they prevented the increase in DNA adducts induced by E_2; the MIX-treated group also showed the maximum reduction in tumor size. MMI/T_3 reduced the formation of DNA adducts in response to E_2 treatment and partially blocked the antineoplastic effect of MIX, as measured by tumor size (MMI/T_3 + MIX + E_2). The weak antineoplastic effect of MMI/T_3 alone was not significant.

Discussion

In this study, we demonstrated that in the DMBA mammary cancer model, both iodine and iodide can diminish and delay tumor initiation and incidence, and they decrease tumor size in frank tumors. We also showed that the antineoplastic iodide effect was blocked if LPO activity was inhibited (MMI/T_3), suggesting that iodide needs to be oxidized to exert this effect. In previous reports, we demonstrated that 0.05% I_2 is enough to prevent 60–70% of the MNU-induced mammary tumors (García-Solís *et al.* 2005); however, in the DMBA model, drinking water

Table 2 Effect of thyroid hormones, iodine, iodide, and/or methimazole on incidence, latency, and tumorigenesis rate

Treatment	No. of rats with cancer	%	Cancer latency (week)	Tumorigenesis
Control	0/10	0	0	0
T_4	0/10	0	0	0
0.1% KI	0/10	0	0	0
MIX	0/10	0	0	0
DMBA	10/10	100	7	1.9 ± 0.4
DMBA + T_4	8/10	80	7	1.4 ± 0.3
DMBA + T_3	8/8	100	7	1.8 ± 0.7
DMBA + MMI/T_3	7/8	87.5	8	1.5 ± 0.4
DMBA + 0.05% KI	8/10	80	7	1.9 ± 0.4
DMBA + 0.05% I_2	7/10	70	8	1.0 ± 0.3*
DMBA + 0.07% I_2	5/10	50	10*	1.0 ± 0.2*

*$P < 0.05$; significantly different from DMBA group.

Table 3 Effect of moderately high concentrations of iodine/iodide and/or methimazole/triiodothyronine (T_3) on tumorigenesis and tumor volume

	Tumorigenesis	Tumor volume
DMBA	1.8 ± 0.7	2.4 ± 1.1
0.1%KI	1.3 ± 0.6	0.1 ± 0.1*
MIX	1.3 ± 0.6	0.07 ± 0.05*
Lugol	1.0 ± 0.0	0.02 ± 0.02*
MMI/T_3	1.8 ± 1.0	2.1 ± 1.2
MM/T_3+KI	1.2 ± 0.4	1.4 ± 0.9
MMI/T_3+MIX	1.5 ± 0.6	0.8 ± 0.5

*$P < 0.05$; significantly different from DMBA group.

containing 0.1% or more iodine or iodide is necessary to observe this effect. It is well established that DMBA is more aggressive than MNU in generating malignant tumors, and the DMBA model is considered to be more similar to breast cancer with respect to the PAH-initiation mechanisms (Al-Dhaheri *et al*. 2008). It is possible that a larger quantity or different types of iodometabolites are necessary to decrease the progression of these DMBA-induced cancers, or that PAHs produce different types of iodine-sensitive tumor cells. In the first case, more than one mechanism or iodine component may be triggered or generated by the iodine/iodide supplement. Several groups have postulated that the antineoplastic I_2 effect could be mediated by direct antioxidant/oxidant action at the mitochondrial level or by the formation of iodolipid intermediates such as 6-IL or iodohexadecanal (Dugrillon *et al*. 1994, Pisarev *et al*. 1994, Shrivastava *et al*. 2006, Rosner *et al*. 2010). Our group has demonstrated that 6-IL formation in solid tumors (MNU) or cancerous cells (MCF-7) is associated with elevated concentrations of its precursor AA, and that 6-IL formation is independent of peroxidases when the supplement is I_2 (Arroyo-Helguera *et al*. 2006, 2008, Aceves *et al*. 2009). DMBA tumors were previously reported to contain high concentrations of AA and/or its metabolites (i.e. prostaglandins; Tan *et al*. 1974, Rillema & Mulder 1978).

In relation to molecular mechanisms, our results showed that iodine induces significant expression of PPARγ in DMBA-treated mammary glands and frank tumors, but not in normal mammary gland, and that this increase correlates with the activation of caspase-3 and the induction of DNA rupture (TUNEL), suggesting that PPARγ could be a crucial mediator of the antineoplastic iodine effect. These results agree with our previous data showing that the apoptotic effect of iodine or 6-IL is accompanied by the significant induction of PPARγ in both *in vitro* and *in vivo* models

(Aceves *et al*. 2009, Nuñez-Anita *et al*. 2009) and that 6-IL is a specific activator ligand of this receptor (Nuñez-Anita *et al*. 2009). Moreover, a recent report shows that pretreatment with rosiglitazone – a potent agonist of PPARγ – synergizes the anticancer activity of cisplatin and minimizes cisplatin-induced nephrotoxicity by stimulating PPARγ expression in DMBA-induced tumors (Tikoo *et al*. 2009).

Iodine supplementation was also accompanied by significant increases in NIS, PEN, and LPO in mammary tumors, which contrast with the null effect in normal mammary gland or with the classical inhibition of NIS and TPO expression observed in normal thyroid gland (Anguiano *et al*. 2007). One possible explanation is that under normal conditions, high doses of iodine decrease the expression of these genes, as part of a mechanism to regulate thyroid function, whereas in cancer situations, the upregulation of these proteins by iodine might be strongly associated with tumoral redifferentiation. A study in thyroid cancer cell lines has shown that high doses of I_2 increase the expression of p53, p21, and Bcl-xL, suggesting that iodine might promote the process of cell redifferentiation (Liu *et al*. 2010). These antitumor effects have also been attributed to PPARγ activation. There is evidence that PPARγ agonists promote cell redifferentiation, which is reflected in the upregulation of specific differentiation markers (NIS, TSH receptor, LPO, and thyroglobulin) and downregulation of a dedifferentiation marker (CD97) in thyroid cancer (Park *et al*. 2005, Aiello *et al*. 2006). Additional
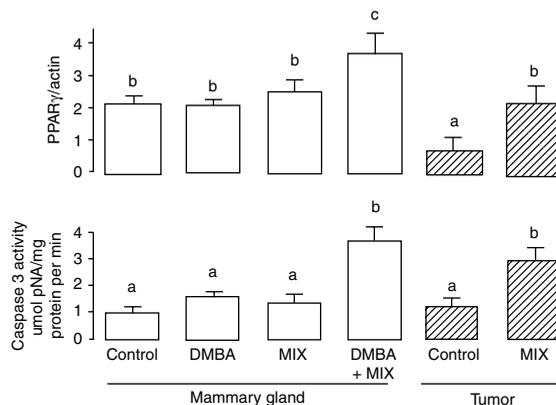

Figure 3 PPARγ induction and caspase-3 activity. Mammary glands and tumors of control or DMBA-injected animals treated or not with iodine MIX for 22 weeks were analyzed for PPARγ mRNA expression by quantitative RT-PCR. Actin mRNA was amplified to check for RNA quantity and integrity. The experiments were repeated three times with independent RNA samples. Caspase-3 activity was analyzed by enzymatic assay ($n=5$). Values are expressed as mean $\pm$ s.d. Means with different letters are significantly different ($P < 0.05$).
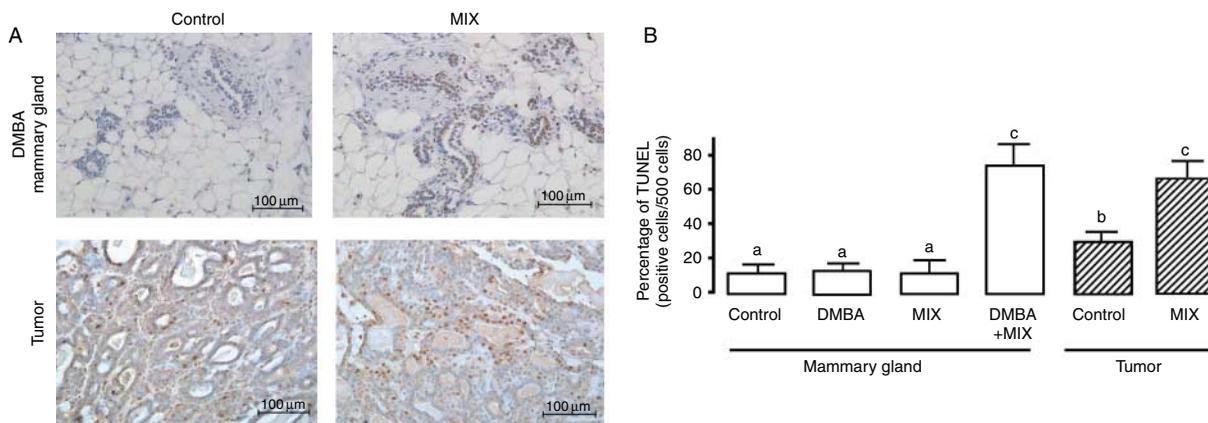
Figure 4 Apoptosis rate. Formalin-fixed, paraffin-embedded sections of mammary glands and tumors treated or not with iodine MIX for 22 weeks were analyzed for apoptosis. (A) Representative sections of TUNEL-positive cells revealed with diaminobenzidine (brown stain) and counterstained with hematoxylin (purple stain; magnification 20×), (B) TUNEL-positive cell quantification. Values are expressed as mean±s.D. Means with different letters are significantly different ($P<0.05$).

experiments are required to confirm this hypothesis with respect to breast cancer.

Exposure to high iodine concentration is a potential risk for thyroid physiology (leading to hypothyroidism or autoimmune disease) as well as for general health (retinal damage; Paul *et al*. 1988). However, careful examination shows that pathological responses occurred at low or moderate iodide intake in patients with underlying or evident thyroid pathology (e.g. Hashimoto's thyroiditis, history of treatment for Graves' diseases, etc.), but in normal subjects only with higher doses of iodine or iodide (>20 mg/day; Robison *et al*. 1998, Burgui *et al*. 2001). No damaging effects were reported in either the human or animal studies that used therapeutic I_2 concentrations (3–15 mg/day; Ghent *et al*. 1993, Kessler 2004, Anguiano *et al*. 2007, Aceves & Anguiano 2009). In this study, our results showed that the thyroid epithelium response was directly proportional to the iodide concentration: iodide or iodine concentrations of 0.05% or less did not modify the cytoarchitecture (epithelium/lumen ratio), but 0.1% KI or Lugol decreased this ratio with the tissue exhibiting thinner thyroid epithelium and increased luminal volume, which suggested a hypothyroid-like situation. However, after 22 weeks of supplementation with all the iodine or iodide concentrations tested, there was no evidence of apoptotic thyrocytes (caspase-3 activity) or changes in T_3 or TSH circulating levels, indicating that at these iodine/iodide doses, the thyroid gland was able to make physiological adjustments to maintain the normal thyroid status.

Another important finding in this study is the participation of LPO in the antineoplastic effect of iodide. Previously, it was reported that DMBA tumors

contain LPO activity (Anderson *et al*. 1984); in this study, we demonstrated that this enzyme is expressed and that its inhibition results in a long- (20 weeks) or short-term (2 weeks) decrease in the antineoplastic effect of iodide. It is well established that LPO is a powerful peroxidase capable of oxidizing iodide even
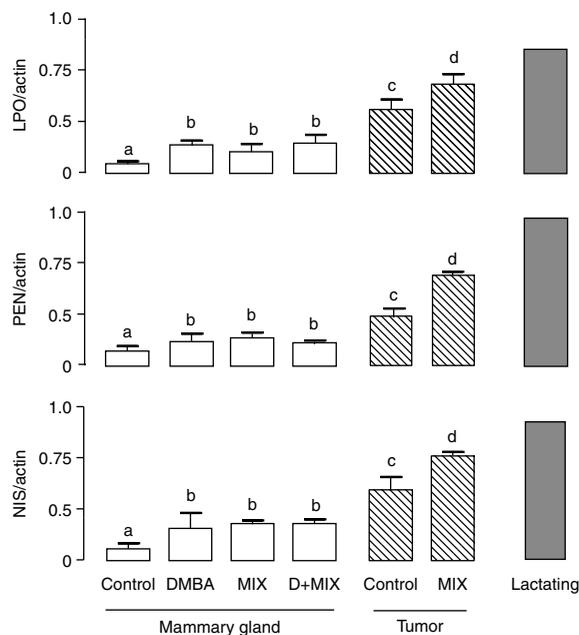

Figure 5 LPO, PEN, and NIS expression. Mammary glands and tumors of control or DMBA-injected animals treated or not with iodine MIX for 22 weeks were analyzed for mRNA expression by quantitative RT-PCR. Actin mRNA was amplified to check for RNA quantity and integrity. Values are expressed as mean ±s.D.; the experiments were repeated three times with independent RNA samples. Means with different letters are significantly different ($P<0.05$). One sample of lactating mammary gland was used as positive control.
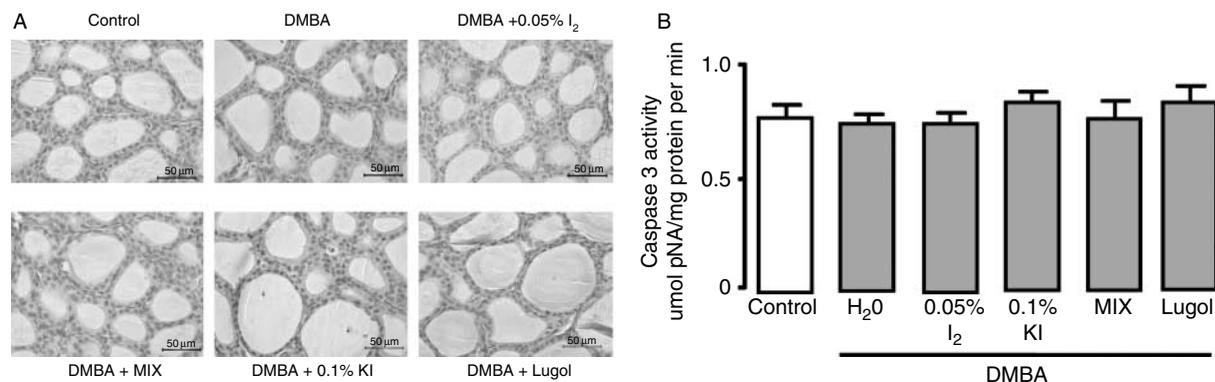
Figure 6 Thyroid gland integrity. Thyroid glands from control and DMBA-treated animals after 22 weeks with or without iodine supplement. (A) H&E-stained, 5 μm, paraffin-embedded sections (magnification 40×), (B) Caspase-3 activity, analyzed by enzymatic assay.

more efficiently than TPO (Guo *et al.* 2004, Davies *et al.* 2008). The presence of LPO in pregnant and lactating mammary glands is considered to be an adaptive mechanism to ensure an adequate supply of iodine to the neonate (Memphan 1987). This enzyme has been reported to be present at a very low concentration or even absent in non-lactating mammary glands and in some tumor cell models such as MNU tumors or MCF-7 and MDA-MD231 cells (Anguiano *et al.* 2007, Arroyo-Helguera *et al.* 2008); however, the present data show that LPO is expressed in precancerous and cancerous DMBA-treated mammary cells. Furthermore, preliminary data from our laboratory demonstrate the presence of LPO in samples of early and advanced human breast cancer (unpublished data). These findings become particularly relevant since LPO can oxidize natural or synthetic estrogens to catechol estrogens (Cavalieri *et al.* 1997, Lovstad 2006). The resulting estrogenic quinones have been shown to react with DNA to form mutagenic adducts that can initiate or promote cancer (Cavalieri *et al.* 1997). To follow this line of thinking we asked whether LPO, in the presence of moderately high levels of E_2 and/or iodine (MIX), could be contributing to tumor growth and/or to the number of DNA adducts in frank tumors. Our data showed several novel results: firstly, mammary glands from DMBA-treated animals (preneoplastic cells) contain the highest levels of DNA adducts of all groups, confirming a recent report that the DNA in these glands is extensively damaged (Wang *et al.* 2011); secondly, at this dose E_2 treatment does not modify the tumor growth rate, but it does increase the number of DNA adducts, indicating that tumor cells are able to metabolize natural estrogens and generate DNA adducts, increasing cellular instability; thirdly, MMI/T_3 prevents the increase of

these E_2-DNA adducts, showing that LPO has an important role in their formation; and finally, that iodine treatment decreases the number of pre-established adducts and prevents the formation of E_2-generated adducts, suggesting the existence of specific iodine-sensitive cells (cells with a high content of adducts?),
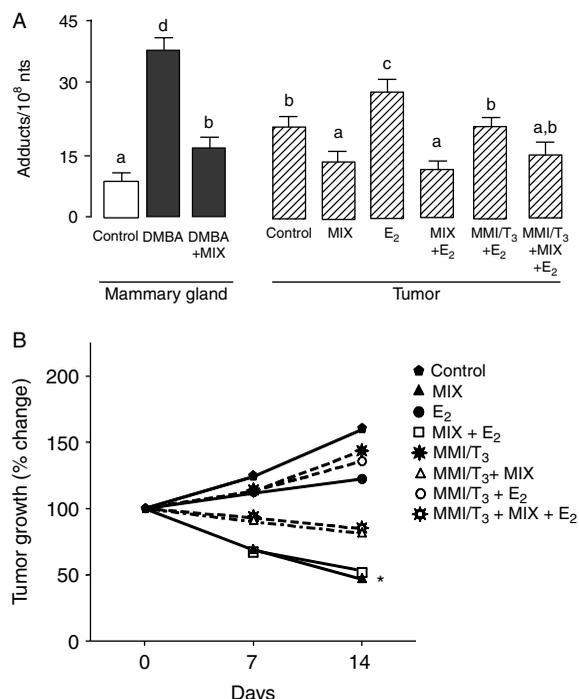

Figure 7 Effect of iodine, estrogen, and lactoperoxidase inhibition on number of DNA adducts and tumor growth. After 10 weeks of DMBA treatment, animals with frank tumors were treated with 17β-estradiol (E_2; 0.5 μg/day), 0.05% I_2 + 0.05% KI (MIX), and/or 0.05% methimazole + 3 μg/ml T_3 (MMI/T_3) for 2 weeks. (A) DNA adduct content in control and DMBA-treated (damaged or frank tumors) mammary glands. Means with different letters are significantly different ($P < 0.05$). (B) Percent change in tumor growth. *$P < 0.05$ compared with control.

and the competition between iodide and estrogen for LPO (higher affinity of iodide versus E_2 for LPO?). Together, these data strongly suggest a complex interaction between E_2 and iodine in mammary carcinogenesis. In this context, it is well established that moderately high concentrations of estrogens (such as proestrus levels) promote cancer progression, but that chronic, high levels of E_2 (characteristic of pregnancy) prevent both its initiation and progression (Nandi *et al*. 1995, Sivaraman & Medina 2002). Many authors have proposed that these effects are independent of nuclear receptors, suggesting catechol estrogens as an important element (Lareef *et al*. 2005). This notion agrees with the report of Cavalieri's group showing that higher levels of E_2-DNA adducts are present in the urine of breast cancer patients and women at high risk for this disease (Cavalieri & Rogan 2010). Although our study cannot exclude the participation of other enzymes (CYP1, CYP2, etc.) in DNA adduct generation, LPO is clearly implicated. Additional studies on the effects of iodine in cells with high levels of DNA adducts and its influence on the production of catechol estrogens, both dependent on and/or independent of LPO are necessary; however, our present data lead us to propose that iodine MIX (0.05% I_2/0.05% KI) could be an excellent component in breast cancer therapy, exerting apoptotic effects and inhibiting the generation of DNA adducts without any side effects on the thyroid or general physiology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

O S and G D contributed equally to this work and carried out the cellular and molecular studies, participated in the animal procedures, and drafted the manuscript; P P, E D M S, and M E G carried out the DNA adduct analysis and helped to draft the manuscript; B A participated in the design of the study and performed the statistical analysis, and C A participated in the study design and coordination. All authors read and approved the final manuscript.

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