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Regulation of NADPH oxidase NOX4 by delta iodolactone (IL-δ) in thyroid cancer cells

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ABSTRACT

Introduction: Iodine is not used only by the thyroid to synthesize thyroid hormones but also directly influences a number of thyroid parameters such as thyroid proliferation and function. Several iodinated lipids, biosynthesized by the thyroid, were postulated as intermediaries in the action of iodide. Among these, iodolactone (IL-δ) and 2-iodohexadecanal (2-IHDA) have shown to inhibit several thyroid parameters. The antiproliferative effect of IL-δ is not restricted to the thyroid gland. IL-δ exhibits anti-tumor properties in breast cancer, neuroblastoma, glioblastoma, melanoma and lung carcinoma cells suggesting that IL-δ could be used as a chemotherapeutic agent. Moreover in a colon cancer cell line (HT-29), IL-δ induced cell death, and this effect was mediated by reactive oxygen species (ROS) generation. The aim of the present study was to analyze the sources of reactive oxygen species induced by IL-δ and to explore the contribution of ROS induced by IL-δ on cell proliferation and apoptosis.

Methodology and results: Cancer thyroid follicular (WRO) and papilar (TPC-1) cells lines were treated with IL-δ. Proliferation and apoptosis was analyzed. IL-δ caused a significant loss of cell viability on WRO and TPC-1 cells in a concentration dependent manner and induced apoptosis after 3 h of treatment. Furthermore, IL-δ (10 μM) increased ROS production (39% WRO and 20% TPC-1). The concomitant treatment of WRO and TPC-1 cells with Trolox or NAC plus IL-δ abrogated the augment of ROS induced by IL-δ exposure. Additionally Trolox and NAC reversed the effect of IL-δ on cell proliferation and apoptosis. Only in WRO cells IL-δ upregulates NADPH oxidase NOX4 expression, and siRNA targeted knock-down of NOX4 attenuates ROS production, apoptosis ($p < 0.05$) and the inhibitory effect of IL-δ on cell proliferation and PCNA expression ($p < 0.05$).

Conclusions: The antiproliferative and pro-apoptotic effect of IL-δ is mediated by different mechanisms and pathway involving different sources of ROS generation depending on the cellular context.

1. Introduction

Different factors have been proposed to be involved in thyroid function and proliferation such as thyrotropin (TSH), growth factors, iodide, radiation, etc. Previous studies have shown that, iodine excess inhibits thyroid cell proliferation and thyroid function through the synthesis of an organified compound. Several compounds, biosynthesized by the thyroid, were postulated as intermediaries in the action of iodide. Among these, two iodolipids: 5-hydroxy-6 iodo-8, 11, 14- eicosatrienoic delta lactone (IL-δ) and 2 iodohexadecanal (2-IHDA) have shown to inhibit several thyroid parameters and its participation in thyroid autoregulation have also been suggested (Pisarev and Gartner, 2000; Panneels et al., 2009).

IL-δ mimics the inhibitory effects of iodide on thyroid cell proliferation (Pisarev et al., 1992; Dugrillon et al., 1994), goiter growth (Pisarev et al., 1988; Thomasz et al., 2010a), iodide uptake (Chazenbalk et al., 1988), H_2O_2 production (Krawiec et al., 1988), and cell membrane transport of glucose and amino acids, although

Abbreviations: DPI, Diphenylene iodonium; DUOX 1, dual oxidase 1; DUOX 2, 2dual oxidase 2; IL-δ, Iodolactone; 2-IHDA, 2 iodohexadecanal; TSH, thyrotropin; TPC-1, Cancer thyroid papilar cells; WRO, Cancer thyroid folicular cells.

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not all iodine inhibitory effects are reproduced by IL-δ (Thomasz et al., 2010b).

Boeynaems and Hubbard (1980) have reported the conversion of exogenous free arachidonic acid into IL-δ in rat thyroid and Dugrillon et al. (1994) demonstrated that this compound is synthesized by the Human gland. Iodide can generate IL-δ only in cells expressing NIS and peroxidases. Several studies have reported that iodide needs to be oxidized by peroxidases, and these iodo-compound induce cytotoxic effects (Boeynaems and Hubbard 1980; Turk et al., 1983; Ekholm and Bjorkman, 1997). The synthesis of iodolipids is not restricted to the thyroid gland. The presence of IL-δ has been reported in normal and tumoral mammary gland from rats fed with continuous I_2 supplements in the diet (Aceves et al., 2009) and in MCF-7 cells (Arroyo-Helguera et al., 2006). It was demonstrated that molecular iodine (I_2), but not iodide (I^-), exerts antineoplasic actions on diverse tissues and this effect may be due to the synthesis of intracellular iodolipids (Shrivastava et al., 2006; Arroyo-Helguera et al., 2008; Nava-Villalba et al., 2015). Moreover, IL-δ displayed a 4-fold more potent antiproliferative effect on breast cancer cells than I_2 (Arroyo-Helguera et al., 2006). It was also described an antiproliferative effect of I_2 and IL-δ in several human cancer cell lines through a mitochondrial mediated apoptosis mechanism (Aranda et al., 2013; Rösner et al., 2016). IL-δ exhibits anti-tumor properties in breast cancer, neuroblastoma, glioblastoma, melanoma and lung carcinoma cells. Moreover in a colon cancer cell line (HT-29), IL-δ induced cell death, and this effect was mediated by reactive oxygen species (ROS) generation (Thomasz et al., 2013). Together, these precedents suggest that IL-δ could be used as a chemotherapeutic agent for the treatment of cancer tissues, alone or in combination with another therapy.

ROS are highly reactive O_2 metabolites, including superoxide radical and hydrogen peroxide (H_2O_2) that are involved in several physiological processes. Thyroid hormone synthesis requires hydrogen peroxide as a substrate and a peroxidase enzyme (TPO) catalyzes the process. In the thyroid gland, H_2O_2 is generated by NADPH oxidases (NOXs), dual oxidase 1 (DUOX 1) and dual oxidase 2 (DUOX 2) (Leseney et al., 1999; Song et al., 2007, Rigutto et al., 2009). DUOX are located at the apical plasma membrane of the thyrocyte, and they produce H_2O_2 in the extracellular colloid space (Dupuy et al., 1989, 1999). In addition to DUOX 1 and DUOX 2, human tyrocytes also express an intracellular reactive oxygen species (ROS) generating system, NADPH oxidase 4 (NOX4) (Ohye and Sugawara, 2010; Ameziane-El-Hassani et al., 2016). The different localization of NOX/DUOX appears to be related to their different functions and this localization suggests a role of NOX4 in thyroid cell signaling, (Baboir, 1999; Bedard and Krause, 2007; Lambeth, 2007; Weyemi et al., 2010).

The aim of the present study was to analyze the sources of reactive oxygen species induced by IL-δ and to explore the contribution of ROS induced by IL-δ on cell proliferation and apoptosis.

2. Materials and methods

2.1. Cell culture

The human WRO cell line was cultured in RPMI 1640 medium containing 10% FBS and penicillin (100 U/ml) and the human TPC-1 cell line was cultured in Coons' modified F-12 medium (50%) and DMEM-high glucose (50%). Cells cultures were maintained in a temperature and humidity controlled incubator at 37 °C with air and 5% CO_2. The medium was replaced with fresh medium every 2–3 days. Cells were harvested with trypsin-EDTA and seeded on 24-and 96 well plates or in 60 mm Tissue-Culture dishes for experimental purposes.

2.2. Chemical synthesis of IL-δ

IL-δ was synthesized with a modification of the method of Monteagudo et al. (1990). Briefly, to a solution of AA (65 mg) in acetonitrile (0.8 mL) was added a solution of iodine (156 mg) in acetonitrile (8 mL) at 4 °C. The solution was kept under N_2, stirred for 4 h at room temperature and protected from light. The solution containing crude product was concentrated under low nitrogen flow to 0.5 ml and separated on silica gel column and preparative TLC using the solvent system CH_2Cl_2/MeOH (97.5:2.5). The IL-δ synthesized was concentrated under low nitrogen flow. Before use the IL-δ was diluted in RPMI, sonicated and introduced into the culture medium to a final concentration of 10 μM. IL-δ is stable under the present experimental conditions (Thomasz et al., 2010b).

2.3. Cell viability

Cells were cultured on 96 well plates for 24 h in RPMI 1640 supplemented with 10% FBS. After 24 h, cells were further incubated for 48 and 72 h in 10% FBS medium in the presence of various compounds. Viability of WRO and TPC cells was evaluated using MTT assay. This assay is based on the cleavage of the tetrazolium salt MTT to a dark blue formazan product by mitochondrial dehydrogenase in viable cells. The absorbance of viable cells was measured in a Spectra Microplate Reader with a test wavelength of 570 nm.

2.4. Assay of ROS production

In order to determine the quantity of ROS produced by WRO and TPC cells, the H_2O_2 concentration within the cells was assayed using the 2′, 7′-dichlorfluorescein-diacetate (DCFH-DA) a well-established compound used to detect and quantify intracellular produced ROS. DCFH-DA is freely permeable across the membranes; upon entering the cell, the acetate groups are hydrolyzed, creating a membrane impermeable form of the dye (DCFH). Hydrogen peroxide and peroxides produced by the cell oxidize DCFH to yield a quantifiable fluorogenic compound 2′, 7′-dichlorofluorescein (DCF), representing the level of ROS present in the cell, which can be detected by fluorescent microscopy.

Cells (1.0×10^6) were incubated with10 μM of DCFH-DA for 20 min at 37 °C, and relative ROS units were determined by fluorescence at $\lambda_{excitation}$: 485/20 nm and $\lambda_{emission}$: 530/25 nm. An aliquot of the cell suspension was lysed, and protein concentration was determined. The results are expressed as arbitrary absorbance units/mg protein.

2.5. Measurement of mitochondrial ROS

ROS generation by mitochondria in living cells was analyzed with the mitochondrial superoxide indicator MitoSOX™ Red (Invitrogen). For assays, cells were treated with IL-δ for 3 h and incubated with 5 μM MitoSOX-Red for 10 min at 37 °C. After washing twice with PBS, mitochondrial ROS were determined by fluorescence Ex/Em: 510/580 nm. The relative MitoSOX™ intensity were normalized to changes in protein content and expressed as fold change with respect to unstimulated control.

2.6. Western blot analysis

Cells were seeded in 60-mm dishes and incubated with different compounds for the time indicated in the text. Proteins were extracted in lysis buffer RIPA (50mMTris–HClpH7.4, 150 mMNaCl, 1% NonidetP4 0.01% SDS, 0.5 %d eoxycholate), supplemented with PMSF 0.5 mM and protease inhibitor cocktail (Sigma-Aldrich).

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Proteins were electrophoresed on 10% SDS-polyacrylamide gel (SDS-PAGE), prior to transfer to polivinylidene difluoride membranes (PVDF) (Millipore, Bedford, MA), in a semidry transfer cell at 18 V for 1 h. Membranes were blocked with 0.2% Tween 20 (Sigma) and 5% BSA for 1 h at RT and then incubated overnight at 4 °C using monoclonal or polyclonal antibodies. Immunoblotting was carried out with monoclonal anti c-Nox-4 antibody (dilution 1:2000, Abcam), polyclonal anti PCNA antibody (dilution 1:200, Santa Cruz Biotechnology), and anti-β- in phosphate buffer saline solution (PBS) with 0.2% Tween 20 (Sigma) and 5% BSA. Immunochemical detection of PCNA or Nox-4 levels using a specific antibody and anti-rabbit IgG antibody and conjugated with peroxidase. Specific proteins were visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, USA). For loading controls immunoblotting was carried out with monoclonal anti-β-actin antibody (1:2000, sigma). Densitometric analysis was performed using the NIH ImageJ analysis Software (1.40 g Wayne Rasband, National Institute of Health, USA) and results were corrected for protein loading by normalization for β-actin expression.

2.7. Caspase-3 activity

Caspase-3 activity was determined with the caspase-3 Assay kit, according to the manufacturer's instructions (Sigma CASP-3-C, Sigma-Aldrich, St. Louis, Mo, USA). This assay is based on the spectrophotometric detection of the Ac-DEVD-pNA substrate after cleavage. Cells were harvested in lysis buffer [50 mm HEPES, 5 mMdithiothreitol (DTT), 5 mN CHAPS, 10 μg/mL pepstatin, benzamidine 2.5 mM, aprotinina 10 μg/mL, pepstatin 1 μg/mL, 0,5 mMphenylmethylsulfonylfluoride (PMSF), pH 7.4] and homogenized with a Teflon-glass homogenizer. Lysates were clarified by centrifugation at 10,000 × *g* for 5 min, and clear lysates containing 100 μg proteins were incubated with caspase-3 substrate, at 37 °C for 3 h. The concentration of the p-nitroaniline (pNA) released from the substrate is calculated from the absorbance values at 405 nm after incubating the plate at 37 °C for 90 min. The activity, expressed as micromoles of p-nitroaniline per minuteper milliliter, was calculated with a p-nitroaniline calibration curve. A positive control of caspase-3 and an inhibitor-treated cell lysate control (for measuring the nonspecific hydrolysis of the substrate) were added to the plate.

2.8. Apoptosis assay by nuclear morphology

At the indicated times after IL-δ treatment, the cells were washed with PBS, and changes in cell morphology were examined by staining slides with the Hoechst dye 33,342 (5 μM/ml in PBS) reagent, during 10 min at room temperature. After staining, the slides were dried thoroughly, rinsed in PBS, and nuclear morphology was observed using a fluorescence microscope. The percentage of apoptotic cells was determined by counting the number of nuclei showing chromatin condensation and fragmentation characteristic of apoptosis after observing a total of at least 100 cells.

2.9. RT-PCR analysis

Extraction and purification of RNA were performed following the TRIzol method. Following reverse transcription using the Superscript II reverse transcriptase (Invitrogen) cDNA (1 μl, 1/10) was used in each PCR reaction in a total volume of 25 μl, with specific primers for the target molecules with the PCR Master Mix (Promega).

NOX 4: forward 5′ -CGGTGCTATTCCTCATGATCAC-3′, reverse 5′-AATCTGGGCTCTTCCATACAAA-3′; CATALASE: forward 5′- TCATATACCTGTGAACTGTG-3′ reverse 5′-ATAGAATGCCCGCACCTGAG-3′; SOD1: forward 5′-AAGGCCGTGTGCGTGCTGAA-3′, reverse 5′-CAAGTCTCCAACATGCCTCT-3′; SOD2: forward 5′- TCACATCAACGCGCAGATCA-3′, reverse 5′ GGCTTCCAGCAACTCCCCTT -3′.

The PCR cycling parameters were as follows (the annealing temperatures and cycle number were optimized for each pair of primers): initial denaturation at 94 °C for 2min (one cycle), denaturation at 94 °C for 30 s, annealing for 60 s and extension at 72 °C for 1min, followed by a 10 min extension at 72 °C after the last cycle.

All PCR products were separated on 2% agarose gel electrophoresis and visualized with Gel Red. Images of the gels were analyzed by densitometry using the Image J software.

2.10. Immunocytochemistry

Sub confluent cells grown in 60 mm dishes with or without IL-δ during 3 h, were fixed in 100% methanol for 5 min at −20 °C. Cells were then washed with PBS and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min. Cells were further Incubated in 3% hydrogen peroxide for 20 min, washed and blocked with 2% BSA in PBS for 30 min. Cells were then incubated at 4 °C overnight with anti-NOX-4 (Abcam, dilution 1:200) antibody. Inmunodetection was performed by the use of the Cell Marque Kit according to the manufacturer's instructions. Peroxidase staining was revealed in 3,3′-diaminobenzidine. Negative controls were performed as described above except that the primary antibody was not included.

2.11. Catalase and Superoxide Dismutase activity

Catalase activity was measured by monitoring the disappearance of H_2O_2 at 240 nm. The reaction mixture contained 30 mg protein, 50 mM potassium phosphate buffer (pH 7.8), and 15 mM H_2O_2. Changes in absorbance were measured for 1 min. The specific activity was calculated as units/mL (mmol/[min mL]).

Superoxide Dismutase (SOD) activity was determined with the Superoxide Dismutase Activity Assay Kit (Abcam Colorimetric). This kit uses WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by a colorimetric method at 450 nm.

2.12. Transfection of small interfering RNAs

Cells were seeded in P60 plates and transfected with 50 nmol/ml of siRNA or a control non targeting scrambled oligonucleotide using siPORTneoFX Transfection Agent (Ambion) according to the manufacturer's instructions with specific human siRNA against NOX4 (NOX4 si RNA, Santa Cruz) or scrambled oligonucleotide siRNA control (sc si RNA, Ambion, Austin, TX).

2.13. Statistical analysis

Experiments were repeated 4–5 times. Results are expressed as mean ± SEM. Statistical analysis of the results was made by one way ANOVA followed by Student-Newman-Keuls test. Differences were considered significant at $p < 0.05$.

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Table 1
Dose dependence of cell viability.

	Control	ILδ 1 μM	ILδ 10 μM	ILδ 30 μM
WRO	100	90 ± 5	75 ± 5**	70 ± 3**
TPC	100	95 ± 5	82 ± 7*	80 ± 5*

Effect of IL-δ on WRO and TPC cells survival. Cells were plated and cultured in 10% FBS-RPMI. The next day, media were removed and cells were incubated for 72 h with 10% FBS-RPMI containing IL-δ at the indicated concentrations.The viability was evaluated using the MTT assay. Cells were treated with MTT (0.5 mg/ml) and incubated for 1 h at 37 °C. Then the absorbance was measured at 570 nm and the results were expressed as percentage of control cell. Data are expressed as means ± SEM from four independents experiments * $p < 0.05$ versus control, **$p < 0.01$ and $p < 0.001$ versus control.

3. Results

3.1. IL-δ inhibits cell proliferation and induces apoptosis

We first analyzed the effect of IL-δ on cell proliferation and apoptosis in two thyroid cancer cell lines WRO (follicular) and TPC-1 (papillar).

The viability was determined by MTT assay. As shown in Table 1, IL-δ caused a significant loss of cell viability on WRO and TPC-1 cells in a concentration dependent manner. As a marker of cell proliferation, PCNA was examined by Western blots. IL-δ caused a significant loss of cell viability and decrease of PCNA expression after

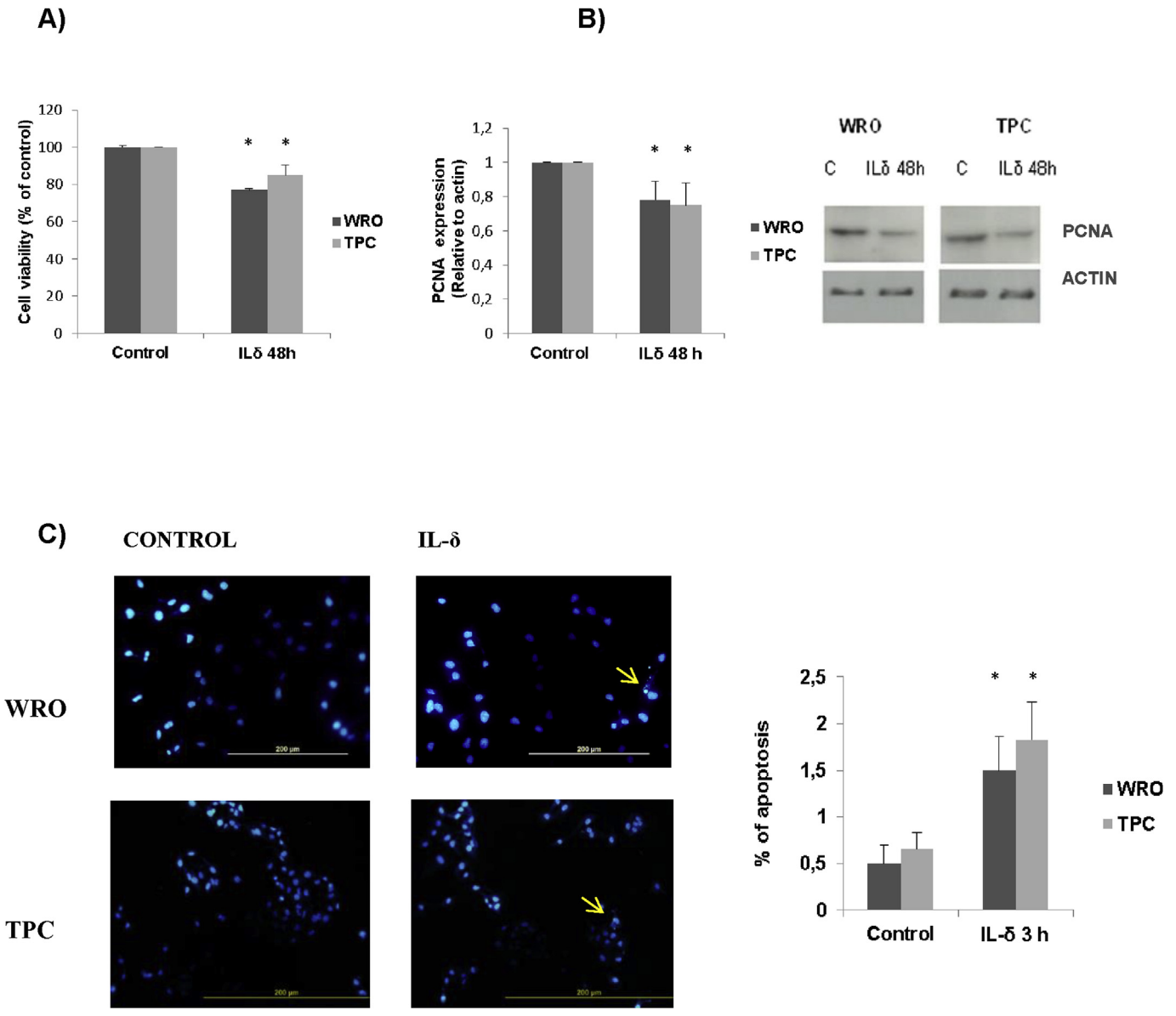

Fig. 1. IL-δ induces WRO and TPC cell death. (A) Effect of IL-δ on WRO and TPC cells survival. Cells were plated and cultured in 10% FBS-RPMI. The next day, media were removed and cells were incubated for 48 h with 10% FBS-RPMI containing IL-δ at the indicated concentrations.The viability was evaluated using the MTT assay. Cells were treated with MTT (0.5 mg/ml) and incubated for 1 h at 37 C. Then the absorbance was measured at 570 nm and the results were expressed as percentage of control cells. (B) Western blot of PCNA expression in cultured WRO and TPC cells. Cells were incubated with 10 μM of IL-δ for 48 h. Right panel: Immunochemical detection of PCNA levels using a specific antibody. Left panel: Quantification of PCNA levels by densitometry scanning of the inmunoblots. Values were normalized with an anti-β-actin antibody. (C) Morphological changes induced by IL-δ. WRO and TPC cells were treated for 3 h with 10 μM of IL-δ. Percentage of cells with nuclear apoptotic morphology was quantified. Cells were staining with Hoechst and observed by fluorescence microscopy. Arrows denote apoptotic morphological changes. Magnification X 200 and X 400. Data are expressed as means ± SEM from four independents experiments * $p < 0.05$ versus control, **$p < 0.01$ and $p < 0.001$ versus control.

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48 h of treatment (Fig. 1A and B). For the following studies a concentration of 10 μM of IL-δ was employed.

IL-δ induced apoptosis after 3 h of treatment in WRO and TPC-1 cell lines. The abnormalities of cell morphology were examined using a fluorescence microscope after Hoechst staining. Our results show that IL-δ induced a significant increase around 3 (WRO) and 2.7 (TPC-1) fold of apoptotic nuclei evidenced by morphological changes such as: condensation of nuclear chromatin along the perimeter of the nucleus; circular or oval nuclei become increasingly lobular and eventually fragment into multiple subnuclei (Fig. 1C). Since caspases play an important role in early apoptosis, we investigated the activation of executive caspase-3 in response to IL-δ treatment. Caspase-3 activity was significantly increased in WRO and TPC-1 cell by IL-δ after 3 h of treatment (Table 2).

3.2. IL-δ induces ROS generation: Correlation with its antiproliferative and pro-apoptotic effect

Since oxidative stress has been associated with apoptosis, we next analyzed if IL-δ stimulates ROS generation in WRO and TPC-1 cells. Substantial cellular ROS accumulation was also detected in WRO and TPC-1 cells by using the cell-permeable DCFH-DA reagent. Cells were incubated with 10 μM IL-δ during 0, 0.5, 1, 3, 4.5 and 24 h before the addition of DCFH-DA, and the intracellular ROS accumulation was assessed by the DCFH-DA-derived fluorescence.

As shown in Fig. 2A, IL-δ significantly increased intracellular ROS levels reaching a maximal level of 39% in WRO cell after 1 h and 20% in TPC-1 cell after 3 h. In addition, mitochondria seemed to contribute to the anion superoxide production in TPC-1 cells exposed to IL-δ treatment, as demonstrated by using the fluorogenic probe MitoSOX Red (Fig. 2B).

To analyze the contribution of ROS on IL-δ induced apoptosis and inhibition of cell proliferation, cells were treated with IL-δ plus Trolox 100 μM or NAC 2.5 mM (radical scavengers). The concomitant treatment of WRO and TPC-1 cells with Trolox or NAC plus IL-δ abrogated the augment of ROS induced by IL-δ exposure (Fig. 2C).

Fig. 2D and E shows cell viability and caspase-3 activity respectively. Trolox and NAC reversed the effect of IL-δ on cell proliferation and apoptosis indicating that ROS are involved in these processes ($p < 0.05$).

3.3. IL-δ stimulates antioxidant system: catalase and SOD

Catalase and SOD mRNA were analyzed by RT-PCR. Fig. 3A show that IL-δ treatment decreased SOD 1 expression by a 30% in WRO cell ($p < 0.01$). No changes were observed for SOD 2 and catalase expression. In TPC-1 cells no changes for SOD1 and SOD2 expression were detected, while a significant inhibition of catalase expression (23%) after 3 h of treatment was observed (Fig. 3B). Then, we decided to analyze whether this effect on gene expression had a correlation with an enzymatic activity modulation. Catalase and SOD activities were measured at different times after IL-δ treatment. Table 3 shows SOD and catalase activity. In WRO cells, IL-δ decreased significantly SOD activity, and after 3 h of treatment, IL-δ increased catalase activity. On the other hand, in TPC-1 cells, we observed a decreased in SOD activity ($p < 0.01$) without changes in catalase activity. These results indicate the involvement of these enzymes in the imbalance of redox-sensitive signaling pathway.

Table 2
Caspase-3 Activity measured as pmol of pNA released/min/ml.

ILδ Treatment	Caspase-3 activity	
	WRO	TPC
0 h	7.5 ± 1.3	7.0 ± 0.9
0.5 h	8.9 ± 1.1	8.9 ± 0.8
1 h	10.6 ± 1.4	10.3 ± 1.0
3 h	12.6 ± 1.5*	12.3 ± 0.8*
24 h	7.7 ± 1	6.9 ± 0.5

Caspase-3 activity was measured after 0.5, 1, 3 and 24 h of ILδ (10 μM) treatment in WRO and TPC cells. Data are expressed as means ± SEM from four independents experiments * $p < 0.05$ versus control.

3.4. IL-δ induces NOX4 expression in a thyroid cancer follicular cell line: WRO

The NADPH oxidases (NOXs and DUOXs) generate ROS intracellularly in a wide variety of tissues. To investigate the effect of IL-δ on NADPH oxidase-dependent ROS generation we inhibit NADPH oxidases/flavoenzymes with Diphenylene iodonium (DPI), an inhibitor of NADPH oxidases/flavoenzymes. ROS generation induced by IL-δ in WRO cells was inhibited by DPI (Fig. 4A, $p < 0.05$). Interestingly intracellular ROS levels were not diminished by DPI in TPC-1 cells, suggesting that the prevalent ROS source in TPC-1 cells is not a NOX enzyme.

Since NOX4 is an important source of ROS, we decided to examine if the effect of IL-δ was mediated by this enzyme. For this purpose, we analyzed the NOX4 mRNA levels in WRO and TPC-1 cells. As expected, NOX4 gene is also expressed in these cell lines, but IL-δ only upregulated NOX4 expression in WRO cells (Fig. 4B). In addition, protein level was upregulated after IL-δ exposure as shown by Western blot analysis (Fig. 4C).

3.5. siRNA targeted knock-down of NOX4 attenuates apoptosis and the antiproliferative effect induced by IL-δ

To determine the role of NOX4 in the response induced by IL-δ, we performed interference RNA experiments. For this purpose, we used a scramble siRNA or NOX4 siRNA in WRO cells incubated with or without IL-δ for 3 h. The silencing effect of this siRNA was confirmed by Western blotting and immunocytochemistry. The amount of NOX4 protein detected was dramatically lower in NOX4 siRNA-transfected WRO cells than in scrambled siRNA-transfected WRO cells. Densitometryc analyses showed that IL-δ significantly enhanced the amount of NOX4 protein in scrambled siRNA-transfected WRO cells but not in NOX4 siRNA-transfected WRO cells (Fig. 5A and B).

To further characterize the role of NOX4, we analyzed whether NOX4 silencing can also influence the IL-δ generated intracellular ROS in WRO cells. IL-δ treatment induced ROS accumulation in scrambled siRNA-transfected WRO cells (29%) but not in NOX4 siRNA-transfected WRO cells (Fig. 6A). These findings indicate that NOX4 plays a critical role in ROS production by IL-δ in WRO cells.

Since IL-δ stimulates NOX4 expression, inhibits cell proliferation and induces cell death, we analyze the relevance of NOX4 in these processes in WRO cells. As shown in Fig. 6B and C, IL-δ inhibited cell proliferation and PCNA expression ($p < 0.05$), while this effect was significantly attenuated in siRNA targeted NOX4 silencing WRO cells.

Moreover, when we analyze the apoptosis, untreated cells transfected with either scrambled or NOX4 siRNAs showed a similar pattern of caspase-3 activity and fluorescent staining. IL-δ led to an increase in the percentage of apoptotic cells (Fig. 7A) and

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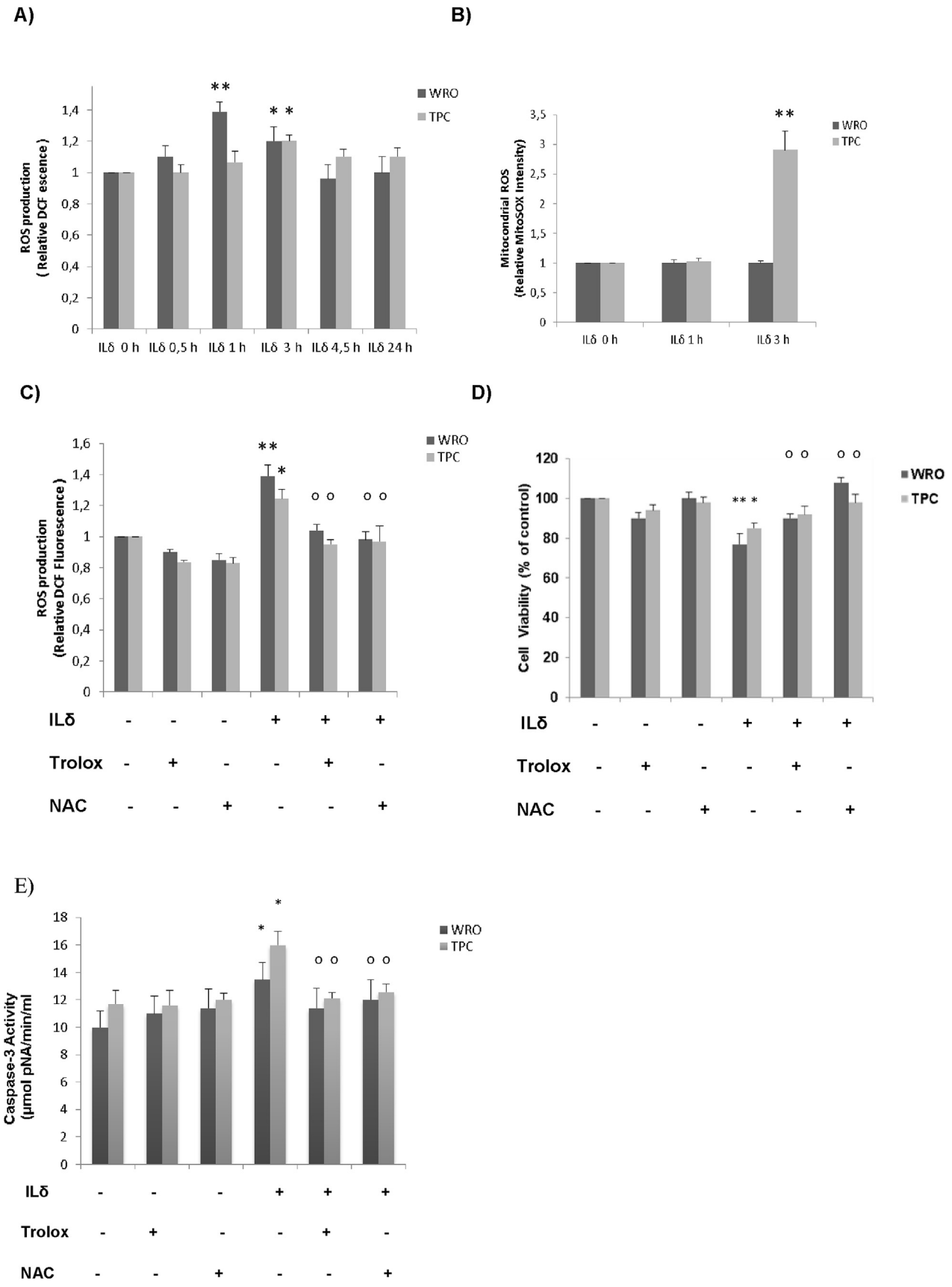

Fig. 2. Effects of IL-δ on induced intracellular ROS generation in WRO and TPC cells. Intracellular accumulation of ROS was assayed by fluorescence analysis using 2′-7′-dichloro-fluorescein diacetate (DCFH-DA) Results were expressed as the ratio of DCF fluorescence/control fluorescence under each treatment. (A) Level of intracellular ROS after IL-δ (10 μM) treatment for 0, 0.5, 1, 3, 4.5 and 24 h. (B) Measurement of mitochondrial O_2°-generation. O_2°-accumulation was evaluated by fluorescence analysis using fluorescence MitoSOX Red

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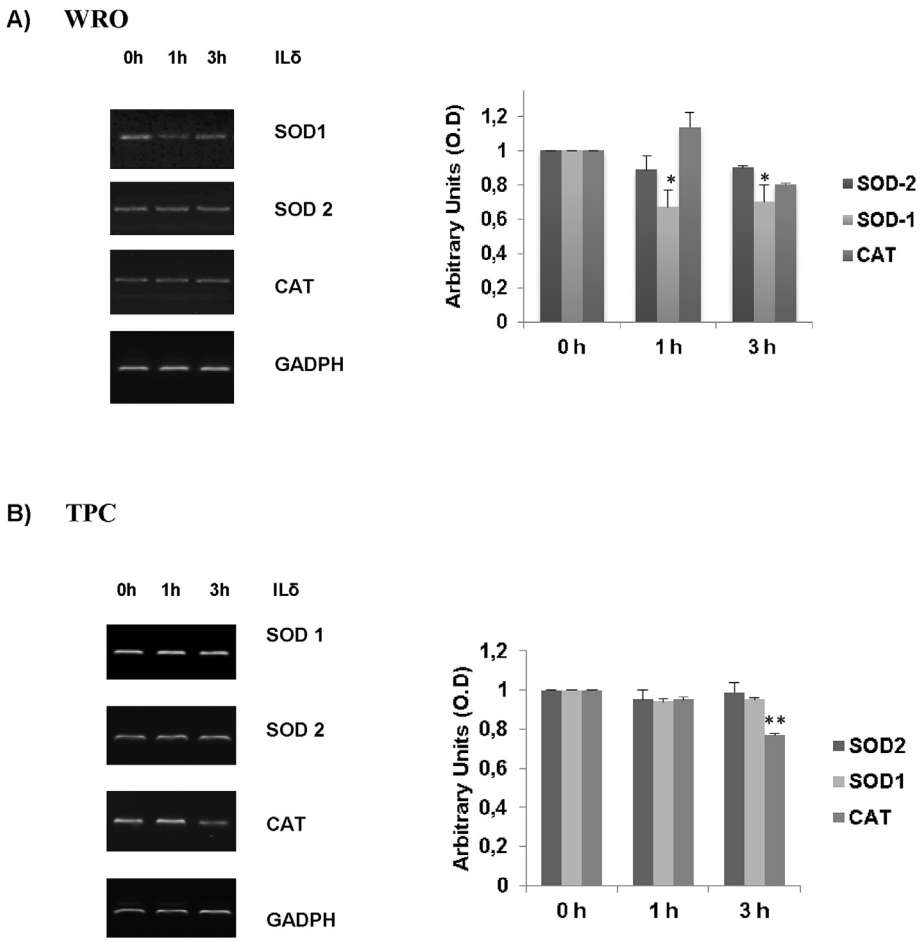

Fig. 3. Effect of IL-δ on antioxidant system. (A) Effect of IL-δ on catalase, SOD 1 and SOD 2 expression in WRO cells. (B) Effect of IL-δ on catalase, SOD 1 and SOD 2 expression in TPC cells. Cells were incubated with 10 μM IL-δ for 0, 1 and 3 h and total RNA was then purified. Catalase, SOD 1 and SOD 2 mRNA was quantified as described. RT-PCR from one representative experiment is shown in the left panel and quantification by densitometric scanning of the gel is shown in the right panel. Data are expressed as the level of on catalase, SOD 1 and SOD 2 mRNA relative to GADPH mRNA. (C) Intracellular ROS generation. Intracellular accumulation of ROS was assayed by fluorescence analysis using 2′-7′-dichlorofluorescein diacetate (DCFH-DA) in WRO and TPC cell after 10 μM IL-δ treatment with or without DPI (20 μM). Each value represents the mean ± SEM of four independent experiments. *$p < 0.05$ versus control, **$p < 0.01$ and ***$p < 0.001$ versus control. Control value was taken as 1.

Table 3
Catalase (CAT) and Superoxide Dismutase (SOD) activity after IL-δ treatment.

UE/mg protein		0 h	1 h	3 h
WRO	CAT	1.23 ± 0.34	1.21 ± 0.36	1.93 ± 0.30*
	SOD	2.00 ± 0.13	1.71 ± 0.10*	1.60 ± 0.09**
TPC-1	CAT	1.25 ± 0.33	1.60 ± 0.33	1.29 ± 0.40[a]
	SOD	3.27 ± 0.16	1.89 ± 0.42*	1.32 ± 0.35**

Treatments were carried on during 0, 1 and 3 h after IL-δ 10 μM treatment. The enzymatic activity is expressed as EU/mg protein and the results are expressed as the mean ± SEM of four independent experiments. *$p < 0.05$, **$p < 0.01$ versus control and [a] $p < 0.05$ (WRO versus TPC-1). EU, enzymatic units; CAT, catalase; SOD, superoxide dismutase.

caspase-3 activity (Table 4) in scrambled-siRNA transfected WRO cells ($p < 0.05$). SiRNA targeted NOX4 silencing significantly attenuated IL-δ mediated caspase-3 activity and the percentage of apoptotic cells (Fig. 7A and B and Table 4).

Taken together, these results indicate that NOX4 up-regulation plays a critical role in ROS production, cell proliferation and cell death induced by IL-δ in WRO cells.

4. Discussion

Iodinated derivatives from arachidonic acid, inhibit several thyroid parameters such as cell proliferation (Pisarev et al., 1992;

probe. WRO and TPC cells were treated with IL-δ (10 μM) for 0, 1 and 3 h and incubated with 5 μM MitoSOX Red probe for 10 min. Results were expressed as relative fluorescence. (C) Effect of Trolox and NAC on ROS generation. Cells were treated with IL-δ (10 μM) plus Trolox 100 μM and NAC 2.5 mM. (D) Rol of ROS on IL-δ induced cell death. Cell survival was evaluated with MTT assay. WRO and TPC cells treated with IL-δ (10 μM), Trolox 100 μM, NAC 2.5 mM or IL-δ (10 μM) plus Trolox 100 μM and NAC 2.5 mM during 48 h. (E) Caspase-3 activity measured as pmol of pNA released/min/ml. Cells were treated with IL-δ plus Trolox 100 μM and NAC 2.5 mM during 3 h and caspase-3 was measured. Data are expressed as means ± SEM from four independents experiments. *$p < 0.05$ versus control, **$p < 0.01$ and p***<0.001 versus control.

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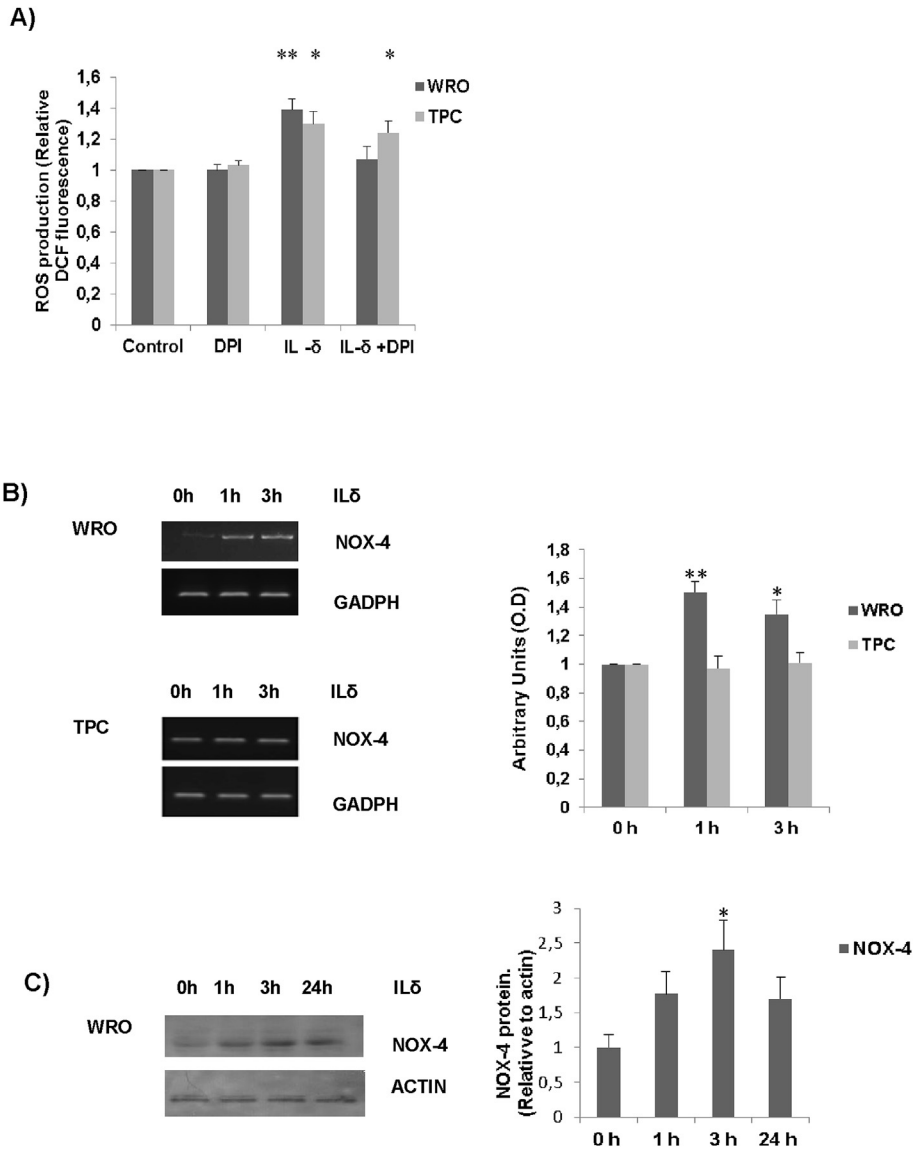

Fig. 4. IL-δ upregulates NOX-4 expression in WRO cell. (A) Effect of IL-δ on NOX-4 expression in WRO and TPC cells. Cells were incubated with 10 μM IL-δ for 0, 1 and 3 h and total RNA was then purified. NOX-4 mRNA was quantified. RT-PCR from one representative experiment is shown in the right panel and quantification by densitometric scanning of the gel is shown in the left panel. Data are expressed as the level of NOX-4 mRNA relative to GADPH mRNA. (B) Western blot of NOX-4 in WRO cells incubated with 10 μM IL-δ for 0, 1, 3 or 24 h. Each value represents the mean ± SEM of four independent experiments. *$p < 0.05$ versus control, **$p < 0.01$ and ***$p < 0.001$ versus control. Control value was taken as 1.

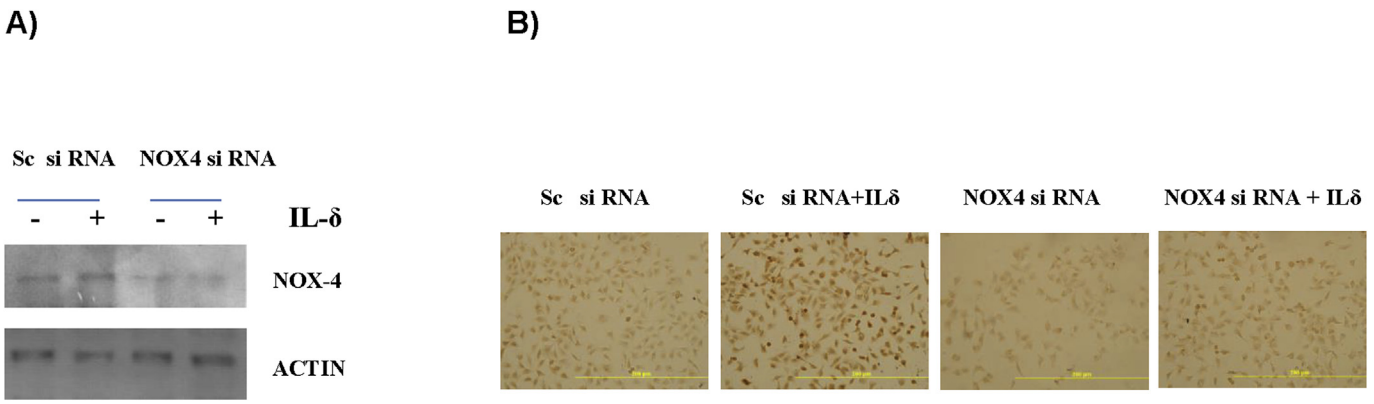

Fig. 5. siRNA target knock-down of NOX-4. NOX-4 transcription levels were analyzed by western blot (A) and immunocytochemistry (B). Cells were transfected with either an unspecific siRNA (Scrambled siRNA) or with the specific NOX-4 siRNA. 48 h after transfection cells were treated with IL-δ 10 μM (3 h) and NOX-4 expression was analyzed.

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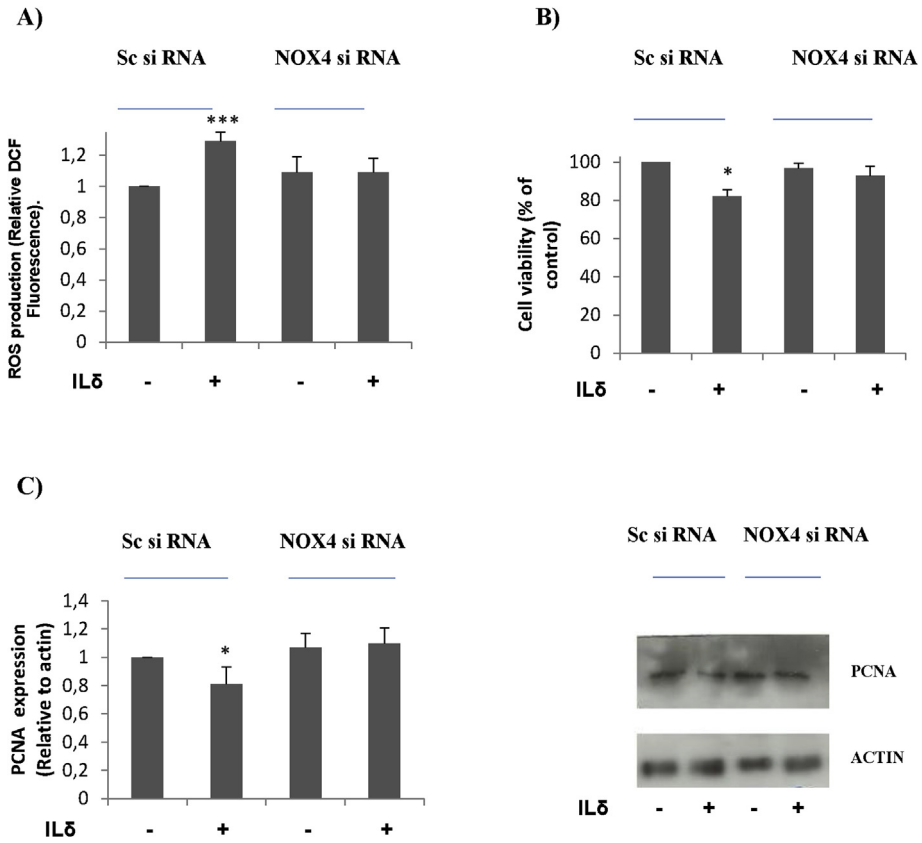

Fig. 6. NOX-4 is involved in IL-δ inhibit cell proliferation in WRO cell. (A) Intracellular production of ROS was assayed by fluorescence analysis using 2′-7′-dichlorofluorescein diacetate (DCFH-DA) in WRO cells transfected with either an unspecific siRNA (Scrambled -sc- siRNA) or the specific NOX-4 siRNA and treated during 1 h with or without IL-δ. (B) Cell viability was analyzed by MTT assay after 48 h of ILδ 10 μM treatment in WRO transfected with either an unspecific siRNA (Scrambled -sc- siRNA) or the specific NOX-4 siRNA. (C) Western blot analysis of PCNA expression was analyzed after 48 h of IL-δ 10 μM treatment in WRO transfected with either an unspecific siRNA (Scrambled -sc- siRNA) or the specific NOX-4 siRNA. Quantification by densitometric scanning of the gel is shown in the left panel and a representative experiment is shown in the right panel. Each value represents the mean ± SEM of four independent experiments. *$p < 0.05$ versus control, **$p < 0.01$ and $p^{***}<0.001$ versus control.

Dugrillon et al., 1994), iodide uptake (Chazenbalk et al., 1988), H_2O_2 production (Krawiec et al., 1988), goiter growth (Pisarev et al., 1988) and inositol-1,4,5-triphosphate (IP3) formation (Dugrillon and Gartner, 1995). It has also been shown that IL-δ and I_2 have antiproliferative effects in neuroblastoma, glioblastoma, melanoma, lung carcinoma cells, mammary carcinoma (MCF-7), colon cancer cell line, HT-29 and prostate cell lines ((Rösner et al., 2010; Thomasz et al., 2013; Aranda et al., 2013). Our studies demonstrated that IL-δ inhibited cell proliferation and induced apoptosis through reactive oxygen species (ROS) in HT-29 cells (Thomasz et al., 2013). It has also been demonstrated that the abnormal production of ROS may trigger cell cycle arrest and apoptosis (Sauer et al., 2001). At this time, we demonstrated that IL-δ induced loss of cell viability on human cancer cell line (WRO) and (TPC-1) in a concentration dependent manner and increased ROS generation. This inhibition on cell proliferation was correlated with a significant decrease in PCNA expression. Nuclear morphological changes revealed that there was a significant percentage of apoptosis, when WRO and TPC-1 cells were exposed to IL-δ. Activation of caspase-3, which is a prominent marker of apoptosis, was also induced by IL-δ. These results are consistent with previous reports which demonstrated that iodine and IL-δ induce apoptosis in *in vitro* studies in several human cancer cell lines (Langer et al., 2003; Arroyo-Helguera et al., 2008; Rosner et al., 2010; Nava-Villalba and Aceves, 2014). Different studies support the notion that the antiproliferative effects of IL-δ are mediated by different pathways. IL-δ can act as a ligand of peroxisome proliferator activator receptor (PPAR) and trigger the caspase apoptosis pathway (Aranda et al., 2013). IL-δ is a specific ligand of PPARs with almost 6-fold higher affinity than AA, and activates specifically the PPAR gamma isoform. These data suggest that IL-δ/PPAR gamma could participate in the anti-proliferative and pro-apoptotic effect of IL-δ. In addition, IL-δ decrease PCNA expression, and it could be due, in part, to an arrest of the cell cycle. In this study, we observed an increase of ROS levels and proposed the ROS mediated pathway to explain this idea, but further studies are required to analyze the molecular mechanism of IL-δ in normal and pathological condition.

It is interesting to mention that since IL-δ uptake does not required NIS transporter nor TPO activity it could be a useful coadjuvant in prevention or cancer treatment.

We decided to analyze the sources of reactive oxygen species induced by IL-δ and to explore the contribution of ROS induced by IL-δ on cell proliferation and apoptosis.

In the present study, we described that treatment with IL-δ increased ROS generation. We observed a decrease in SOD activity and specifically, in WRO cells we observed an increase in catalase activity in response to IL-δ (3 h) treatment which contributes with the decrease of ROS.

As we demonstrated, blocking of ROS production (with a

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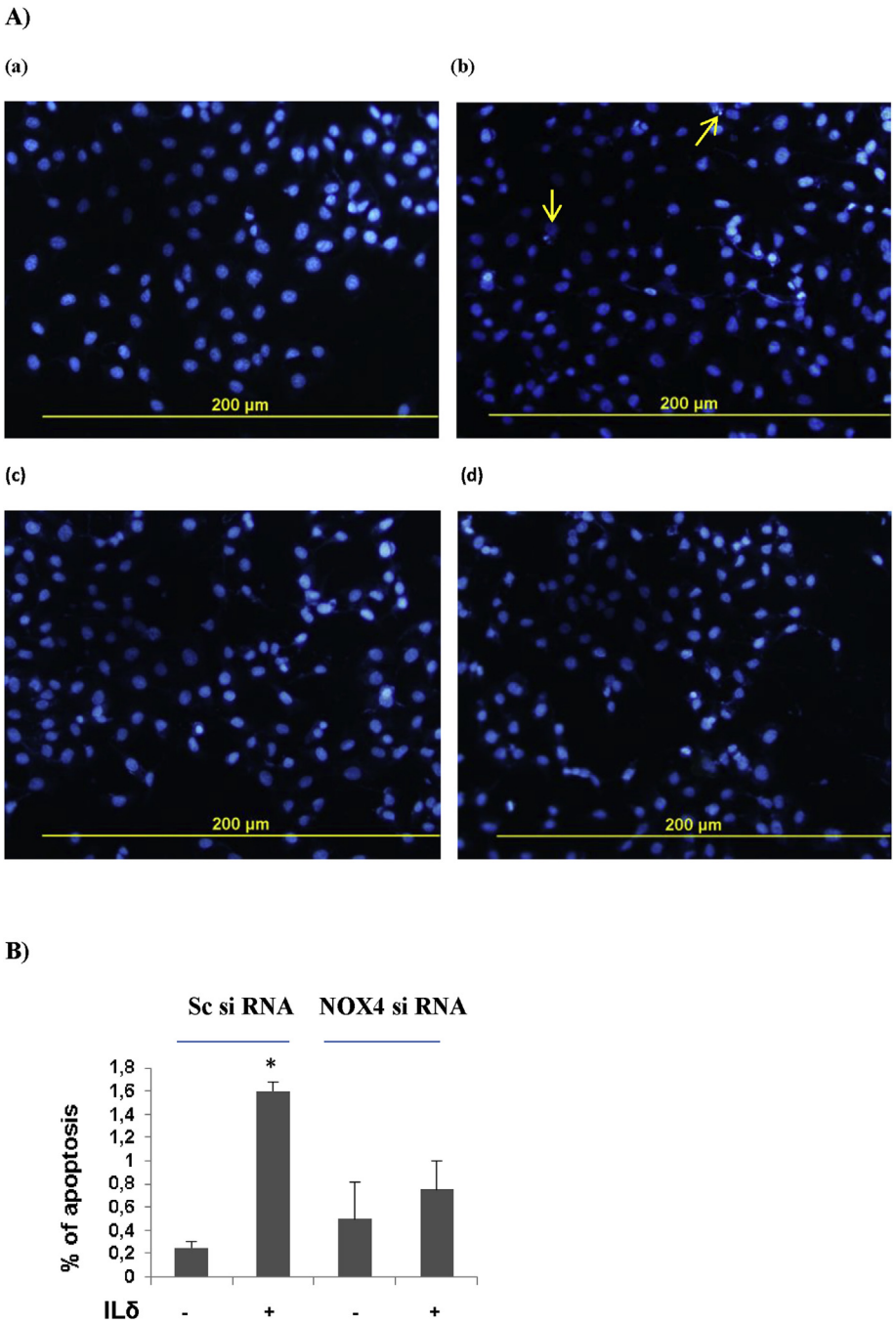

Fig. 7. siRNA target knock-down of NOX-4 attenuates IL-δ induced apoptosis in WRO cell. Cells were transfected with either an unspecific siRNA (Scrambled -sc- siRNA) or the specific NOX-4 siRNA. (A) Morphological changes induced by ILδ (10 µM) after 3 h of treatment. Percentage of cells with nuclear apoptotic morphology was quantified. sc-siRNA (a), sc-siRNA + IL-δ (b), NOX-4 siRNA (c), NOX-4 siRNA + IL-δ (d). Cells were staining with Hoechst and observed by fluorescence microscopy. Magnification X 400. Data are expressed as means ± SEM from four independents experiments * $p < 0.05$ versus control, ** $p < 0.01$ and *** $p < 0.001$ versus control.

scavenger such as Trolox or NAC) resulted in an inhibition of apoptosis and cell survival returned to control levels. It can also be concluded that ROS are a critical mediators of IL-δ on inhibition of cell proliferation and induced cell apoptosis.

In contrast with DUOXs, NOX4 generates H_2O_2 and O_2° in intracellular compartments such as the endoplasmatic reticulum, mitochondria or nucleus and is constitutively active (Leto et al., 2009; Carvalho and Dupuy, 2013). NOX4 is a H_2O_2 generator located inside the thyroid cell and Weyemi et al. (2010) have demonstrated the expression of NOX4 and its partner p22phox in normal and thyroid cancer tissues. They found that NOX4 expression is positively regulated by TSH stimulation, suggesting a role of ROS in TSH signaling.

ROS are overproduced in cancer cells (Quinn et al., 2006; Lambeth, 2007), and it was shown that inhibitors of NOXes decrease cell proliferation (Brar et al., 2002). It was also

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Table 4
Caspase-3 Activity measured as pmol of pNA released/min/ml.

Treatment	Caspase-3 activity
Scrambled si RNA	10.0±0.58
Scrambled si RNA+ILδ	12.7±0.27*
NOX4 si RNA	10.4±0.69
NOX4 si RNA+ILδ	11.1±0.62

siRNA target knock-down of NOX-4 attenuates ILδ induced apoptosis in WRO cell. Cells were transfected with either an unspecific siRNA (Scrambled siRNA) or the specific NOX-4 siRNA. Caspase-3 activity was measured after 3 h of ILδ (10 μM) treatment. Data are expressed as means ± SEM from four independents experiments * $p < 0.05$ versus control.

demonstrated that NOX4 expression is upregulated in thyroid cancer, particularly in papillary thyroid cancers, suggesting that this H_2O_2-generating system could be involved in thyroid cancer development or progression mechanism (Weyemi et al., 2010, 2012; Azouzi et al., 2017).

In our *in vitro* study with WRO cells, inhibition of NADPH oxidase activity with DPI abolished ROS production and subsequent apoptotic cell death in IL-δ treated cells. This suggests that the intracellular ROS generation by NADPH oxidase activations was directly involved in the apoptotic mechanism of IL-δ.

Here, we report that NOX4 protein plays a key role in the overproduction of ROS and in the control of proliferation and apoptosis induced by IL-δ in WRO cells. SiRNA to NOX4 prevent the increase in intracellular ROS, indicating that NOX4 was responsible for ROS generation (Fig. 8).

The data of the present study provide strong evidence that NOX4 is involved in IL-δ induced apoptosis and inhibition of cell proliferation. IL-δ treated WRO cells undergo apoptosis characterized by nuclear morphological changes and caspase-3 activation. The loss of NOX4 expression in WRO cells transfected with the NOX4 siRNA reversed the anti-proliferative effect of IL-δ and markedly reduced the apoptotic events induced by IL-δ.

Inhibition of NADPH oxidase activity with DPI didn't abolish ROS production and IL-δ didn't regulate NOX4 expression in TPC-1 cells. Weyemi et al. (2010) observed a higher level of NOX4 expression in papillary thyroid cancer samples compared with those of follicular thyroid cancer. These results could explain the absence of NOX4 regulation by IL-δ in TPC-1 cells. In these cells, there was also a mitochondrial ROS increase and a reduction of SOD activity. These results suggest that the role of ROS in IL-δ signaling is not mediated through NOXes in the TPC-1 cell line. In fact, different pathway could be acting with different intracellular sources of H_2O_2 production and compartments with specific functions. The anti-proliferative effect of IL-δ could be involving mitochondria-mediated ROS production. These results are in line with others studies demonstrating the antiproliferative/cytotoxic effect of I_2 that involve disruption of the mitochondrial transmembrane potential, a critical event in mitochondrial-mediated apoptosis (Rösner et al., 2010, Shrivastava et al., 2006; Yang et al., 2000). Additionally, the decreased in SOD activity may contribute to the increase in ROS production. Sun et al. (2011), demonstrated that Povidone-iodine induced apoptosis through inhibiting SOD activity (Fig. 8).

At the current state of data, it is most likely that the anti-proliferative effect of IL-δ is mediated, in part, by different mechanisms and pathway involving different sources of ROS generation depending on the cellular or tissue context. The regulation of NOX4 expression by IL-δ in WRO cell and the involvement mitochondria-mediated ROS production in TPC-1 cells may reflect different pathways and ways of regulation of cell proliferation and apoptosis induced by IL-δ.

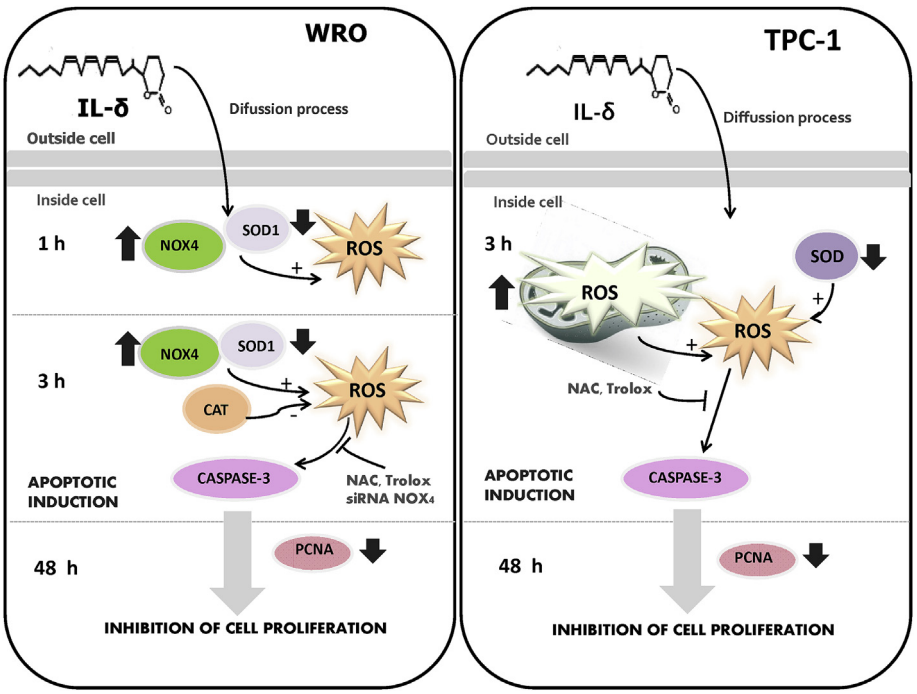

Fig. 8. Schematic representation of inhibitory effects of IL-δ in thyroid cancer cells (left panel: WRO cells, right panel: TPC-1 cells). IL-δ enters to the cell by a diffusion process and increases the ROS levels by different mechanism. In WRO cells there is an increase in NOX-4 expression and a decrease in SOD activity. After 3 h of IL-δ treatment an increase of catalase activity in response to IL-δ was also observed. The increase in ROS levels inhibit cell proliferation and induce cell death by apoptosis. In TPC-1 cells there is an increase of mitochondrial ROS and a decrease in SOD activity which results in ROS imbalance, inhibition of cell proliferation and apoptosis induction. NOX-4: NADPH oxidase 4, NAC: *N*-Acetylcysteine, Trolox: antioxidant, SOD: superoxide dismutase, CAT: catalase.

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In conclusion, ROS mediate IL-δ effects although the source differs depending on the cellular context.

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References

Aceves, C., García-Solís, P., Arroyo-Helguera, O., Vega-Riveroll, L., Delgado, G., Anguiano, B., 2009. Antineoplastic effect of iodine in mammary cancer: participation of 6-iodolactone (6-IL) and peroxisome proliferator activated receptors (PPAR). Mol. Cancer 8, 33–42.

Ameziane-El-Hassani, R., Schlumberger, M., Dupuy, C., 2016. NADPH oxidases: new actors in thyroid cancer? Nat. Rev. Endocrinol. 12, 485–494.

Aranda, N., Sosa, S., Delgado, G., Aceves, C., Anguiano, B., 2013. Uptake and antitumoral effects of iodine and 6-iodolactone in differentiated and undifferentiated human prostate cancer cell lines. Prostate 73, 31–41.

Arroyo-Helguera, O., Anguiano, B., Delgado, G., Aceves, C., 2006. Uptake and antiproliferative effect of molecular iodine in the MCF-7 breast cancer cell line. Endocr. Relat. Cancer 13, 1147–1158.

Arroyo-Helguera, O., Rojas, E., Delgado, G., Aceves, C., 2008. Signaling pathways involved in the antiproliferative effect of molecular iodine in normal and tumoral breast cells: evidence that 6-iodolactone mediates apoptotic effects. Endocr. Relat. Cancer 15, 1003–1011.

Azouzi, N., Cailloux, J., Cazarin, J.M., Knauf, J.A., Cracchiolo, J., Al Ghuzlan, A., Hartl, D., Polak, M., Carré, A., El Mzibri, M., Filali-Maltouf, A., Al Bouzidi, A., Schlumberger, M., Fagin, J.A., Ameziane-El-Hassani, R., Dupuy, C., 2017. NADPH oxidase NOX4 is a critical mediator of BRAFV600E-induced downregulation of the sodium/iodide symporter in papillary thyroid carcinomas. Antioxid. Redox Signal 26, 864–887.

Baboir, B.M., 1999. NADPH oxidase: an update. Blood 93, 1464–1476.

Bedard, K., Krause, K.H., 2007. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol. Rev. 87, 245–313.

Boeynaems, J.M., Hubbard, W.C., 1980. Transformation of arachidonic acid into an iodolactone by the rat thyroid. J. Biol. Chem. 255, 9001–9004.

Brar, S.S., Kennedy, T.P., Sturrock, A.B., Huecksteadt, T.P., Quinn, M.T., Whorton, A.R., Hoidal, J.R., 2002. An NAD(P)H oxidase regulates growth and transcription in melanoma cells. Am. J. Physiol. Cell Physiol. 282, 1212–1224.

Carvalho, D.P., Dupuy, C., 2013. Role of the NADPH oxidases DUOX and NOX4 in thyroid oxidative stress. Eur. Thyroid. J. 2, 160–167.

Chazenbalk, G.D., Valsecchi, R.M., Krawiec, L., Burton, G., Juvenal, G.J., Monteagudo, E., Chester, H.A., Pisarev, M.A., 1988. Thyroid autoregulation. Inhibitory effects of iodinated derivatives of iodinated derivatives of arachidonic acid on iodine metabolism. Prostaglandins 36, 163–172.

Dugrillon, A., Gartner, R., 1995. Delta-iodolactones decrease epidermal growth factor-induced proliferation and inositol-1,4,5-trisphosphate generation in porcine thyroid follicles, a possible mechanism of growth inhibition by iodide. Eur. J. Endocrinol. 132, 735–743.

Dugrillon, A., Uedelhoven, W.M., Pisarev, M.A., Bechtner, G., Gartner, R., 1994. Identification of a δ-iodolactone in iodide treated human goiter and its inhibitory effect on proliferation on human thyroid follicles. Horm. Metab. Res. 26, 465–469.

Dupuy, C., Kaniewski, J., Dème, D., Pommier, J., Virion, A., 1989. NADPH-dependent H_2O_2 generation catalyzed by thyroid plasma membranes. Studies with electron scavengers. Eur. J. Biochem. 185, 597–603.

Dupuy, C., Ohayon, R., Valent, A., Noel-Hudson, M.S., Deme, D., Virion, A., 1999. Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cDNAs. J. Biol. Chem. 274, 37265–37269.

Ekholm, R., Bjorkman, U., 1997. Glutathione peroxidase degrades the intracellular hydrogen peroxide and thereby inhibits intracellular protein iodination in thyroid ephitelium. Endocrinology 138, 2871–2878.

Krawiec, L., Chazenbalk, G.D., Puntarulo, S.A., Burton, G., Boveris, A., Valsecchi, R.M., Pisarev, M.A., 1988. The inhibition of PB125I formation in calf thyroid caused by 14-iodo-15-hydroxy-eicosatrienoic acid is due to decreased H_2O_2 availability. Horm. Metab. Res. 20, 86–90.

Lambeth, J.D., 2007. Nox enzymes, ROS, and chronic disease: an example of antagonistic pleiotropy. Free Radic Biol. Med. 43, 332–347.

Langer, R., Burzler, C., Bechtner, G., Gartner, R., 2003. Influence of iodide and iodolactones on thyroid apoptosis. Evidence that apoptosis induced by iodide is mediated by iodolactones in intact porcine thyroid follicles. Exp. Clin. Endocrinol. Diabetes 111, 325–329.

Leseney, A.M., Dème, D., Legué, O., Ohayon, R., Chanson, P., Sales, J.P., Carvalho, D.P., Dupuy, C., Virion, A., 1999. Biochemical characterization of a Ca 2+/NAD(P)H-dependent H_2O_2 generator in human thyroid tissue. Biochimie 81, 373–380.

Leto, T.L., Morand, S., Hurt, D., Ueyama, T., 2009. Targeting and regulation of reactive oxygen species generation by Nox family NADPH oxidases. Antioxid. Redox Signal 11, 2607–2619.

Monteagudo, E.S., Caro, H.N., Veleiro, A.S., Pisarev, M.A., Burton, G., 1990. Synthesis and characterization of iodinated derivatives of arachidonic acid. Anales de la Asociación de Química de Argentina 78, 31–36.

Nava-Villalba, M., Aceves, C., 2014. 6-iodolactone, key mediator of antitumoral properties of iodine. Prostagl. Other Lipid Mediat. 112, 24–33.

Nava-Villalba, M., Nuñez-Anita, R.E., Bontempo, A., Aceves, C., 2015. Activation of peroxisome proliferator-activated receptor gamma is crucial for the antitumor effect of 6-iodolactone. Mol. Cancer 14, 168–172.

Ohye, H., Sugawara, M., 2010. Dual oxidase, hydrogen peroxide and thyroid diseases. Exp. Biol. Med. 235, 424–433.

Panneels, V., Juvenal, G., Boeynaems, J.M., Dumont, J.E., Van Sande, J., 2009. Iodide effects on the thyroid. In: Preedy, V.R., Burrow, G.N., Watson, R. (Eds.), Comprehensive Handbook on Iodine: Nutritional, Endocrine and Pathological Aspects. Oxford Academic Press, pp. 99305–99316.

Pisarev, M.A., Gartner, R., 2000. Autoregulatory action of iodine. In: Braverman, L.E., Uitger, R.D. (Eds.), The Thyroid, 9th ed. Lippincott, Philadelphia, pp. 85–90.

Pisarev, M.A., Chazenbalk, G.D., Valsecchi, R.M., Burton, G., Krawiec, L., Monteagudo, E., et al., 1988. Thyroid autoregulation. Inhibition of goiter growth and of cAMP formation in rat thyroid by iodinated derivatives of arachidonic acid. J. Endocrinol. Invest. 1, 669–674.

Pisarev, M.A., Bocanera, L.V., Chester, H.A., Juvenal, G.J., Pregliasco, L.B., Krawiec, L., 1992. Effect of iodoarachidonates on thyroid FRTL-5 cells growth. Horm. Metab. Res. 24, 558–561.

Quinn, M.T., Ammons, M.C., Deleo, F.R., 2006. The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. Clin. Sci. 111, 1–20.

Rigutto, S., Hoste, C., Grasberger, H., Milenkovic, M., Communi, D., Dumont, J.E., Corvilain, B., Miot, F., De Deken, X., 2009. Activation of dual oxidases Duox1 and Duox2: differential regulation mediated by camp-dependent protein kinase and protein kinase C-dependent phosphorylation. J. Biol. Chem. 284, 6725–6734.

Rösner, H., Torremante, P., Möller, W., Gärtner, R., 2010. Antiproliferative/cytotoxic activity of molecular iodine and iodolactones in various human carcinoma cell lines. No interfering with EGF-signaling, but evidence for apoptosis. Exp. Clin. Endocrinol. Diabetes 118, 410–419.

Rösner, H., Wolfgang, M., Groebner, S., Torremante, P., 2016. Antiproliferative/cytotoxic effects of molecular iodine, povidone-iodine and Lugol's solution in different human carcinoma cell lines. Oncol. Lett. 12, 2159–2162.

Sauer, H., Wartenberg, M., Hescheler, J., 2001. Reactive oxygen species as intracellular messengers during cell growth and differentiation. Cell Physiol. Biochem. 11, 173–186.

Shrivastava, A., Tiwari, M., Sinha, R.A., Kumar, A., Balapure, A.K., Bajpai, V.K., Sharma, R., Mitra, K., Tandon, A., Godbole, M.M., 2006. Molecular iodine induces caspase-independent apoptosis in human breast carcinoma cells involving mitochondria-mediated pathway. J. Biol. Chem. 28, 19762–19771.

Song, Y., Driessens, N., Costa, M., De Deken, X., Detours, V., Corvilain, B., Maenhaut, C., Miot, F., Van Sande, J., Many, M.C., Dumont, E., 2007. Roles of hydrogen peroxide in thyroid physiology and disease. J. Clin. Endocrinol. Metab. 92, 3764–3773.

Sun, P., Zhao, J.M., Luo, Z.C., Zhang, P., Chen, P., Zhang, X.L., Luo, S., Yang, D.B., Tan, J., Zhou, Y., Liu, T., Liu, Y.F., Wen, Y., 2011. Diluted povidone-iodine inhibits tumor growth through apoptosis-induction and suppression of SOD activity. Oncol. Rep. 27, 383–388.

Thomasz, L., Oglio, R., Randi, A.S., Fernandez, M., Dagrosa, M.A., Cabrini, R.L., Juvenal, G.J., Pisarev, M.A., 2010a. Biochemical changes during goiter induction by methylmercaptoimidazol and inhibition by delta-iodolactone in rat. Thyroid 20, 1003–1013.

Thomasz, L., Oglio, R., Dagrosa, M.A., Krawiec, L., Pisarev, M.A., Juvenal, G.J., 2010b. 6 Iodo-delta-lactone reproduces many but not all the effects of iodide. Mol. Cell. Endocrinol. 323, 161–166.

Thomasz, L., Oglio, R., Rossich, L., Villamar, V., Perona, M., Salvarredi, Pisarev, M.A., Juvenal, G.J., 2013. 6 Iodo-d-lactone: a derivative of arachidonic acid with antitumor effects in HT-29 colon cancer cells. Prostagl. Leukot. Essent. Fat. Acids 88, 273–280.

Turk, J., Henderson, W.R., Klebanoff, S.J., Hubbard, W.C., 1983. Iodination of arachidonic acid mediated by eosinophil peroxidase, myeloperoxidase and lactoperoxidase. Identification and comparison of products. Biochim. Biophys. Acta 751, 189–200.

Weyemi, U., Caillou, B., Talbot, M., Ameziane-El-Hassani, R., Lacroix, L., Lagent-Chevallier, O., Al Ghuzlan, A., Roos, D., Bidart, J.M., Virion, A., Schlumberger, M., Dupuy, C., 2010. Intracellular expression of reactive oxygen species-generating NADPH oxidase NOX4 in normal and cancer thyroid tissues. Endocr. Relat. Cancer 17, 27–37.

Weyemi, U., Lagente-Chevallier, O., Boufraqech, M., Prenois, F., Courtin, F., Caillou, B., Talbot, M., Dardalhon, M., Al Ghuzlan, A., Bidart, J.M., Schlumberger, M., Dupuy, C., 2012. ROS-generating NADPH oxidase NOX4 is a critical mediator in oncogenic H-Ras-induced DNA damage and subsequent senescence. Oncogene 31, 1117–1129.

Yang, C.F., Shen, H.M., Ong, C.N., 2000. Intracelular thiol depletion causes mitochondrial permeability transition in ebselen-induced apoptosis. Arch. Biochem. Biophys. 380, 319–330.

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