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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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