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Effects of 2-iodohexadecanal in the physiology of thyroid cells

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ABSTRACT

Iodide has direct effects on thyroid function. Several iodinated lipids are biosynthesized by the thyroid and they were postulated as intermediaries in the action of iodide. Among them, 2-iodohexadecanal (2-IHDA) has been identified and proposed to play a role in thyroid autoregulation. The aim of this study was to compare the effect of iodide and 2-IHDA on thyroid cell physiology. For this purpose, FRTL-5 thyroid cells were incubated with the two compounds during 24 or 48 h and several thyroid parameters were evaluated such as: iodide uptake, intracellular calcium and H_2O_2 levels. To further explore the molecular mechanism involved in 2-IHDA action, transcript and protein levels of genes involved in thyroid hormone biosynthesis, as well as the transcriptional expression of these genes were evaluated in the presence of iodide and 2-IHDA. The results obtained indicate that 2-IHDA reproduces the action of excess iodide on the "Wolff-Chaikoff" effect as well as on thyroid specific genes transcription supporting its role in thyroid autoregulation.

1. Introduction

Iodide plays an important role in thyroid growth and physiology. Not only it is essential for thyroid hormone biosynthesis but also directly influences a number of thyroid parameters such as thyroid proliferation and function (Dumont et al., 1992). Inhibitory actions of iodide include those on iodide organification (Wolff-Chaikoff effect), hormone secretion, cyclic 3′,5′-adenosine monophosphate generation, thyroglobulin proteolysis, glucose and aminoacid transport, protein and RNA biosynthesis, thyroid blood flow, thyroid growth, etc., (Pisarev and Gartner, 2000; Panneels et al., 2009; Gérard et al., 2009). The capability of the intracellular iodide levels to modulate thyroid function has been termed **thyroid autoregulation.**

Since most of the inhibitory effects of iodide on thyroid function and growth are reversed by the thionamide drugs, which block the action of thyroid peroxidase, methylmercaptoimidazole (MMI) or propylthiouracil (PTU), it was proposed that an organic iodocompound, called XI, might be the mediator in the autoregulatory mechanism (Van Sande et al., 1975). The nature of the candidate proposed for XI has been the subject of extensive research. Different compounds have been proposed to be this mediator, such as iodinated peptides (Lissitzky et al., 1961) including Tg (Sellitti and Suzuki, 2014) or T_3 (Juvenal et al., 1981).

The biosynthesis of iodolipids has been observed in the thyroid gland of several species and their participation in thyroid autoregulation has been suggested. Boeynaems and Hubbard (1980) have reported that the addition of iodide and arachidonic acid to rat thyroid tissue resulted in the formation and release of 5-hydroxy-6 iodo-8, 11, 14-eicosatrienoic delta lactone (IL-δ). In addition, Dugrillon et al. (1994) demonstrated that IL-δ is synthesized by the human thyroid gland. In 1990 Pereira et al. demonstrated α-iodohexadecanal (2-IHDA) as the major iodolipid formed when horse thyroid slices were incubated *in vitro* with radioiodide (Pereira et al., 1990). These results were confirmed in dog and rat thyroid tissue (Panneels et al., 1996). Interestingly, both IL-δ and 2-IHDA mimic some inhibitory effects of iodide on several thyroid parameters (Pisarev and Gartner, 2000; Panneels et al., 2009).

The aim of the present study was to systematically analyze the

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effect of 2-IHDA on the regulation of several thyroid parameters involved in thyroid hormone synthesis.

2. Material and methods

2.1. Cell culture

FRTL-5 rat thyroid cells (ATTC CRL-8305) were kindly provided by Dr. L.D. Kohn and the Interthyr Research Foundation (MD, USA). Cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, 50:50 v/v) (GIBCO, Invitrogen Corporation, USA) supplemented with 5% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina), bovine TSH (1 mU/mL), hydrocortisone (3.62 ng/mL), transferrin (5μg/mL), insulin (10 mg/mL), somatostatine (10 ng/mL) and glycil-L-histidyl-L-lysine acetate (10 ng/mL) (6H media). Cell cultures were kept at 37 °C in 5% CO_2-95% air atmosphere in a humidified incubator. When cells reached confluence, they were cultured in the same media except without TSH (5H media). After 3 days of quiescence, cells were incubated with the different compounds for the time indicated in the text.

2.2. Iodide uptake and efflux

Cells were incubated in the absence (control) or the presence of TSH (500 μU/ml), TSH plus KI (5-30 μM) or TSH plus 2-IHDA (5-30 μM) for 24 and 48 h. Cells were washed and further incubated in DMEM/F12 media containing 1 μM NaI supplemented with 0.5 μCi carrier free $Na^{125}I$ (17Ci/mg, Perkin Elmer) at 37 °C. After 30 min, cells were washed rapidly with ice-cold buffered HBSS plus 1 μM NaI. Nonspecific iodide accumulation was determined in the presence of 1 mM $KClO_4$ and this value has been subtracted from the values presented. Cells were solubilized with 0.5 mL of 0.1 N NaOH and its radioactivity was measured. Results are expressed as dpm/min/μg protein. Protein content was measured using the Lowry method. To measure iodide efflux, after iodide load, media was removed and replaced every 2 min with 0.5 mL of fresh buffered HBSS, containing 1 μM NaI. Results are presented as percentage of the total radioactivity remaining at the indicated times.

2.3. ^{3}H-2-deoxy-glucose uptake

Glucose uptake was measured as previously described (Krawiec et al., 1991) with minor modifications. Cells were washed with ice-cold glucose-free Krebs-Ringer-Hepes (KRH) buffer and incubated in KRH buffer supplemented with 0.5 μCi of 2-(1,2-^{3}H)-deoxy-D-glucose (^{3}H-DOG) (30.2 Ci/mmol, Perkin Elmer) and 100 μM deoxyglucose for 20 min at 37 °C. The incubations were stopped by washing the cells three times with ice-cold KRH containing 100 mM glucose. Nonspecific uptake was determined by addition of 100 mM glucose to the incubation buffer. Cells were solubilized in 0.1 N NaOH. Aliquots were taken for measurement of total radioactivity and referred to protein content determined by the Lowry method.

2.4. H_2O_2 production

H_2O_2 was determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, USA). Briefly, two hundred microliters of the Amplex Red reaction mixture (100 μM Amplex Red and 0.2 U/ml horse radish peroxidase in phosphate buffered saline, pH 7.4 (PBS)) was added to the cells following a short incubation period at 37 °C. Amplex Red conversion to resorufin was measured at emission of 595 nm (excitation 485 nm) using a DTX 880 Beckman Coulter microplate reader. Results are reported in arbitrary fluorescence units normalized to the protein content.

2.5. Intracellular calcium levels

Cells were treated with Fura 2-AM (Molecular Probes, Invitrogen, USA) for 30 min at 4 °C and then washed three times with PBS. Fluorescence was monitored at 340 nm excitation and 525 nm emission wavelengths using DTX 880 Beckman Coulter microplate reader. Results are reported in arbitrary fluorescence units normalized to the protein content.

2.6. Western blot

Total proteins were extracted in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 0.5% deoxycholate), supplemented with PMSF 0.5 mM and protease inhibitor cocktail (Sigma-Aldrich).

Total proteins (30 μg) were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes (Nazar et al., 2012). Membranes were blocked with 5% non-fat dry milk in PBS containing 0.2% Tween 20 (Sigma) for 1 h at RT and then incubated overnight at 4 °C with polyclonal anti-Tg (A0251; DakoCytomation. Dilution 1:10,000), polyclonal anti-NIS (kindly provided by Dr. Nancy Carrasco. Dilution 1:5000), monoclonal anti-TPO (sc-58432; Santa Cruz. Dilution 1:300), polyclonal anti-DUOX2 (sc-134442; Santa Cruz. Dilution 1:200), monoclonal anti-Pax8 (sc-81353; Santa Cruz. Dilution 1:150), polyclonal anti-NKX2-1 (sc-13040; Santa Cruz. Dilution 1:150), polyclonal anti-FOXE1 (sc-16392; Santa Cruz. Dilution 1:150), polyclonal anti-β-actin (sc-1616; Santa Cruz. Dilution 1:500) antibodies Membranes were washed, incubated for 1 h at RT with peroxidase-labeled secondary anti-rabbit or anti-mouse secondary antibodies (1:5000; Santa Cruz), and visualized with the enhanced chemiluminescence method. Densitometric analysis was performed using the ImageJ Software (National Institute of Health, USA) and results were corrected for protein loading by normalization for β-actin expression.

2.7. RNA extraction and real-time PCR

Extraction and purification of total RNA were performed using the TRIzol reagent. 2.5 μg RNA was used as a template for cDNA synthesis with Oligo$(dT)_{20}$ using the Superscript III reverse transcriptase (Invitrogen). cDNA (1 μl, 1/10 dilution) was used in each PCR reaction in a total volume of 25 μl, with specific primers for the target molecules and the Real Time Master Mix (Promega). Real-time PCR was carried out using a Rotor-gene Q analyzer (Qiagen). Specific primers pairs for each gene analyzed and temperature of annealing are listed below: *Nis*: forward 5′-GCTGTGGCATTGTCATGTTC-3′ and reverse 5′-TGAGGTCTTCCACAGTCACA-3′ (57 °C); *Tg*: forward 5′-GAATTGCTGGCAGATGTTCAG-3′and reverse 5′-GGGCACTGAGCTCCTTGTAG-3′ (58 °C); *Tpo*: forward 5′-TCTGGCATCACTGAACTTGC-3′and reverse 5′-CGGTGTTGTCACAGATGACC-3′ (61 °C); *Duox1*: forward 5′-GGCCATCAGTATGTCTTCG-3′ and reverse 5′-CGCTGATCTCCACCTTCTTC-3′ (61 °C); *Duox2*: forward 5′-CAAATCGTCCATGGGTGCC-3′and reverse 5′-TCCACAGTTGTCAGAAATAG-3′ (54 °C); *Nkx2-1*: forward 5′-GGTGCCCTCTGGCCCTATAG-3′ and reverse 5′-GCTTGTCGATGATGCCTTTT-3′ (57 °C); *Foxe1*: forward 5′-TTCGTGTCTGCCATGTGAGC-3′ and reverse 5′-GCCCACGTCCAAAGCAAATC-3′ (61 °C); *Pax8*: forward 5′-GGCCACCAAATCTCTGAGCC-3′ and reverse 5′-TGGGAATCGATGCTCAGTCG-3′ (61 °C); *Gadph*: forward 5′-ACAGCAACAGGGTGGTGGAC-3′ and reverse 5′-TTTGAGGGTGCAGCGAACTT-3′ (57 °C). Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method using *Gadph* as internal control.

2.8. Transfection assays

FRTL-5 cells were grown in 24-well plates to approximately 80% confluency. After 3 days of TSH-starving, cells were transfected using TransFast Transfection Reagent (Promega) as specified by the manufacturer. Cells were cotransfected with 0.25 μg of luciferase reporter-promoter constructs and 0.25 μg of β-galactosidase reporter vector. Luciferase activities were determined using a Luciferase Assay System (Promega), according to the manufacturer's recommended protocol and normalized relative to the levels of β-galactosidase activity. The following promoter constructs were used: pNIS 2.8-Luc (Costamagna et al., 2004), p420-TPO (Ortiz et al., 1999), pTgLuc-688 (Shimura et al., 2001), pTx43 (Duox1) and pTx41 (Duox2) (Pachucki et al., 2004), CNS87 (Pax8) (Nitsch et al., 2010), pFOXE1Δ 0 (Brancaccio et al., 2004) and T/EBP -4.11 (Nkx2-1) (Oguchi and Kimura, 1998).

2.9. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described (Nicola et al., 2010). Briefly, cells were fixed in 1% formaldehyde for 10 min; nuclei were obtained and lysed in immunoprecipitation (IP) buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% NP-40, 1.0% Triton X-100 and protease inhibitors cocktail]. The lysates were sonicated at 4 °C under conditions determined empirically to yield DNA fragments of an average size of about 500 bp. Supernatants were diluted 5-fold in IP buffer and precleared by adding salmon sperm DNA-saturated Protein A/G Plus Agarose (Santa Cruz) for 30 min at 4 C. Precleared chromatin was immunoprecipitated with 3 μg non-specific control IgG or 3 μg specific antibody directed against PAX8 (sc-377181; Santa Cruz), FOXE1 (sc-16391; Santa Cruz), or NKX2-1 (sc-13040; Santa Cruz). Immune complexes were allowed to bind to salmon sperm DNA-saturated Protein A/G PLUS-Agarose during 4 h at 4 °C under rotation. Immunoprecipitates were washed and DNA was purified using Chelex-100 (Bio-Rad Laboratories, Hercules, CA). DNA was quantitated by qPCR. Relative fold increases were calculated according to the equation: $2^{-[(Ct.input - Ct.target) - (Ct.input - Ct.mock)]}$. Specific primers pairs for each gene analyzed and temperature of annealing are listed: *Nis* proximal promoter: forward 5′-TGAGCCGTCCATAGCCTTTC-3′and reverse 5′-GCACCCTTTCGGTCCTGG-3′ (57 °C); *Nis* NUE enhancer: forward 5′-GCGCACTCCATCCAGATCAT-3′ and reverse 5′-TTGGCCTCTTGGTGGTGAAG-3′ (57 °C); *Tg*: forward 5′-CTAGCCTCACATTTCTTGTCCCC-3′ and reverse 5′-GGGGTAGGACCAGTGTCCCATCT-3′ (54 °C); *Tpo* forward 5′-TGCTAAGTCTGGGCTGTGTG-3′ and reverse 5′- GGACAAGAAAGGGACCTGGG-3′ (55 °C); *Duox2* foward: 5′-AAGTGCATCCAGGAAGGTG-3′ and reverse 5′-TGAACATCCTGGGCAGGAGTG-3′ (54 °C); *Pax8* enhancer: forward 5′-TGAACTATCCAGCATCGCACA-3′ and reverse 5′-AGTGTTCAAGCTGGGGCTTT-3′ (55 °C).

2.10. 2-IHDA synthesis

2-IHDA was prepared according to the procedures described by Ohayon et al. (1994).

2.11. Statistical analysis

Statistical analyses were performed using ANOVA followed by Student-Newman-Keuls test for multiple comparisons. Data are expressed as mean ± SEM. Differences were considered significant at $P < 0.05$.

3. Results

3.1. 2-IHDA inhibits iodide and glucose uptake

Table 1 shows that 2-IHDA inhibited radioiodide uptake in a concentration-dependent manner. 2-IHDA at concentrations of 30, 10 and 5 μM reduced radioiodide uptake by 88%, 51% and 44% at 24 h of incubation, respectively. Under similar conditions KI caused a reduction of radioiodide uptake of 65% at 30 μM, 45% at 10 μM; while at 5 μM the reduction was 43%. After 48 h of treatment, the inhibitory effect was more pronounced, being 90% (30 μM), 71% (10 μM) and 60% (5 μM) for 2-IHDA while KI showed a similar, although less pronounced, effect than the iodolipid.

Because the intracellular concentration of iodide involves a dynamic balance between influx and efflux processes, we studied the effect of 2-IHDA on this last parameter. An increase of about 20% at early time-points (2 and 4 min) was observed when cells were incubated with 10 and 30 μM of 2-IHDA (Fig. 1 A). No significant effect was observed with 5 μM of 2-IHDA. KI increased slightly the rate of radioiodide efflux although the change did not reach statistical significance (Fig. 1 B).

To determine whether the effect of 2-IHDA on radioiodide uptake could reflect a more generalized effect on the plasma membrane transport, we studied its effect on ^{3}H-DOG uptake. Table 2 shows that 2-IHDA inhibited ^{3}H-DOG uptake, in a time and concentration-dependent manner. Although KI showed the same inhibitory effect, the percentage of inhibition was smaller than that of 2-IHDA. At the highest concentrations 2-IHDA completely abolished the effect of TSH on ^{3}H-DOG uptake at 24 h of incubation. At 48 h, 2-IHDA-treated cells showed lower ^{3}H-DOG uptake than control cells.

3.2. Calcium levels and H_2O_2 production are modulated by 2-IHDA

An important effect of TSH on thyroid hormone synthesis involves the stimulation of calcium intracellular levels and H_2O_2 production, which are required for the covalent incorporation of iodide into thyroglobulin. The addition of low concentrations of 2-IHDA stimulated intracellular calcium levels by 52% compared to TSH-treated cells (Table 3A). Regarding H_2O_2 production, low concentrations of 2-IHDA stimulated its generation, 72.4% (5 μM), 54.2% (10 μM), while the highest concentration had an inhibitory effect, 50.7% (30 μM) (Table 3B). KI showed a similar, although less pronounced effect than 2-IHDA (Table 3 A and B).

Table 1
Effects of different concentrations of KI and 2-IHDA on iodide uptake in FRTL-5 cells.

	I^- uptake (pmol/μg protein)	
	24 h	48 h
Control	3.18 ± 0.23	5.04 ± 1.10
TSH (T)	15.73 ± 1.01$^{°°°}$	35.98 ± 2.99$^{°°°}$
T + 2-IHDA 5 μM	10.20 ± 1.33***	17.35 ± 0.81***
T + 2-IHDA 10 μM	9.35 ± 0.41***	12.84 ± 0.55***
T + 2-IHDA 30 μM	4.68 ± 0.37***	8.06 ± 0.80***
T + KI 5 μM	10.39 ± 0.46***	19.21 ± 1.60***
T + KI 10 μM	10.05 ± 0.85***	17.47 ± 1.22***
T + KI 30 μM	7.60 ± 0.94***	12.97 ± 1.26***

Cells were preincubated for 24 or 48 h with the different compounds, and the iodide uptake was measured as described in Material and Methods. Each value is the average of 5 experimental determinations by quadruplicate. Results are expressed as the mean ± SEM of each group. $^{°°°}P < 0.001$ vs control (absence of TSH); $^{***}P < 0.001$, vs. TSH.

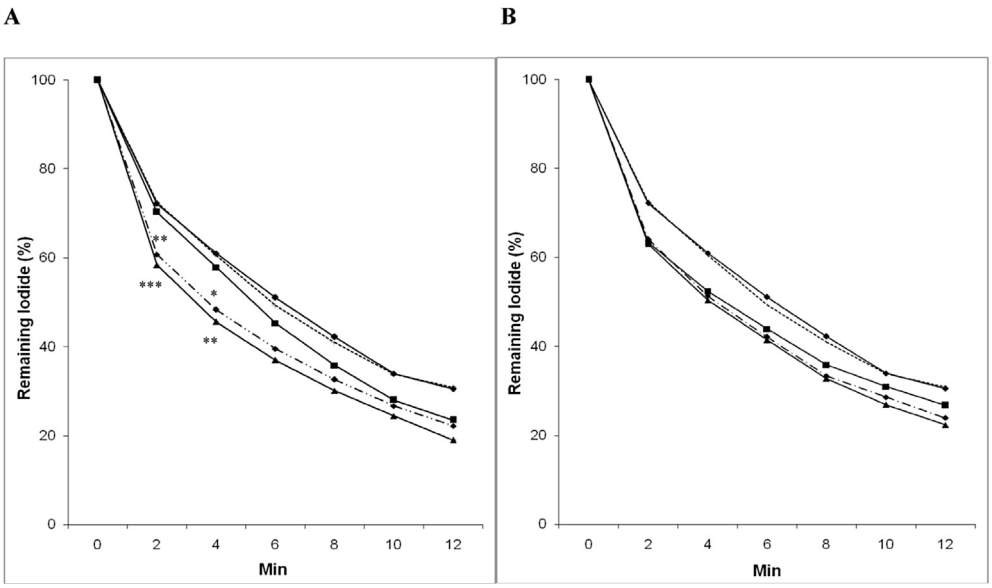

Fig. 1. 2-IHDA affects the efflux of I^- from FRTL-5 cells. Cells were incubated for 48 h in 5H (control, ——◆——), 6H medium (TSH, - - - -), 6H with 2-IHDA (**A**) (5 μM ——■——; 10 μM –··–◆–··–; 30 μM ——▲——) or KI (**B**) (5 μM ——■——; 10 μM –··–◆–··–; 30 μM ——▲——). I^- efflux was measured as described in Materials and Methods. Each point is the average of close triplicates. *P < 0.05, **P < 0.01, ***P < 0.001, vs. TSH.

3.3. 2-IHDA regulates the expression of genes involved in thyroid hormones synthesis

To further explore the molecular mechanism involved in 2-IHDA effect on thyroid function, mRNA levels of genes coding for proteins involved in thyroid hormone synthesis were measured by RT-qPCR. Fig. 2 and Table S1 show that 2-IHDA strongly inhibits the TSH-stimulated mRNA expression of NIS, TPO, Tg, and Duox1 in a concentration-dependent manner. Moreover, low concentrations of 2-IHDA (5 μM) significantly increase Duox2 mRNA expression (754.8%), while high concentrations of 2-IHDA (30 μM) reduced Duox2 mRNA expression. KI showed similar but less pronounced effects to those of 2-IHDA.

Since the transcription factors PAX8, NKX2-1 and FOXE1 are involved in the regulation of thyroid specific genes expression, their mRNA levels were measured at 48 h of 2-IHDA and KI treatment. As shown in Fig. 2, 2-IHDA diminished the expression of *Foxe-1* and *Pax 8* while it did not significantly affect the levels of *Nkx2-1*. At 24 h a similar effect was observed, although less pronounced (results not shown); nevertheless, a stimulation of *Nkx2-1* by 2-IHDA was found: 82.6% (10 μM) and 119.6% (30 μM).

Additionally, we determined whether the changes in mRNAs levels in response to 2-IHDA correlated with changes in protein levels. Western blot analysis showed that TSH treatment increases the protein levels of NIS, TPO, Tg, Pax8 and FOXE 1, but not for NKX2-1 and DUOX2. Consistently, 2-IHDA inhibited protein expression in response to TSH except on DUOX2 and NKX2-1 in which the effects were concentration dependent (Fig. 3). No effects were observed on β-actin levels.

Table 2
Effects of different concentrations of KI and 2-IHDA on [^{3}H] 2-Deoxy-D-glucose uptake.

	^{3}H DOG uptake (d.p.m./μg protein)	
	24 h	48 h
Control	11.38 ± 0.32	23.88 ± 0.58
TSH (T)	14.72 ± 0.40$^{°°°}$	36.33 ± 0.67$^{°°°}$
T + 2-IHDA 5 μM	13.03 ± 0.39*	33.19 ± 0.51**
T + 2-IHDA 10 μM	11.34 ± 0.28***	30.77 ± 0.49***
T + 2-IHDA 30 μM	9.09 ± 0.29***	18.23 ± 0.29***
T + KI 5 μM	13.11 ± 0.41*	33.48 ± 0.58*
T + KI 10 μM	12.43 ± 0.37*	31.05 ± 0.61***
T + KI 30 μM	11.84 ± 0.31	26.88 ± 0.58***

Each value is the average of 3 experimental determinations by quadruplicate. Results are expressed as the mean ± SEM of each group. $^{°°°}$P < 0.001 vs control (absence of TSH); *P < 0.05, **P < 0.01, ***P < 0.001, vs. TSH.

Table 3
Effects of different concentrations of KI and 2-IHDA on Ca^{2+} and H_2O_2 production in FRTL-5 cells.

	%
A	
Control	100.0
TSH (T)	75.4 ± 6.9
T + 2-IHDA 5 μM	114.6 ± 4.2**
T + 2-IHDA 10 μM	102.5 ± 7.5
T + 2-IHDA 30 μM	75.4 ± 4.1
T + KI 5 μM	107.5 ± 7.4*
T + KI 10 μM	100.7 ± 4.6
T + KI 30 μM	93.5 ± 7.2
B	
Control	100.0
TSH (T)	81.8 ± 1.1
T + 2-IHDA 5 μM	141.1 ± 4.2***
T + 2-IHDA 10 μM	126.2 ± 7.5***
T + 2-IHDA 30 μM	40.3 ± 4.1***
T + KI 5 μM	121.4 ± 7.7***
T + KI 10 μM	108.2 ± 1.6*
T + KI 30 μM	76.9 ± 3.5

Cells were preincubated for 48 h with the different compounds, and Ca^{2+} **(A)** and H_2O_2 **(B)** was measured as described in Materials and Methods. Each value is the average of 3 experimental determinations by triplicate. Results are expressed as the mean ± SEM of each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. TSH.

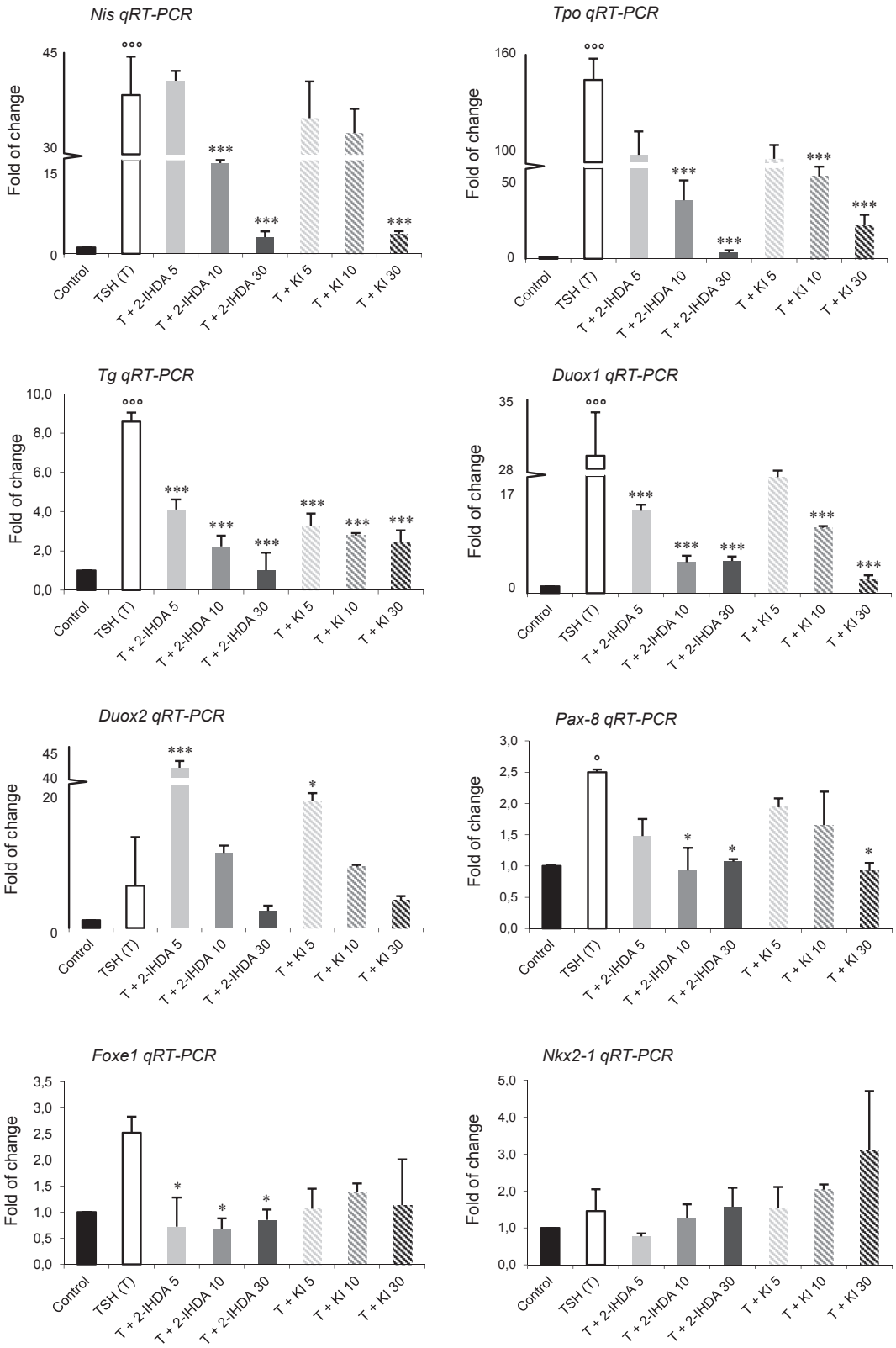

Fig. 2. KI and 2-IHDA affects the expression of thyroid specific genes. Cells were preincubated for 48 h with the different compounds, and the expression of thyroid specific genes was measured as described in Section 2. Relative expression values indicate the relative change in mRNAs expression levels, evaluated by qRT-PCR analysis, between treated cells and control cells, normalized with glyceraldehyde-3-phosphate dehydrogenase. Each value is the average of 4–5 experimental determinations by triplicate. Results are expressed as the mean ± SEM of each group. °°°P < 0.001, °P < 0.05 vs. control; *P < 0.05, **P < 0.01, ***P < 0.001 vs. TSH.

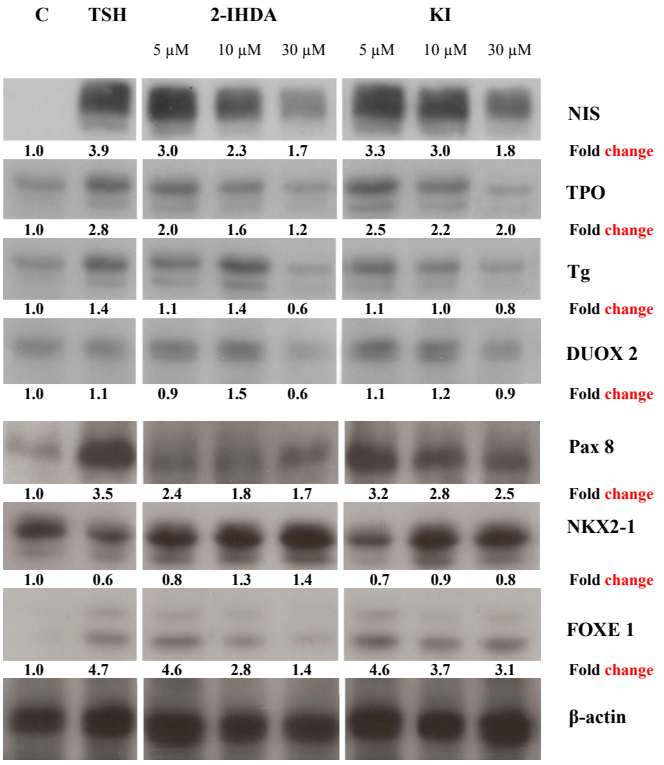

Fig. 3. 2-IHDA regulates thyroid-specific proteins expression. Representative Western Blot of thyroid specific proteins using a specific antibody and antirabbit IgG antibody conjugated with peroxidase. Protein levels were quantified by densitometry scanning of the immunoblots. Values were normalized with an anti-β-actin antibody; control (5H) value was taken as 1.

3.4. 2-IHDA regulates thyroid-specific gene expression at the transcriptional level

To investigate the molecular mechanism involved in the effects of 2-IHDA on the transcriptional regulation of thyroid specific gene expression, we assessed the effect of the iodolipid on the promoter activity of different genes involved in thyroid hormone biosynthesis. Luciferase reporter DNA constructs containing regulatory elements required for TSH response of different genes were transiently transfected into FRTL-5 cells and assayed for transcriptional activity in response to TSH and different doses of 2-IHDA or KI. As shown in Fig. 4 and Table S2, TSH stimulated the promoter activity of *Nis, Tg, Tpo* and *Duox1*, whereas 2-IHDA induced a significant inhibition of the TSH stimulated transcriptional activation in a concentration-dependent manner (Fig. 4). In the case of *Duox2*, TSH stimulated its transcriptional activity but 2-IHDA had a dual effect depending on the concentration assayed, similar to its effect on mRNA levels (Fig. 4). KI had a similar action at high concentrations, although less pronounced.

To further investigate the mechanism by which 2-IHDA regulates the expression of genes involved in thyroid hormonogenesis, we examined the association between the transcription factors Pax8, NKX2-1 and FOXE1 and its promoter regions using ChIP assays. In response to 2-IHDA a decreased binding of Pax8 to the promoter region of different thyroid specific genes was observed (Fig. 5). Conversely, the binding of NKX2-1 was stimulated. Regarding FOXE1, we detected an increased binding to *Tg* and *Tpo* promoters but only at the highest concentration while in *Duox2* promoter an increase was caused by the higher concentration of the iodolipid and a slight inhibition was found with 10 μM. As with the other parameters, KI showed a lower inhibitory effect (Fig. 5 and

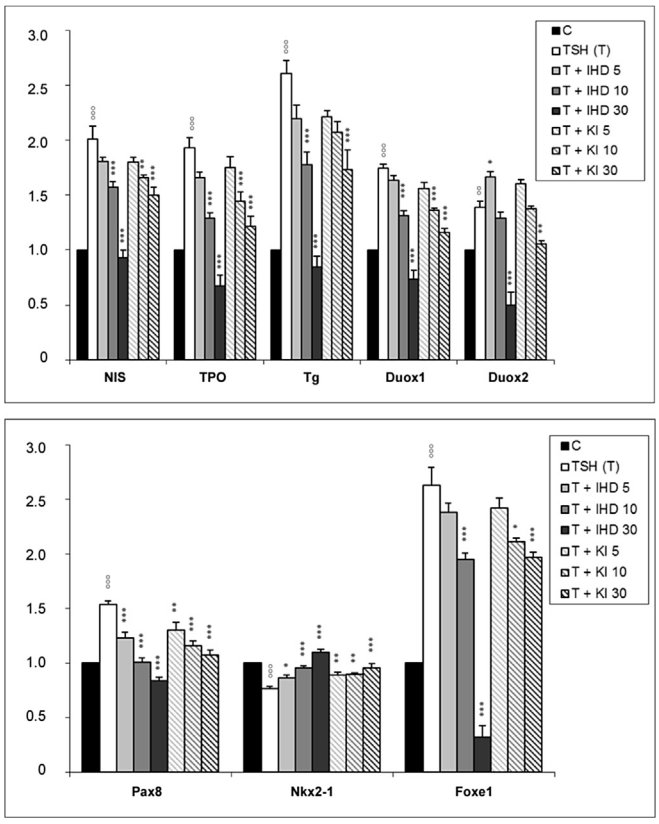

Fig. 4. 2-IHDA modulates thyroid-specific gene promoter activity. FRTL-5 cells were transiently transfected with different thyroid-specific genes promoters constructs linked to the reporter gene luciferase (Luc). Cells were treated with the different compounds as indicated in Materials and Methods for 24 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity (100%) for each construct. Each treatment was done by triplicate. The data shown are the mean ± SEM of 4–6 different experiments. . °°P < 0.01, °°°P < 0.001 vs control; *P < 0.05, **P < 0.01, ***P < 0.001, vs. TSH.

Table S3).

4. Discussion

Iodine is an essential constituent of thyroid hormones but also an important regulator of thyroid follicular cell proliferation and function. Besides the well-known role on the "Wolff-Chaikoff" effect, other functional parameters and thyroid growth are regulated by iodide (Juvenal et al., 2011). Iodolipids have been proposed as the intermediates of iodide action (Table S4). Particularly 2-IHDA has been reported as a potential mediator of the Wolff-Chaikoff effect (Panneels et al., 1994). Our previous results have demonstrated that 2-IHDA has an antigoitrogenic activity, decreasing the intracellular levels of cAMP, thus reducing the number of cells and the glandular epithelial height (Thomasz et al., 2010a). Moreover 2-IHDA has an inhibitory effect on FRTL-5 thyroid cell proliferation mediated by cell cycle arrest and apoptosis (Thomasz et al., 2015).

Our results show that KI and 2-IHDA inhibit radioiodide uptake, although the action of the iodolipid is more potent at equal concentrations. Interestingly we observed that 2-IHDA increased iodide efflux at an early time point. Recently Calil-Silveira et al. (2016) reported that iodide exposure at higher doses than those used here, increased iodide efflux in PCCl3 cells. Pendrin could be involved in this effect since iodide excess increased pendrin expression and its plasma membrane localization. In agreement, we observed that 2-IHDA treatment stimulates pendrin mRNA levels and its promoter

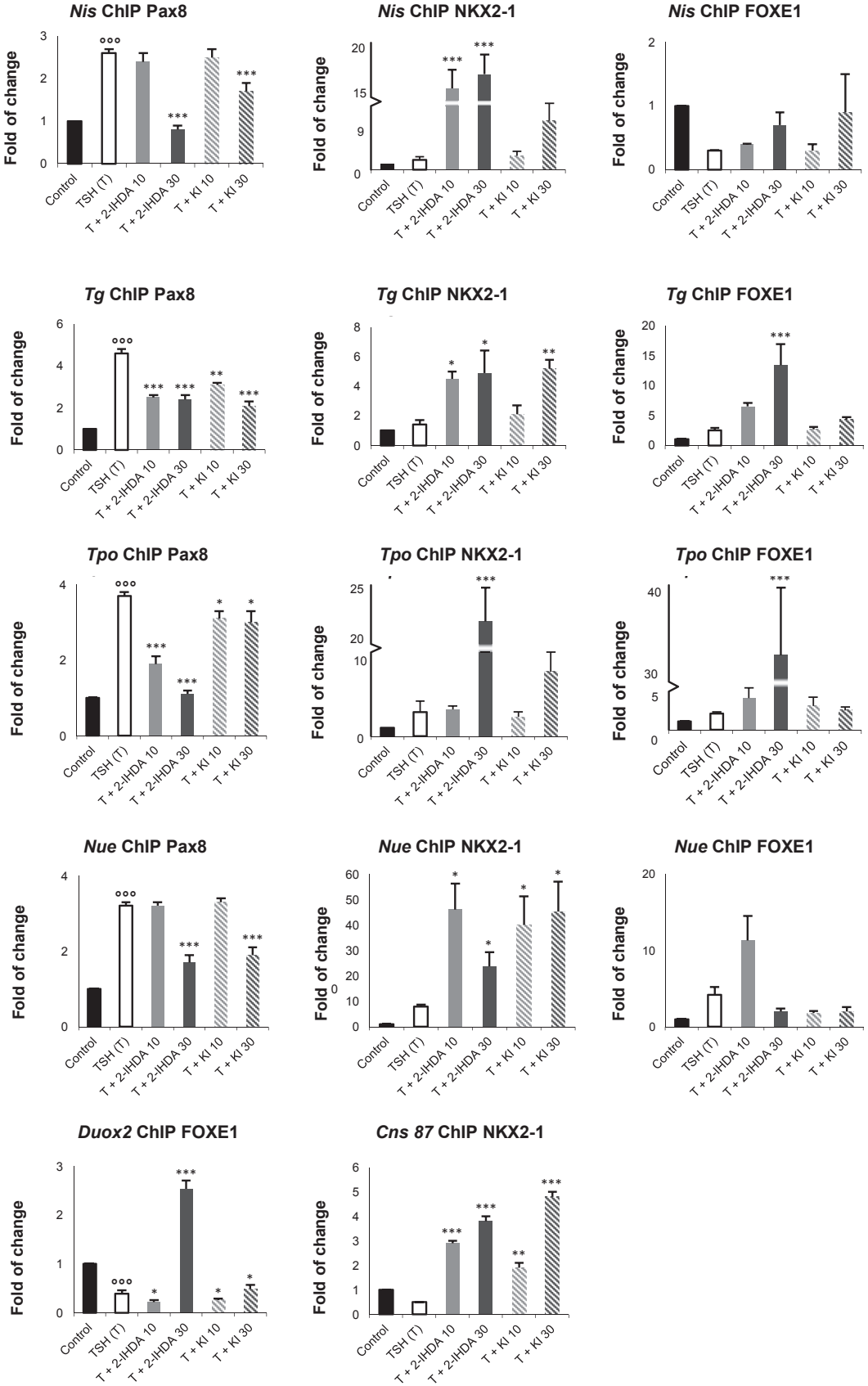

Fig. 5. 2-IHDA modulates thyroid transcription factors association to thyroid-specific gene promoter. Chromatin immunoprecipitation (ChIP) were performed as described in Section 2. Immunoprecipitated DNA was measured by real-time PCR and is reported as relative fold of increase (Transcription factor IP/Total input), taking arbitrarily the control relation as 1.0. Each value is the average of 3–4 experimental determinations by triplicate. Results are expressed as means ± SEM. °°°P < 0.001, vs. control; *P < 0.05, **P < 0.01, ***P < 0.001 vs. TSH.

activity (data not shown) although a role of Anoctamin-1 was also postulated in this process (Twyffels et al., 2014). However, this effect on iodide efflux could not explain the strong inhibition of radioiodide uptake observed with the iodolipid indicating a possible effect on NIS protein as it was previously suggested (Grollman et al., 1986). Controversial results have been reported regarding the effect of excess iodide on NIS mRNA expression. Huang et al. (2011) found stimulation, others found no effect (Eng et al., 2001; Leoni et al., 2011; Arriagada et al., 2015) while most found an inhibition (Uyttersprot et al., 1997; Eng et al., 1999; Spitzweg et al., 1999; Maier et al., 2007; Leoni et al., 2008; Fiore et al., 2009; Thomasz et al., 2010b; Serrano-Nascimento et al., 2012, 2016). The discrepancy might be attributed to the different models employed, iodide doses, or to species-specific differences in NIS gene regulation. Interestingly, the study of Scipioni et al. (2007) showed that NIS protein expression *in vivo* is modulated by iodine supply; no differences in TSH serum levels were found between patients from iodine-sufficient and iodine-deficient areas of Italy, indicating a direct regulation by the halogen. We observed a more pronounced decrease in NIS mRNA caused by 2-IHDA compared to the effect of KI. This decrease takes place at the transcriptional level since there is a reduction of NIS promoter activity. Our results are in agreement with data showing that the effect of high iodide concentrations on the suppression of *Nis* gene expression is caused by the decreased binding of Pax8 to its promoter region (Suzuki et al., 2010; Serrano-Nascimento et al., 2016).

One question that arises from these results is whether the inhibitory effect of 2-IHDA on TSH-stimulated iodide uptake could be a generalized action on different transport processes. Filetti et al. (1986) demonstrated that excess iodide inhibits not only the transport of iodide but also the accumulation of other metabolites. Previously we have demonstrated that three other iodo-compounds: the free acid (14-iodo-15-hydroxy-6-eicosatrienoic acid), its omega lactone (IL-ω) (Krawiec et al., 1991) and IL-δ (Thomasz et al., 2010b) mimic the action of iodide on 2-deoxy-D-glucose uptake. Consistently, we observed that 2-IHDA reduced ^{3}H-DOG uptake, showing an additional effect on membrane transport.

In order to synthesize thyroid hormones iodide must be oxidized and bound to Tg. This process is catalyzed by TPO in the presence of DUOX-produced H_2O_2. TSH induces H_2O_2 generation through the increase of intracellular calcium levels. Iodide inhibits its own oxidation and binding to proteins due to an inhibition of H_2O_2 generation by DUOX proteins (Corvilain et al., 1988; Morand et al., 2003). Significantly, DUOX activity is very low in thyroid from patients with diffuse toxic goiter treated with iodine before surgery (Cardoso et al., 2001). In porcine thyroid membranes 2-IHDA inhibits Duox1 and Duox2 expression (Ohayon et al., 1994), and in cultured dog thyroid cells 2-IHDA decreases H_2O_2 production (Panneels et al., 1994). In agreement with Corvilain et al. (2000), we have observed a dual effect of 2-IHDA; at low concentrations it causes a stimulation of calcium and H_2O_2 levels, while an inhibition occurs at higher concentrations, supporting the role of 2-IHDA as the mediator on the Wolff-Chaikoff effect. It must be noted that Duox2 plays a major role in Tg iodination as iodide organification defect are caused by biallelic mutations in the *Duox2* gene (Moreno et al., 2002; Varela et al., 2006).

Another important protein involved in iodide organification which is also regulated by 2-IHDA is TPO. Its expression was found to be modulated by high and physiological concentrations of iodide *in vitro* (Morand et al., 2003; Leoni et al., 2008; Serrano-Nascimento et al., 2016) and *in vivo* studies (Uyttersprot et al., 1997; Eng et al., 1999; Maier et al., 2007). Regarding *Tg*, other gene down-regulated by 2-IHDA, exposure to a high dose of iodide reduces its expression in PCCl3 cells (Leoni et al., 2008; Serrano-Nascimento et al., 2016) and in bovine follicles (Thomasz et al., 2010b) while no effect was found by others (Pregliasco et al., 1996; Uyttersprot et al., 1997; Suzuki et al., 1998; Eng et al., 1999; Kostic et al., 2009).

The transcriptional expression of genes involved in thyroid hormonogeneis is regulated by several thyroid transcription factors, including Pax 8, NKX2-1 and FOXE1 (Fernández et al., 2015). 2-IHDA inhibited the expression of Pax 8 and FOXE 1. On the contrary, the expression of NKX2-1 was stimulated by 2-IHDA while it was inhibited by TSH. It is well known the stimulatory effect of TSH on Tg, NIS, TPO and DUOX 2 expression; however its role in the regulation of the expression of thyroid-specific transcription factors is still unclear. While the expression of Pax 8 and FOXE 1 is stimulated by TSH/cAMP (Van Renterghem et al., 1996; Ortiz et al., 1999), the effect on NKX2-1 seems to be different. In FRTL-5 cells its expression is down-regulated by the addition of either TSH or forskolin (Shimura et al., 2001) but Van Renterghem et al. (1995) found no effect in primary cultures of dog thyrocytes.

Concerning the TSH-induced binding of transcription factors to thyroid specific genes promoters, 2-IHDA reduced the interaction of Pax 8 with the promoters in agreement with Serrano-Nascimento et al. (2016) showing that Pax 8 binding to *Nis* upstream enhancer is reduced by iodide treatment; however Leoni et al. (2011) did no detect any effect. Conversely 2-IHDA stimulated NKX2-1 and FOXE 1 binding on almost all promoters/enhancers. The stimulation of NKX2-1 binding agrees with its gene expression stimulation, but surprisingly FOXE 1 binding was stimulated while its expression was inhibited. It was demonstrated that, although FOXE1 is stimulated by TSH, its expression inversely correlates with differentiation of thyroid follicular cells, interfering with transcriptional activation by NKX2-1 and Pax-8 on *Tg, TPO* and *Duox2* promoter, but not on *Nis* promoter, according with no effect of 2-IHDA on FOXE 1 binding (Zannini et al., 1997; Perrone et al., 2000; Fernández et al., 2013).

Besides iodolipids, several compounds have been postulated to be the mediators of the iodide-induced thyroid autoregulation. It was shown that Tg is able to inhibit thyroid-specific gene expression including thyroid transcription factors. However unlike iodide, Tg stimulates cell proliferation and this effect is also observed in non-thyroid cells (Sellitti and Suzuki, 2014). Regarding T_3, few evidences suggest that thyroid hormone may have direct actions on the thyroid cell (Pisarev, 1985). Two iodolipids, IL-δ and 2-IHDA were suggested to be involved in thyroid autoregulation. IL-δ does not reproduce all the effects of iodide. The iodolipid inhibited the stimulatory effect of EGF on porcine thyroid cell proliferation but did not reproduce the inhibitory effects of KI on cAMP accumulation (Dugrillon et al., 1990). In porcine follicles (Gartner et al., 1997) and in *in vivo* studies in rats (Thomasz et al., 2010c) IL-δ did not regulate TGF-β1 synthesis, as it was expected since iodide increases its expression (Yuasa et al., 1992; Cowin et al., 1992; Thomasz et al., 2010c).

Our results support a role of 2-IHDA as the intermediary of iodide, the XI compound, in the autoregulatory process. However, the possibility that more than one compound could participate in thyroid autoregulation cannot be ruled out. In summary, we present evidence demonstrating that 2-IHDA reproduces the action of excess iodide on the "Wolff-Chaikoff" effect as well as on thyroid specific genes transcription supporting its role in thyroid autoregulation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.08.036.

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