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Inhibitory effects of 2-iodohexadecanal on FRTL-5 thyroid cells proliferation

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ABSTRACT

Although thyroid gland function is mainly under the control of pituitary TSH, other factors, such as iodine, play a role in this process. The thyroid is capable of producing different iodolipids such as 6-iodo-deltalactone and 2-iodohexadecanal (2-IHDA). It was shown that these iodolipids mimic some of the inhibitory effects of excess iodide on several thyroid parameters.

Objectives: To study the effect of 2-IHDA on cell proliferation and apoptosis in FRTL-5 cells.

Results: FRTL-5 cells were grown in the presence of TSH and treated with increasing concentrations of KI and 2-IHDA (0.5, 5, 10 and 33 μM) for 24, 48 and 72 h. Whereas KI inhibited cell proliferation only at 33 μM after 72 h of treatment, 2-IHDA inhibited in a time and concentration dependent manner. Analysis of cell cycle by flow cytometric DNA analysis revealed an accumulation of cells in G1 phase induced by 2-IHDA. The expression of cyclin A, cyclin D1 and cyclin D3 were reduced after treatment with 2-IHDA whereas CDK4 and CDK6 proteins were not modified. 2-IHDA induced a dynamic change in cytoplasmic to nuclear accumulation of p21 and p27 causing these proteins to be accumulated mostly in the nucleus. We also observed evidence of a pro-apoptotic effect of 2-IHDA at highest concentrations. No significant effect of KI was observed.

Conclusion: These results suggest that the inhibitory effects of 2-IHDA on FRTL-5 thyroid cell proliferation are mediated by cell cycle arrest in G1/S phase and cell death by apoptosis.

1. Introduction

It is well known that pituitary TSH exerts the major task in the regulation of thyroid function (Dumont et al., 1992). However, this gland is capable of certain degree of autonomy, independently of TSH control. Iodine plays an important role in thyroid physiology and biochemistry. It is essential for hormone biosynthesis since it is the limiting factor in normal conditions and it also directly influences a number of thyroid parameters such as thyroid proliferation and function (Panneels et al., 2009; Pisarev and Gärtner, 2000). It is known that iodide can decrease basal thyroid function and impair its response to different stimulators such as TSH, LATS, cyclic nucleotides, prostaglandins, etc. Many biochemical parameters were found to be impaired, including iodide transport, iodide organification/the Wolff–Chaikoff effect, amino acid incorporation into protein, adenylate cyclase activity, glucose oxidation, lactate production, etc. Regarding thyroid growth it has been shown that in hypophysectomized rats low iodine diet (LID) can induce an increase in thyroid weight and epithelial height (Chapman, 1941). Bray (1968) described that hypophysectomized rats fed with a LID developed a larger goiter produced by exogenous TSH than those animals that received a normal diet. Moreover, experimental studies have shown that in rats under a LID, goiter appears well before TSH increases (Berthier and Lemarchand-Béraud, 1978; Matsuzaki and Suzuki, 1975; Naeije et al., 1978). The increased sensitivity to TSH could therefore explain the coexistence of goiters with normal, or very slightly elevated, levels of circulating TSH in certain areas of endemic goiters (Hellstern et al., 1978; Pisarev et al., 1970; Young et al., 1975). On the other hand, excess iodine can sometimes produce effects opposite to those of LID.

The capability of the intracellular content of iodide to modulate the gland function may be defined as **thyroid autoregulation**. Since most of these effects of KI are blocked by the addition of methimazole and PTU it was proposed that iodine has to be converted to an intracellular organic (XI) compound in order to exert its

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inhibitory action (Halmi and Stuelke, 1956; Van Sande et al., 1975). There is still some controversy regarding the nature of the organic intermediates involved in iodine action on the thyroid. Iodinated lipids are biosynthesized by the thyroid gland. Among these, 6-iodo-5-hydroxy-8,11,14-eicosatrienoic acid δ-lactone (IL δ) and 2-iodohexadecanal (2-IHDA) have been shown to inhibit several thyroid parameters and its participation in thyroid autoregulation has been suggested (Panneels et al., 2009). Moreover it was demonstrated that IL δ prevents goiter growth in rats (Pisarev et al., 1988, 1994) and that, *in vitro*, it inhibits thyroid cell proliferation in pigs (Dugrillon and Gärtner, 1995; Dugrillon et al., 1990), humans (Dugrillon et al., 1994) and FRTL-5 cells (Pisarev et al., 1992). Regarding 2-IHDA, in *in vitro* studies no growth suppressing effect could be demonstrated by measuring thymidine incorporation in primary cultures of dog thyroid cells (Panneels et al., 1994a) but we have demonstrated that it has an antigoitrogenic action (Thomasz et al., 2010a). Therefore the objective of this work was analyze the role of 2-IHDA on cell proliferation and to study the mechanisms involved in such regulation. We chose to use the FRTL-5 normal rat thyroid cell line because it reproduces *in vitro* many aspects of the behavior of normal thyroid *in vivo* (Kimura et al., 2001).

2. Methodology

2.1. Cell culture

FRTL-5 cell line was cultured in DMEM-F12 containing 5% FBS and the six hormone mixture [6H medium: cortisol (3.6 ng/ml), transferrin (5 μg/ml), glycyl-L-histydil-L-lysine acetate (10 ng/ml), 10 ng/ml somatostatin, insulin (10 μg/ml), and bovine TSH (1 mU/ml)]. Cells were harvested with trypsin–EDTA and seeded on 24- and 96-well plates or in Tissue-Culture 60 mm dishes for experimental purposes. Cultures were maintained in a temperature and humidity controlled incubator at 37 °C with air and 5% CO2.

2.2. Cell viability

The viability of the cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay. FRTL-5 cells were seeded in 96 well plates at 5×10^3 cells/well and cultured for 2 days in 6H medium or in 5H medium (without TSH). Then the cells were incubated for 24, 48 and 72 h in 6H medium in the presence of various compounds. The viability of FRTL-5 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.

2.3. Cell cycle analysis

Cells were plated and cultured in 5% FBS–6H for 48 h. Then the medium was removed and cells were incubated for 24, 48 and 72 h with 5% FBS–6H (TSH) containing 2-IHDA at 10 and 33 μM.

Cell cycle analysis was performed by propidium iodide (PI) staining. Cells were trypsinized, collected by centrifugation and washed with ice-cold PBS before fixing in 70% ethanol at 4 °C. Fixed cells were resuspended in 0.2 ml PBS containing 50 μg/ml RNase I (Sigma, R4875) and 60 μg/ml PI (Sigma, P4170). Cells were incubated at room temperature for 30 min and then analyzed by flow cytometry on a FACSCalibur (Becton Dickinson Immunocytometry Systems). TSH and FBS starved cells were used as a control of G1 arrest. Ten thousand cells were measured *per* experimental condition and analyzed with WinMDI and Cylchred 1.0.2 software (Cardiff University, UK).

2.4. Detection of p21 and p27 by immunocytochemistry

Subconfluent cells grown in 60 mm dishes with or without 2-IHDA during 24 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, incubated in 3% hydrogen peroxide for 20 min, washed and blocked with 2% BSA in PBS for 30 min. Slices were then incubated at 4 °C overnight with anti p21 (mouse monoclonal (22), Calbiochem, dilution 1:200) and p27 (rabbit polyclonal, Millipore, dilution 1:200) antibodies. Immunodetection 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 discussed earlier except the primary antibody was not included. P27 and p21 expression was evaluated from both cytoplasmic and nuclear staining. Semi-quantitative analysis of staining distribution was scored as negative (–), positive (+) and positive (++) according with the percentage of cells showing immunoreactivity. Negative indicates the complete absence or weak staining in <1% of the cells, (+) indicates positive staining in 1–50% of the cells and (++) indicates positive staining in >50% of the cells. Positive cytoplasms and positive nuclei for p27 and p21 were counted in a total of at least 100 cells. Three independent experiments were performed with triplicate per condition.

2.5. Western blot analysis

Cells were seeded in 60-mm dishes and incubated with different compounds for the time indicated in the text. Cells were scraped in ice cold PBS and lysed in cold RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NonidetP4 0.1% SDS, 0.5% deoxycholate), supplemented with PMSF 0.5 mM and protease inhibitor cocktail (Sigma-Aldrich).

Thirty micrograms of total proteins was electrophoresed on 10 and 7.5% polyacrylamide gels and transferred to PVDF membranes. Immunoblotting was carried out with monoclonal anti c-jun antibodies (dilution 1:200, Dako), and polyclonal anti c-fos (dilution 1:200, Calbiochem), anti Bax/Bcl-2 (dilution 1:500, Calbiochem) and anti cyclin D1, D2, D3, A (dilution 1:200, Santa Cruz) in phosphate buffer saline solution (PBS) with 0.2% Tween 20 (Sigma) and 5% BSA. Membranes were washed, incubated for 1 h at RT with peroxidase-labeled secondary antirabbit antibody or secondary antimouse antibody (1:4000; Amersham), and visualized with the enhanced chemiluminescence method. Densitometric analysis was performed using the VisionWorks Life Science Software. Results were corrected for protein loading by normalization for β-actin expression (Sigma).

2.6. Caspase-3 activity

Caspase-3 activity was determined with the caspase-3 Assay kit, according to the manufacturer's instructions (Sigma). 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 mM dithiothreitol (DTT), 5 mN CHAPS, 10 μg/ml pepstatin, benzamidine 2.5 mM, aprotinin 10 μg/ml, pepstatin 1 μg/0.5 mM phenylmethylsulfonylfluoride (PMSF), pH 7.4] and homogenized with a Teflon-glass homogenizer. Lysates were clarified by centrifugation at $10{,}000 \times g$ 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. The activity, expressed as micromoles of p-nitroaniline per minute per milliliter, was calculated with a p-nitroaniline calibration curve. A positive control of caspase-3 and an inhibitor of caspase-3

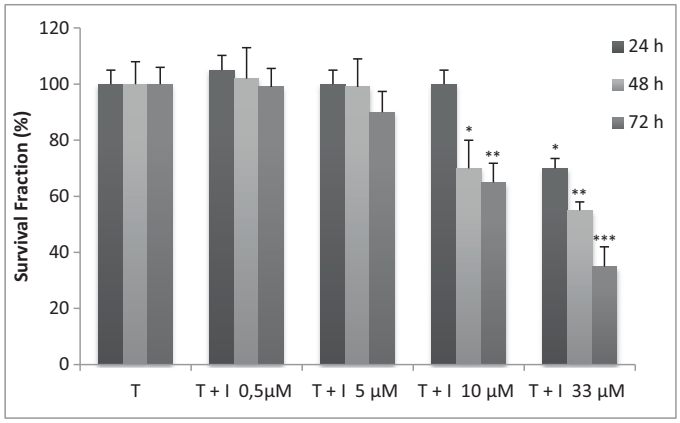


Fig. 1. Effect of 2-IHDA on cell proliferation by MTT assay. Cells were plated and cultured in 6H medium as described in Materials and methods section for 48 h. Then the medium was removed and cells were incubated for 24, 48 and 72 h with TSH (T) containing 2-IHDA (T + I) at the indicated concentrations. TSH value was taken as 100. Results are expressed as the mean ± SD from four independent experiments. * $p < 0.05$ vs. TSH, ** $p < 0.01$ vs. TSH, *** $p < 0.001$ vs. TSH.

(200 mmol/l inhibitor Acetyl-Asp-Glu-Val-Asp-al [Ac-DEVD-CHO]) were added to the plate.

2.7. Apoptosis assay by nuclear morphology

Apoptosis was assessed using fluorescence staining. Cells were incubated with 100 µl PBS containing 2 µl of MIX buffer (propidium iodide, 0.2 mg/ml; Hoechst 33342, 0.03 mg/ml; fluorescein diacetate, 0.75 mg/ml) at room temperature for 5 min. Fluorescein diacetate and propidium iodide were used to stain viable and dead cells, respectively. Hoechst 33342 was used to evaluate differences between normal and apoptotic nuclei. Fluorescent microscopy was used to identify the percentage of propidium iodide-impermeable cells having condensed/fragmented nuclei (apoptotic). 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.

2.8. Statistical analysis

Significance of the differences among groups was calculated by an ANOVA plus a Student–Newman–Keuls. For all statistical analyzes, a probability value of 0.05 was considered significant. At least three independent assays were performed.

3. Results

3.1. Effect of 2-IHDA and KI on cell viability

As shown in Fig. 1, exposure to 2-IHDA at 10–33 µM for 24, 48, and 72 h caused FRTL-5 cell growth inhibition in a concentration- and time-dependent manner. The results shown in Fig. 1 indicate that 2-IHDA at 10 µM for 24 h did not markedly inhibit cell growth but at 33 µM caused 30% inhibition at 24 h. Ten and 33 µM of 2-IHDA caused a 30% and 45% inhibition at 48 h, and 35% and 65% inhibition of cell growth at 72 h. 2-IHDA concentrations less than 10 µM had no inhibitory effects. The homolog molecules palmitic acid and hexadecanal did not produce any significantly effects (5% and 12% respectively at 33 µM). $KClO_4$ (1 mM) an inhibitor of NIS symporter had no interference on 2-IHDA action at 72 h [TSH: 100%; $KClO_4$: 93.1%; TSH + 2-IHDA (10 µM): 69.2% ($p < 0.01$ vs. TSH); TSH + 2-IHDA (33 µM): 40.3% ($p < 0.001$ vs. TSH); TSH + 2-IHDA (10 µM) + $KClO_4$:

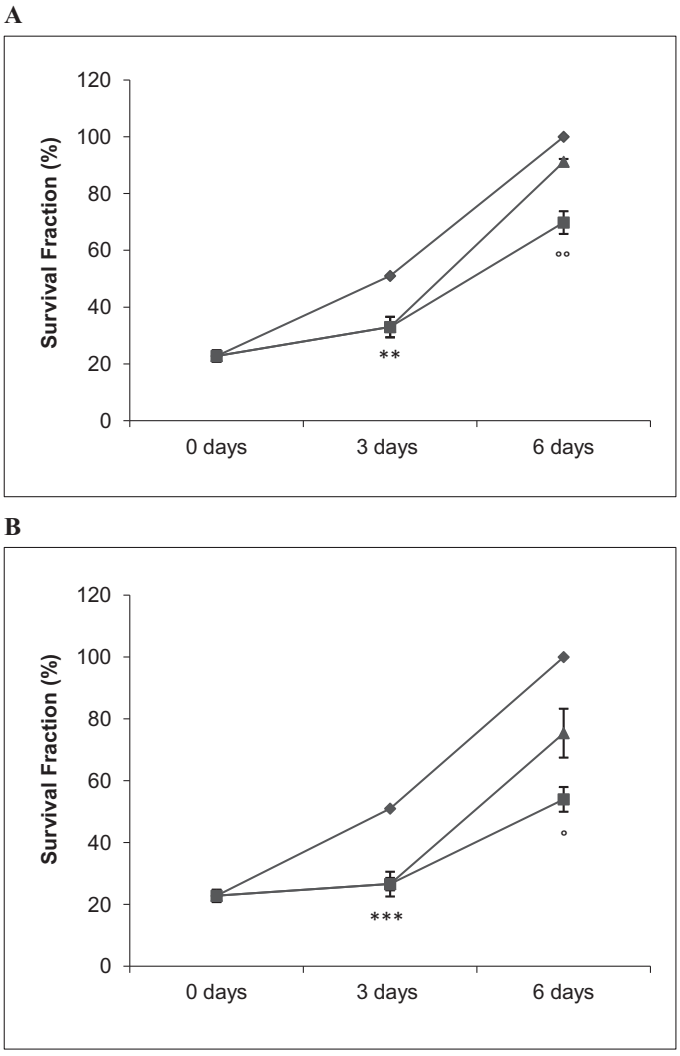


Fig. 2. Reversibility of 2-IHDA action. FRTL-5 cells were cultured in the presence of TSH (♦); TSH + 10 µM 2-IHDA (·) (A); TSH + 33 µM 2-IHDA (·) (B). Two groups were switched after 3 days from TSH + 10 µM 2-IHDA (▲) (A) or TSH + 33 µM 2-IHDA (▲) (B) to 6H medium. TSH value at 6 days was taken as 100. Results are expressed as the mean ± SD from four independent experiments, ** $p < 0.01$ vs. (♦), *** $p < 0.001$ vs. (♦), ° $p < 0.5$ vs. (▲), °° $p < 0.01$ vs. (▲).

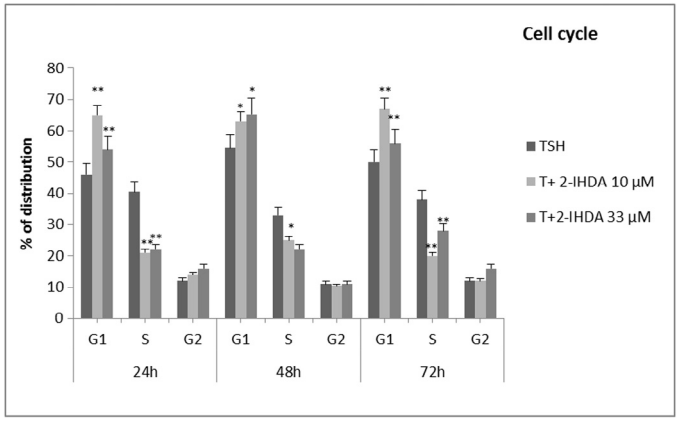


Fig. 3. Flow cytometry analysis. Cells were plated and cultured in 6H for 48 h. Then the medium was removed and cells were incubated for 24, 48 and 72 h with TSH (6H) containing 2-IHDA at the indicated concentrations. Results are expressed as the mean ± SD from four independents experiments * $p < 0.05$ vs. TSH, ** $p < 0.01$ vs. TSH, *** $p < 0.001$ vs. TSH.

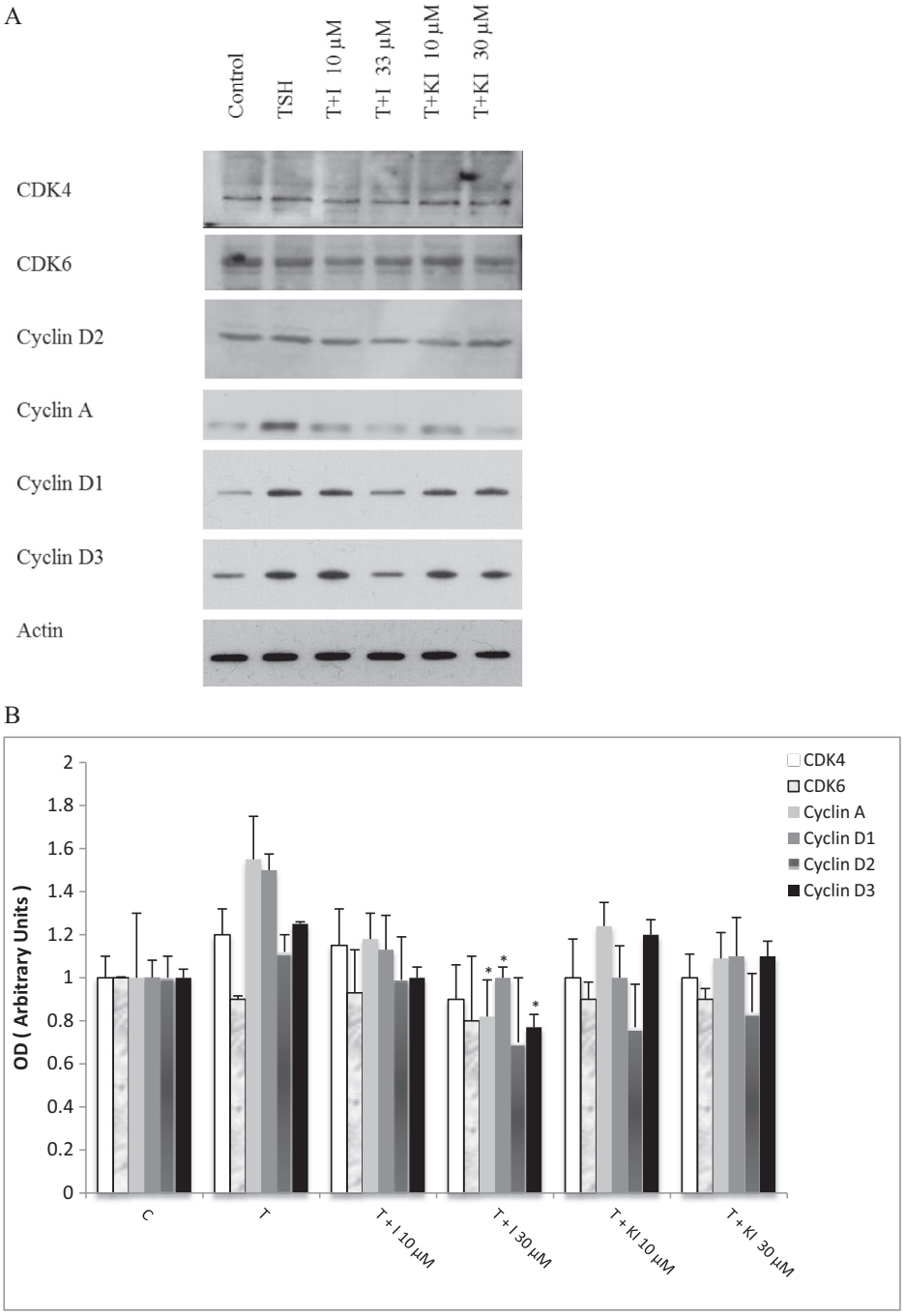


Fig. 4. Effects of 2-IHDA on cell cycle regulators. Cells were plated and cultured in 6H medium for 48 h. Then the medium was removed and cells were incubated for 24 h without TSH (control, 5H) or with TSH (T) containing 2-IHDA (T + I) or KI (T + KI) at the indicated concentrations. (A) Immunochemical detection of cyclin A, D1, D2, D3, CDK4/6 and actin levels using a specific antibody and antirabbit IgG antibody conjugated with peroxidase. (B) Protein level quantification by densitometry scanning of the immunoblots. Values were normalized with an anti-β-actin antibody. Control (5H) value was taken as 1. Results are expressed as means ± SEM from three independent experiments, $^{*}p < 0.05$ vs. TSH.

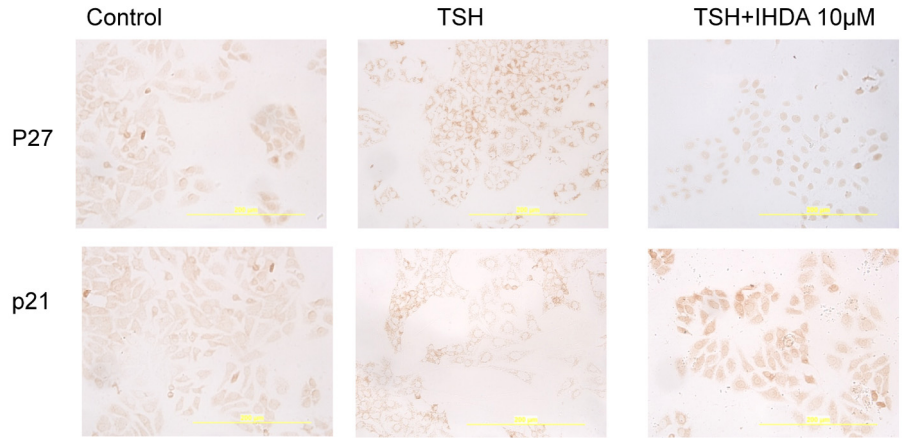
68.3% ($p < 0.01$ vs. TSH); TSH + 2-IHDA (33 µM) + $KClO_4$: 39.5% ($p < 0.001$ vs. TSH)]. Regarding KI only the highest dose at 72 h has effect (27% inhibition, $p < 0.01$) (D). For the following studies concentrations of 10 µM and 33 µM of 2-IHDA were employed.

The possibility that the cells might recover from the 2-IHDA action was also explored. As shown in Fig. 2, when cells were maintained during 3 days in medium containing TSH plus 2-IHDA their growth was significantly inhibited. At this point they were switched to 6H medium devoided of 2-IHDA and they resumed growth at normal rate.

Contrary to its effects on FRTL 5 cells 2-IHDA had a lower effect on thyroid carcinoma cell (WRO and TPC cell lines) proliferation (results not shown).

3.2. Cell cycle analysis

Analysis of 2-IHDA treated FRTL-5 cells by flow cytometry revealed that the percentage of G1 phase cells increased upon treatment with 2-IHDA, concomitant with a reduction of the cell population in S phase at the three times studied (Fig. 3). 2-IHDA ex-



	Control		TSH		TSH + 2-IHDA 33 μM		TSH + 2-IHDA 10 μM	
	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus
P27	++	++	++	-	+	++	++	++
P21	++	++	++	-	+	++	++	++

Fig. 5. 2-IHDA regulates the subcellular localization of p21 and p27. (A) Cells were plated and cultured in 6H medium for 48 h. Then the medium was removed and cells were incubated for 24 h without TSH (control, 5H), TSH or TSH containing 2-IHDA and immunostained using anti-p21 antibody and anti-p27 antibody. (B) Semi-quantitative analysis of staining distribution of p21 and p27 was scored as negative (–), positive (+) and positive (++) according to the percentage of cells showing immunoreactivity as described in Materials and methods section.

posures from 10 to 33 μM resulted in a range of approximately 65% of cells at G1 phase compared with 50% of TSH treated cells at G1 phase. These results showed that 2-IHDA treatment inhibited entry into S-phase, arresting FRTL-5 cells in the G0/G1 phase.

2-IHDA induces nuclear translocation of p21 and p27 and regulates the expression of cyclins induced by TSH.

Cell cycle progression from G1 to S-phase is primarily controlled by the D-type cyclins in association with CDK4/6. To further understand the molecular events underlying the observed G1 arrest, we next examined the effects of 2-IHDA on key regulatory molecules including cyclins, CDK4/6, p21 and p27.

As illustrated in Fig. 4, TSH increased the level of cyclin A (1.6), cyclin D1 (1.5), and cyclin D3 (1.3) protein expression, but did not significantly affect the levels of cyclin D2. The levels of cyclin A, cyclin D1 and cyclin D3 were reduced after treatment with 2-IHDA whereas CDK4 and CDK6 proteins were not modified.

Since the CDK activity can be negatively regulated by CKIs, the levels of p21 and p27 protein were thus assayed in the 2-IHDA-treated FRTL-5 cells. After treatment with TSH p21 and p27 were detected in the cytoplasm. In contrast 2-IHDA induced a dynamic change in cytoplasmic to nuclear accumulation of p21 and p27 causing these proteins to be accumulated mostly in the nucleus (Fig. 5).

3.3. *c-fos and c-jun expression*

As shown in Fig. 6, 2-IHDA inhibited the expression of c-jun protein at early times. No effect was observed on c-fos expression.

3.4. *2-IHDA induces apoptosis in FRTL-5 cells*

In addition to its inhibitory effect on cell proliferation 2-IHDA at 33 μM induced apoptosis in FRTL-5 cells, as evidenced by morphological features of programmed cell death such as pyknosis, karyorrhexis, cell shrinkage and cell blebbing (4%) ($p < 0.01$) (Fig. 7A and B). KI had a negligible effect.

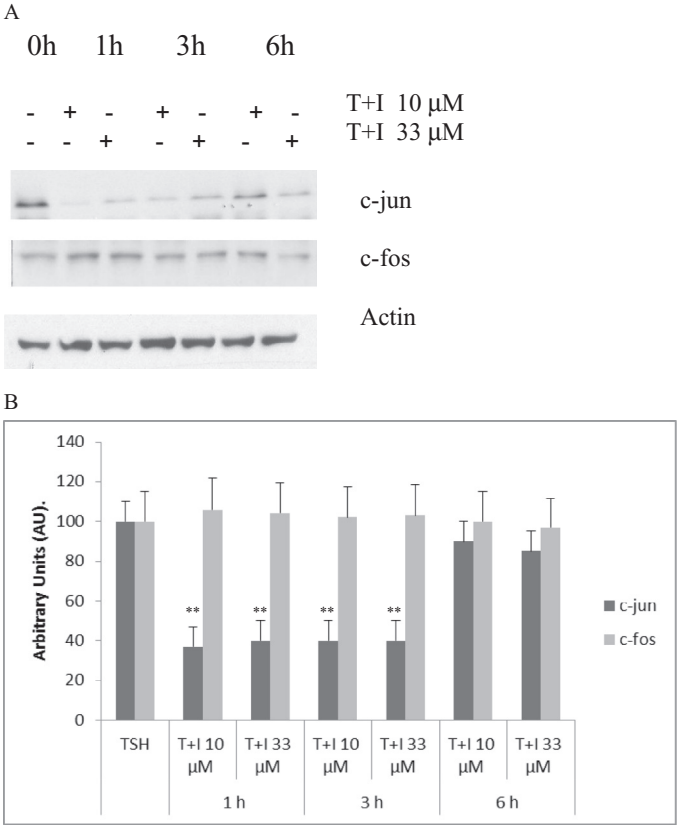


Fig. 6. Western blot of c-jun and c-fos expression in cultured FRTL-5 cells. Cells were plated and cultured in 6H medium for 48 h. Then the medium was removed and cells were incubated with TSH (T) containing 10 μM and 33 μM of 2-IHDA (T + I) for 0, 1, 3, 6 and 24 h. Results are means ± SEM from three independent experiments. (A) Immunochemical detection of c-jun, c-fos and actin levels using a specific antibody and antirabbit IgG antibody conjugated with peroxidase. (B) Quantification of c-jun and c-fos levels by densitometry scanning of the immunoblots. Values were normalized with an anti-β-actin antibody. TSH value was taken as 100. Results are expressed as means ± SEM from three independent experiments, $^{*}p < 0.05$ vs. control.

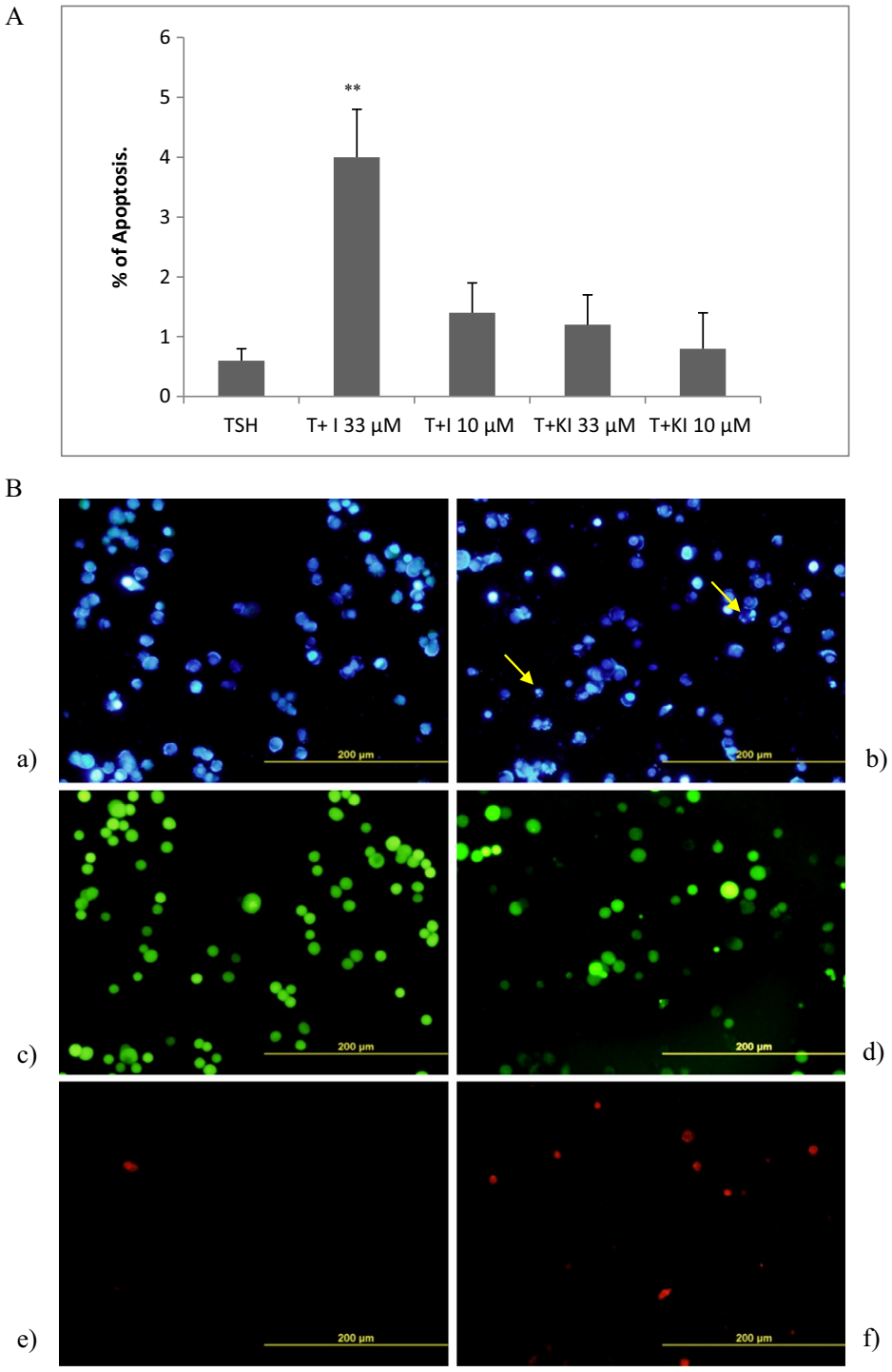


Fig. 7. Effects of 2-IHDA on cell apoptosis. Cells were plated and cultured in 6H medium for 48 h. Then the medium was removed and cells were incubated with TSH containing 2-IHDA (T + I) or KI (T + KI) at the indicated concentrations for 24 h. (A) Apoptotic rate. Data are expressed as the number of apoptotic nucleus per 100 cells of three independent experiments at ×200 magnification (**$p < 0.01$ vs. TSH). (B) Representative micrographs of 2-IHDA induced changes of cell morphology after 24 h of treatment. Cells were staining with Hoechst (blue), DAF (green) and PI (red) and observed by fluorescence microscopy. Apoptotic nuclei (arrowheads) exhibited peripheral chromatin clumping, blebbing, and fragmentation (a,b), cytoplasm of living cells were labeled with DAF (c,d) and necrotic cells were labeled with IP (e,f). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To further confirm that 2-IHDA has an apoptotic effect we measured the abundance of caspase 3 which is associated with the initiation of the apoptotic process. At 24 h caspase-3 activity was significantly increased by around 138% ($p < 0.01$) (Fig. 8A), while KI had no effect. At short times an effect on caspase-3 activity was observed after 6 h (109%) with 33 µM of 2-IHDA ($p < 0.01$) (Fig. 8B). The effect of 2-IHDA on Bax (an apoptosis promoter) to Bcl-2 (an apoptosis inhibitor) ratio was also analyzed. Thirty-three micromoles of 2-IHDA caused a significant increase in the ratio of Bax/Bcl-2 after 6 h of treatment (Fig. 9A and B) ($p < 0.01$). These events might be involved in the apoptotic death induced by 2-IHDA.

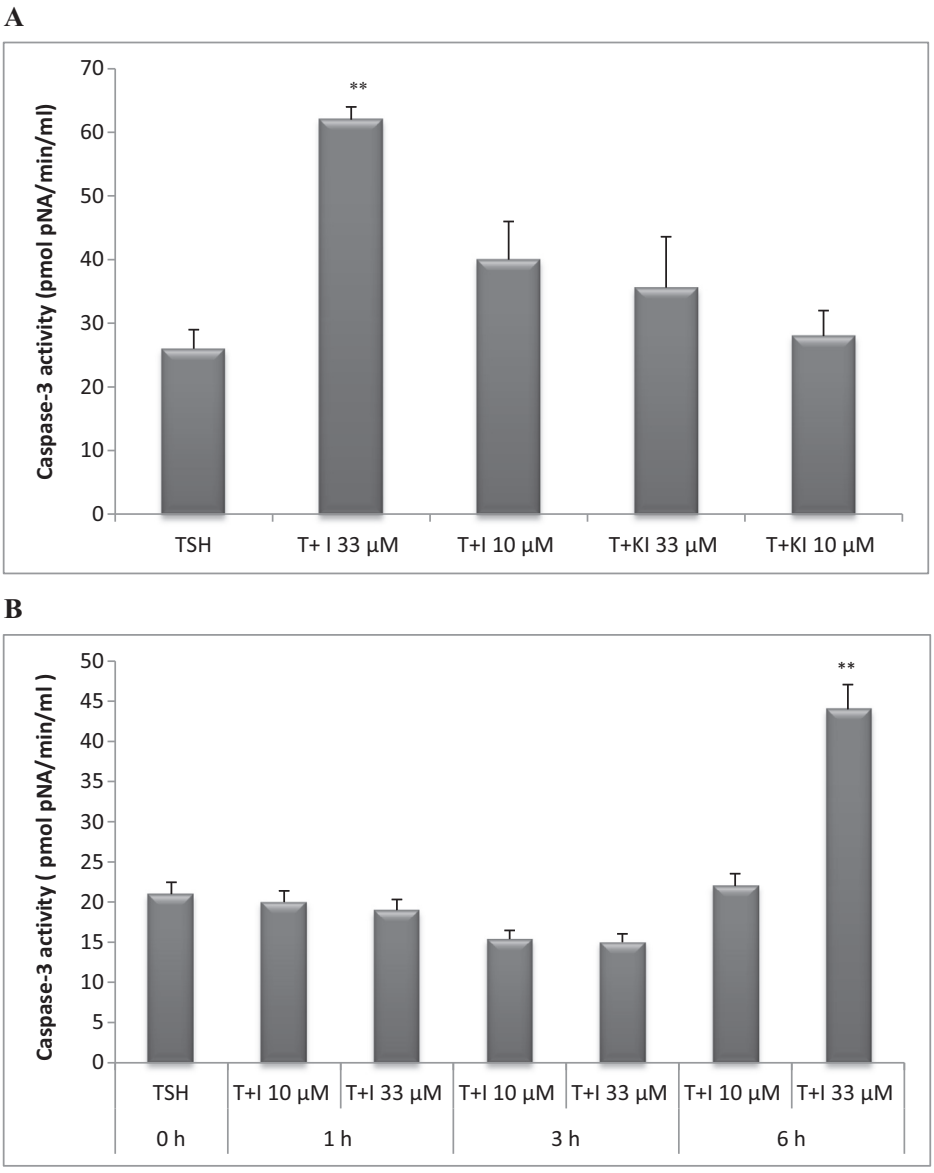


Fig. 8. Caspase-3 activity measure as pmol of pNA released/min/ml. Cells were plated and cultured in 6H medium for 48 h. Then the medium was removed and cells were incubated with TSH containing 2-IHDA (T + I) or KI (T + I) at indicated concentrations for 24 h (**p < 0.01 vs. TSH) (A), or cells were incubated with TSH containing 2-IHDA 10 µM and 33 µM (T + I) during 0, 1, 3 and 6 hours (**p < 0.01 vs. 0 µM 2-IHDA) (B). Data are expressed as mean ± SD of four independent experiments.

4. Discussion

Thyroid growth is mainly regulated positively by two distinct mitogenic signals, one elicited by TSH acting through its G protein coupled receptor and the other elicited by growth factors acting through their receptor tyrosine kinases (Roger et al., 2010). Negative control is exerted by iodide through an iodinated molecule XI. Several candidates have been proposed for XI (Panneels et al., 2009). The biosynthesis of iodolipids has been observed in the thyroid gland from several species and their participation in thyroid autoregulation has been suggested.

2-iodohexadecanal (2-IHDA) was isolated as the major iodolipid formed in horse thyroid slices incubated *in vitro* with radioiodide (Pereira et al., 1990) while Panneels et al. (1996) have shown the synthesis of this iodocompound in cultured dog thyroid cells. *In vivo* the synthesis of this compound in rat thyroid after the intraperitoneal injection of KI has been demonstrated (Pereira et al., 1990). Its biosynthesis is likely to involve the addition of iodine to the vinyl ether group of plasmalogens present in the basolateral membrane.

In porcine thyroid membranes 2-IHDA inhibits DUOX1 and DUOX2 (Ohayon et al., 1994), and in cultured dog thyroid cells 2-IHDA decreases H_2O_2 production (Panneels et al., 1994a) and cAMP accumulation by directly inhibiting the activity of adenylate cyclase (Panneels et al., 1994b). 2-IHDA would be the mediator of two important regulatory mechanisms in the thyroid gland: the Wolff–Chaikoff effect and inhibition of adenylate cyclase (Panneels et al., 2009).

Regarding growth effects we have recently demonstrated that this compound has antigoitrogenic activity, decreasing the intracellular levels of cAMP, reducing the number of cells and the glandular epithelial height (Thomasz et al., 2010a). 2-IHDA was not only able to prevent the growth of MMI induced goiter, but also caused the involution of performed goiter. The iodolipid caused a significant reduction in goiter weight after 3 days compared with

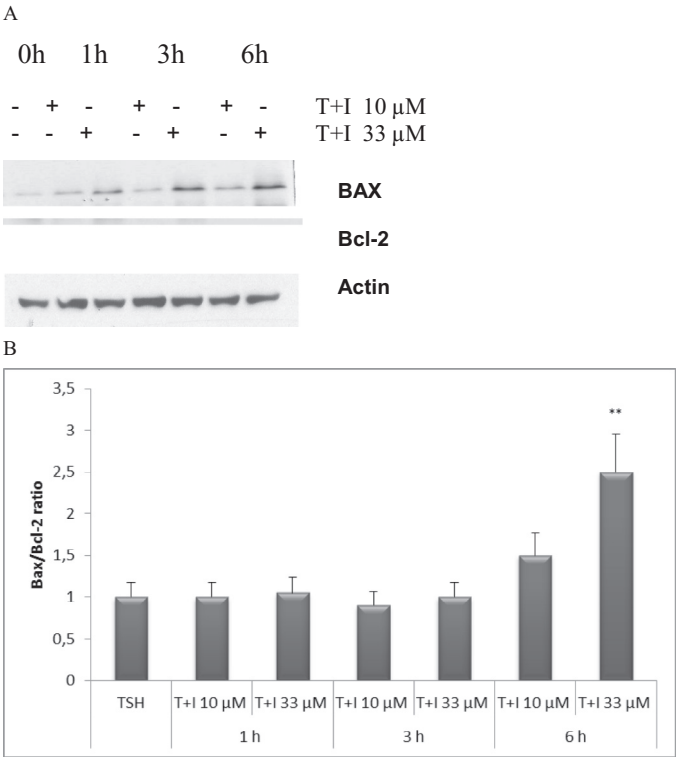


Fig. 9. Western blot analysis of Bax and bcl-2 expression in FRTL-5 cells. Cells were plated and cultured in 6H medium for 48 h. Then the medium was removed and cells were incubated with TSH containing 10 μM and 33 μM of 2-IHDA (T + I) for 0, 1, 3 and 6 h. (A) Immunochemical detection of Bax, Bcl-2 and actin levels using a specific antibody. (B) Quantification of Bax and Bcl-2 levels by densitometry scanning of the immunoblots, values were normalized and are expressed as Bax/Bcl-2 ratio. TSH value was taken as 1. Results are expressed as the mean ± SEM.

the spontaneous involution and iodide failed to alter this value. On the other hand in *in vitro* studies no growth suppressing effect could be demonstrated by measuring thymidine incorporation in cultured dog thyroid cells (Panneels et al., 1994a). Therefore, the present studies were performed in order to further clarify the action of 2-IHDA on cell proliferation utilizing the FRTL-5 cell line model.

It was shown that treatment with 2-IHDA resulted in inhibition of cell proliferation in a concentration-dependent manner, without any detectable effects of the homolog molecules palmitic acid and hexadecanal. KI only at the highest dose at 72 h had an effect, suggesting a direct action of this compound. Moreover, nuclear magnetic resonance analyses have shown that after 2 days of incubation 2-IHDA remains stable (results not shown). In *in vitro* studies it has been demonstrated also that iodine inhibits thyroid cell proliferation although at higher concentrations than in our study (Becks et al., 1988; Smerdely et al., 1993; Tramontano et al., 1989). It is important to remark that excess iodide does not alter TSH binding with its receptor (Uchimura et al., 1979) and that the growth inhibition by iodide occurs, at least, in part, at the level of the cAMP pathway (Pisarev and Itoiz, 1972). Moreover, previous *in vitro* (Panneels et al., 1994b) and *in vivo* (Thomasz et al., 2010b) studies showed that 2-IHDA inhibits cAMP formation.

2-IHDA had a lower effect on thyroid tumor cell proliferation (results not shown). IL-δ, the other iodolipid postulated to have a role in thyroid autoregulation, inhibits thyroid and non thyroid carcinoma cell proliferation (Langer et al., 2003; Rösner et al., 2010; Thomasz et al., 2013). Moreover it was shown that in the mammary gland, which organifies iodide (Arroyo-Helguera et al., 2006), the synthesis of IL-δ takes place (Aceves et al., 2009). On the contrary we have observed that 2-IHDA reproduces the effects of iodide on thyroid specific gene transcription (to be published elsewhere) while IL-δ had a moderate effect (Thomasz et al., 2010b). These results would indicate that 2-IHDA is the main compound in thyroid autoregulation while IL-δ has a broader effect and not restricted to the thyroid but this hypothesis remains to be elucidated.

Cyclins and CDKs play important roles in cell cycle regulation (Roger et al., 2010). Cyclin D1 and cyclin D3, member of the D-type family of G1 cyclins, regulate G1 progression in mammalian cells and TSH induces their expression or activates their complex with CDK4 (Motti et al., 2003; Paternot et al., 2006). Cyclin D3 is more specifically involved in TSHr/cAMP pathway in thyrocytes (Depoortere et al., 1998; Motti et al., 2003; Paternot et al., 2006), and cAMP-dependent stimulation of the activity of cyclin D3–CDK complexes is involved in the pathogenesis of human thyroid goiter and adenoma (Roger et al., 2010). We, therefore, determined the protein expression of cell cycle factors closely related to G1/S checkpoints, including cyclins (cyclins A, D1, D2 and D3) and CDKs (CDK4/6).

Our results suggest that the antiproliferative effect of 2-IHDA was, at least in part, due to an arrest of the cell in the G1/S phase of the cell cycle. The arrest was associated with the down-regulation of cyclins and nuclear accumulation of cell cycle inhibitors such as p21 and p27.

On the other hand, we have shown that 2-IHDA decreased c-Jun expression. Several studies demonstrated that addition of TSH or cAMP analog rapidly induces immediate early gene expression (c-fos, c-jun and c-myc) in FRTL-5 cells with different time courses, suggesting an important role of their gene product on thyroid cell function (Dumont et al., 1992; Kambe et al., 1996). The inhibition of c-jun expression could be involved in the antiproliferative effect of 2-IHDA and reduced cyclin D1 expression. But more studies are necessary to understand the molecular mechanism of action involved in 2-IHDA inhibits cell proliferation.

Activation of the caspase-3 pathway is a hallmark of apoptosis. An increase in the percentage of apoptotic cells and caspase-3 activity was observed after 6 and 24 h of treatment with 33 μM of 2-IHDA. The Bcl-2 family proteins (pro-apoptotic and anti-apoptotic proteins) are critical regulator of the apoptotic pathway. The Bcl-2 family of anti-apoptotic proteins (*e.g.*, Bcl-2) and pro-apoptotic proteins (*e.g.*, Bax) are the central regulators of caspase activation and cellular life-and-death switch (Rolland and Conradt, 2010). Alteration of the ratio of Bcl-2 to Bax is significant in determining whether apoptosis occurs (Tait and Green, 2010).

We investigated the possible involvement of Bax/Bcl-2 in 2-IHDA action; Western blot analysis showed that Bax was up-regulated after 33 μM of 2-IHDA treatment and Bcl-2 expression was diminished after 6 h of treatment, thereby an increase in the Bax/Bcl-2 ratio was observed.

We conclude, therefore, that 2-IHDA inhibits cell growth and also higher doses induces death by apoptosis, resulting in morphological changes typical of apoptotic death, still evident by chromatin condensation and fragmentation, increased Bax/Bcl-2 ratio and caspase-3 activity.

In summary in the present study we demonstrate that 2-IHDA inhibits FRTL-5 thyroid cell proliferation. We have demonstrated that 2-IHDA induces arrest of cell cycle progression in the G1/S phase. On the other hand 2-IHDA induces cell death by apoptosis.

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