

High Iodine Concentration Attenuates *RET/PTC3* Oncogene Activation in Thyroid Follicular Cells

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Background: Papillary thyroid carcinoma (PTC) is frequently associated with a *RET* gene rearrangement that generates a *RET/PTC* oncogene. *RET/PTC* is a fusion of the tyrosine kinase domain of *RET* to the 5' portion of a different gene. This fusion results in a constitutively active MAPK pathway, which plays a key role in PTC development. The *RET/PTC3* fusion is primarily associated with radiation-related PTC. Epidemiological studies show a lower incidence of PTC in radiation-exposed regions that are associated with an iodine-rich diet. Since the influence of excess iodine on the development of thyroid cancer is still unclear, the aim of this study is to evaluate the effect of high iodine concentrations on *RET/PTC3*-activated thyroid cells.

Methods: PTC3-5 cells, a rat thyroid cell lineage harboring doxycycline-inducible *RET/PTC3*, were treated with 10^{-3} M NaI. Cell growth was analyzed by cell counting and the MTT assay. The expression and phosphorylation state of MAPK pathway-related (Braf, Erk, pErk, and pRet) and thyroid-specific (natrium-iodide symporter [Nis] and thyroid-stimulating hormone receptor [Tshr]) proteins were analyzed by Western blotting. Thyroid-specific gene expression was further analyzed by quantitative reverse transcription (RT)-polymerase chain reaction.

Results: A significant inhibition of proliferation was observed, along with no significant variation in cell death rate, in the iodine-treated cells. Further, iodine treatment attenuated the loss of Nis and Tshr gene and protein expression induced by *RET/PTC3* oncogene induction. Finally, iodine treatment reduced Ret and Erk phosphorylation, without altering Braf and Erk expression.

Conclusion: Our results indicate an antioncogenic role for excess iodine during thyroid oncogenic activation. These findings contribute to a better understanding of the effect of iodine on thyroid follicular cells, particularly how it may play a protective role during *RET/PTC3* oncogene activation.

Background

THYROID CARCINOMAS are one of the most prevalent malignancies in the endocrine system; of these, 70% correspond to the papillary histomorphological type (1). Despite the vast number of studies examining papillary thyroid carcinoma (PTC) oncogenesis, the initial molecular events in its development are still not well established.

It is well known that 60% of the PTCs are related to alteration of *RET*, *RAS*, or *BRAF* genes (2,3). *RET* gene rearrangements, in particular, are observed in 20% of the PTCs (4). In thyroid follicular cells, *RET* activation occurs after a pericentric translocation in chromosome 10 which leads to a chimera formed by a 5' heterologous gene and the 3' tyrosine kinase domain of *RET* (5). This rearrangement leads to the constitutive activation of the MAPK pathway. In the thyroid follicular cell, the biological effects of the *RET/PTC* rearrangements, which are dependent on *RET/PTC*-RAS-

BRAF-MAPK pathway activation, include incremental proliferation and loss of function (6,7).

The identified *RET/PTC* isoforms can vary according to the extent of the 5' segment (5); the most prevalent are *RET/PTC1* and *RET/PTC3* (3,8). *RET/PTC1* is generally found in sporadic and adult carcinomas, whereas *RET/PTC3* is generally found in pediatric PTC (9). Even though pediatric PTCs are relatively rare, exposure to radiation can promote the increase of PTC incidence in children (10). Recently, epidemiological studies have revealed that PTC incidence in response to radiation exposure is more pronounced in iodine-deficient regions than in regions with sufficient or excess iodine, indicating an important role for iodine in PTC incidence (11,12).

Iodine itself plays an important role in the normal thyroid follicular cell, regulating thyroid cell differentiation and proliferation (13,14). However, little is known about the molecular mechanisms underlying the effect of excess iodine in radiation-exposed thyroid cells. This study analyzes the effect

of high iodine concentrations in RET/PTC3 oncogene-activated rat normal thyroid cells.

Materials and Methods

Cell culture and RET/PTC3 oncogene induction

PTC3-5 cells (a gift from Dr. James A. Fagin from Sloan-Kettering Memorial Institute, NY), which are PCCI3 (rat normal thyroid) cells harboring a doxycycline (DOX)-inducible RET/PTC3 oncogene (15), were maintained in H4 complete medium consisting of Ham-F12 supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA), 1 mIU/mL bovine thyroid-stimulating hormone (Sigma, St. Louis, MO), 10 μ g/mL insulin (Sigma), 5 μ g/mL apotransferrin (Sigma), 10 nM hydrocortisone (Sigma), and antibiotics, in a humidified 5% CO₂ incubator at 37°C. Cells transfected with RET/PTC3 subcloned into the PUHG10-3 vector, which has a heptamirized tetO and CMV promoter (15), and the PUHD172-neo vector, which directs high-level expression of the reverse tetracycline transactivator protein (7), were selected by treatment with 1 μ g/mL neomycin (Invitrogen) and 1 μ g/mL hygromycin (Invitrogen). In the RET/PTC3 group, RET/PTC3 expression was induced by treatment with 1 μ g/mL DOX (Calbiochem, San Diego, CA). The other group was treated with DOX and 10⁻³ M NaI (Merck, Darmstadt, Germany) and called the RET/PTC3-iodine group. PTC3-5 cells cultured without DOX (control group) but in presence of 10⁻³ M NaI were the control-iodine group. All groups were cultured for 0, 24, 48, 72, and 120 hours, respectively. We also tested the effect of different iodine concentrations in RET/PTC3-induced thyroid cells. This study complied with the guidelines of the University of Sao Paulo Institute of Biomedical Sciences Ethical Committee.

Cell proliferation and viability

Cell counting. Cells were plated at a concentration of 5 × 10⁴ cells per well. At the indicated time, cells were trypsinized to release them from the culture dish. The supernatant from previously collected cells was used to inactivate the trypsin. After centrifugation, the cell pellet was resuspended in complete medium, followed by cell counting with a Neubauer hemacytometer. The number of dead cells was determined by staining with 4% Trypan Blue (Invitrogen).

MTT assay. Cells were plated at a density of 1 × 10⁴ cells per well in 96-well plates. At the times indicated above, 0.125 mg/mL MTT (Invitrogen) was added to the cells. After 3 hours, the medium was removed, and the cells were resuspended in 100 μ L of isopropanol/0.04 M HCl, followed by measurement of absorbance at 595 nm in a spectrophotometer (Spectra Max Plus; Molecular Devices, Sunnyvale, CA) (16). Three independent assays were performed in triplicate.

Cell viability analysis by Annexin versus propidium iodide. Cells treated with different concentrations of iodine (10⁻⁷, 10⁻⁵, and 10⁻³ M) were analyzed using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Sigma). Cells were harvested from six-well plates, resuspended in 0.3 mL of phosphate-buffered saline, and treated with 5 μ L of Annexin V-FITC conjugate for 15 minutes. This was followed by the addition of 10 μ L of propidium iodide

solution and immediate analysis by flow cytometry (Easy-Cyte Mini System; Guava Technologies, Hayward, CA). Cells that were early in the apoptotic process were stained with the Annexin V-FITC conjugate alone. Live cells showed no staining with either the propidium iodide solution or the Annexin V-FITC conjugate. Necrotic cells were stained by both propidium iodide and the Annexin V-FITC conjugate. The assay was realized in triplicates.

Gene expression by quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was generated using 3 μ g of total RNA, in the presence of 100 ng/ μ L of random primers, 10 U RNase inhibitor, 1 mM dNTP mix, 10 mM DTT, 1 × reverse transcription (RT) buffer, and 200 U M-MLV transcriptase (Invitrogen) to total volume of 20 μ L. For quantitative RT-polymerase chain reaction, 50 ng of each cDNA, 10 μ L SYBRGreen PCR Master Mix (Applied Biosystems, Foster City, CA), and primers for the thyroid-specific genes sodium-iodide symporter (*Nis*) and thyroid-stimulating hormone receptor (*Tshr*) were used in a sequence detection system (Applied Biosystems). Ribosomal protein L19 (*Rpl19*) expression was used as an internal control (17).

Western blotting and antibodies

Twenty micrograms of each sample was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transblotted to nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ). The membranes were incubated with specific primary antibodies in a blocking solution (5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20), followed by incubation with the corresponding peroxidase-conjugated secondary antibody. The antigens were visualized using the luminol reagent, and the band intensities were quantified using Scion Image software (Scion Corporation, Frederick, MA).

For the quantitative protein expression assays, the following antibodies were used: NIS (a gift from Dr. Sissy M. Jhiang from Ohio State University), TSHr (sc-13936; Santa Cruz Biotechnology, Santa Cruz, CA), BRAF (sc-166; Santa Cruz Biotechnology), ERK (sc-94; Santa Cruz Biotechnology), pRET (sc-20252R; Santa Cruz Biotechnology), pERK (sc-7383; Santa Cruz Biotechnology), and, as a control, α -tubulin (sc-5286; Santa Cruz Biotechnology).

Statistical analysis

The results of the cell proliferation, gene, and protein expression assays are presented as the mean \pm standard deviation. Statistical analysis included analysis of variance followed by the Bonferroni *t*-test to compare results between groups. Differences were considered significant at *p* < 0.05.

Results

Treatment effects of different iodine concentrations in RET/PTC3-induced thyroid cells

To observe which iodine concentration had the strongest effect on the RET/PTC3-induced thyroid cells, PTC3-5 cells were treated with different concentrations of NaI, including 10⁻⁷ M as the physiological concentration, 10⁻⁵ M as an

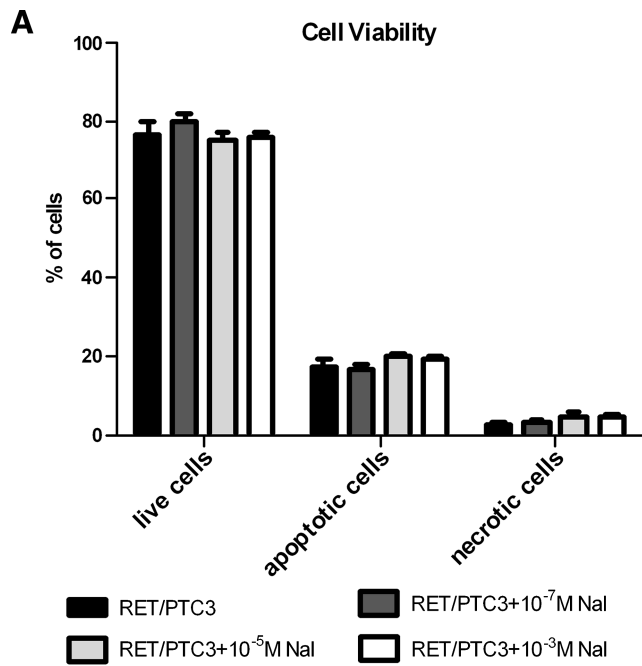
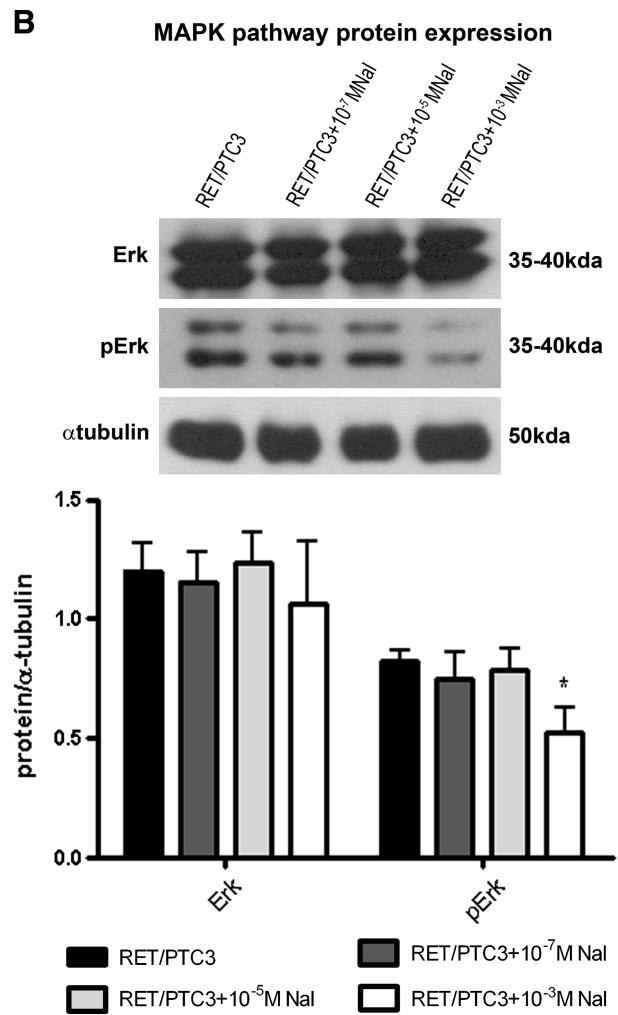


FIG. 1. The effect of treatment with different concentrations of iodine (10^{-7} , 10^{-5} , and 10^{-3} M) on cell viability and MAPK pathway protein expression in *RET/PTC3*-induced thyroid cells. PTC3-5 cells with *RET/PTC3* oncogene induction treated with different NaI concentrations were submitted to Annexin versus propidium iodide staining (A); no alteration in cell viability was observed. Expression of the proteins Erk, pErk, and α -tubulin in PTC3-5 cells with *RET/PTC3* oncogene induction and simultaneously treated with crescent NaI concentrations (B). The results are representative of two independent reactions, performed in duplicate. * $p < 0.05$ versus *RET/PTC3*. PTC, papillary thyroid carcinoma.



intermediary concentration, or 10^{-3} M as the high iodine concentration, simultaneously with oncogene induction for 72 hours. Analysis for apoptosis or necrosis showed no significant alteration in cell death for any treatment (Fig. 1A).

Western blot analysis using specific antibodies for Erk, pErk, and α -tubulin was used to compare their expression in *RET/PTC3*-induced thyroid cells treated with different iodine concentrations. Treatment with 10^{-3} M NaI resulted in reduced Erk phosphorylation (28%), with no alteration of Erk expression (Fig. 1B), while none of the other iodine concentrations had any significant effect on the expression of either protein.

Effect of treatment with high iodine concentrations on thyroid cell proliferation and viability in RET/PTC3-induced thyroid cells

The proliferation and cell death rate of thyroid follicular cells in culture in the presence of high concentrations of iodine are shown in Figure 2. Cells treated with 10^{-3} M NaI (control-iodine cells) experienced a 20% reduction in proliferation over 48 hours, but the proliferative capacity recovered after 120 hours. A 48% reduction in proliferation that lasted until 120 hours was observed in *RET/PTC3*-induced cells. The combined *RET/PTC3*-induced plus 10^{-3} M NaI-treated cells (*RET/PTC3*-iodine) experienced a 30% reduction in proliferation that lasted until the end of the treatment, when compared to *RET/PTC3*-induced cells (Fig. 2A). A similar result

was observed using the MTT assay, with the more significant result observed at 72 hours. A 39% reduction in proliferation was observed in control-iodine group, whereas the *RET/PTC3* group experienced a 63% reduction, and the *RET/PTC3*-iodine group presented a 40% reduction, when compared to *RET/PTC3* group (Fig. 2B).

Conversely, an incremental increase in cell death was observed in the control-iodine cells as compared to the control group (Fig. 2C). The number of dead cells greatly increased in the *RET/PTC3* group over time as compared to the control group. These data agree with a prior study by Wang *et al.* that showed this phenomenon in thyroid cells induced by *RET/PTC3* expression (15). Interestingly, approximately the same number of dead cells was maintained in the *RET/PTC3*-iodine group as in the *RET/PTC3* group (Fig. 2C).

Effect of treatment with a high concentration of iodine on the MAPK pathway in RET/PTC3-induced thyroid cells

To evaluate the effect of iodine on MAPK pathway effectors, Braf and Erk expression was analyzed along with the phosphorylation state of Erk and Ret (Fig. 3). When the *RET/PTC3* oncogene was not induced, no difference was seen in the expression of these MAPK effectors in the control-iodine group compared to the control group. However, when *RET/PTC3* was activated, increased phosphorylation of Ret (98%)

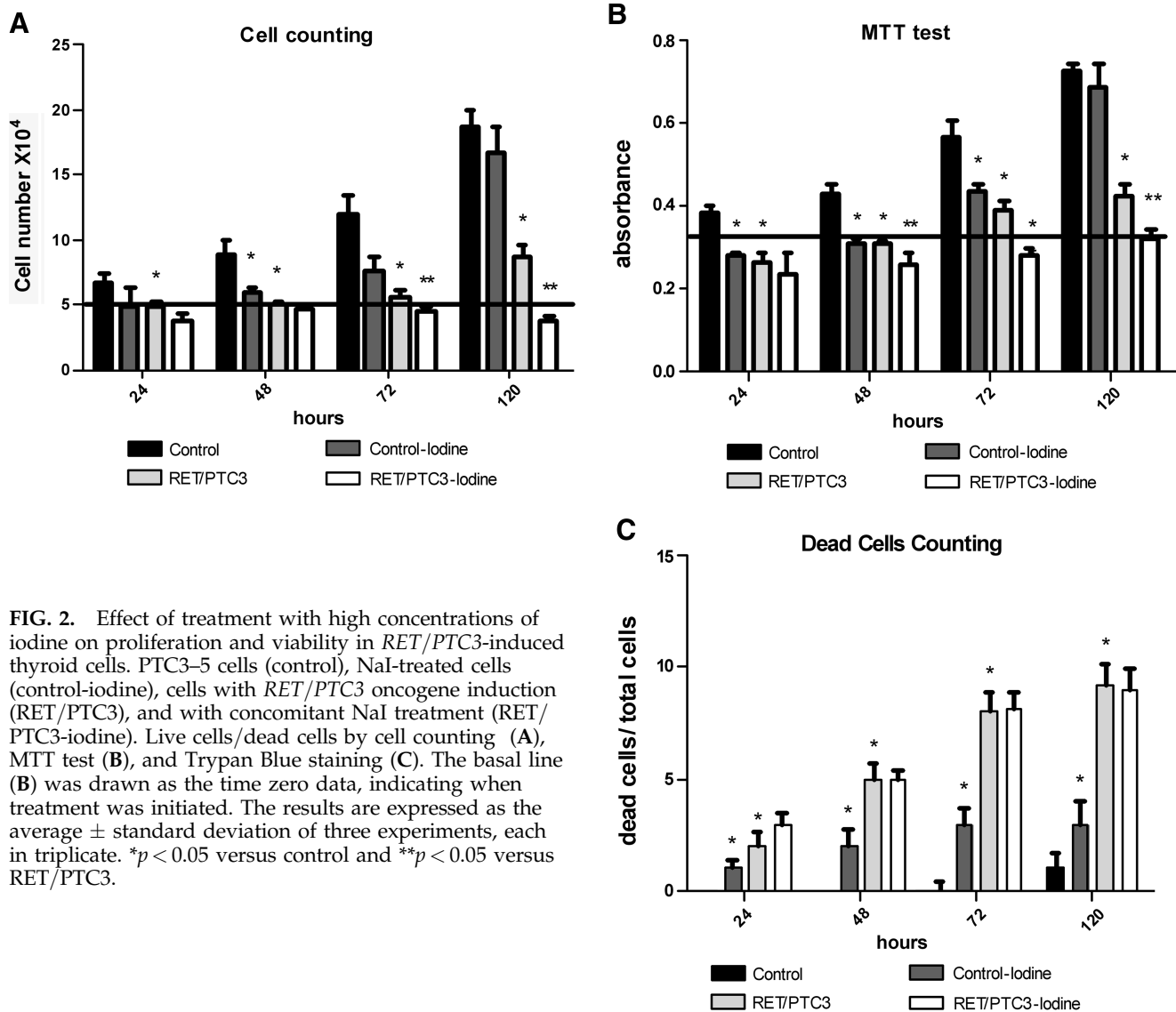


FIG. 2. Effect of treatment with high concentrations of iodine on proliferation and viability in *RET/PTC3*-induced thyroid cells. PTC3-5 cells (control), NaI-treated cells (control-iodine), cells with *RET/PTC3* oncogene induction (*RET/PTC3*), and with concomitant NaI treatment (*RET/PTC3*-iodine). Live cells/dead cells by cell counting (A), MTT test (B), and Trypan Blue staining (C). The basal line (B) was drawn as the time zero data, indicating when treatment was initiated. The results are expressed as the average \pm standard deviation of three experiments, each in triplicate. * $p < 0.05$ versus control and ** $p < 0.05$ versus *RET/PTC3*.

and Erk (56%) was observed in the *RET/PTC3* group. However, no difference was observed in the expression of Braf or Erk protein in the 48- or 72-hour treatment, when compared to the control group (Fig. 3A). Alternatively, treatment of *RET/PTC3*-induced cells with iodine decreased phosphorylation of Ret by 17% and, more importantly, reduced phosphorylation of Erk by 26%. Moreover, no significant change in Erk or Braf expression was observed in the *RET/PTC3*-iodine group as compared to the *RET/PTC3* group (Fig. 3).

Effect of treatment with a high concentration of iodine on the expression of specific thyroid genes in *RET/PTC3*-induced thyroid cells

Nis and *Tshr* mRNA expression is reduced at 48 hours in the control-iodine group of thyroid follicular cells, and it returns to normal by the 120-hour treatment (Fig. 4). A reduction in the expression of *Nis* mRNA was observed in the *RET/PTC3* group beginning at 24 hours and lasting to 120 hours. The results observed with the expression of *Tshr* mRNA were similar.

An incremental increase in *Nis* mRNA expression was observed in the *RET/PTC3*-iodine group already at 24 hours and was maintained until 120 hours (24 hours: 16%, 48 hours: 93%, 72 hours: 48%, and 120 hours: 64%) (Fig. 4). On the other hand, *Tshr* expression was increased in 48 hours (31%) and 72 hours (17%), while an additional reduction was observed in 120 hours (12%) (Fig. 4).

A reduction in *Nis* protein expression observed in the control-iodine group was reversed by 72 hours. *Nis* protein expression was also significantly reduced in the *RET/PTC3* group, but this effect was inhibited in the *RET/PTC3*-iodine cells (Fig. 5). The expression of the *Tshr* protein followed a pattern similar to that observed for *Nis* protein expression, with the exception of an observed decrease in *Tshr* expression in the 72-hour treatment.

Discussion

This study observed that treatment with a high concentration of iodine can modulate the effect of oncogene activation in thyroid follicular cells. The *RET/PTC* fusion plays a key role in PTC progression by inducing the loss of specific

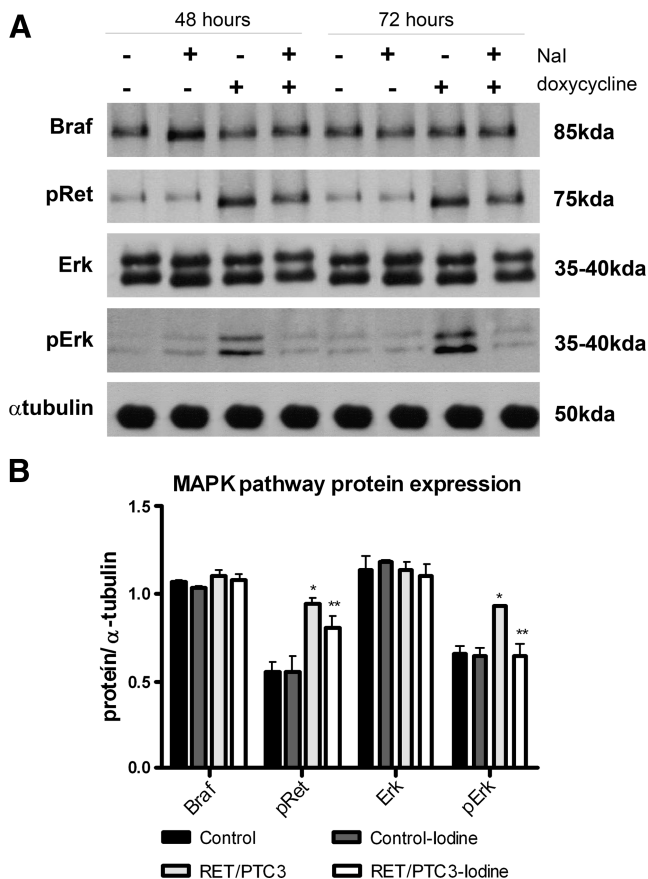


FIG. 3. The effect of treatment with high concentrations of iodine in MAPK pathway protein expression in *RET/PTC3*-induced thyroid cells. Expression of the proteins Braff, Erk, pErk, and α -tubulin in PTC3-5 cells (control), PTC3-5 NaI-treated (control-iodine) cells, cells with *RET/PTC3* oncogene induction (*RET/PTC3*), or in cells with *RET/PTC3* oncogene induction and simultaneous NaI treatment (*RET/PTC3*-iodine). The assay was conducted at 48 and 72 hours by Western blotting (A). Representative graphs showing the relative expression of the MAPK pathway effectors at 72 hours (B). Representative results of three independent reactions, performed in duplicate. * $p < 0.05$ versus control and ** $p < 0.05$ versus *RET/PTC3*.

thyroid cell characteristics (6,15). This oncogene-controlled induction system represents an important tool for understanding the early steps in thyroid oncogenesis.

Previous studies have shown an intimate relationship between iodine availability and thyroid carcinoma incidence in radiation-exposed regions (12). In this study, we show that the 10^{-3} M iodine concentration decreases *RET/PTC3*-induced cell proliferation with no increase in cell death when compared to the other iodine concentrations tested. This concentration has also been previously tested in studies concerning the effect of excess iodine on thyroid follicular cells (17,18), and it also seems to have an interesting effect in oncogene-activated cells.

Previous studies showed that *RET/PTC3*-activated cells exhibit a reduction in cell number and an incremental increase in the number of dead cells (15). Interestingly, iodine treatment during *RET/PTC3* induction diminishes the cell number even further, suggesting that this effect is not caused by increased

cell death (Fig. 2C). Further, these effects are similar to the inhibitory effect of excess iodine on normal follicular cell proliferation (19,20), which, together with the inhibition of thyroid hormone synthesis (Wolff Chaikoff effect), are known as the autoregulatory effects of iodine (21). The prolonged iodine treatment of *RET/PTC3*-induced cells in our study resulted in the loss of the iodine effect on proliferation; this phenomenon is well known in normal follicular cell differentiation as the escape from the iodine autoregulatory effect (22,23).

RET/PTC3 oncogene activation constitutively activates the MAPK pathway, which plays an important role in malignant transformation in the thyroid (6). *RET/PTC3* induction increases the phosphorylation of Ret and Erk, which is the MAPK pathway effector, with no change in the expression of the Braff or Erk protein. This effect is inhibited by treatment with high iodine concentrations, indicating that the MAPK pathway is negatively controlled by high iodine concentrations during oncogenic activation of thyroid cells. Interestingly, the inhibition of the MAPK pathway is more pronounced at the 10^{-3} M iodine concentration than in the other concentrations (Fig. 1).

Thyroid follicular cells are characterized by the expression of specific genes, including those that are involved in iodine metabolism and thyroid physiology (such as *Nis* and *Tshr*) (24). *Nis* plays a key role in iodine transport via the thyrocyte, and it is one of the most important thyroid-specific differentiation markers (25). The loss of functional *Nis* expression is a well-documented event that occurs during thyroid tumorigenesis (26,27). In our study, *RET/PTC3* oncogene induction drastically reduced *Nis* mRNA and protein expression. Interestingly, treatment with high concentrations of iodine simultaneously with *RET/PTC3* oncogene activation leads to a transitory increase in *Nis* mRNA (Fig. 4) and protein (Fig. 5) expression.

The regulation of *Nis* expression depends upon the integrity of the Tsh-Tshr-cAMP signaling pathway (28). *RET/PTC3* induction reduces *Tshr* mRNA expression; however, this reduction can be briefly inhibited by high concentrations of iodine, as observed in experiments examining the expression of *Tshr* mRNA (Fig. 4) and protein (Fig. 5) in this study. These results indicate that iodine could maintain cellular responsiveness to Tsh and preserve iodine transport by *Nis* through the cell membrane during *RET/PTC3* oncogene activation in thyroid cells. In addition to increasing *Tshr* expression, iodine also led to a reduction in cell proliferation, indicating that other growth factor pathways may be involved in iodine-related effects in the thyroid follicular cells (17,29,30).

The attenuating effects of treatment with high iodine concentrations on genes involved in iodine metabolism are not sustained, suggesting that iodine treatment transiently delays loss of thyroid function during oncogenic activation. Our data also show that iodine can function as a MAPK pathway inhibitor, modulating levels of the component proteins of this pathway during *RET/PTC3* activation (Fig. 2). Recent studies have shown that U0126, a drug known to inhibit the MAPK pathway, reduces pErk levels and restores thyroid gene expression, including *Nis* and *Tshr* (31), in a PTC cell lineage, which is similar to the effects observed in the *RET/PTC3*-induced cells treated with iodine.

Epidemiological studies have demonstrated that regions with sufficient or excessive iodine consumption that have been exposed to radiation have a lower incidence of pediatric PTC compared to iodine-deficient regions (12). Stable iodine is

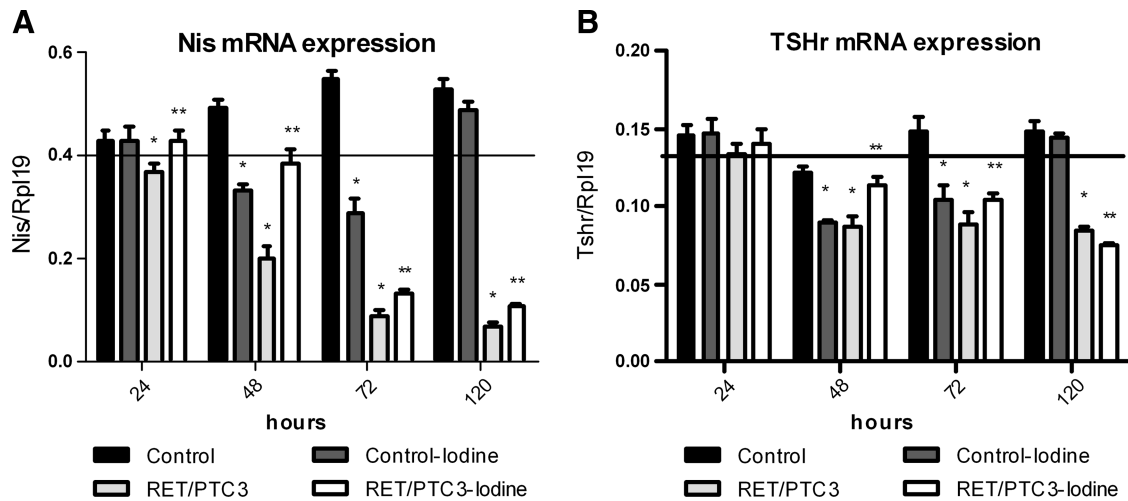


FIG. 4. The effect of treatment with high concentrations of iodine on expression of thyroid-specific genes. PTC3-5 cells with *RET/PTC3* induction in the presence of doxycycline (1 $\mu\text{g}/\text{mL}$) and NaI (10^{-3} M) for 24, 48, 72, and 120 hours. mRNA expression of Nis (A) and Tshr (B) normalized with RPL19 gene expression in PTC3-5 cells (control), PTC3-5 NaI-treated (control-iodine) cells, cells with *RET/PTC3* oncogene induction (RET/PTC3), or in cells with *RET/PTC3* oncogene induction and simultaneous NaI treatment (RET/PTC3-iodine). The basal line indicated time zero data, at the time treatment was initiated. The results are expressed as average \pm standard deviation of three experiments, performed in triplicate. * $p < 0.05$ versus control and ** $p < 0.05$ versus RET/PTC3. Nis, sodium-iodide symporter; Tshr, thyroid-stimulating hormone receptor.

also used prophylactically to protect thyroid follicular cells from incorporation of radioactive iodine (11,32,33). Further, the role played by high iodine concentrations appears to be wider than simply protecting follicular cells from radioactive iodine entrance; it also acts in thyroid cell growth and modulates the expression of several important genes involved in thyroid physiology and transformation.

Conclusion

Treatment with high iodine concentrations can delay oncogenic activation effects, reduce cell growth, and rescue

thyroid-specific gene and protein expression. Excess iodine can act as a protective agent in thyroid follicular cells during *RET/PTC3* activation, thereby attenuating the oncogenic process in the thyroid.

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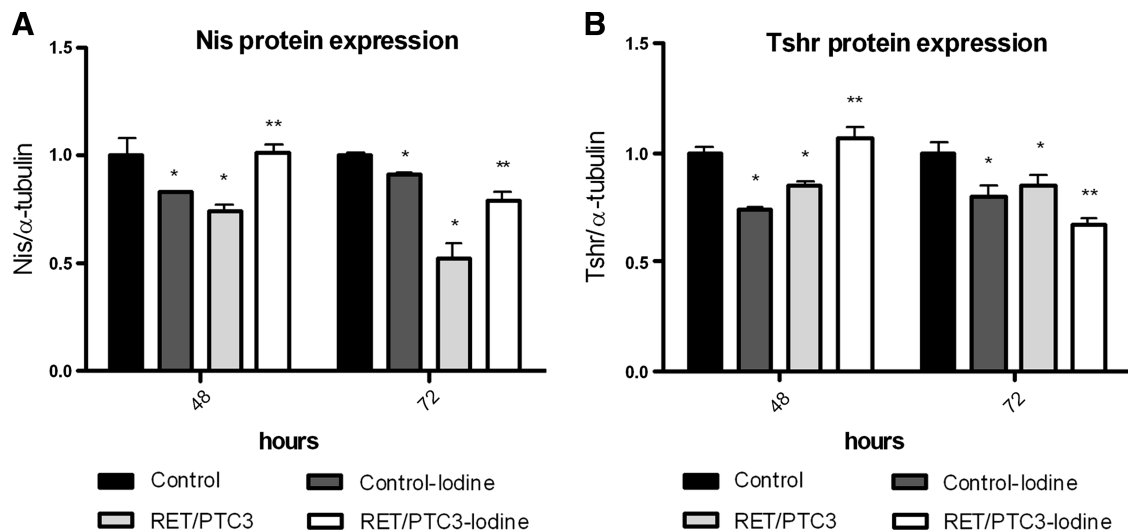


FIG. 5. The effect of treatment with high concentrations of iodine on thyroid-specific protein expression. Semiquantitative expression of Nis and Tshr proteins in PTC3-5 cells (control), PTC3-5 NaI-treated (control-iodine) cells, cells with *RET/PTC3* oncogene induction (RET/PTC3), or in cells with *RET/PTC3* oncogene induction and simultaneous NaI treatment (RET/PTC3-iodine) normalized with α -tubulin protein expression. Graph showing Nis (A) and Tshr (B) expression at 48 hours and 72 hours from results of three independent Western blotting experiments performed in duplicate. * $p < 0.05$ versus control and ** $p < 0.05$ versus RET/PTC3.

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Disclosure Statement

The authors declare no conflict of interest that would prejudice the impartiality of this scientific work.

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