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Abstract

Different factors are involved in thyroid function and proliferation such as thyrotropin (TSH), insulin, growth factors, iodide, etc. TSH and IGF1/insulin increase proliferation rate and stimulate genes involved in thyroid differentiation. In the present study, we analyze the physiological regulation of NOX4 expression by TSH, insulin and iodine, and the role of NOX4 on thyroid genes expression. Differentiated rat thyroid cells (FRTL-5) were incubated in the presence or absence of TSH/ insulin and TTF2, PAX8, TPO, NIS, NOX4, TGFβ1, FOXO1/3 mRNA levels were examined by Real Time PCR. We showed that TSH and insulin repress NOX4 expression and appears to be inversely correlated with some thyroid genes. SiRNA targeted knockdown of NOX4 increased mRNA levels of TGFβ1, TPO, PAX8, TTF2, FOXO1 and FOXO3. A PI3K inhibitor (LY294002), increases the expression of NIS, TTF2 and FOXO1/3, however PI3K/AKT pathway does not regulate NOX4 expression. We observed that iodine increased NOX4 expression and knockdown of NOX4 reduced ROS and reversed the inhibitory effect of iodine on NIS, TPO, PAX8 and TTF2 expression. Our findings provide strong evidence that NOX4 could be a novel signalling modulator of TSH/insulin pathway and would have a critical role in the autoregulatory mechanism induced by iodine.

Keywords: Thyroid, Nox4, Iodine, ROS, autoregulation

1. Introduction

Thyroid-stimulating hormone (TSH) is a major regulator of thyroid proliferation and function. TSH regulates biosynthesis, storage and secretion of two thyroid hormones, triiodothyronine (T3) and thyroxine (T4). In thyroid, TSH and IGF-1/ insulin exert similar or complementary functions on proliferation and differentiation through several transduction pathways (Kimura et al., 2001).

Thyroid hormones synthesis is the main differentiated function of thyroid gland. It is an oxidative process that requires hydrogen peroxide (H_2O_2) as a substrate and a peroxidase enzyme (TPO) which catalyzes the process. In thyroid gland, H_2O_2 is generated by NADPH oxidases (NOXs), dual oxidase 1 (DUOX1) and dual oxidase 2 (DUOX2) (Leseney et al., 1999, Song et al., 2007, Rigutto et al., 2009). DUOXs are located at the apical plasma membrane of the thyrocyte, and they produce H_2O_2 in the extracellular colloid space (Dupuy et al., 1989; Dupuy et al., 1999). In addition to DUOX1 and DUOX2, human normal thyrocytes also express an intracellular reactive oxygen species (ROS) generating system, NADPH oxidase 4 (NOX4) (Weyemi et al., 2010; Ohye et al., 2010; Ameziane-El-Hassani et. al., 2016). These enzymes belong to the NOX/DUOX family, which consists of seven members including five NADPH oxidases (NOX1, NOX2, NOX3, NOX4 and NOX5) and two DUOXs (DUOX1 and DUOX2). NOX4 is the only NOX with a constitutive ROS-generating activity that depends directly on its gene expression (Ameziane-El-Hassani et al., 2016).

NOX4 is primarily localized in intracellular compartments such as the endoplasmic reticulum, perinuclear regions and mitochondria, but was also detected at the plasma membrane and focal adhesions (Ushio-Fukai, 2009). In the thyroid, it is located intracellularly and at the plasma membrane (Fortunato et al., 2013; Santos et al., 2013;

Weyemi et al., 2013). These findings would suggest a role in intracellular signaling mediated by ROS.

ROS generation by NOX4 has been implicated in high rates of proliferation and tumor progression (Ohye et al., 2010), although the role of NOX4 in the thyroid has not been elucidated. Some studies suggest that it might participate in thyroid dedifferentiation and in cancer pathogenesis (Weyemi & Dupuy, 2013; Azouzi et al., 2017). Previous reports suggest that NOX4 plays a key role in transforming growth factor beta 1 (TGFβ1) action in many cell types, both in vitro (Carmona-Cuenca et al., 2008; Boudreau et al., 2012) and in vivo (Hecker et al., 2009). Recently, it has been shown that ROS generation by NOX4 is a critical mediator of the down regulation of the sodium iodide symporter (NIS) in $BRAF^{V600E}$ mutated thyroid cell lines (Azouzi et al., 2017). Moreover, the knockdown of NOX4 significantly reduced the TGFβ1 effect on cell viability in human thyroid follicular carcinoma cells but not in differentiated rat thyroid cells (Oglio et al., 2018). These results suggest a role of NOX4 depending on the state of thyroid cellular differentiation.

There are evidences that NOX4 modulates downstream insulin signaling by a redox sensitive mechanism (Mahadev et al., 2004). In thyroid cells, insulin and thyrotropin (TSH) synergistically induce proliferation and differentiation (Maciel et al., 1988). Since the interaction of TSH/ insulin signaling has been analyzed (Kimura et al., 2001, Song et al., 2010, Roger et al., 2010) there are certain aspects which required further studies. The Forkhead FOXO transcription factors (FOXO) family was suggested as participants in both stimuli (Zaballos & Santisteban, 2013). The PI3K pathway controls the transcriptional regulation and subcellular location of FOXOs (Franz et al., 2016). Moreover, it has been shown that changes in ROS levels induced by NOX4 modulate FOXO activities in others tissues (Klotz et al., 2015). In the thyroid gland it has been shown that FOXO1 and

FOXO3a regulate proliferation, apoptosis and DNA damage repair (Karger et al., 2009, Klagge et al., 2011, Zaballos et al., 2013, Franz et al., 2016).

The role of NOX4 in thyroid physiology is not completely understood. Therefore, we analyzed the physiological regulation of NOX4 expression by TSH, insulin and iodine. Next, we studied the contribution of NOX4 on the modulation of thyroid genes expression and FOXOs transcription factors in a differentiated rat thyroid cells (FRTL-5) and the correlation between NOX4 and thyroid dedifferentiation.

2. Materials and methods

2.1 Cell culture

The differentiated rat thyroid cells (FRTL-5) were cultured in Dulbecco`s modified Eagle`s medium (DMEN) (Gibco) supplemented with 5% fetal bovine serum (FBS; Natocor, Argentina), and six hormones mixture (6H: 1 nM hydrocortisone, 5 µg/mL transferrin, 10 ng/mL somatostatin, 10 ng/mL glycl-L-histidyl-L-lysine acetate, 1 mUI/mL TSH and 10 µg/mL insulin; Sigma). All cells were supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin and were kept in humidified atmosphere of 5 % CO2 at 37 ºC. Cells were harvested with TRYPSIN (0.25 trypsin 0.25%-EDTA 0.02% solution), seeded on 6 and 24 well plates or in Tissue-Culture 60 mm dishes for experimental purposes. The effect of hormones was studied by starving near-confluent cells from TSH and insulin in the presence of 5% serum.

2.2 Assay of ROS production

In order to determine the ROS levels produced by FRTL-5 cells, the H_2O_2 concentration within the cells was determined using the 2',7'-dichlorfluorescein-diacetate

(DCFH-DA, Sigma) a well-established compound used to detect and quantify intracellular produced ROS. DCFH-DA is freely permeable across the membranes; after entering the cell, the acetate group is hydrolyzed, creating a membrane impermeable form of the dye (DCFH). Hydrogen peroxide and peroxides produced by the cell oxidize DCFH to yield a quantifiable fluorogenic compound 2',7'-dichlorofluorescein (DCF), representing the level of ROS present in the cell, which can be detected by fluorescent microscopy.

Cells (1.0×10^6) were incubated with 10 μM of DCFH-DA for 20 min at 37ºC, and relative ROS units were determined by fluorescence at $\lambda_{excitation}$: 485/20 nm and $\lambda_{emission}$: 530/ 25nm. An aliquot of the cell suspension was lysed, and protein concentration was determined by Lowry et al., 1951. The results are expressed as arbitrary absorbance units/mg protein.

2.3 Western blot analysis

Cells were seeded in 60-mm dishes and incubated with different compounds for the time indicated in the text. Proteins were extracted in lysis buffer RIPA (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet P4 0.01% SDS, 0.5% deoxycholate), supplemented with PMSF 0.5 mM and protease inhibitor cocktail (Sigma-Aldrich). Proteins were electrophoresed on 10% SDS-polyacrylamide gel (SDS-PAGE), prior to transfer to polivinylidene difluoride membranes (PVDF) (Millipore, Bedford, MA), in a semidry transfer cell at 18 V for 1h. Membranes were blocked with 0.2% Tween 20 (Sigma) and 5% BSA for 1 h at RT and then incubated overnight at 4 ºC using monoclonal or polyclonal antibodies. Immunoblotting was carried out with monoclonal anti c-Nox-4 antibody (Abcam Cat# ab133303, RRID:AB_11155321) dilution 1:2000 and monoclonal anti-β-actin antibody (Sigma-Aldrich Cat# A2668, RRID:AB_258014) dilution 1:2000 in

phosphate buffer saline solution (PBS) with 0.2% Tween 20 (Sigma) and 5% BSA. Following incubation with horseradish peroxidase (HRP) conjugated mouse anti-rabbit IgG (Thermo Fisher Scientific Cat# 45-000-683, RRID:AB_2721111). Specific proteins were visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, USA) and densitometric analysis was performed using the NIH ImageJ analysis Software (1.40g Wayne Rasband, National Institutes of Health, USA).

2.4 RNA extraction and real-time PCR

Extraction and purification of RNA were performed following the TRIzol method. 2.5µg RNA was used as a template for cDNA synthesis with Oligo$(dT)_{20}$ using the Superscript III reverse transcriptase (Invitrogen). cDNA (1µl, 1/10) was used in each PCR reaction in a total volume of 25µL, 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´ (63ºC); TPO: forward 5´-TCTGGCATCACTGAACTTGC-3´ and reverse 5´-CGGTGTTGTCACAGATGACC-3´ (61ºC); TTF2: forward 5´-TTCGTGTCTGCCATGTGAGC-3´ (61ºC) and reverse 5´-GCCCACGTCCAAAGCAAATC-3´; PAX8: forward 5´-GGCCACCAAATCTCTGAGCC-3´ and reverse 5´-TGGGAATCGATGCTCAGTCG-3´ (61ºC); NOX4, forward, 5´-TTCTCAGGTGTGCATGTAGC-3´ and reverse, 5´-CGGAACAGTTGTGAAGAGAAGC-3´ (63ºC); TGFβ1, forward, 5´-CTGCTGACCCCCACTGATAC-3´, and reverse, 5´-GCCCTGTATTCCGTCTCCTT-3´ (62ºC); FOXO1: forward 5´-GGTGAAGAGTGTGCCCTACTT-3´ and reverse 5´-CTTGCCTCCCTCTGGATTGA-3´(60 ºC); FOXO3: forward 5´-CGGCTCACTTTGTCCCAGAT-3´ and reverse 5´-CGGCTCATTTGTCCCAGAT-3´ (60ºC); GPx-1: forward 5´-

CCTCAAGTACGTCCGGCCTG-3´ and reverse 5´-CAACATCGTTGCGACACACC-3´ (57 ºC); GADPH: forward 5´-ACAGCAACAGGGTGGTGGAC-3´ and reverse 5´-TTTGAGGGTGCAGCGAACTT-3´ (57ºC). Relative changes in gene expression were calculated according to the $2^{-\Delta\Delta Ct}$ method using GADPH as internal control.

2.5 Transfection of small interfering RNAs

Cells were seeded in Tissue-Culture 60 mm dishes and transfected with 50 nmol/mL of siRNA or a control non targeting scrambled oligonucleotide using siPORTneoFX Transfection Agent (Ambion) according to the manufacturer's instructions with specific human siRNA against NOX4 (NOX4 siRNA, Santa Cruz Biotechnology) or scrambled oligonucleotide siRNA control (sc siRNA, Ambion, Austin, TX).

2.6 Statistical analysis

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

3 Results

3.1 Thyrotropin and Insulin suppress NOX4 expression

Thyrotropin and IGF1/insulin are important hormones in thyroid regulation. They stimulate the expression of genes involved in thyroid differentiation. To study the physiological regulation of NOX4 by TSH and insulin, a differentiated rat thyroid cell line (FRTL-5) was used. Ambesi-Impiobato et al., demonstrated that FRTL-5 cells maintained several of biochemical markers of the thyroid follicular cells (Ambesi-Impiobato et al., 1980). Amount them we may mention that it requires thyrotropin (TSH) to proliferate, have

physiologic iodine uptake, synthesize thyroglobulin and thyroperoxidase, form follicular structures and are non-tumorigenic (Ambesi-Impiobato et al., 1987).

FRTL-5 cells were incubated in medium supplemented with/without TSH and insulin and NOX4 expression was analyzed. In the absence of either TSH or insulin, as well as in untreated cells, NOX4 mRNA levels were increased by 4, 3 and 5 fold respectively compared to cells treated with TSH plus insulin (Figure 1A).

3.2 NOX4 expression is inversely correlated with thyroid genes expression

In order to analyse the contribution of NOX4 to thyroid specific genes expression, interference RNA experiments were performed. NIS, TPO and PAX8 expression were decreased in the absence of TSH and/or insulin, compared to cells treated with TSH plus insulin (Figure 2A), while NOX4 expression increased significantly. In siRNA targeted knockdown of NOX4 experiments, TPO ($p<0.05$), PAX8 ($p<0.01$) and TTF2 ($p<0.05$) mRNA levels were increased. Although siRNA targeted knockdown of NOX4 did not change NIS mRNA in FRTL-5 cells treated with TSH plus insulin, whereas NIS mRNA levels were increased in the absence of TSH and/or insulin (Figures 2B, C, D, E, F).

3.3 TGFβ1 induces NOX4 mRNA expression

We analyzed by Real Time PCR NOX4 and TGFβ1 expression and the same pattern of expression takes place for these genes under the different treatments (Figure 3A). To investigate if TGFβ1 mediates NOX4 expression, FRTL-5 cells were incubated with a selective inhibitor of TGFβ1 receptor, SB-431542. As shown in Figure 3B, SB-431542 completely inhibited NOX4 expression; while siRNA targeted knockdown of NOX4 increased TGFβ1 mRNA levels (Figure 3C). These data indicate that TGFβ1 positively

regulated NOX4 expression, which leads to a NOX4 driven feedback that mediates, in part, TGFβ1 expression.

3.4 NOX4 regulates FOXOs mRNA expression

Since FOXO1 is highly expressed in differentiated thyroid cells compared to thyroid tumor cells, and TSH treatment decreases FOXO1 expression (Zaballos and Santisteban, 2013) we investigated whether NOX4 expression was involved in this process. TSH and insulin treatment decreased FOXO1 and FOXO3a expression. In the absence of TSH and insulin, FOXOs mRNA levels were significantly increased. Interference RNA experiments were performed and FOXO1 and FOXO3a mRNA levels were evaluated. As shown in Figure 4, the NOX4 knockdown increases significantly FOXO1 and FOXO3a mRNA expression in FRTL-5 cells treated with TSH plus insulin. Instead, in starved cells, there were no changes of FOXO1 and FOXO3a mRNA levels.

3.5 PI3K/AKT is not involved with NOX4 mRNA expression

Since TSH plus insulin suppress NOX4 expression, we decided to study whether the PI3K signaling pathway is involved in this effect. FRTL-5 cells were treated with a specific inhibitor of the PI3K pathway (LY294002) and NOX4 mRNA levels were determined. Figure 5A shows, that the repression of NOX4 mRNA by TSH plus insulin was not reversed by LY294002 suggesting that PI3K/AKT pathway was not involved in NOX4 expression. Insulin and TSH are important hormones for thyroid function and both stimulate the expression of thyroid specific genes. LY294002 treatment increased NIS expression and also TTF2, FOXO1 and FOXO3 mRNA levels. This inhibitor had no effect on TPO and PAX8 mRNA levels (Figure 5B, C, D, E, F, G, H).

3.6 NOX4 expression is increased by iodine

Cells were incubated with KI 10 and 100 µM during 3 and 24 h. The intracellular ROS accumulation was assessed by the DCFH-DA-derived fluorescence. Substantial cellular ROS accumulation was also detected in FRTL-5 cells incubated with iodine. To investigate the cellular source of ROS generated by iodine excess in proliferating FRTL-5 cell line, we inhibited NADPH oxidases/flavoenzymes with Diphenylene iodonium (DPI). DPI completely blocked the increase of ROS induced by iodine excess suggesting the involvement of a NOX enzyme (Figure 6A, B).

Since NOX4 is an important source of ROS, we studied if the effect of iodine was mediated by this enzyme. For this purpose, Real Time PCR and Western Blot analysis were performed. As shown in Figure 6C, KI (10 and 100 µM) treatment increased NOX4 mRNA levels and protein expression in FRTL-5 cells. Next, we performed interference RNA experiments, using a scramble siRNA or NOX4 siRNA in FRTL-5 cells incubated with or without KI (10 and 100 µM) for 24 h (Figure 6D). As shown in Figure 6E and F, KI treatment induced ROS accumulation in scrambled siRNA-transfected cells but not in NOX4 siRNA transfected cells. These findings indicate that NOX4 plays a critical role in ROS production induced by KI in FRTL-5 cells.

3.7 SiRNA targeted knock-down of NOX4 reverses the inhibitory effect of iodine on NIS, TPO, PAX8 and TTF2 mRNA expression

To further characterize the role of NOX4 in the KI effect on thyroid function, we analyzed mRNA levels of NIS, TPO and the transcriptions factors PAX8 and TTF2 by RT-PCR. KI treatment (10 and 100 µM) inhibited by 60-70 % NIS, TPO and PAX8 expression while TTF2 was reduced by 50 % with 100 µM of KI. SiRNA targeted knock-down of

NOX4 reversed the inhibitory effect of iodine excess on NIS, TPO, PAX8 and TTF2 mRNA levels (Figure 7). These findings indicate that NOX4 plays a critical role on the inhibitory effect of iodine excess.

3.8 Selenium regulated NOX4 expression

Since Selenium dependent enzymes are very important for the modulation of thyroid ROS levels, we investigated the effect of Selenium (Se) on glutathione peroxidase (GPx), NOX4, TGFβ1 and some thyroid specific genes expression. The selenite concentrations (0.01–0.1μM) used here were within physiological range (Demelash et al., 2004) with no unwanted cytotoxic effects (Leoni et al., 2016; Oglio et al., 2018). Since TSH is required for GPx expression in FRTL-5 cells (Björkman and Ekholm, 1995; Villette et al., 1998), the following experiments were done with TSH and in the presence or absence of insulin.

As shown in Figure 8A, no significant changes in GPx-1 mRNA levels were detected in Selenium-treated FRTL-5 cells. Interesting, we observed an increase in GPx-1 expression when cells were cultured without insulin ($p < 0.05$). The addition of Selenium caused an increase in NOX4 mRNA expression in the presence of TSH and insulin. Contrary, NOX4 and TGFβ1 expression decrease when cells were cultured with Selenium in the absence of insulin. As shown in Figure 8B, treatment with 0.1 μM of Selenium in the presence of TSH and insulin increased NIS expression while TPO, TTF-2 and PAX-8 expression do not change significantly. Instead, in cultured cells without insulin, we observed a non-significant decrease of thyroid genes expression.

4 Discussion

Different factors have been proposed to be involved in the regulation of thyroid function and proliferation such as TSH, insulin, growth factors, iodide, etc. Thyrotropin and IGF1/insulin increase the proliferation rate and stimulate genes involved in thyroid differentiation.

In this study, we showed that TSH and insulin repress NOX4 expression and regulate differentiated thyroid gene expression. It is possible that NOX4 participates in thyroid dedifferentiation since it is repressed by thyrotropin and insulin. Besides it is inversely correlated with differentiated thyroid genes expression. Starved cells resemble a dedifferentiation condition where NOX4 was increased and thyroid-specific gene expressions were decreased. Moreover, knockdown of NOX4 increased the expression of TTF2, PAX8 and TPO. However, the mechanism by which thyrotropin and insulin represses NOX4 expression remains to be elucidated. This results show that NOX4 expression must be finely regulated, in contrast to thyroid tumor cells, in which the uncontrolled proliferation of the cells is accompanied by an increased expression of NOX4 (Weyemi & Dupuy, 2013). The expression level of NOX4 is increased in thyroid tumors which might participate in thyroid dedifferentiation and cancer pathogenesis (Weyemi & Dupuy, 2013; Azouzi et al., 2017). Moreover, NOX4 has been implicated in tumor progression (Boudreau et al., 2012). Present and past studies would indicate that NOX4 acts as a modulator of differentiation in thyroid normal cells. On the contrary, during cancer developed it may stimulate tumor promoter, uncontrolled growth, metastasis and epithelial-mesenchymal transition (EMT).

Previous reports suggest that NOX4 plays a key role in TGFβ1 effect in lung mesenchymal cells and breast epithelial cells (Hecker et al., 2009; Boudreau et al., 2012).

Here, we observed that TGFβ1 positively regulated NOX4 expression. The knockdown of NOX4 increased TGFβ1 mRNA levels suggesting a loop between NOX4 and TGFβ1 which mediates, at least in part, TGFβ1 expression. In thyroid cells, more studies are required to provide further evidence about the link between TGFβ1 and NOX4. In coincidence with NOX4, different studies demonstrated that thyroid tumors have high levels of TGFβ1 (Kimura et al., 1999; Morosini et al., 1996; Matsuo et al., 2010).

Other genes that are increased after NOX4 knockdown are FOXO1 and FOXO3. FOXOs have emerged as important tumor suppressors, promoting cell cycle arrest, apoptosis and DNA damage repair (Franz et al. 2016). They may be novel players in cellular stress response (Karger et al., 2009). In agreement with the literature, our results show that TSH and insulin treatment decreased FOXO1 and FOXO3 expression and are transcriptionally regulated by PI3K/AKT signaling pathway (Karger et al., 2009; Zaballos & Santisteban, 2013). In our model, we have shown that PI3K/AKT pathway does not regulate NOX4 expression. However, NOX4 knockdown increases FOXO1 and FOXO3 expression after TSH and insulin treatment. These preliminary results would suggest that NOX4 could regulate FOXOs expression in a PI3K/AKT dependent mechanism. Further studies are required to clarify this question.

Iodine is another factor that regulates thyroid functions and growth. Previous studies have shown that, iodine excess inhibits several thyroid parameters, such as iodide uptake, H_2O_2 formation, cell proliferation, and inhibit the expression of certain genes within the thyroid (Pannels et al., 2009, Thomasz et al., 2010, Rossich et al., 2016). Several studies have demonstrated that iodine excess induce ROS production, intracellular oxidation levels, and therefore cell toxicity (Many et al., 1992, Golstein & Dumont, 1996, Poncin et al., 2008). Here, we report for the first time that iodine increases NOX4 expression and its

silencing prevents the increase of intracellular ROS induced by iodine excess. Moreover, NOX4 knockdown reverses the inhibitory effect on NIS, TPO, PAX8 and TTF2 mRNA expression induced by iodine excess. Therefore, we proposed that NOX4 protein plays a key role in the overproduction of ROS induced by iodine excess and is involved in the regulation of thyroid specific genes expression in FRTL-5 cells.

On the other hand Serrano et al., 2014, demonstrated that the rapid inhibitory effect of high concentrations of iodine on NIS expression and function involved the participation of PI3K/Akt signaling pathway. We suggest that PI3K/Akt signaling pathway is downstream NOX4 activation since the inhibition of the PI3K/Akt pathway by LY294002 did not change NOX4 expression. We hypothesize that iodine increased the expression of NOX4 which induces the overproduction of ROS and this could be crucial for stimulating Akt phosphorylation and reduction of NIS expression in thyroid cells. In addition, PI3K/Akt signaling pathway seems to regulate TTF2 expression since that preincubation of thyroid cells with LY294002 increased its expression.

Iodine excess increases TGFβ1 mRNA (Yuasa et al., 1992) and protein expression (Grubeck-Loebenstein et al., 1989). The present results demonstrated that excess iodine increases NOX4. Since inhibition of TGFβ1 by SB-431542 decrease NOX4 expression it is likely that NOX4 could participate in the sequence as follow: Iodine → TGFβ1→ NOX4 → ROS.

In thyroid, Selenium content is crucial for its preservation and plays an essential role in regulating thyroid function (Beckett and Arthur, 2005; Kohrle et al., 2005). It has been shown that Selenium increases the synthesis and the activity of the glutathione peroxidase (GPx) in cultures thyroid cells and in other cells types (Villette et al., 1998; Bjorkman and Ekholm, 1995). The GPx has a major role in regulating intracellular

physiological levels of hydrogen peroxide (H_2O_2) in the thyroid cell (Song et al., 2007). In our experimental model, Selenium treatment did not increase GPx-1 expression. However, we cannot rule out the effect of Selenium on other Selenoproteins present in thyroid, such as others GPx isoenzymes, thioredoxin reductases (TRs) and iodothyronine deiodinases (Ds) (Beckett and Arthur, 2005). Furthermore, the addition of Selenium caused an increase in NOX4 mRNA expression in the presence of TSH and insulin. This result could be interpreted as a cellular adaptive response to changes in redox metabolism. A different mechanism was observed in the absence of insulin. The finding of reduced NOX4 expression could be associated with the decreased of TGFβ1 expression. Further studies are required to clarify this point. Our data would suggest that Selenium has different effect related to the presence or absence of insulin in the coulter medium.

On the other hand, our results demonstrate that the addition of Selenium increased NIS expression, according with Leoni et al., who demonstrated that Se increases TSH induced NIS expression and activity in rat thyroid cells (Leoni et al., 2016).

The data presented provides strong evidence for a novel signaling modulator of TSH/ insulin pathway. First, NOX4 seems to have control over thyroid differentiation. This point is evidenced by the following results: 1) TSH/insulin represses NOX4 mRNA expression; 2) the level of NOX4 appears to be inversely correlated with some thyroid genes. Second, NOX4 could have a critical role in the autoregulatory mechanism induced by iodine. This follows from: 1) iodine excess induces ROS through NOX4, 2) silencing NOX4 reverses the inhibitory effect of iodine on thyroid genes.

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Conflicts of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Legends to figures

Figure 1: Thyrotropin and Insulin suppress NOX4 expression. (A) Proliferating FRTL-5 cells were cultured with TSH plus insulin, TSH alone, insulin alone or in the absence of both hormones and NOX4 mRNA levels were determined by real time quantitative PCR. Data are presented as the means ± S.D of 3 experiments; *= $p<0.05$; **= $p<0.01$ and ***= $p<0.001$ as compared to +TSH +Insulin.

Figure 2: NOX4 expression is inversely correlated with thyroid genes expression. Proliferating FRTL-5 cells were cultured with TSH plus insulin, TSH alone, insulin alone or in the absence of both hormones and thyroid genes mRNA levels were determined by real time quantitative PCR. **(A)** Effect of TSH and insulin on NOX4, NIS, TTF2, PAX8 and TPO expression. Data are presented as the means ± S.D of 3 experiments; *= $p<0.05$; **= $p<0.01$ and ***= $p<0.001$ as compared to +TSH +Insulin. **(B – F)** Effect of SiRNA target knockdown of NOX4 on thyroid genes expression. Cells were transfected with siRNA NOX4 or scrambled (Scr) and NOX4, PAX8, TTF2, NIS and TPO mRNA levels were determined by real time quantitative PCR. Data are presented as the means ± S.D of 3 experiments; *= $p<0.05$, and **= $p<0.01$ as compared to respective Scr (Control).

Figure 3: TGFβ1 induces NOX4 expression. Proliferating FRTL-5 cells were cultured with TSH plus insulin, TSH alone, insulin alone or in the absence of both hormones and **(A)** mRNA levels of NOX4 and TGFB1 were determined by real time quantitative PCR. **(B)** Effect of 10 µM of SB-431542 on NOX4 mRNA levels in FRTL-5 cells. Cells were transfected with siRNA NOX4 or scrambled (Scr) and TGFB1 mRNA levels were

determined by real time quantitative PCR (C). Data are presented as the means ± S.D of 3 experiments; *= p <0.05, and **= p <0.01 as compared to respective control.

Figure 4: NOX4 regulated FOXOs mRNA expression. (A, B) Cells were transfected with siRNA NOX4 or scrambled (Scr) and cultured with TSH plus insulin, TSH alone, insulin alone or in the absence of both hormones for 24 h. FOXO1 and FOXO3a mRNA levels were determined by real time quantitative PCR. Data are presented as the means ± S.D of 3 experiments; a = p <0.05 and aa =p <0.01 as compared to Scr Control (+TSH+ insulin); b =p <0.05 and bb=p <0.01 as compared to siRNA NOX4 Control (+TSH+ insulin) and *= p <0.05, and **= p <0.01 as compared to respective Scr condition.

Figure 5: PI3K/AKT is not involved with NOX4 mRNA expression. Proliferating FRTL-5 cells were treated in the presence or absence or both, of Insulin and TSH for 24 h. An inhibitor of the PI3K pathway (30 µM, LY29004) was added during the last 3 hours of treatment. **(A)** Effect of PI3K/AKT pathway on NOX4, **(B)** TGFβ1, **(C)** TTF2, **(D)** PAX8, **(E)** NIS, **(F)** TPO, **(G)** FOXO1, and **(H)** FOXO3a mRNA levels were determined by real time quantitative PCR. Data are presented as the means ± S.D of 3 experiments; a= p<0.05 and aa= p<0.01 as compared to Vehicle Control (+TSH+ insulin); b = p <0.05 and bb= p<0.01 as compared to LY29004 Control (+TSH+ insulin) and *= p<0.05, and **= p <0.01.

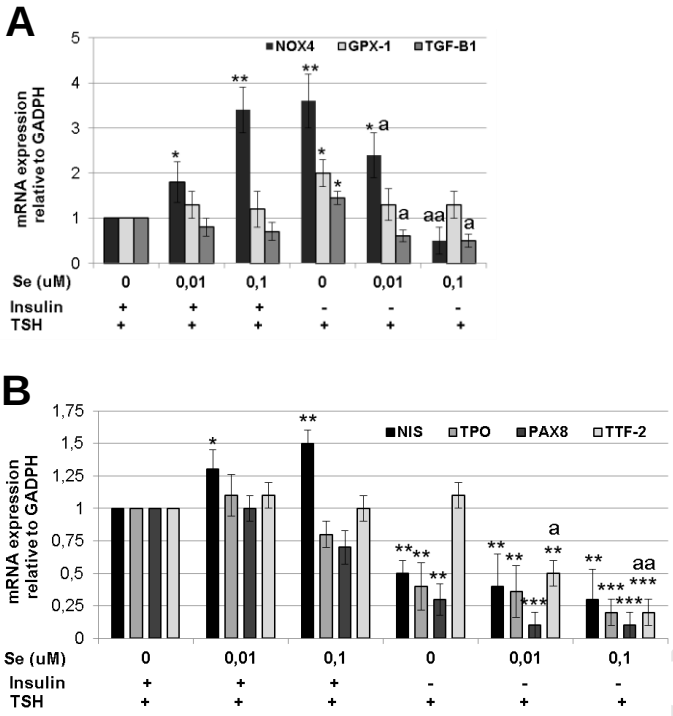
Figure 6: NOX4 expression is increased by iodine. **(A, B)** Diphenylene iodonium (DPI) abolish KI-induced ROS production. Intracellular ROS levels were detected by using DCFH2-DA. Proliferating FRTL-5 cells were incubated during 24 h with KI (10 and 100 µM) and in the presence or absence of NADPH oxidase/flavoenzyme inhibitor DPI (20 µM) in the last 15 min of treatment. Results represent the means ± S.D of 4 experiments of

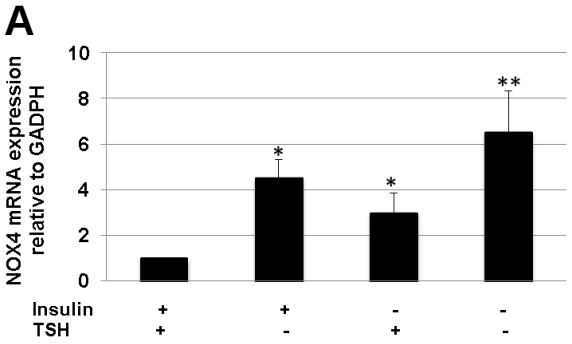
duplicated dishes. *= p < 0.05 and **= p <0.01; as compared Control (+TSH); aa= p< 0.01 as compared to KI 100 µM; b= p < 0.05 and bb= p < 0.01 as compared to KI 10 µM. **(C)** KI induces expression of NOX4 protein in FRTL-5 cells. NOX4 expression was detected by Western blot. Results represent the means relative to B-actin control ± S.D; **= p <0.01 (KI versus TSH). **(D)** FRTL-5 cells were transfected with siRNA NOX4 or scrambled (Scr) and treated with KI. After 24 h, NOX4 mRNA levels were determined by real time quantitative PCR. Data are presented as the means ± S.D of 3 experiments; **= p <0.01. **(D)** NOX4 increases intracellular ROS levels. FRTL-5 cells were incubated during 24 h with KI (10 and 100 µM) in the presence or absence of siRNA NOX4 and intracellular ROS levels were detected by using DCFH2-DA. Results represent the means ± S.D of 4 experiments of duplicated dishes. **= p <0.01 (KI versus TSH); aa= p< 0.01, a= p<0.05 as compared to KI 100 µM; b= p< 0.05 and bb = p< 0.01 as compared to KI 10 µM.

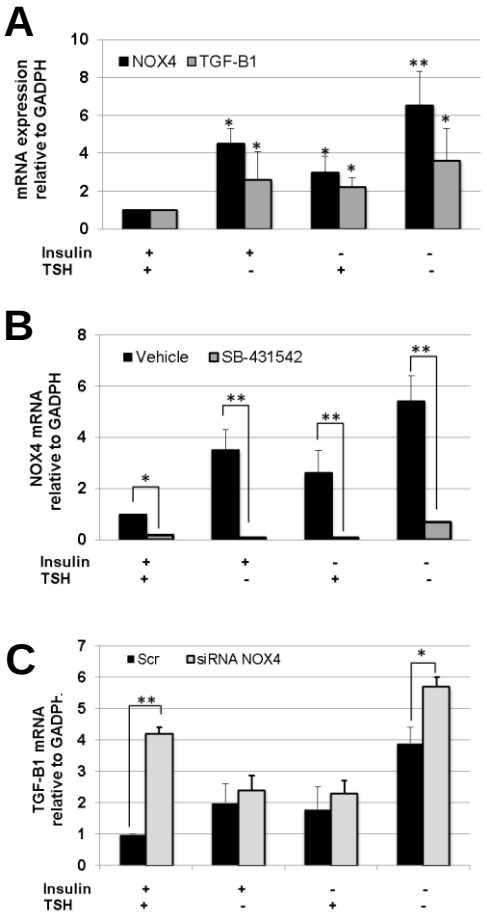
Figure 7: SiRNA targeted knock-down of NOX4 reverses the inhibitory effect of iodine on specific thyroid genes expression. FRTL-5 cells were transfected with siRNA NOX4 or scrambled (Scr) and treated with KI (10 and 100 µM) for 24 h. **(A)** PAX8, **(B)** TTF2, **(C)** TPO and **(D)** NIS mRNA levels were determined by real time quantitative PCR. Data are presented as the means ± S.D of 3 experiments; *= p<0.05, **= p <0.01 and ***= p <0.001 KI versus Scr TSH control; a= p <0.05 as compared to Scr KI (10µM) and b= p<0.05 as compared Scr KI (100µM).

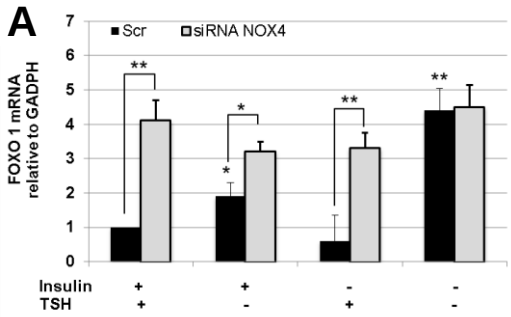
Figure 8: Effect of sodium selenite (Se) on in the expression of glutathione peroxidase (GPx), NOX4, TGFβ1 and thyroid specific genes. Proliferating FRTL-5 cells were pre incubated in the presence or absence of 0.01 and 0.1 µM of Se for 24 h. The effect of hormones was studied by starving near-confluent cells from insulin in the presence of 5% serum. **(A)** Effect of Se on NOX4

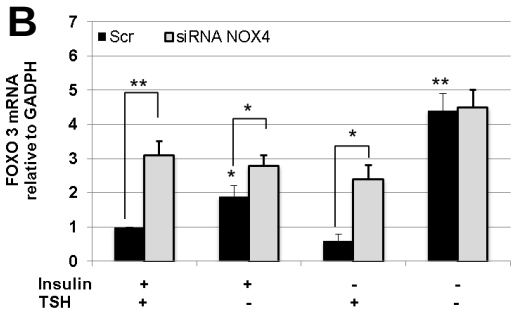
TGFβ1 and GPX-1 mRNA expression. NOX4, TGFβ1and GPX-1 mRNA levels were determined by real time quantitative PCR. **(B)** Effect of Se on NIS, TPO, PAX8 and TTF-2 expression. Thyroid genes expression were determined by real time quantitative PCR. Data are presented as the means ± S.D of 3 experiments. *= $p<0.05$; **= $p<0.01$ and ***= $p<0.001$ as compared to +TSH +insulin; a = $p<0.05$ and aa =$p<0.01$ as compared to TSH alone (+TSH –insulin).

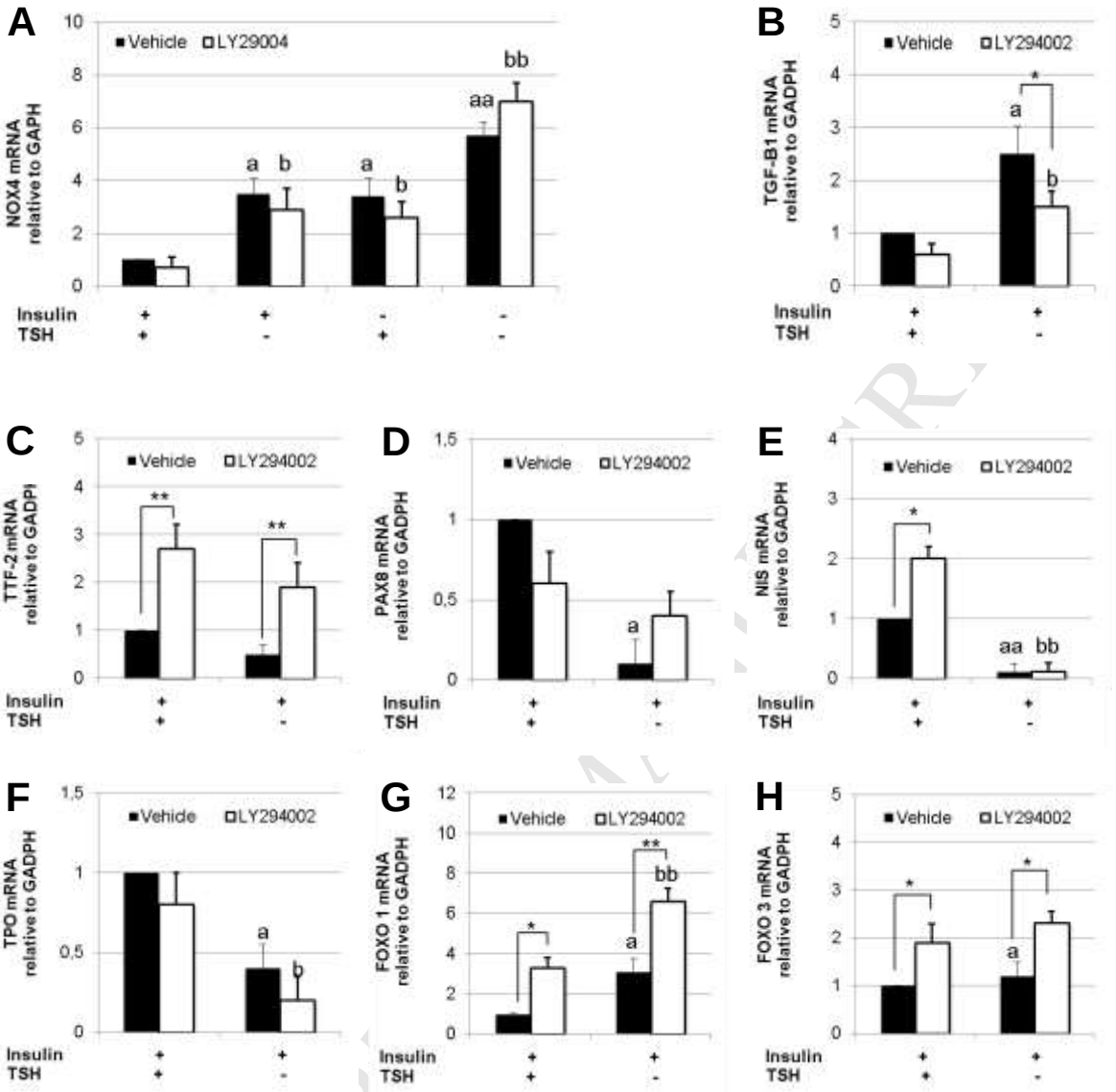








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NOX4 mRNA relative to GAPH
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TSH
B
Vehicle
LY294002
TGF-B1 mRNA relative to GADPH
C
TTF-2 mRNA relative to GADPH
D
PAX8 mRNA relative to GADPH
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NIS mRNA relative to GADPH
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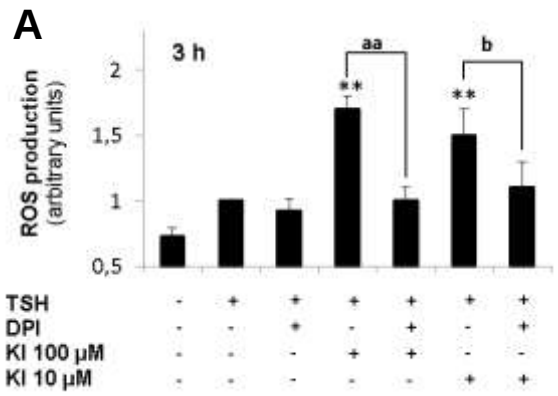
FOXO 1 mRNA relative to GADPH
H
FOXO 3 mRNA relative to GADPH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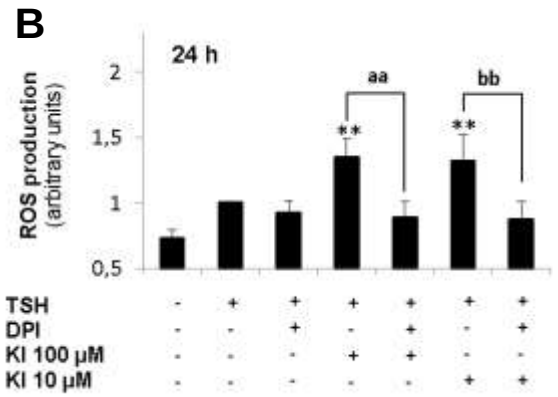
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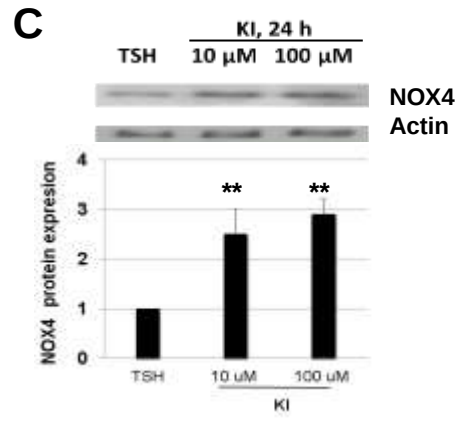
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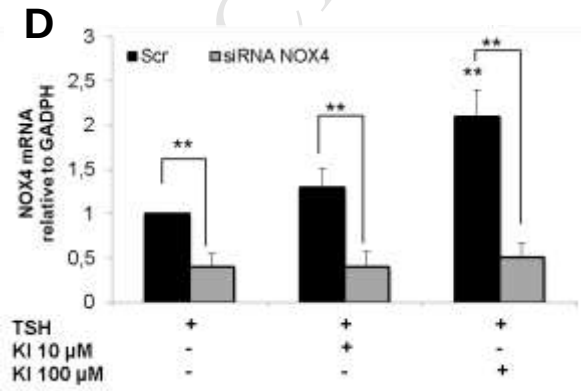
100 uM
KI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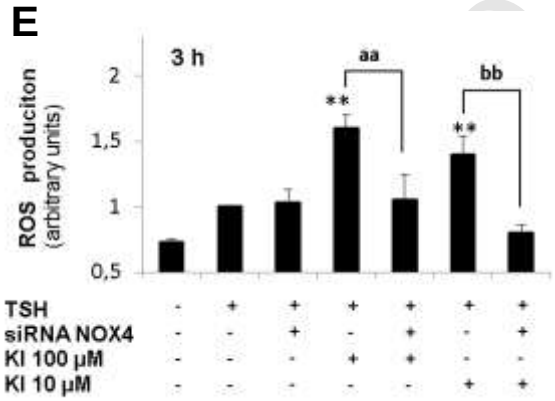
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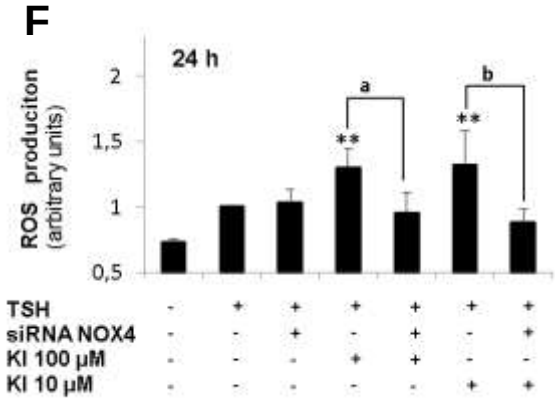
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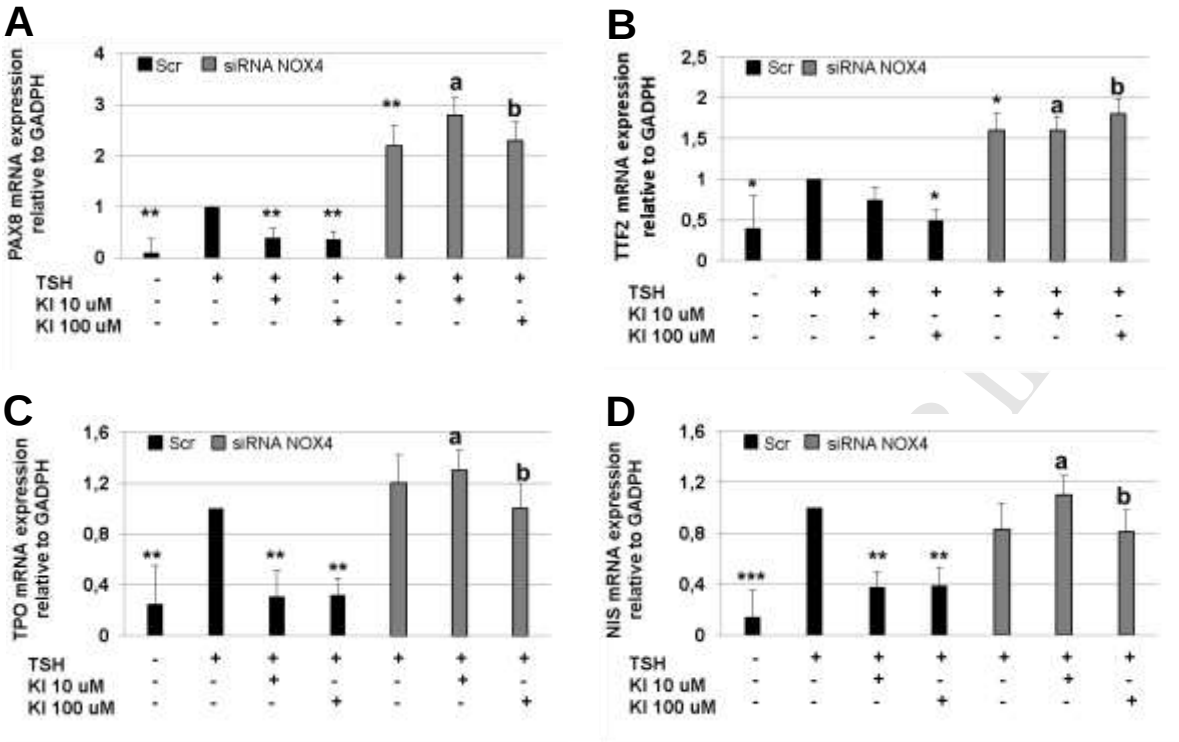

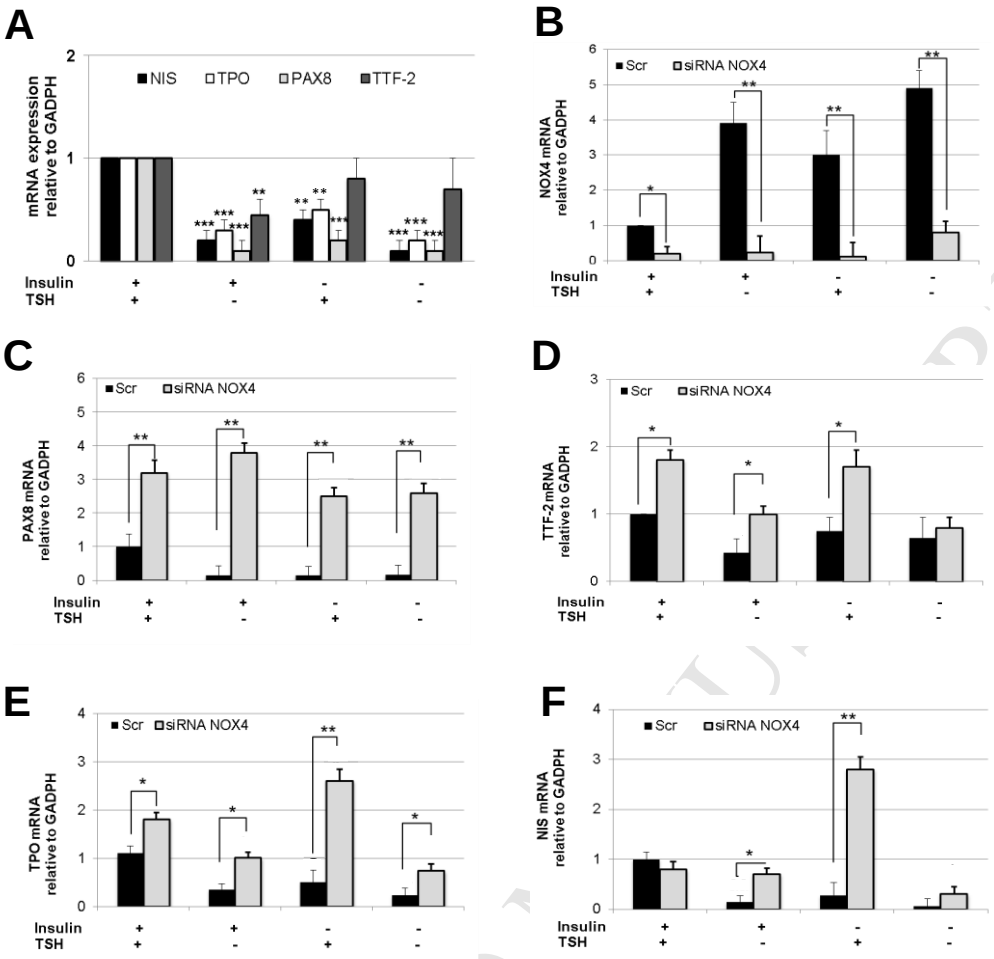

NOX4 seems to have control over thyroid differentiation.

TSH/insulin represses NOX4 mRNA expression;

The level of NOX4 appears to be inversely correlated with some thyroid genes.

NOX4 could have a critical role in the autoregulatory mechanism induced by iodine.