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Excess iodide downregulates Na^+/I^- symporter gene transcription through activation of PI3K/Akt pathway

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A B S T R A C T

Transcriptional mechanisms associated with iodide-induced downregulation of NIS expression remain uncertain. Here, we further analyzed the transcriptional regulation of NIS gene expression by excess iodide using PCCl3 cells. NIS promoter activity was reduced in cells treated for 12–24 h with 10^{-5} to 10^{-3} M NaI. Site-directed mutagenesis of Pax8 and NF-κB cis-acting elements abrogated the iodide-induced NIS transcription repression. Indeed, excess iodide (10^{-3} M) excluded Pax8 from the nucleus, decreased p65 total expression and reduced their transcriptional activity. Importantly, p65-Pax8 physical interaction and binding to NIS upstream enhancer were reduced upon iodide treatment. PI3K/Akt pathway activation by iodide-induced ROS production is involved in the transcriptional repression of NIS expression. In conclusion, the results indicated that excess iodide transcriptionally represses NIS gene expression through the impairment of Pax8 and p65 transcriptional activity. Furthermore, the data presented herein described novel roles for PI3K/Akt signaling pathway and oxidative status in the thyroid autoregulatory phenomenon.

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1. Introduction

Iodide (I^-) is an essential component of thyroid hormones. Active I^- accumulation in the thyroid follicular cell is mediated by the Na^+/I^- symporter (NIS), a basolateral plasma membrane glycoprotein that plays a essential role in thyroid physiology (Portulano et al., 2014). NIS mediates I^- transport in several tissues other than the thyroid gland, e.g., salivary glands, stomach, small intestine and lactating breast, suggesting an important role of NIS in I^- homeostasis (Nicola et al., 2015). Insufficient dietary I^- causes hypothyroidism and subsequently goiter, stunted growth, retarded psychomotor development and even irreversible mental retardation (Zimmermann, 2009).

Thyrotropin (TSH) is the primary regulator of I^- uptake and NIS expression in thyroid cells. TSH-induced cyclic adenosine monophosphate (cAMP) production stimulates I^- transport by increasing NIS gene transcription (Kogai et al., 1997, Ohno et al., 1999). In fact, elevation of cAMP levels induces the transcriptional activity of several transcription factors that stimulates NIS gene expression (Kogai et al., 2006). The regulatory region controlling rat NIS gene expression contains a proximal promoter located within nucleotides −110 and −420 relative to the transcription start site, and a strong TSH-responsive far-upstream enhancer (NUE) between nucleotides −2264 and −2495 (Tong et al., 1997, Chun et al., 2004). The NUE region contains two thyroid transcription factor-1

Abbreviations: NIS, Na^+/I^- Symporter; TSH, thyroid stimulating hormone or thyrotropin; NUE, NIS Upstream Enhancer; NF-κB, Nuclear Factor-kappa B; Pax8, Paired Box 8; RPL19, ribosomal protein L19; TSHR, thyrotropin receptor; IκB-α, NF-κB inhibitor alpha; IL-6, interleukin 6; TPO, thyroid peroxidase; Tg, thyroglobulin; NAC, N-acetylcysteine; ROS, Reactive oxygen species; PI3K, Phosphoinositide 3-kinase; Akt, Protein kinase B; ChIP, Chromatin Immunoprecipitation; PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase.

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(TTF-1)-binding sites with uncertain roles in the control of NIS expression (Ohno et al., 1999), two paired box 8 (Pax8)-binding sites (Ohno et al., 1999), and a cAMP-response element (CRE)-like site, required for TSH responsiveness (Chun et al., 2004). Nicola et al., 2010 demonstrated that upon activation, the transcriptionally active NF-κB subunit p65 translocates to the nucleus and synergizes with Pax8 to regulate NIS gene transcription. Recently, Galrao et al., 2014 identified a novel enhancer region located between nucleotides −1887 to −2152 bp in the human NIS promoter and observed that its hypermethylation was associated with reduced NIS gene expression in thyroid tumors.

Although thyroid function is tightly regulated by TSH, excess I^- is a negative regulator of thyroid hormone synthesis (Serrano-Nascimento et al., 2014). High concentrations of I^- reduce thyroid hormone synthesis and secretion, a phenomenon known as the Wolff-Chaikoff effect (Wolff and Chaikoff, 1948). Indeed, elevated intracellular I^- concentrations trigger an intrinsic autoregulatory mechanism that protects thyroid cells against I^- overload leading to the inhibition of I^- transport and, in turn, restores thyroid hormonogenesis (Braverman and Ingbar, 1963, Grollman et al., 1986). Several mechanisms have been proposed to explain the molecular basis of the "escape" from the Wolff–Chaikoff effect. Inhibition of I^- uptake in response to excess I^- has been associated with a transcriptional inhibition of NIS expression (Eng et al., 1999). However, the transcriptional mechanism associated with the "escape" from the Wolff-Chaikoff effect remains to be fully characterized. Recent studies evidenced that excess I^- also regulates NIS expression through post-transcriptional mechanisms (Serrano-Nascimento et al., 2014, 2010, 2012, Leoni et al., 2011).

Several signaling pathways are involved in the regulation of NIS expression in thyrocytes (Garcia and Santisteban, 2002, Zaballos et al., 2008, Corvilain et al., 1994). Indeed, we recently demonstrated the involvement of the PI3K/Akt cascade in the excess I^--induced post-transcriptional regulation of NIS expression. Our data indicated that excess I^- increases mitochondrial superoxide production in PCCl3 thyroid cells, which in turn activates PI3K/Akt signaling pathway thus regulating NIS expression (Serrano-Nascimento et al., 2014). Therefore, we proposed a novel role for PI3K/Akt signaling, linking thyroid oxidative state to the "escape" from the Wolff-Chaikoff effect.

Here, we aimed to further investigate the molecular events involved in the downregulation of NIS expression in response to excess I^- treatment using PCCl3 cells, a highly differentiated thyroid cell line. We observed that excess I^- downregulates NIS expression at transcriptional level repressing the TSH-induced NIS promoter activity. High concentrations of I^- reduced NIS gene expression by reducing the transcriptional activity of the transcription factors Pax8 and the NF-κB subunit p65. In addition, we demonstrated that excess I^- activated PI3K/Akt pathway through increased reactive oxygen species (ROS) production leading to a downregulation of NIS gene expression. Thus, our results provide novel evidence regarding thyroid oxidative state and the I^--induced autoregulatory mechanism that reduces NIS expression.

2. Materials and methods

2.1. Reagents

$Akt_{1/2}$ kinase inhibitor ($Akti_{1/2}$), wortmannin, LY294002, BAY 11-7082, 2-Phenyl-1,2-benzisoselenazol-3-(2H)-one (Ebselen), N-acetylcysteine (NAC), 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2-DCFDA), actinomycin D, 2-mercapto-1-methyl-imidazole (MMI), bovine serum albumin (BSA) and sodium iodide were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

The rat thyroid-derived highly differentiated PCCl3 cell line was cultured in Ham's F12 media supplemented with 5% fetal bovine serum (Life Technologies, Carlsbad, CA), 1 mU/ml bovine TSH, 10 μg/ml bovine insulin, 5 μg/ml bovine transferrin and 10 nM hydrocortisone (Sigma Chemical Co.) in a 5% CO_2 humidified atmosphere at 37 °C. Cells were treated with 10^{-3} M NaI for the indicated periods of time. Sodium iodide concentration was selected based on previous studies that aimed to further characterize the inhibitory effects of excess I^- on NIS expression and activity (Leoni et al., 2011,Eng et al., 2001,Leoni et al., 2008). Moreover, additional experiments were performed in PCCl3 cells treated with 10^{-7} to 10^{-3} M NaI for 24 h.

When chemical inhibitors were used, PCCl3 cells were preincubated with dimethyl sulfoxide (DMSO; vehicle) or specific inhibitors at the indicated concentrations for 1 h before NaI treatment. To investigate the participation of iodinated compounds in the I^--induced inhibitory effects, PCCl3 cells were preincubated with the thyroid peroxidase inhibitor methimazole (10^{-3} M), for 1 h before NaI treatment for 24 h. To assess the role of intracellular I^- excess, cells were concomitantly treated with equal concentrations of NaI (10^{-3} M) and $NaClO_4$ (10^{-3} M), a competitive NIS inhibitor (Dohan et al., 2007). To evaluate the role of TSH on the I^--induced effects, PCCl3 cells were cultured for 4 days in fresh media without TSH and supplemented with 0.2% calf serum (starving condition) (Nicola et al., 2010). Thereafter, cells were treated with the indicated concentration of NaI in the presence or absence of 1 mU/ml TSH for 24 h.

2.3. Transient transfections and gene reporter assays

PCCl3 cells were transiently transfected in 24-well plates with 0.64 μg of the indicated luciferase reporter constructs/well using LipofectAMINE 2000 reagent (Life Technologies, Carlsbad, CA) following manufacturer's recommendation. Twenty four hours after transfection, cells were treated with NaI at the indicated concentrations and periods of time. Luciferase activity was evaluated using a Luciferase Assay System (Promega, Madison, WI) according to manufacturer's recommendation. To assess transfection efficiency, cells were co-transfected with 0.16μg/well of the normalization reporter pCMV-β-galactosidase (Promega, Madison, WI).

The constructs linked to luciferase used in the transfection experiments were: 1) −2854 to +13 bp DNA fragment of the rat NIS promoter (pNIS) (Garcia and Santisteban, 2002); 2) −2495 to −2264 bp DNA fragment of the rat NIS promoter enhancer cloned 5′ upstream to the thymidine kinase promoter (pNUE); 3) site-directed mutants of NUE region (pNUE-A MT, pNUE-C MT, pNUE-κB MT) (Ohno et al., 1999, Nicola et al., 2010); 4) −168 to +36 bp DNA fragment of the rat thyroglobulin promoter (pTg) (Velez et al., 2006); 5) −429 to +3 bp DNA fragment of the rat thyroperoxidase promoter (pTPO) (Nazar et al., 2012); 6) the expression vector encoding Flag-tagged Pax8 (Pax8-Flag) and the Pax8 reporter vector (Cp5-luc) containing five Pax8 binding sites (Baratta et al., 2009); and 7) the NF-κB reporter vector (5× κB-Luc) containing five κB consensus sites, which was obtained from Clontech Laboratories (Palo Alto, CA).

2.4. RNA extraction and real-time PCR analysis

Total RNA was purified using Trizol following the manufacturer's instructions. Real-Time PCR amplifications were performed using Platinum SYBR Green qPCR Super Mix-UDG (Life Technologies, Carlsbad, CA). Gene-specific primers sequences are described in Supplemental Table 1. Relative changes in gene expression were

calculated using the $2^{-\Delta\Delta Ct}$ method and Rpl19 as internal control whose expression did not significantly alter under the experimental conditions (Supplemental Fig. 1) (Leoni et al., 2008). Each pair of primers resulted in a single peak after the melting curves analysis, indicating that a single cDNA fragment was amplified.

2.5. Protein isolation and western blot analysis

Total protein extraction was performed as described before (Eng et al., 2001). Briefly, PCCl3 cells were harvested in RIPA buffer [50 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). For isolation of cytoplasmic and nuclear proteins, PCCl3 cells were harvested in buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF] and incubated on ice for 15 min. The lysate was centrifuged and the supernatant was collected as cytoplasmic fraction. The nuclear pellet was resuspended in buffer C [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] and incubated on ice for 20 min. The lysate was centrifuged and the supernatant was collected as nuclear fraction. To evaluate proteins phosphorylation, PCCl3 cells were lysed in ice-cold lysis solution containing 1% Triton X-100 in PBS supplemented with 1 mM $NaOV_3$, 1 mM NaF, 0.1 mM PMSF and protease inhibitor cocktail.

Proteins were resolved by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were blocked and incubated with primary antibodies diluted in 3% BSA, 0.1% Tween 20, Tris-buffered saline overnight at 4 °C. Equal loading was evaluated by stripping and re-probing the same membrane with the indicated loading controls. The purity of cytoplasmic/nuclear extracts was evaluated assessing the expression of cytoplasmic and nuclear markers. Primary antibodies, source and dilutions are presented in Supplemental Table 2. Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Jackson Immuno Research Laboratories (West Grove, PA). Proteins blots were visualized by using the Amersham Biosciences enhanced chemiluminescence detection system (Buckinghamshire, UK). Blot densitometry was analyzed using the ImageJ Software (National Institutes of Health, Bethesda, MD).

2.6. Immunofluorescence analysis

PCCl3 cells grown on coverslips were fixed with 2% paraformaldehyde in PBS for 20 min and further permeabilized with PBS containing 0.05% Triton X-100 for 15 min. Cells were incubated 1 h at room temperature with anti-p65 antibody (1:100) and anti-Pax8 antibody (1:50). After washing, cells were incubated with anti-rabbit FITC-conjugated or anti-mouse TRITC-conjugated secondary antibodies (Sigma Chemical Co., St. Louis, MO) for 1 h in darkness. Cells were visualized in a ZEISS Axiovert 100 M fluorescence microscope (Carl Zeiss, Germany). Images were processed with ImageJ Software and Photoshop CS6 (Adobe, San Jose, CA).

2.7. Co-immunoprecipitation

Nuclear fractions from PCCl3 cells were isolated and further lysed in RIPA buffer. Nuclear proteins (300 μg) were precleared for 20 min at 4 °C with Protein A/G PLUS (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitations were performed with 5 μg polyclonal anti-p65 or control rabbit IgG antibodies overnight at 4 °C under constant rotation. Immune complexes were allowed to bind to Protein A/G PLUS-Agarose during 4 h at 4 °C under constant rotation. Agarose beads were washed with RIPA buffer, resuspended in 2× sample buffer and boiled. Pax8 levels in the immunoprecipitated material were evaluated by Western blot analysis, as indicated above.

2.8. Chromatin immunoprecipitation (ChIP)

After treatment, PCCl3 cells were crosslinked in culture media containing 1% formaldehyde. Nuclei were isolated and lysed in 50 mM Tris–HCl (pH 8), 10 mM EDTA, and 1% SDS. Genomic DNA was broken by sonication and 10-fold diluted in IP Dilution Buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 0.5% Nonidet P-40]. Immunoprecipitation was performed with 2 μg of affinity-purified mouse monoclonal anti-p65 antibody or control mouse IgG. Immune complexes were purified with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were washed four times with IP Dilution Buffer containing 0.1% SDS; twice with High Salt IP Wash Buffer [50 mM Tris–HCl (pH 7.5), 500 mM NaCl, 5 mM EDTA, 0.1% SDS, and 1% Triton X-100] and once with TE [10 mM Tris–HCl (pH 8), 1 mM EDTA]. DNA was purified using Chelex-100 (Bio-Rad, Richmond, CA). Immunoprecipitated DNA was quantified by real-time PCR using primers spanning NUE region (Nicola et al., 2010). Relative fold of change was calculated according to the equation: $2^{-[(Ct.input - Ct.target) - (Ct.input - Ct.mock)]}$.

2.9. Measurement of reactive oxygen species (ROS) production

ROS production was evaluated using the ROS-sensitive probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2-DCFDA). PCCl3 cells untreated or treated with 10^{-6} to 10^{-3} M NaI for 24 h were incubated with 5 μM carboxy-H2-DCFDA probe for 20 min. Moreover, in additional experiments PCCl3 cells were concomitantly treated with 10^{-3} M NaI and Ebselen or NAC for 24 h, to evaluate the efficiency of these drugs on the blockade of ROS production. Thereafter, the cells were incubated with 5 μM carboxy-H2-DCFDA and analyzed by flow cytometry. The fluorescence of 10^4 events per sample was acquired using Guava Easy Cyte flow cytometer and analyzed with Guava Express Pro Software (EMD Millipore, Billerica, MA). Immunofluorescence microscopy analysis was performed as described above.

2.10. Statistical analysis

All data were reported as means ± SEM. Data was subjected to normality test (Kolmogorov–Smirnov) and to one-way or two-way analysis of variance (ANOVA) followed by Student-Newman-Keuls or Bonferroni post-tests, respectively, as described in figure legends. Comparisons between two groups were made using two-tailed Student's unpaired t test. Statistical analysis was performed by using GraphPad Prism (GraphPad Software, San Diego, CA). Differences were considered statistically significant at p-value < 0.05.

3. Results

3.1. Excess I^- represses NIS gene expression at transcriptional level

We have previously demonstrated that excess I^- rapidly reduces NIS mRNA expression through post-transcriptional mechanisms including reduction of NIS mRNA poly(A) tail length, NIS mRNA half-life and NIS mRNA association to ribosomes (Serrano-Nascimento et al., 2010, 2012). In order to investigate the involvement of transcriptional mechanisms in the regulation of NIS gene expression by excess I^-, we evaluated NIS mRNA expression in PCCl3 thyroid cells treated with NaI for different periods of time (30 min–24 h) in the presence or absence of the RNA synthesis

inhibitor actinomycin D (ActD). As previously reported (Serrano-Nascimento et al., 2012), excess I^- rapidly reduced NIS mRNA expression despite the presence of actinomycin D (Fig. 1A). This result supports an acute post-transcriptional regulation of NIS mRNA content by excess I^-. Moreover, in agreement with previous observations (Uyttersprot et al., 1997, Eng et al., 1999, Leoni et al., 2011), we observed that excess I^- treatment for longer periods of time (12 and 24 h) significantly reduced the expression of NIS mRNA (Fig. 1A). However, conversely to the results observed after 30 min of treatment, long-term excess I^- effects were abrogated in the presence of actinomycin D (Fig. 1A). Together, these data suggest that excess I^- also regulates NIS gene expression through transcriptional mechanisms.

3.2. Excess I^- downregulates NIS promoter activity

To evaluate the transcriptional repression of NIS gene expression in response to excess I^-, we analyzed the effect of I^- treatment in PCCl3 cells transiently transfected with a luciferase reporter construct containing a 2.8-kb DNA fragment of the rat NIS promoter (pNIS). I^- treatment for 30 min did not modify NIS promoter activity (Fig. 1B); however, a significant repression was observed after 12 and 24 h of treatment (Fig. 1B). In addition, we observed a significant downregulation of NIS promoter activity in cells treated with lower concentrations of NaI (10^{-5} and 10^{-4} M) for 24 h (Fig. 1C). It is worth noting that I^- treatment did not modify the luciferase activity in PCCl3 cells transiently transfected with the empty pGL3 vector (data not shown). Additionally, the concomitant treatment of PCCl3 cells with $NaClO_4$ and NaI prevented the reduction of NIS mRNA content and NIS promoter activity induced by excess I^- exposure (Fig. 1D–E). Thus, our data suggest that intracellular excess I^- triggers the inhibitory transcriptional effect on NIS mRNA expression.

Thereafter, we assessed the effect of I^- on the TSH-responsive NIS upstream enhancer region (NUE). For this purpose, PCCl3 cells were transiently transfected with a reporter containing the NUE region linked to the thymidine kinase promoter (pNUE, Fig. 2A) and further treated with NaI for different periods of time. Although no alteration of pNUE reporter activity was detected after excess I^- treatment for 30 min, a significant reduction of luciferase activity was observed after 12 and 24 h of I^- treatment (Fig. 2B). Moreover, we observed a concentration-dependent reduction of pNUE activity in response to I^- treatment for 24 h (Fig. 2C).

The NUE region contains several cis-regulatory elements required for full TSH-responsiveness. Site-directed mutagenesis studies indicated that both the Pax8 binding sites and the CRE-like sequence are required for the induction of NUE activity in response to TSH (Chun et al., 2004). Therefore, to determine the transcription factors involved in the inhibitory effect of excess I^- on NIS promoter activity, we tested the activity of pNUE mutants whose Pax8 (pNUE-A MT and pNUE-C MT) or NF-κB (pNUE-κB MT) binding sites were removed by site-directed mutagenesis. As reported (Chun et al., 2004), the mutant missing the CRE-like binding site did not show significant TSH-induced transcriptional activity (data not shown). Interestingly, excess I^- did not modulate the transcriptional activity of pNUE mutants missing Pax8 binding sites A or C as well as the NF-κB binding site (Supplemental Fig. 2A–C). Altogether, these results suggest that Pax8 and NF-κB are needed for NIS gene transcription repression induced by excess I^- treatment.

3.3. MMI treatment abrogates the inhibitory effects of excess iodide

The inhibitory effects of excess I^- are usually reversed by compounds that inhibit thyroid peroxidase (TPO)-mediated I^- organification, such as methimazole (MMI) and propylthiouracil (PTU) (Grollman et al., 1986, Leoni et al., 2011). Some studies have suggested that intracellular TPO-produced iodocompounds, which may inhibit TSH-induced adenylate cyclase activity, mediates the “escape” from the Wolff-Chaikoff effect (Panneels et al., 1994, Thomasz et al., 2010a). Therefore, we evaluated NIS mRNA expression in PCCl3 cells incubated with NaI in the presence of MMI for 24 h. Our results demonstrated that excess I^- did not modulate NIS mRNA expression in the presence of MMI (Fig. 3A). This result suggests that iodocompounds may participate as negative regulators of NIS gene expression.

3.4. Excess I^- downregulates NIS gene expression through the impairment of TSH pathway

In thyroid cells, TSH stimulates I^- uptake through activation of the TSH receptor (TSHR) and intracellular increase of cAMP levels. Therefore, we investigated TSHR expression in response to excess I^-. Our data indicated that I^- treatment reduced TSHR mRNA and protein expression (Supplemental Fig. 3A–B). The effect of excess I^- on TSHR mRNA was abrogated in the presence of MMI (Supplemental Fig. 3C). Moreover, we evaluated whether excess I^--induced NIS transcriptional repression was dependent on the TSH action. Hence, PCCl3 cells were TSH-starved for 4 days before excess I^- treatment either in the absence or presence of TSH for 24 h. As expected, TSH treatment significantly increased NIS mRNA expression and NIS promoter activity in TSH-deprived cells (Fig. 3B–C). Even though, the magnitude of TSH stimulation was lower in the presence of NaI (Fig. 3B–C). It is worth noting that excess I^- significantly reduced NIS mRNA expression even in the absence of TSH (Fig. 3B). However, the inhibitory effect of excess I^- on NIS promoter activity was only observed in the presence of TSH (Fig. 3C). Therefore, our data suggest that excess I^- may repress TSH signaling leading to a transcriptional reduction of NIS expression.

3.5. Excess I^- reduces Pax8 transcriptional activity and leads to Pax8 nuclear exclusion

The transcription factor Pax8 is essential for the expression of different thyroid differentiation markers involved in the thyroid hormones synthesis, including NIS expression (Antonica et al., 2012). Indeed, TSH-induced NIS expression requires Pax8 binding to the NIS upstream enhancer (Ohno et al., 1999, Chun et al., 2004). Our data suggested that Pax8 may be involved in the suppression of NIS promoter activity induced by excess I^-, since the pNUE site-directed mutants lacking Pax8 binding sites (pNUE-A MT and pNUE-C MT) were insensitive to I^- treatment (Supplemental Fig. 2A–B). Interestingly, we did not observe significant changes in Pax8 total protein levels in I^--treated PCCl3 cells (Fig. 4A). However, excess I^- shuttled Pax8 content from the nuclear to the cytoplasmic fraction as observed by immunoblot analysis of isolated cytoplasmic and nuclear proteins (Fig. 4B–C). Similar results were obtained when Pax8 localization was analyzed by immunofluorescence (Fig. 4D, white arrows). Additionally, we evaluated the effect of I^- treatment on Pax8-dependent gene transcription using a Pax8-responsive reporter containing five Pax8-binding sites linked to luciferase (Cp5-luc). As shown in Fig. 4E excess I^- significantly impaired Pax8-dependent Cp5-luc reporter activity after 12 and 24 h of treatment.

To investigate whether I^- treatment modulates Pax8 expression at post-translational level, TSH-starved PCCl3 cells were co-transfected with the Pax8 reporter Cp5-luc together with a vector constitutively expressing Flag-tagged Pax8. Immunoblot analysis of whole cell extracts demonstrated that Pax8 is extremely reduced in TSH-starved cells. Moreover, we observed equal exogenous Pax8 protein expression in control and I^--treated TSH-starved

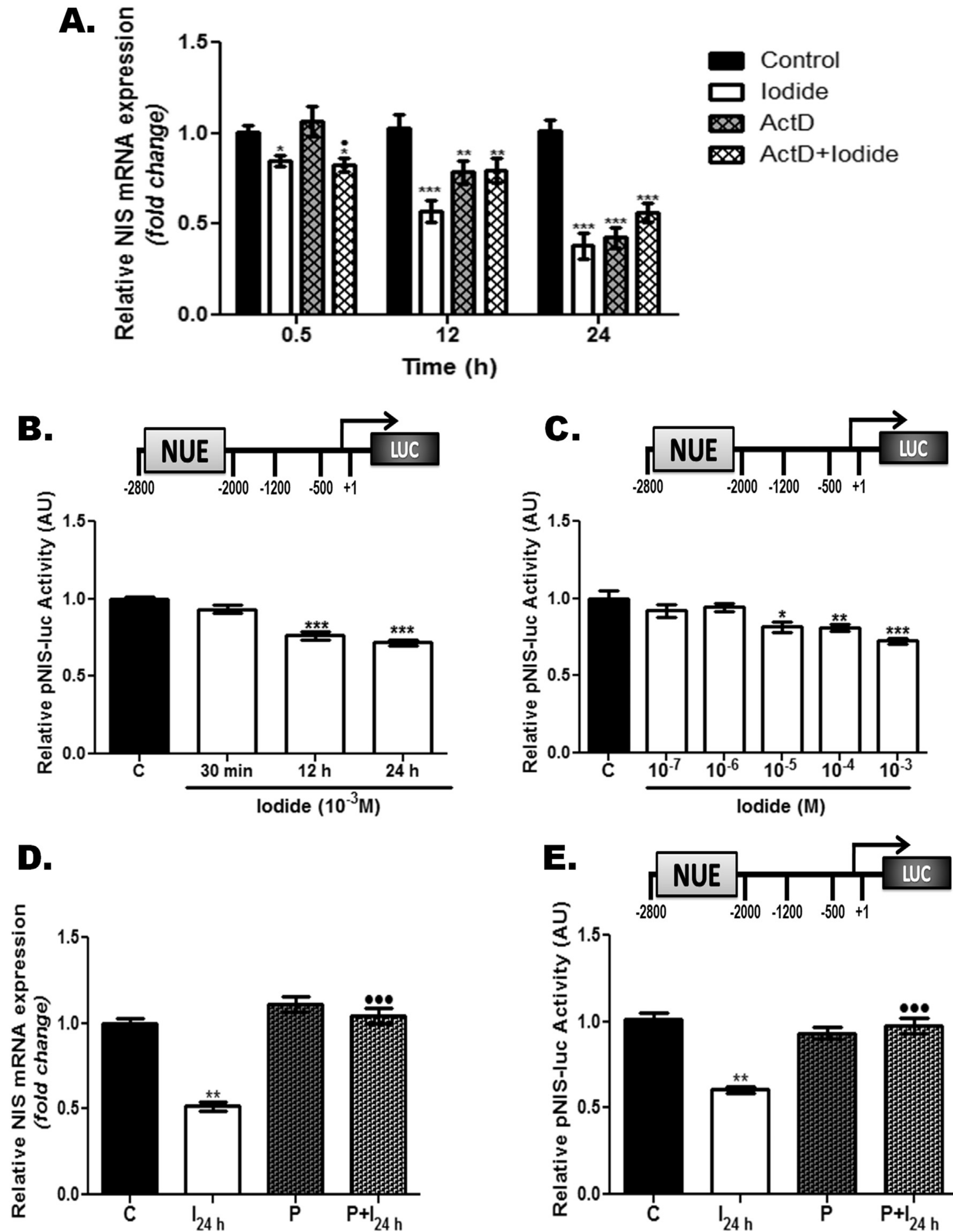

Fig. 1. Excess I^- represses NIS mRNA expression through post-transcriptional and transcriptional mechanisms. (A) Relative NIS mRNA expression evaluated by real-time PCR. PCCl3 cells were incubated with actinomycin D (4 μM) for 1 h before the treatment with NaI (10^{-3} M) for 30 min, 12 and 24 h. Results are indicated as fold change relative to the mRNA levels of cells treated with vehicle (DMSO). Six independent experiments were performed in duplicate (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control cells, • P < 0.05 vs ActD-treated cells (ANOVA, Student–Newman–Keuls). (B) PCCl3 cells were transiently transfected with the pNIS, which is schematically represented in the upper panel, and treated with 10^{-3} M NaI for the indicated periods of time. Results are expressed as luciferase activity normalized to that of β-galactosidase and relative to the activity of untreated cells (control), in arbitrary units (AU). Six independent experiments were performed in triplicate (n = 6). ***P < 0.001 vs. C (ANOVA, Student–Newman–Keuls). (C) pNIS-transfected PCCl3 cells were treated with different concentrations of NaI (10^{-7} M to 10^{-3} M) for 24 h. Results are expressed as luciferase activity normalized to that of β-galactosidase and relative to the activity of untreated cells (control), in arbitrary units (AU). Six independent experiments were performed in duplicate (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 vs. C (ANOVA, Student–Newman–Keuls). (D) Relative NIS mRNA expression evaluated by real-time PCR. PCCl3 cells were incubated with $NaClO_4$ (10^{-3} M) for 1 h before the treatment with NaI (10^{-3} M) for 24 h. Results are indicated as fold change relative to control cells. Five independent experiments were performed in duplicate (n = 5). **P < 0.01 vs. C, ••• P < 0.001 vs. $I_{24\ h}$. (E) pNIS-transfected PCCl3 cells were treated with $NaClO_4$ (10^{-3} M) for 1 h before the treatment with NaI (10^{-3} M) for 24 h. Results are expressed as luciferase activity normalized to that of β-galactosidase and relative to the activity of control cells, in arbitrary units (AU). Four independent experiments were performed in triplicate (n = 4). **P < 0.01 vs. C, ••• P < 0.001 vs. $I_{24\ h}$. (ANOVA, Student–Newman–Keuls).

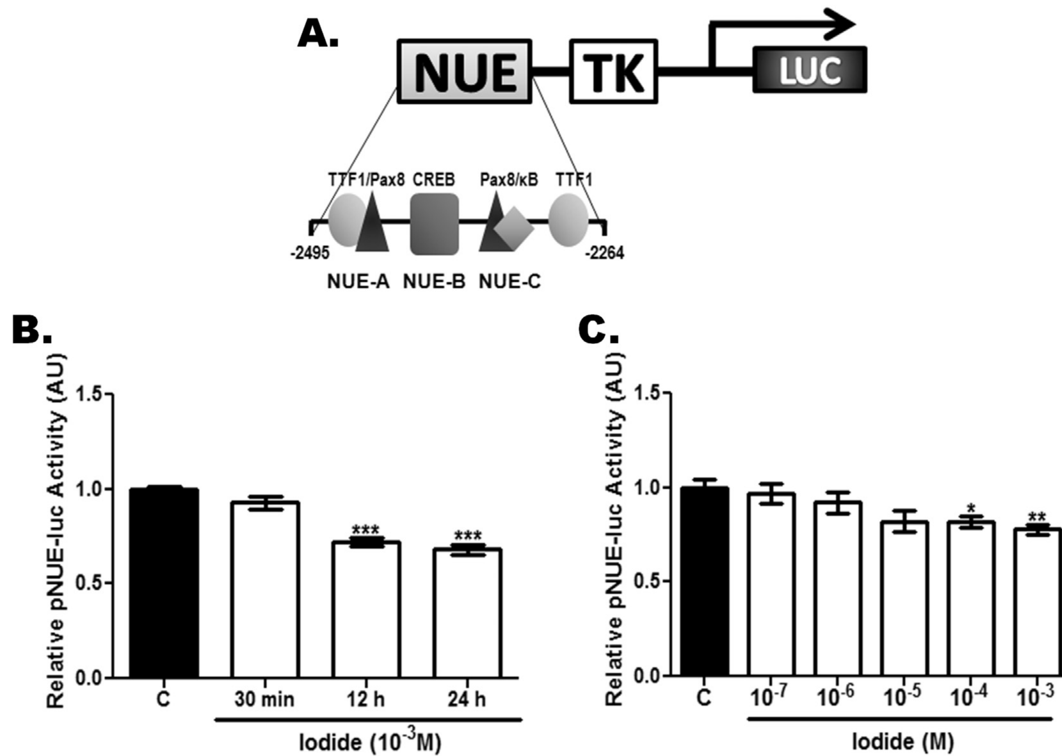

Fig. 2. Excess I⁻ represses the transcriptional activity of the far upstream enhancer NUE. (A) Schematic representation of the construct pNUE containing the −2495 to −2264 bp DNA fragment of the rat NIS far upstream enhancer cloned 5′ upstream to the thymidine kinase (TK) promoter linked to luciferase (LUC), indicating transcription factors binding sites. (B) PCCl3 cells were transiently transfected with the pNUE reporter and treated with 10^{-3} M NaI for the indicated periods of time. Results are expressed as luciferase activity normalized to that of β-galactosidase and relative to the activity of untreated cells (control), in arbitrary units (AU). Six independent experiments were performed in triplicate (n = 6).***P < 0.001 vs. C (ANOVA, Student–Newman–Keuls). (C) pNUE-transfected PCCl3 cells were treated with different concentrations of NaI (10^{-7} M to 10^{-3} M) for 24 h. Six independent experiments were performed in triplicate (n = 6).*P < 0.05, **P < 0.01 vs. C (ANOVA, Student–Newman–Keuls).

transfected cells (Fig. 4F). As expected, Pax8 over-expression significantly increased Pax8 reporter activity (Fig. 4G). Interestingly, I⁻ treatment significantly reduced exogenous Pax8-induced Cp5 reporter activity in co-transfected PCCl3 cells (Fig. 4G).

In addition, we evaluated Pax8 transcriptional activity in response to I⁻ treatment by assessing the expression of the Pax8-regulated thyroid differentiation genes thyroperoxidase (TPO) and thyroglobulin (Tg) (Di Palma et al., 2003). In agreement with the I⁻-induced decreased Pax8 transcriptional activity, TPO promoter activity and mRNA expression were decreased in I⁻-treated cells (Supplemental Fig. 4A–B). Similar data were obtained on Tg promoter activity and mRNA expression (Supplemental Fig. 4C–D).

These data demonstrate that excess I⁻ repress NIS gene expression by down regulating Pax8 transcriptional activity. Moreover, even though I⁻ treatment has not diminished Pax8 total content, the impairment of its transcriptional activity by I⁻-induced post-translational modifications cannot be ruled out.

3.6. Excess I⁻ reduces NF-κB-dependent NIS gene transcription

Consistently with the role of Pax8 modulating NIS gene expression, it has been demonstrated that the NF-κB subunit p65 interacts with Pax8 to synergistically induce NIS gene expression (Nicola et al., 2010). Here, we observed that I⁻ treatment did not modulate NIS promoter activity when the κB consensus site is mutated (pNUE-κB MT) (Supplemental Fig. 2C), leading us to study the effect of excess I⁻ on p65 protein expression. We observed a significant reduction of p65 protein expression in total lysates of PCCl3 cells incubated with NaI for 12 and 24 h (Fig. 5A). In agreement, immunofluorescence analysis showed reduced p65 expression in response to I⁻ treatment (Fig. 5B). Additionally, we evaluated NF-κB-dependent gene transcription in PCCl3 cells transiently transfected with the NF-κB-responsive vector 5× κB-Luc containing five multimerized κB-binding sites linked to luciferase. High concentration of I⁻ reduced 5× κB-Luc reporter activity after 12 and 24 h treatment (Fig. 5C). Consistently, we observed a significant downregulation of the NF-κB-targets NF-κB inhibitor (IκB)-α and interleukin (IL)-6 mRNA expression after I⁻ treatment (Supplemental Fig. 5A–B).

To further investigate the role of NF-κB signaling in the I⁻-induced downregulation of NIS gene expression, we evaluated NIS promoter activity in response to excess I⁻ in the presence of the NF-κB pathway inhibitor BAY 11-7082. The treatment with BAY 11-7082 reduced pNIS reporter activity in control cells, suggesting that NF-κB signaling is required for full TSH-responsiveness (Fig. 5D). Moreover, blockage of NF-κB signaling abrogated the inhibitory effect triggered by I⁻ on NIS promoter activity (Fig. 5D). Altogether, our data suggest that excess I⁻ represses NIS transcriptional expression by impairing TSH-activated NF-κB transcriptional activity.

3.7. Excess I⁻ impairs Pax8 and p65 interaction and binding to NIS promoter

Nicola et al., 2010 suggested a physical interaction and functional synergy between the transcription factor Pax8 and the NF-κB subunit p65 for transcriptional activation of the NIS gene. Consistently with the downregulation of NIS gene expression induced by excess I⁻, our data demonstrated that excess I⁻ reduced the physical interaction between Pax8 and p65 in PCCl3 cells (Fig. 6A).

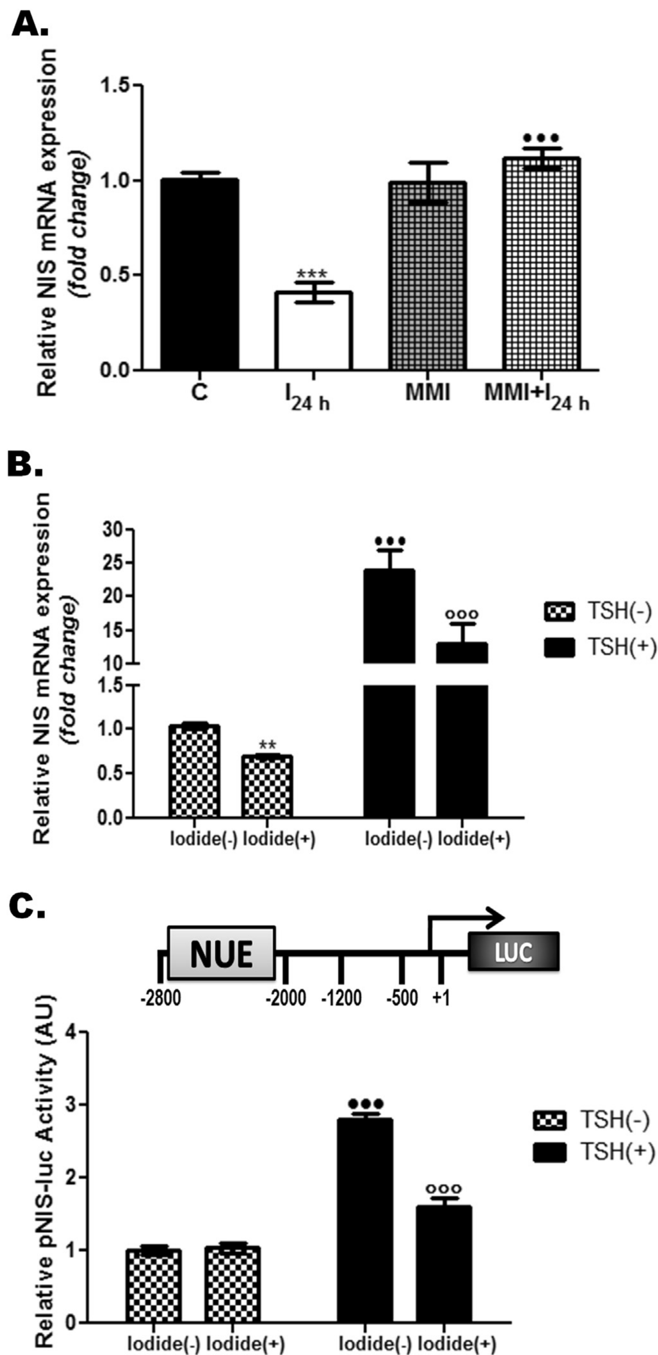

Fig. 3. TSH pathway is involved in the transcriptional downregulation of NIS mRNA expression by I^- excess treatment. (A) PCCl3 cells were incubated with MMI (10^{-3} M) 1 h before NaI treatment (10^{-3} M) for 24 h. Relative NIS mRNA levels were evaluated by real-time PCR. Values are indicated as fold change relative to the mRNA levels of untreated cells. Six independent experiments were performed in duplicate (n = 6). ***P < 0.001 vs. C, ●●● P < 0.001 vs. $I_{24\ h}$ (ANOVA, Student–Newman–Keuls). (B) TSH-starved PCCl3 cells were treated with 10^{-3} M NaI in the presence or absence of TSH (1 mIU/ml) for 24 h. Relative NIS mRNA expression was evaluated as described in (A). Four independent experiments were performed in duplicate (n = 4). **P < 0.01 vs. TSH(−)NaI(−); ●●● P < 0.001 vs. TSH(−)NaI(−) and TSH(−)NaI(+); °°° P < 0.001 vs. TSH(+)NaI(−) (Two-way ANOVA, Bonferroni). (C) TSH-starved PCCl3 cells were transiently transfected with the rat NIS promoter construct pNIS, which is schematically represented in the upper panel, and further treated with 10^{-3} M NaI in the presence or absence of 1 mIU/ml TSH for 24 h. Results are expressed as luciferase activity normalized to that of β-galactosidase and relative to the activity of untreated TSH-starved cells, in arbitrary units (AU). Four experiments were performed in duplicate (n = 4). ●●● P < 0.001 vs. TSH(−)Iodide(−) and TSH(−)Iodide(+),°°° P < 0.001 vs. TSH(+)Iodide(−) (Two-way ANOVA, Bonferroni).

To further test the mechanism involved in I^--induced repression of NIS gene expression, we examined the binding of the transcription factor p65 to NIS promoter using chromatin immuneprecipitation (ChIP) assays (Fig. 6B). Interestingly, NaI treatment for 12 and 24 h significantly reduced p65 binding to the NUE region (Fig. 6C). Hence, these results strongly suggest that excess I^- decreased the TSH-stimulated NIS transcriptional expression by impairing the binding of the abovementioned transcription factors to the NIS promoter.

3.8. Excess I^- inhibits NIS gene expression through Akt activation

Recently, we demonstrated that I^- overload promptly reduced NIS-mediated I^- transport through activation of the PI3K/Akt signaling pathway (Serrano-Nascimento et al., 2014). Therefore, we investigated whether I^--activated PI3K/Akt signaling also modulates NIS transcriptional expression. We observed that the treatment of PCCl3 cells with 10^{-3} M NaI for 24 h significantly increased Akt phosphorylation at Ser-473 (Fig. 7A). Interestingly, lower concentrations of NaI also induced significant activation of Akt phosphorylation in PCCl3 cells (Supplemental Fig. 6A). Additionally, PI3K signaling is involved on Akt activation, since the PI3K inhibitor LY294002 abrogated the I^--induced Akt phosphorylation (Fig. 7A). Thereafter, we observed that blockage of PI3K signaling by LY294002 or wortmannin abrogated the reduction of NIS mRNA expression triggered by excess I^- (Fig. 7B). In accordance, inhibition of PI3K/Akt signaling prevented I^--induced downregulation of NIS promoter activity (Fig. 7C). As already reported (Garcia and Santisteban, 2002, Zaballos et al., 2008), inhibition of PI3K/Akt signaling for 24 h increased TSH-induced NIS gene expression (Fig. 7B–C).

Immunofluorescence studies demonstrated that blockage of Akt phosphorylation abrogated I^--induced reduction of p65 total expression and nuclear Pax8 exclusion (Fig. 7D). Moreover, inhibition of Akt signaling prevented the I^--induced downregulation of Pax8 and NF-κB transcriptional activity (Fig. 7E–F). Altogether, these data suggest that excess I^- impairs NIS transcriptional expression through activation of PI3K/Akt signaling pathway.

3.9. Excess I^--induced reactive oxygen species production repressed NIS gene expression

Although reactive oxygen species (ROS) production is essential for thyroid hormone synthesis (Song et al., 2007), unregulated ROS production causes deleterious effects on thyrocytes (Weyemi et al., 2010). Leoni et al., 2011 demonstrated increased ROS production in PCCl3 cells exposed to excess I^-. Moreover, we recently demonstrated that the post-transcriptional effects triggered by excess I^- that rapidly modulate NIS expression involve increased mitochondrial superoxide production (Serrano-Nascimento et al., 2014). Therefore, we evaluated whether increased ROS production in response to I^- treatment regulates NIS gene transcriptional expression. Using the oxidative stress indicator carboxy-H2-DCFDA, we detected increased ROS production in response to I^- exposure (10^{-3} M) for 24 h (Fig. 8A–B). Flow cytometry analysis also indicated increased ROS levels in cells treated with lower concentrations of NaI (Supplemental Fig. 6B–C).

To evaluate the role of increased ROS production on the I^--induced Akt phosphorylation, PCCl3 cells were treated with ebselen (30 μM), a glutathione peroxidase mimetic and inhibitor of glutathione reductase (Bhowmick et al., 2015) or with NAC (1 mM), a nonspecific ROS scavenger. The concomitant treatment of PCCl3 cells with these drugs and NaI abrogated the augment of ROS production induced by excess I^- exposure (Fig. 8B–C). Interestingly, the activation of PI3K/Akt signaling was dependent on

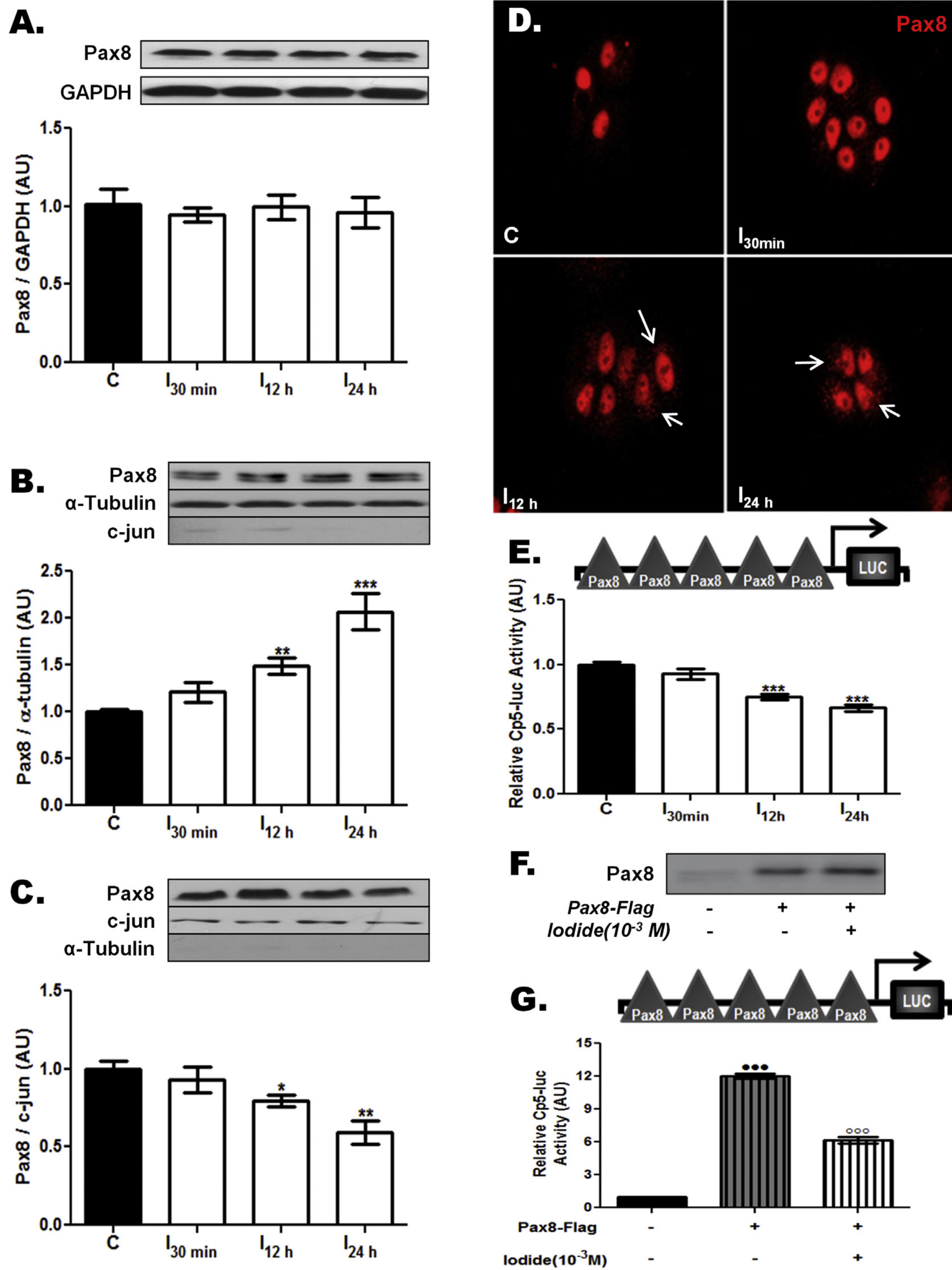

Fig. 4. Excess I^- excludes Pax8 from the nucleus and reduces its transcriptional activity. (A) Western blot analysis of Pax8 total content in PCCl3 cells treated with 10^{-3} M NaI for the indicated periods of time. GAPDH was used as a loading control. Results are expressed as means ± SEM in arbitrary units (AU). Six independent experiments were performed (n = 6). *P > 0.05 (ANOVA, Student–Newman–Keuls). (B and C) PCCl3 cells treated with 10^{-3} M NaI for the indicated periods of time and further processed to isolate cytoplasmic (B) and nuclear (C) proteins. Pax8 expression was evaluated by western blot in both fractions. The cytoplasmic protein α-tubulin and the nuclear protein c-jun were used as loading and purification control. Results are expressed as means ± SEM in arbitrary units (AU). Five independent experiments were performed (n = 5). *P < 0.05, **P < 0.01, ***P < 0.01 vs. control cells (ANOVA, Student–Newman–Keuls). (D) Immunufluorescence analysis assessing Pax8 expression and localization in PCCl3 cells treated with 10^{-3} M NaI for the indicated periods of time. White arrows indicate the presence of Pax8 staining in the cytoplasm. Two independent experiments were performed in triplicate. Magnification 600×. (E) PCCl3 cells were transiently transfected with Pax8-responsive reporter Cp5-luc containing five Pax8-binding sites linked to luciferase (LUC), which is schematically represented in the upper panel. Transfected cells were treated with 10^{-3} M NaI for different periods of time. In the lower panel, results are expressed as luciferase activity normalized to that of β-galactosidase and relative to the activity of untreated cells, in arbitrary units (AU). Six independent experiments were performed (n = 6).***P < 0.001 vs. C (ANOVA, Student–Newman–Keuls). (F) Pax8 total expression in TSH-starved transfected with pcDNA3.1 or Pax8-Flag vectors. Transfected cells were treated with 10^{-3} M NaI for 24 h and Pax8 expression was evaluated by Western Blot. (G) TSH-starved PCCl3 cells were transiently co-transfected with the Pax8 reporter Cp5-luc and the empty vector pcDNA3.1 or a expression vector encoding flag-tagged Pax8 (Pax8-Flag). Co-transfected cells were treated with 10^{-3} M NaI for 24 h. Results are expressed as described in (E). Four independent experiments were performed (n = 4). ••• P < 0.001 vs. pcDNA3.1-transfected cells,°°° P < 0.001 vs. untreated Pax8-Flag-transfected cells (ANOVA, Student–Newman–Keuls).

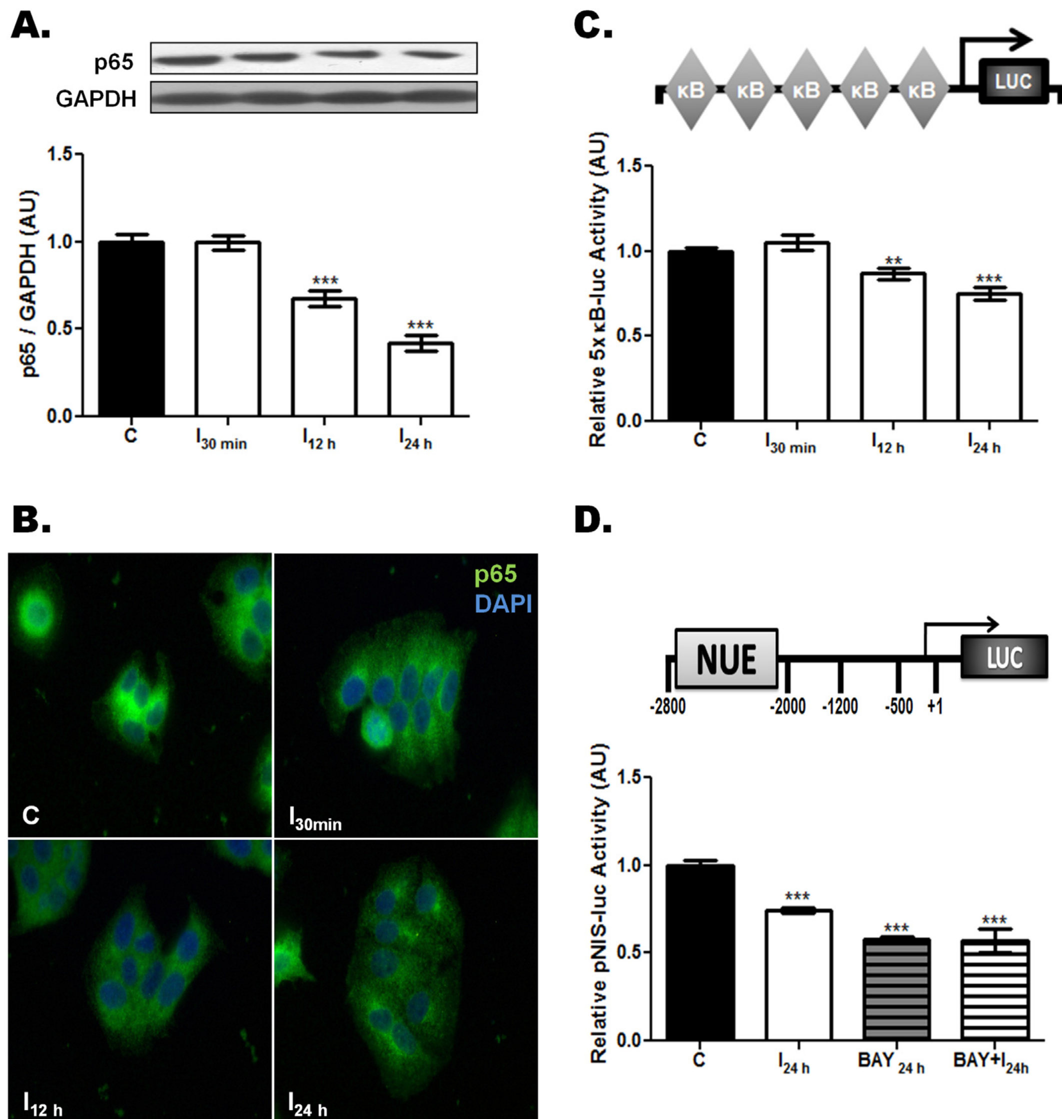

Fig. 5. Excess I⁻ reduces p65 total content and transcriptional activity. A. Western blot analysis of p65 total content in PCCl3 cells treated with 10^{-3} M NaI for the indicated periods of time. GAPDH used as a loading control. Results are expressed as means ± SEM in arbitrary units (AU). Six experiments were performed (n = 6). ***P < 0.001 vs. control cells (ANOVA, Student–Newman–Keuls). (B) Immunufluorescence analysis assessing p65 expression in PCCl3 cells treated with 10^{-3} M NaI for the indicated periods of time. Two independent experiments were performed in triplicate. Magnification 600×. (C) PCCl3 cells were transiently transfected with the NF-κB-responsive vector 5× κB-Luc containing five multimerized κB-binding sites linked to luciferase (LUC), which is schematically represented in the upper panel. Transfected cells were treated with 10^{-3} M NaI for the indicated periods of time. In the lower panel, results are expressed as luciferase activity normalized to that of β-galactosidase and relative to the activity of untreated cells, in arbitrary units (AU). Six independent experiments were performed (n = 6). **P < 0.01, ***P < 0.001 vs. control cells (ANOVA, Student–Newman–Keuls). (D) PCCl3 cells were transiently transfected with the NIS promoter construct pNIS, which is schematically represented in the upper panel. Thereafter, the cells were incubated with BAY 11–7082 (1 μM) for 1 h before 10^{-3} M NaI treatment for 24 h. Results are expressed as described in (C). Four independent experiments were performed (n = 4). ***P < 0.001 vs. control cells (ANOVA, Student–Newman–Keuls).

increased ROS production, since the previous treatment of PCCl3 cells with the ebselen or NAC abrogated the I⁻-induced Akt phosphorylation at Ser-473 (Fig. 9A–B). Moreover, both ebselen and NAC treatment prevented the downregulation of NIS promoter activity induced by I⁻ treatment (Fig. 9C–D). In accordance, both drugs abolished the inhibition of Pax8 and p65 transcriptional activity induced by excess I⁻ exposure (Fig. 9E–H). Taken together, these results strongly suggest that the reduced Pax8/p65 transcriptional activity and the repression of NIS gene expression depend on the activation of PI3K/Akt cascade through increased ROS production induced by excess I⁻ (Fig. 10).

4. Discussion

Our results indicated that excess I⁻ treatment transcriptionally downregulates NIS expression through the impairment of Pax8 and p65 interaction and binding to NIS promoter. Our data also support that I⁻ induces these effects through activation of PI3K/Akt signaling pathway by increased ROS production.

The inhibition of NIS-mediated I⁻ uptake by excess I⁻, known as the Wolff-Chaikoff effect escape, is an adaptive mechanism that reduces the intracellular I⁻ content and guarantees thyroid hormones synthesis restoration (Braverman and Ingbar, 1963, Grollman et al., 1986). Previous studies have suggested that both post-transcriptional and transcriptional events are involved in I⁻-induced downregulation of NIS expression and function (Eng et al.,

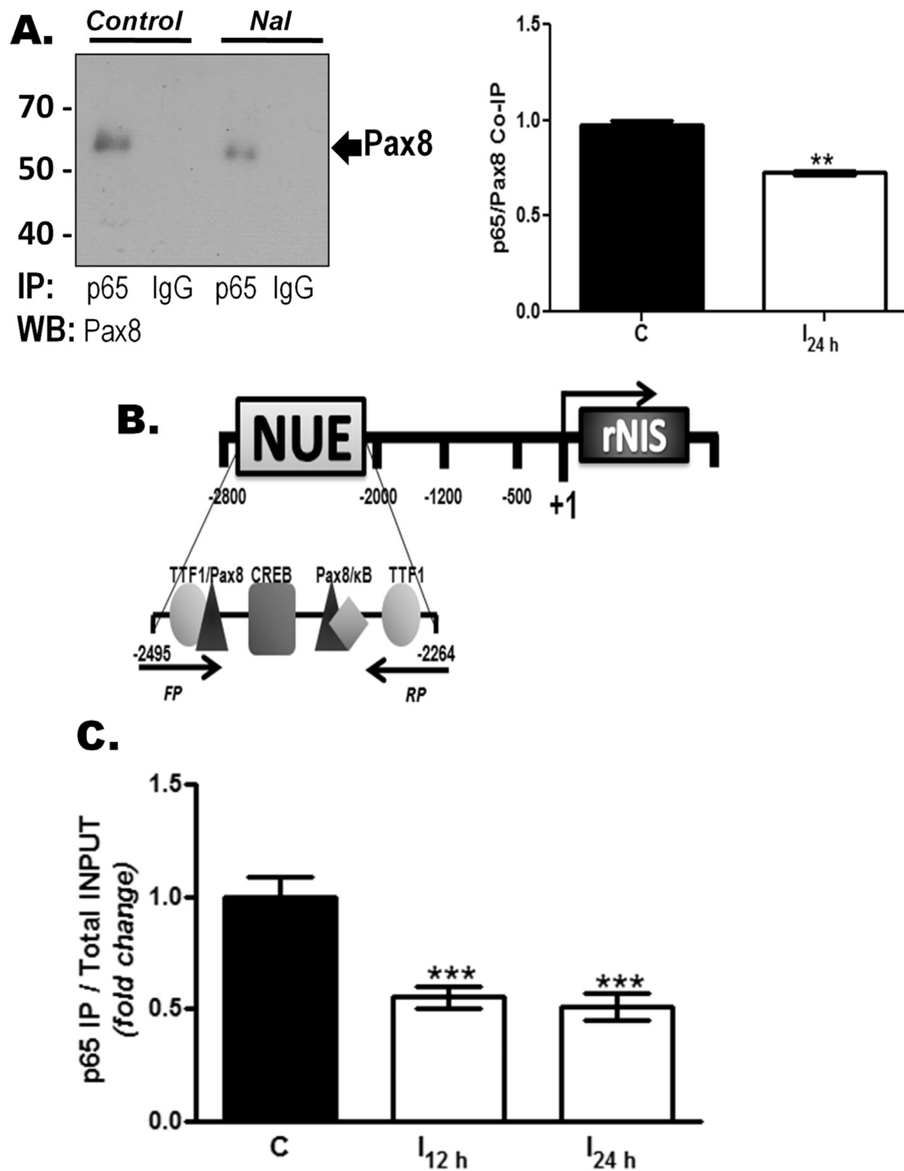

Fig. 6. Excess I⁻ reduces Pax8 and p65 interaction and association to NIS promoter. (A) Immunoprecipitations were performed with rabbit IgG or anti-p65 polyclonal antibody using nuclear lysates from PCCl3 cells incubated with 10^{-3} M NaI for 24 h. Left panel shows a representative immunoblot assessing Pax8 expression in the co-immunoprecipitated material using western blot. Right panel shows the densitometric analysis evaluating p65/Pax8 interaction in control and I⁻-treated cells. Three independent experiments were performed (n = 3). **P < 0.01 vs. untreated cells (Unpaired two-tailed Student's t-test). (B) Schematic representation of the rat NIS promoter. Positions are relative to relative to ATG start site denoted as +1. The location of primer sets (FP – forward primer and RP – reverse primer) used in ChIP experiments is shown. Transcription factors binding sites are indicated. (C) PCCl3 cells were treated with 10^{-3} M NaI for the indicated periods of time before cross-linking and further ChIP assay. DNA amount in immunoprecipitates was determined by real-time PCR as indicated in Material and Methods. Results are expressed as relative fold increase setting untreated cells as 1.0. Relative fold of increase was calculated according to the equation Fold Change = $2^{[(Ct.input-Ct.target) - (Ct.input-Ct.mock)]}$. Results are expressed as means ± SEM. Six independent experiments were performed (n = 6). **P < 0.01, ***P < 0.01 vs. control cells (ANOVA, Student–Newman–Keuls).

1999,Serrano-Nascimento et al., 2010, 2012, Leoni et al., 2011). Although several post-transcriptional mechanisms have been proposed, the molecular mechanisms involved in the transcriptional repression of NIS gene by excess I⁻ are still controversial and not completely understood. Here, we presented novel data indicating that excess I⁻ represses NIS expression at transcriptional level, as the treatment of PCCl3 cells with excess I⁻ reduced NIS promoter activity. Moreover, we demonstrated that the treatment of thyroid cells with actinomycin D abrogated the inhibitory effects of I⁻ exposure on NIS mRNA expression. Furthermore, we evidenced that I⁻-induced downregulation of NIS promoter activity relies on the inhibition of trans-acting factors binding to the far upstream enhancer NUE. In addition, Suzuki et al., 2010 reported that excess I⁻ blocked the stimulatory effect of TSH on the proximal NIS promoter activity by diminishing the binding of protein complexes to the DNA. However, these authors have mainly worked with higher concentration of I⁻ (10 mM) and with constructs 420 bp upstream the NIS transcription start site. In our study, we have used the complete NIS promoter fragment or its upstream enhancer (NUE) and confirmed the I⁻-induced transcriptional repression of NIS gene activity, even with lower concentrations of NaI treatment. In contrast to our observations, Leoni et al., 2011 have not observed a transcriptional repression of NIS gene expression in I⁻-treated PCCl3 cells. However, in a previous study, Leoni et al., 2008 demonstrated a significant reduction of TSHR, Tg, TPO and NIS mRNA expression in PCCl3 cells exposed to excess I⁻. It is worth noting that there was a significant

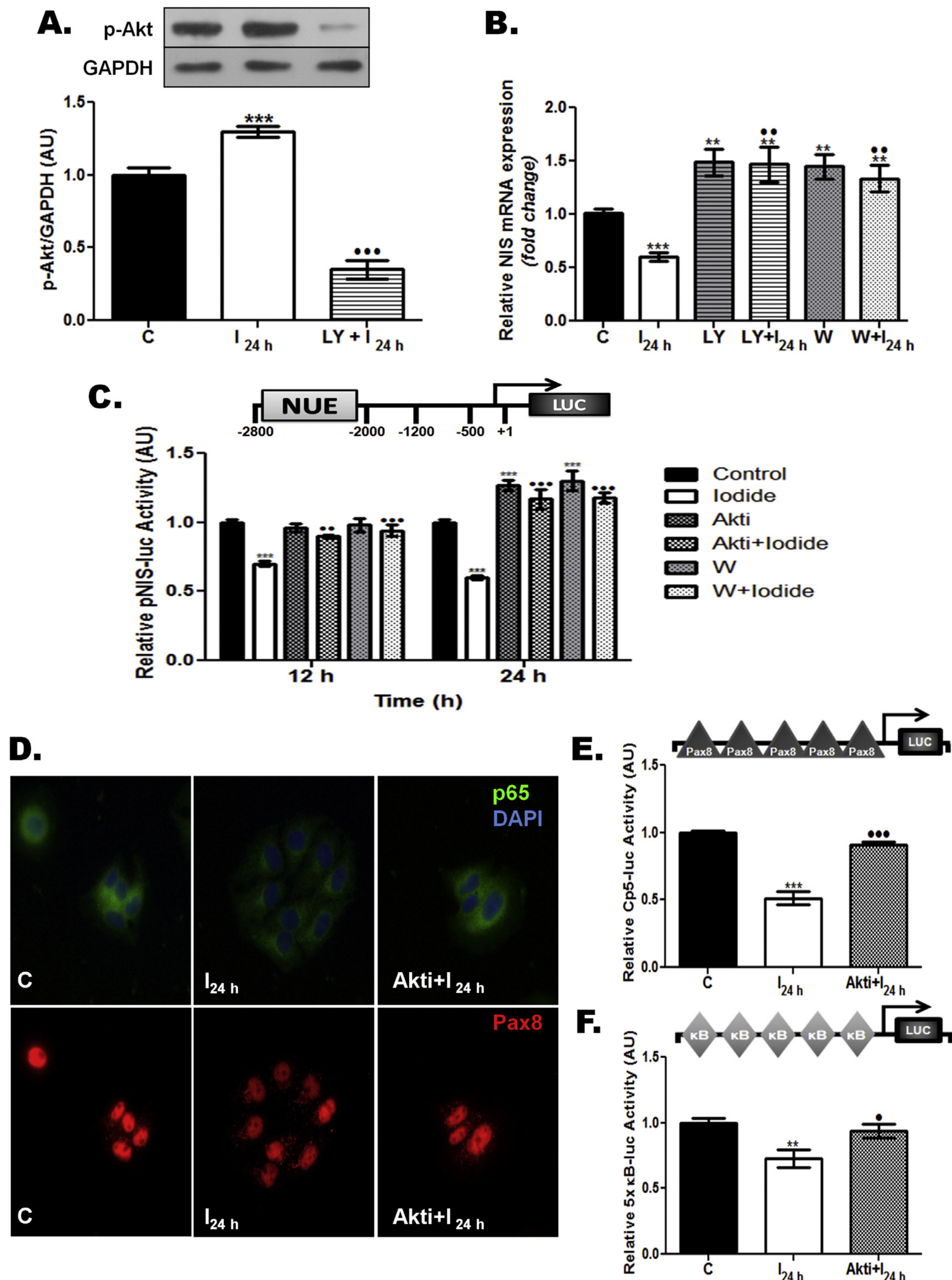

Fig. 7. Excess I⁻-induced PI3K/Akt signaling impairs NIS gene transcription. (A) Representative Western blot analysis assessing Akt phosphorylation at Ser-473 (p-Akt) in PCCl3 cells treated with 10^{-3} M NaI for 24 h. Cells were incubated LY294002 (10 μM) for 1 h before NaI treatment for 24 h. GAPDH was used as a loading control. Six independent experiments were performed (n = 6). ***P < 0.001 vs. control cells; ••• P < 0.001 vs. $I_{24\ h}$ (ANOVA, Student–Newman–Keuls). (B) Relative NIS mRNA expression in PCCl3 cells incubated with LY294002 (10 μM) or Wortmannin (1 μM) for 1 h before NaI treatment for 24 h. Results are indicated as fold change relative to the mRNA levels of untreated cells. Six experiments were performed in duplicate (n = 6). **P < 0.01, ***P < 0.001 vs. control cells; •• P < 0.01 $I_{24\ h}$ (ANOVA, Student–Newman–Keuls). (C) PCCl3 cells were transiently transfected with the NIS promoter construct pNIS, which is schematically represented in the upper panel, and treated with $Akti_{1/2}$ (10 μM) or Wortmannin (1 μM) for 1 h before 10^{-3} M NaI treatment for 24 h. Results are expressed as luciferase activity normalized to that of β-galactosidase and relative to the activity of DMSO-treated cells, in arbitrary units (AU). Six independent experiments were performed (n = 6). ***P < 0.001 vs. control cells; ••• P < 0.001 I (ANOVA, Student–Newman–Keuls). D. Immunofluorescence analysis evaluating p65 and Pax8 expression in PCCl3 cells exposed to 10^{-3} M NaI for 24 h in the presence or absence of $Akti_{1/2.}$ (10 μM). Two experiments were performed in triplicate. Magnification 600×. (E and F) PCCl3 cells were transiently transfected with the Pax8-responsive reporter Cp5-luc or the NF-κB-responsive reporter 5xκB-Luc, which are schematically represented in the upper panel. Thereafter, transfected cells were treated with 10^{-3} M NaI in the presence of 10 μM $Akti_{1/2}$ for 24 h. Results are expressed as indicated in (C). Three independent experiments were performed (n = 3). **P < 0.01, ***P < 0.001 vs. control cells; • P < 0.05, ••• P < 0.001 vs. $I_{24\ h}$ (ANOVA, Student–Newman–Keuls).

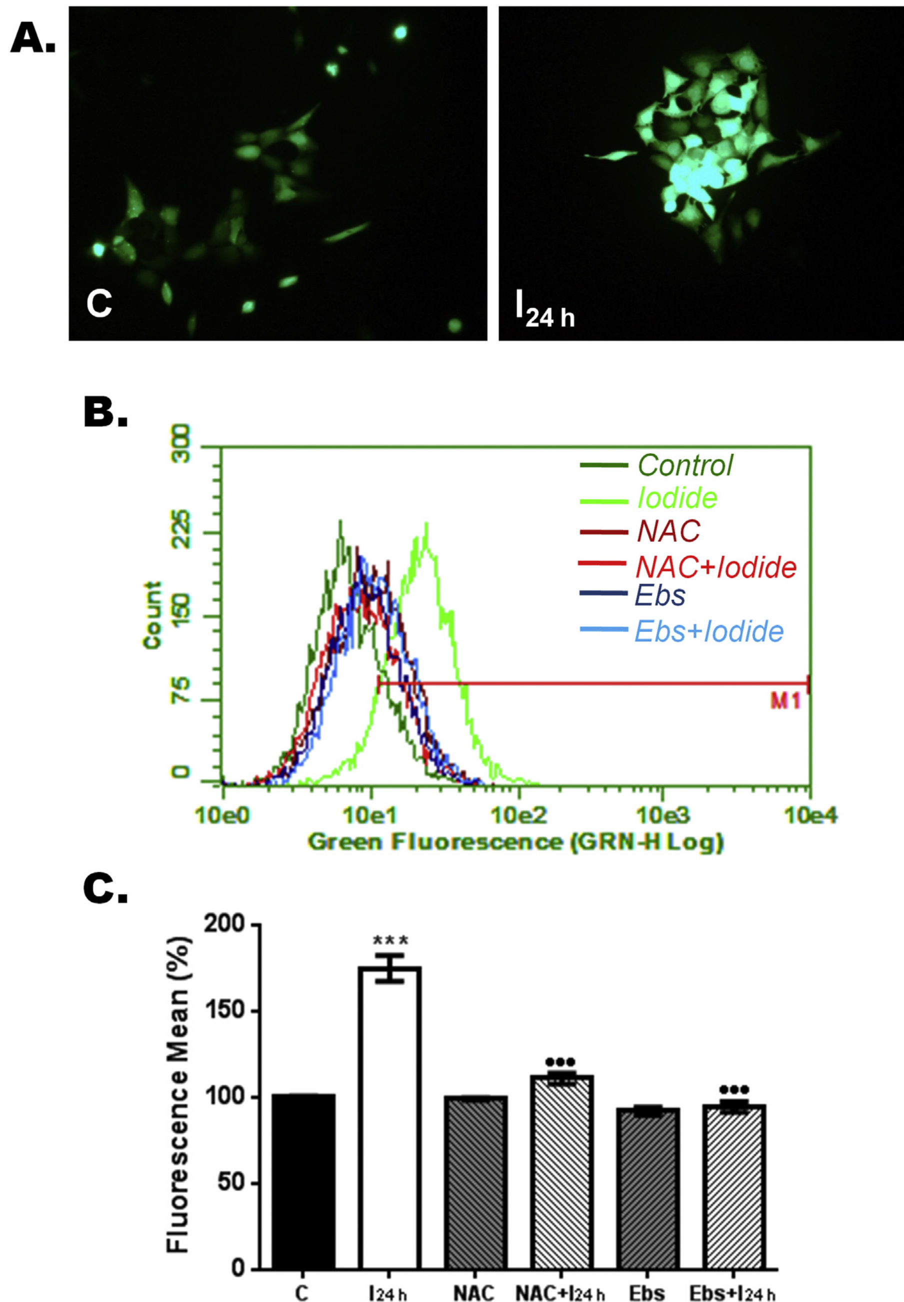

Fig. 8. Excess I⁻ induces reactive oxygen species (ROS) production. (A) Immunofluorescence analysis of ROS production in PCCl3 cells treated with 10^{-3} M NaI for 24 h using the ROS-sensitive probe carboxy-H2-DCFDA (5 μM) for 20 min. ROS production was evaluated in a ZEISS Axiovert 100 M fluorescence microscope. Two independent experiments were performed in triplicate. Magnification 400 × . (B) Representative histogram analysis of flow cytometry analysis of ROS production in PCCl3 cells treated only with NaI (10^{-3} M), or concomitantly treated with NaI and ebselen (Ebs, 30 μM) or NAC (10^{-3} M) for 24 h and incubated with carboxy-H2DCFDA (5 μM) for 20 min. Ten thousand events were evaluated per sample. Four experiments were performed (n = 4). (C) Relative quantification of fluorescence intensity means from flow cytometry data. ***P < 0.001 vs. control cells, ●●● P < 0.001 vs. $I_{24\,h}$ (ANOVA, Student–Newman–Keuls).

difference in the magnitude of I⁻ effect on NIS mRNA expression in the first study in comparison to the last one, although the same cell line and the same high I⁻ concentration were used. The discrepancy between the aforementioned results could be a consequence of the cell line aging, which has been demonstrated to significantly influence thyroid responsiveness to some treatments (Spitzweg et al., 1999, Taniguchi et al., 1998).

The acute post-transcriptional effects induced by excess I⁻ on NIS mRNA expression seem to be triggered by I⁻ *per se* (Serrano-Nascimento et al., 2014, 2010, 2012). However, the abrogation of I⁻-induced effects by MMI treatment suggests that iodocompounds may be involved in the transcriptional repression of NIS gene expression (Grollman et al., 1986, Leoni et al., 2011). Moreover, we observed that excess I⁻ reduced TSHR expression and that this effect was also abrogated in the presence of MMI. These data agree with previous studies supporting an impairment of TSH signaling by I⁻ excess or iodocompounds (Panneels et al., 1994, Thomasz et al., 2010a, 2010b). It is worth noting that both PCCl3 and FRTL5 cells have poor capacity to organify iodide by TPO activity. Therefore, since I⁻ is an extremely reactive anion, other iodocompounds than those related to TPO activity could be formed by elevated intracellular I⁻ concentration.

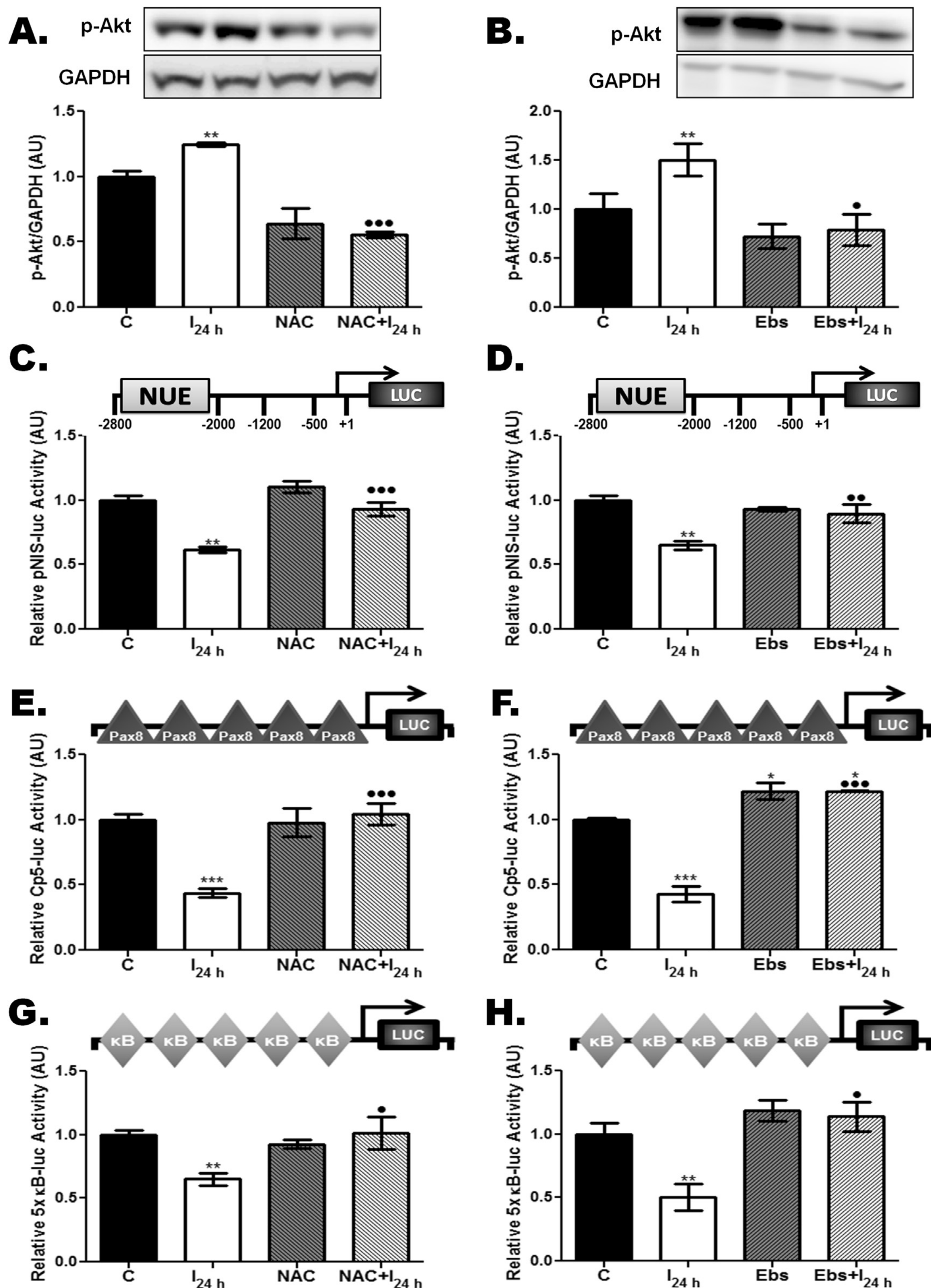

Fig. 9. I^--induced ROS production activates PI3K/Akt signaling cascade. (A–B) Representative Western blot analysis assessing Akt phosphorylation at Ser-473 (p-Akt) in PCCl3 cells treated with iodide and antioxidant agents. Cells were incubated NAC (10^{-3} M) or ebselen (Ebs, 30 μM) for 1 h before NaI (10^{-3} M) treatment for 24 h. GAPDH was used as a loading control. Three experiments were performed in duplicate (n = 3). **P < 0.01 vs. control cells; • P < 0.05, •• P < 0.01 vs. $I_{24\ h}$ (ANOVA, Student–Newman–Keuls). (C–H) PCCl3 cells were transiently transfected with the NIS promoter construct (pNIS; C-D) Pax8-responsive reporter (Cp5-luc; E-F) or the NF-κB reporter (5xκB-Luc, G-H), which are schematically represented in the upper panel. Thereafter, transfected cells were incubated with NAC (10^{-3} M) or ebselen (Ebs, 30 μM) for 1 h and then treated with 10^{-3} M NaI for 24 h. Results are expressed as luciferase activity normalized to that of β-galactosidase and relative to the activity of vehicle-treated cells. Three experiments were performed in triplicate (n = 3).*P < 0.05, **P < 0.01, ***P < 0.001 vs. control cells; • P < 0.05, •• P < 0.01, ••• P < 0.001 vs. I_{24h} (ANOVA, Student–Newman–Keuls).

Since TSH is the main positive regulator of NIS expression and activity (Kogai et al., 1997, Ohno et al., 1999), it was expected that TSH treatment would significantly increase NIS mRNA levels in TSH-deprived cells. Interestingly, excess I^- reduced NIS mRNA

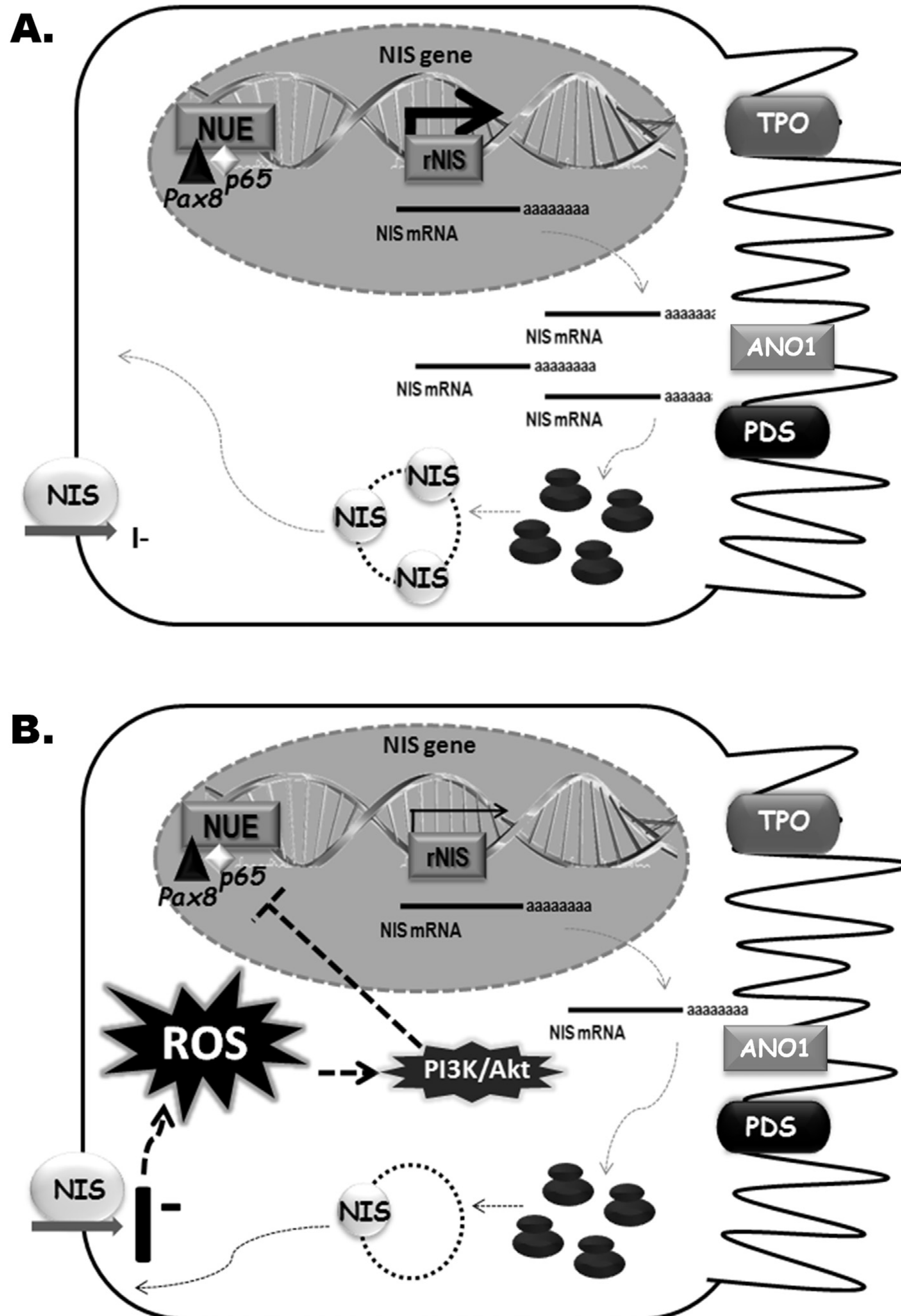

Fig. 10. Putative pathway involved in the transcriptional repression of NIS promoter activity by I^--activated ROS/PI3K/Akt signaling cascade. (A) Under physiological conditions, NIS gene transcription is positively regulated by several transcription factors, such as Pax8 and p65, which interact and transactivate NIS promoter activity. The mature NIS mRNAs are translated in the ribosomes, and vesicles containing NIS protein are directed to the plasma membrane. (B) High intracellular I^- levels increase ROS production, which in turn, activates PI3K/Akt signaling pathway. This cascade decreases NIS mRNA content by reducing NIS gene transcriptional activity through the impairment of Pax8/p65 binding to NIS gene promoter.

expression both in TSH-starved cells and in TSH-treated cells. Even though the magnitude of the reduction of NIS mRNA content induced by I^- treatment was higher in the presence of TSH, the inhibitory effect of I^- in TSH-deprived cells suggests that the excess of this trace element also reduces NIS mRNA expression by mechanisms that are independent on TSH signaling cascade. In contrast, the repression of NIS promoter activity induced by excess I^- was only observed in the presence of TSH, suggesting that I^--induced NIS gene transcriptional repression depends on TSH signaling pathway. These findings reinforce that both post-transcriptional and transcriptional events may contribute to the repression of NIS mRNA expression in response to I^- treatment; however, excess I^--induced NIS transcriptional repression seems to be more prominent after long-term treatment.

In thyroid cells, the transcription factor Pax8 is a nuclear phosphoprotein whose expression and transcriptional activity are regulated by several hormones, including TSH and insulin (Medina et al., 2000). Pax8 plays an important role in the expression of the differentiated phenotype of thyroid follicular cells (Antonica et al., 2012, Di Palma et al., 2003). Furthermore, Pax8 is the main

transcription factor involved in the regulation of NIS expression under physiological and pathological conditions (Ohno et al., 1999, Baratta et al., 2009). Here, we demonstrated that excess I^- impairs Pax8 transcriptional activity, thus reducing NIS gene expression. Moreover, as previously proposed, I^- treatment reduced the expression of Pax8 in the nucleus of thyroid cells without affecting total Pax8 expression (Leoni et al., 2011). In agreement, Ohmori et al., 1999 described that tumor necrosis factor-α negatively regulates Tg expression by inducing the nuclear exclusion of Pax8.

Although TSH-stimulated cAMP/PKA signaling pathway increases Pax8 gene expression and modulates its transcriptional activity, TSH signaling does not modulate neither Pax8 phosphorylation nor its nuclear localization (Mascia et al., 2002, Poleev et al., 1997). Interestingly, excess I^- inhibited the transcriptional activation of the Pax8 reporter Cp5-luc induced by exogenous Pax8 overexpression in TSH-starved thyroid cells suggesting that I^- may induce post-translational mechanisms to control Pax8 transcriptional activity.

NF-κB is an inducible transcription factor involved in the regulation of several genes involved in different cell processes. In the inactive form, the transcriptionally active NF-κB subunits p65 and p50 subunits remain in the cytoplasm bound to IκB proteins. Upon stimulation, IκB proteins are degraded and the transcriptionally active NF-κB subunits translocate to the nucleus to regulate gene expression (Perkins, 2007). In thyroid cells, TSHR activation stimulates NF-κB signaling pathway (Morshed et al., 2009). In fact, Nicola et al., 2010 has identified a strongly conserved binding site for NF-κB p65 subunit in the NUE region and demonstrated that p65 stimulates NIS gene transcriptional activity. In agreement, we observed that blockage of the NF-κB signaling using BAY 11-7082 reduces NIS promoter activity, thus supporting a physiological role of NF-κB controlling NIS expression. Interestingly, excess I^- decreased p65 protein expression and its transcriptional activity, thus indicating that NF-κB signaling is repressed in the presence of high I^- concentrations. In agreement with our observations, previous data indicated that excess I^- reduces the expression of major histocompatibility class I (MHC-I) molecules in thyroid cells by diminishing protein/DNA complexes and interfering with NF-κB nuclear signaling in thyrocytes (Taniguchi et al., 1998).

TSHR activation induces the dissociation of receptor coupled-trimeric G proteins into Gα and Gβγ subunits. Released-Gβγ dimers stimulate Akt phosphorylation in a PI3K-dependent manner (Zaballos et al., 2008). Several studies have demonstrated that PI3K/Akt activation negatively regulates NIS expression and function in thyroid cells (Garcia and Santisteban, 2002, Kogai et al., 2008, Liu et al., 2012). Moreover, we previously demonstrated the involvement of PI3K/Akt signaling mediating the acute effect of excess I^- on NIS expression post-transcriptional downregulation (Serrano-Nascimento et al., 2014). Here, we evidenced the involvement of the PI3K/Akt pathway in the excess I^--induced transcriptional repression of NIS gene expression, since the treatment of thyroid cells with PI3K or Akt inhibitors abrogated the downregulation of NIS promoter activity triggered by I^- treatment. In addition, our data suggested that I^--induced PI3K/Akt signaling activation leads to Pax8 exclusion from the nucleus. In accordance, Zaballos et al., 2008 demonstrated that activation of the PI3K/Akt signaling reduced Pax8 expression in the nucleus of thyroid cells. The authors hypothesized that Pax8 could interact with the transcription factor FoxO1, which is excluded from the nucleus by PI3K/Akt pathway activation (Zaballos et al., 2008). In fact, the PI3K pathway seems to determine the subcellular localization of FoxO proteins through post-translational modifications, as phosphorylation and proteasome degradation (Huang and Tindall, 2007, Zaballos and Santisteban, 2013).

Our data also indicated that PI3K/Akt signaling pathway is involved in the I^--induced inhibition of p65 expression and binding to DNA. Although several studies have assessed the cross-talk between PI3K/Akt and NF-κB signaling pathways, the results are still controversial. According to the type of cell, the activation of PI3K cascade can positively or negatively modulate NF-κB-driven gene expression (Fukao and Koyasu, 2003, Rajaram et al., 2006). Interestingly, in TLR4 expressing cells, the activation of PI3K/Akt pathway negatively regulates the transactivation activity of p65 (Zhang et al., 2007, Guha and Mackman, 2002). Accordingly, the inhibition of PI3K/Akt signaling in these cells enhanced LPS-induced nuclear accumulation of transcriptionally active NF-κB subunits and NF-κB-dependent gene expression. Moreover, Guha and Mackman (Guha and Mackman, 2002) demonstrated that the inhibition of PI3K signaling in monocytes augmented the phosphorylation and degradation of IκB-α, resulting in increased nuclear translocation of NF-κB subunits. Interestingly, in thyrocytes, which express TLR4 (Nicola et al., 2009), the blockade of the PI3K/Akt pathway increased the TSH-induced NF-κB signaling pathway (Morshed et al., 2009).

Reactive oxygen species (ROS) production is essential for thyroid hormone synthesis as these molecules work as cofactors required for TPO-mediated I^- oxidation and organification (Song et al., 2007). In agreement with previous reports (Leoni et al., 2011, Vitale et al., 2000, Arriagada et al., 2015), we observed that excess I^- increased ROS production in a concentration-response manner. These results are also in accordance with studies performed in human thyroid follicles and thyroid slices from other species (Many et al., 1992; Corvilain et al., 2000). Interestingly, our results indicated that increased ROS levels impaired the transcriptional activity of the Pax8 and p65 through stimulation of PI3K/Akt pathway, since the treatment of thyrocytes with ebselen or NAC abrogated both the activation of p-Akt and the inhibitory action of I^- on NIS promoter activity. In accordance, a recent study has demonstrated *in vitro* that the inhibitory effects of I^- on thyrocytes were prevented in the presence of a mixture of catalase, Tiron, and MCI-186. Indeed, the authors concluded that the I^--induced ROS production derived from different thyrocytes organelles (Arriagada et al., 2015). Finally, in addition to the data obtained with NAC and ebselen, we have also demonstrated that the reduction of NIS and TSHR mRNAs expression were abrogated in the presence of MMI, which has also antioxidants properties, reinforcing the importance of ROS production in the induction of I^- effects (Kim et al., 2001).

Oxidative stress modulates the activity of several phosphatases, such as the phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN), which opposes PI3K, leading to inactivation of Akt signaling (Meng et al., 2002, Denu and Tanner, 1998, Downes et al., 2004). Therefore, increased ROS production could activate PI3K/Akt cascade by inhibiting PTEN or other phosphatases activities through the reversible oxidation of catalytic cysteine residues (Lee et al., 2002, Leslie et al., 2003).

Moreover, besides the stimulation of PI3K/Akt signaling cascade, increased ROS levels might have a direct impact on the transcriptional regulation of thyroid gene expression, since the post-translational regulation of thyroid-enriched transcription factors by redox status modulates their binding to DNA (Kambe et al., 1996). Indeed, it has been previously shown that diamide-induced Pax8 oxidation reduced its binding to the Tg promoter, whereas expression of the cellular reducing catalyst thioredoxin restored Pax8 DNA binding (Kambe et al., 1996). Furthermore, intracellular redox state also influences the NF-κB signaling, since sulfhydryl modifying agents inhibit the binding of NF-κB subunits to their cognate DNA binding motifs (Matthews et al., 1996, Toledano and Leonard, 1991). Indeed, oxidant agents trigger post-translational modifications in cysteine residues of NF-κB subunits, such as the S-glutathionylation, which inhibits p65-DNA binding

and gene transcription activation (Lin et al., 2012). Therefore, the reduced Pax8 and p65 transcriptional activity induced by excess I^- may involve oxidative post-translational modifications that impair their binding to DNA and consequently, decrease TSH-induced NIS gene transcription.

In addition, previous studies have shown that thyroid transcription factors are controlled by a multifunctional protein apurinic apyrimidinic endonuclease/redox factor 1 (APE/Ref-1) through its redox activity (Tell et al., 2002). Moreover, it has been demonstrated that APE/Ref-1 strongly cooperates with Pax8 to stimulate human NIS promoter activity (Puppin et al., 2004). Interestingly, it has been shown that TSH increases cytoplasm-to-nucleus translocation of Ref-1 and consequently modulates both Pax8 and TTF1 transcriptional activities (Tell et al., 2000). Therefore, since excess I^- impaired TSH signaling pathway, we cannot exclude a modulation and a role of APE/Ref-1 on the control of NIS promoter activity under I^- treatment condition.

The concentration of NaI (10^{-3} M) used in our experimental conditions might be a limitation of this study. However, this concentration of NaI was previously used in several *in vitro* studies that aimed to investigate the inhibitory effects of I^- on NIS expression, including during the Wolff-Chaikoff effect (Serrano-Nascimento et al., 2014, 2012; Leoni et al., 2011, Eng et al., 2001, Leoni et al., 2008, Takasu et al., 1985). Considering that the present study aimed to further explore the mechanisms involved in the inhibition of NIS mRNA expression by excess I^-, the maintenance of the treatment concentration seemed to be pertinent. Nevertheless, in the present study, we performed additional concentration-response experiments and indicated that lower concentration of NaI significantly reduced NIS promoter activity, increased ROS production and induced Akt phosphorylation in exposed PCCl3 cells. Therefore, even though the focus of our studies was related to a high NaI concentration, the I^--triggered effects were also observed in lower concentrations of I^- treatment. Altogether, these results reinforce that besides its importance as an essential substrate for thyroid hormones synthesis, I^- is an important regulator of thyroid function and gene expression.

The use of a monolayer culture to perform the experiments could be considered another limitation of the study. Nevertheless, PCCl3 cells are highly differentiated thyroid cells that express thyroid-specific genes and thyroid transcription factors (Medina and Santisteban, 2000). In accordance, thyroid monolayer cultures, as FRTL5 or PCCl3 cells, are frequently used in studies that aim to evaluate physiological phenomena and the molecular aspects involved in thyroid differentiation and function (Garcia and Santisteban, 2002, Zaballos et al., 2008, Fernandez et al., 2013, Ruiz-Llorente et al., 2012). Moreover, several molecular mechanisms related to the Wolff-Chaikoff effect were described using these cell lineages (Arriagada et al., 2015, Leoni et al., 2011, Eng et al., 2001, Leoni et al., 2008; Grollman et al., 1986). Finally, our experiments successfully reproduced the results obtained in I^--treated rats as well in thyroid follicles from humans and other species, in which I^- excess treatment reduced NIS mRNA expression and increased ROS production (Leoni et al., 2011; Serrano-Nascimento et al., 2010; Eng et al., 1999; Corvilain et al., 2000; Many et al., 1992). Therefore, since in the present study we aimed to evaluate the molecular mechanisms involved in these effects, the use of this monolayer culture was considered appropriate.

5. Conclusion

In conclusion, our study demonstrated that high concentration of I^- not only reduces NIS-mediated I^- uptake and NIS mRNA expression through post-transcriptional events, but also down-regulates NIS gene expression by repressing Pax8 and p65 transcriptional activity. Moreover, our results indicated that excess I^- repressed NIS gene expression through ROS-induced activation of PI3K/Akt signaling pathway. Altogether, our data add new perspectives linking the role of PI3K/Akt pathway and redox state to the I^--induced thyroid autoregulatory mechanism.

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Disclosure statement

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.02.006.

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