Modulation of sodium iodide symporter expression and function by LY294002, Akti-1/2 and Rapamycin in thyroid cells

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Abstract
The selective increase of Na\(^+\)/I\(^-\) symporter (NIS)-mediated active iodide uptake in thyroid cells allows the use of radioiodine I\(^{131}\) for diagnosis and targeted treatment of thyroid cancers. However, NIS-mediated radioiodine accumulation is often reduced in thyroid cancers due to decreased NIS expression/function. As PI3K signaling is overactivated in many thyroid tumors, we investigated the effects of inhibitors for PI3K, Akt, or mTORC1 as well as their interplay on NIS modulation in thyroid cells under chronic TSH stimulation. PI3K inhibition by LY294002 increased NIS-mediated radioiodide uptake (RAIU) mainly through upregulation of NIS expression, however, mTORC1 inhibition by Rapamycin did not increase NIS-mediated RAIU despite increased NIS protein levels. In comparison, Akt inhibition by Akti-1/2 did not increase NIS protein levels, yet markedly increased NIS-mediated RAIU by decreasing iodide efflux rate and increasing iodide transport rate and iodide affinity of NIS. The effects of Akti-1/2 on NIS-mediated RAIU are not detected in nonthyroid cells, implying that Akti-1/2 or its derivatives may represent potential pharmacological reagents to selectively increase thyroidal radioiodine accumulation and therapeutic efficacy.

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Introduction
The Na\(^+\)/I\(^-\) symporter (NIS) is a transmembrane glycoprotein that is expressed on the basolateral membrane of thyroid follicular cells. NIS-mediated iodide uptake from the circulating bloodstream into the thyroid follicular cells is the first step for the synthesis of iodine-containing thyroid hormones and is the molecular basis for targeted radioiodide ablation of residual thyroid tissue and treatment of recurrent or metastatic thyroid cancer after thyroidectomy. However, about 20% of patients with differentiated thyroid tumors and nearly all patients with poorly differentiated thyroid tumors fail to respond to radioiodide therapy in part due to decreased NIS expression/function (Caillou et al. 1998, Castro et al. 2001, Patel et al. 2002). Thus, it is of clinical importance to investigate NIS modulation by various pharmacological agents with the objective to increase/restore NIS expression/function for better efficacy of radioiodine therapy.

MEK and PI3K signaling pathways are overactivated in many malignant thyroid tumors (Knauf & Fagin 2009, Xing 2010), and NIS expression level is decreased by MAPK or PI3K activation in cultured thyroid cells (Cass & Meinkoth 2000, Knauf et al. 2003, Zaballos et al. 2008). RasV12S35 that preferentially binds to and activates Raf-1/MEK signaling pathway was shown to completely abolish TSH-induced NIS expression in WRT thyroid cells (Cass & Meinkoth 2000). Doxycycline-induced expression of MEK1E217/E221 decreased NIS mRNA levels in PCC13 rat thyroid cells (Knauf et al. 2003). Similarly, overexpression of either G\(_{\text{PVY}}\)
subunit of TSH receptor coupled G proteins (Zaballos et al. 2008) or RasV12C40 (Cass & Meinkoth 2000) that leads to PI3K activation decreased NIS protein levels in rat thyroid cells. Moreover, insulin-like growth factor 1 (IGF1) appears to decrease TSH-induced NIS promoter activity, mRNA, and protein levels through PI3K activation (Garcia & Santisteban 2002). Accordingly, pharmacological inhibitors targeting MAPK/ERK or PI3K signaling may increase or restore NIS expression and activity in thyroid cells.

MEK inhibition by PD98059 increases NIS mRNA and protein levels in thyroid cells expressing the RET/PTC oncogenes as well as in parental thyroid cells, albeit with different magnitude and temporal dynamics (Garcia & Santisteban 2002, Knauf et al. 2003, Vadysirisack et al. 2007b). NIS-mediated radioiodide uptake (RAIU) was transiently decreased at 4 h, but increased at 24 and 48 h with PD98059 treatment despite NIS protein levels were increased to variable extent in PCCl3 thyroid cells expressing RET/PTC1 (Vadysirisack et al. 2007b), suggesting that MEK signaling not only modulates NIS at the expression but also at the activity level. In rat thyroid FRTL-5 cells that are acutely treated with TSH after prolonged TSH deprivation, PI3K inhibition by LY294002 increases NIS promoter activity, mRNA, and protein levels, as well as NIS-mediated RAIU (Garcia & Santisteban 2002, Kogai et al. 2008b). However, under these conditions, Akt inhibition by Akti-1/2 only slightly increased NIS mRNA levels and had little effect on NIS-mediated RAIU (Kogai et al. 2008b). A recent study showed that mTORC1 inhibition by Rapamycin increased NIS expression and NIS-mediated RAIU in PCCl3 cells under acute TSH stimulation (de Souza et al. 2010). To date; however, the effects of LY294002, Akti-1/2, or Rapamycin on NIS modulation in thyroid cells under chronic TSH stimulation have not yet been studied. Moreover, the characterization of interplay between PI3K/Akt and MAPK/ERK signaling pathways on NIS modulation remains elusive.

In this study, we demonstrate that treatment with LY294002 or Rapamycin increases NIS protein levels yet only LY294002 increases NIS-mediated RAIU in thyroid cells under chronic TSH stimulation. Interestingly, treatment with Akti-1/2 significantly increases NIS-mediated RAIU without increasing NIS protein levels, and the effect of Akti-1/2 on NIS protein levels and activity prevailed over LY294002, Rapamycin, or PD98059. Finally, NIS modulation by PI3K/Akt appeared to be different in thyroid cells vs nonthyroid cells, as LY294002 and Akti-1/2 decreased RAIU in nonthyroid cells expressing exogenous NIS. Taken together, PI3K/Akt inhibitors may selectively enhance thyroidal radioiodine accumulation and have potential to improve the efficacy of radioiodine therapy for patients with thyroid cancer.

Materials and methods

Cell culture and reagents

PCCl3 rat thyroid cells were maintained in Kaighn’s medium (Sigma), 5% calf serum, 2 mM glutamine, 1% penicillin–streptomycin, 10 mM NaHCO3, and 6H hormone (1 mU/ml bovine TSH, 10 µg/ml bovine insulin, 10 nM hydrocortisone, 5 µg/ml transferrin, 10 ng/ml somastatin, and 2 ng/ml t-glyceryl-histidylylsine). PCCl3 cells with doxycycline-inducible MEK activation (Knauf et al. 2003) (kindly provided by Drs Jeffrey A Knauf and James A Fagin at Memorial Sloan Kettering Cancer Center) were induced with 2 µg/ml doxycycline for 72 h. HEK293 cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin–streptomycin. LY29402 was purchased from Caymen (Ann Arbor, MI, USA), Akti-1/2 and Rapamycin were purchased from Calbiochem (Gibbstown, NJ, USA). shRNA targeting Akt1 and Akt2 (Hou et al. 2009) with its vector control were a generous gift from Dr Mingzhao Xing at The Johns Hopkins University School of Medicine.

RNA extraction and quantitative real-time PCR (qRT-PCR)

PCCl3 cells were seeded in 100 mm dishes. After treatment with DMSO, LY294002, Rapamycin, or Akti-1/2 for 24 h, cells were washed with PBS twice and total RNA was extracted with a mixture of Trizol reagent (Invitrogen) and chloroform (Sigma). RNA was precipitated with RNase/DNase-free isopropanol and pelleted by centrifuging at 12 000 g for 20 min at 4 °C. The RNA pellet was washed with 70% RNase/DNase-free isopropanol and resuspended in 20 µl DEPC-treated water. Contaminating DNA was removed by DNase I (Invitrogen) digestion. One microgram of extracted RNA was used for the First-Strand Synthesis reverse transcription reaction (Invitrogen) performed according to the manufacturer’s instructions. qRT-PCR was performed in a 20 µl reaction mixture volume, containing cDNA template, Power SYBR Green PCR MasterMix (Applied Biosystems, Carlsbad, CA, USA), and primers. Reaction mixtures were incubated at 50 °C for 2 min followed by an initial activation at 95 °C for 15 min, and then subjected to 40 PCR cycles of denaturation (95 °C for 15 s), annealing (56 °C for 30 s), and extension (60 °C for 1 min) using the ABI
RAIU assay

Cells were pretreated with pharmacological inhibitors for 24 h before the assay. At 24 h, cells were incubated with 2 μCi NaI125 in 5 μM nonradioactive NaI for 30 min at 37 °C with 5% CO2. Cells were then washed with cold Hank’s Balanced Salt Solution two times and then lysed in cold 95% ethanol for 20 min at room temperature. Cell lysate was collected and counted for radioactivity by the gamma-counter (Packard Instruments, Waltham, MA, USA). RAIU not mediated by NIS was excluded by performing the assay in the presence of 3 mM perchlorate (ClO4−), a competitive inhibitor of iodide uptake by NIS. Experiments were performed in triplicates. Our pilot study showed that IC50 of perchlorate for NIS-mediated RAIU is 3 μM (data not shown). For DMSO-treated PCCl3 cells, RAIU ranged from 0.5 to 1.1 pmol/3×10⁴ cells among different experimental trials. For DMSO-treated HEK293 expressing exogenous rNIS, RAIU is about 3.6 pmol/3×10⁴ cells.

Western blot analysis and antibodies

Cells were pretreated with inhibitors for 24 h prior to the analysis. At 24 h, cells were lysed and homogenized using 18½ gauge needles in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin. Protein concentrations were determined by Bradford assay (Bio-Rad) and equal amounts of protein were subjected to the 10% SDS–PAGE gel, then transferred onto the nitrocellulose membrane. The membrane was blocked in 5% dry milk in TBST buffer (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20) and incubated with various primary antibodies for 1 h at room temperature, followed by incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. The signal was later detected using ECL detection reagent (Amersham Pharmacia). Rabbit anti-rNIS antibody PA716 was generously provided by Dr Bernard Rousset (Institut National de la Santé et de la Recherche Médicale). Rabbit anti-Na+/K+-ATPase was generously provided by Dr Beth Lee (The Ohio State University). Rabbit anti-pAkt and anti-pp70S6K1 were purchased from Cell Signaling (Danvers, MA, USA). Rabbit anti-pERK was purchased from Invitrogen. Mouse anti-β-actin was purchased from Abcam (Cambridge, MA, USA). Mouse anti-FLAG monoclonal antibody M2 was purchased from Sigma. Densitometry analysis was performed using Scion image software.

Iodide efflux assay

1125I efflux assay was performed as described (Marsee et al. 2004). Cells were pretreated with pharmacological inhibitors for 24 h, and then incubated in media containing NaI125 as described for RAIU assay. Cells were washed twice and incubated with cold 1 ml HBSS for 2 min. HBSS was then collected and replaced with new medium. This was repeated every 2 min for a total of 10 min. Cells were then lysed with 95% ethanol for 20 min. The total uptake was calculated as the sum of total efflux washes and the lysates. Experiments were performed in triplicates.

Cell surface biotinylation

Cell surface biotinylation was performed as previously described (Vadysirisack et al. 2007b). Cells were pretreated with pharmacological inhibitors for 24 h prior to the assay. Cells were then washed with cold PBS containing 1 mM MgCl2 and 0.1 mM CaCl2 (PBS–Ca/Mg). Cells were incubated with 1 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) in PBS–Ca/Mg for 1 h at 4 °C. The reaction was then quenched using 100 mM glycine in PBS–Ca/Mg for 20 min. Cells were then lysed in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 30 min at 4 °C and homogenized using 18½ gauge needles. Lysates were centrifuged at 14 000 g for 20 min at 4 °C and supernatant containing the whole cell lysate was collected. Bradford assay (Bio-Rad) was used to determine the protein concentration. To separate the biotinylated cell surface proteins from nonbiotinylated proteins, equal amounts of whole cell extract were incubated with avidin-coated agarose beads (Pierce) overnight at 4 °C. The beads were then washed with lysis buffer and protein was eluted with 2× Laemmlı sample buffer at 95 °C for 5 min.
Flow cytometric analysis for cell surface NIS
HEK293 cells were seeded in 100-mm dishes for 24 h, and transfection was performed using FuGene 6 with 5 μg of Flag-tagged rNIS constructs. Cells were harvested by 0.25% trypsin at 48 h after transfection. To detect cell surface NIS protein levels, flow cytometric analysis was performed using nonpermeabilized cells with 1:50 diluted mouse anti-FLAG monoclonal antibody M2 (Sigma–Aldrich Corp.) that recognizes extracellular FLAG peptide, followed by 1:100 diluted FITC-conjugated goat anti-mouse IgG (Sigma–Aldrich Corp.). Cells were then washed and resuspended in fixative buffer (1% paraformaldehyde in PBS) for analysis by a FACS Calibur flow cytometer (BD Biosciences, Eerembodegem, Belgium). Secondary antibody alone was used as a negative control for nonspecific fluorescence.

Statistical analysis
Comparisons between groups were performed using paired t-test; a P value <0.05 was considered to be statistically significant. As the iodide efflux data from the same trial were correlated, a linear mixed effects model with a quadratic term of time was used for the analysis to take account of the correlation of the data. To ensure unbiased hypothesis tests, a covariance structure estimation method for small samples was used to avoid underestimation of experimental error. Holm’s procedure (Holm 1979) was used to correct for the multiple pairwise comparisons to control type I error at 0.05.

Results
Both LY294002 and Rapamycin increased NIS mRNA and protein levels in PCC13 cells under chronic TSH stimulation
NIS modulation by PI3K inhibitor (LY294002) or mTORC1 inhibitor (Rapamycin) has been examined in rat thyroid cells acutely treated with TSH after prolonged TSH deprivation (Garcia & Santisteban 2002, Kogai et al. 2008b, Zaballos et al. 2008, de Souza et al. 2010). In this study, we showed that LY294002 and Rapamycin had similar effect on NIS expression in PCC13 cells under chronic TSH stimulation. By qRT-PCR analysis, NIS mRNA levels were increased 1.7- and 1.8-fold by 24 h treatment with 10 μM LY294002 and 20 nM Rapamycin respectively (Fig. 1A; n = 2). As shown in a representative western blot analysis (Fig. 1B), NIS protein levels were also increased by both LY294002 and Rapamycin with an average of 1.97±0.8-fold (n = 4) and 1.9±0.5-fold

Figure 1 Both LY294002 and Rapamycin increased NIS mRNA and protein levels in PCC13 cells under chronic TSH stimulation. (A) qRT-PCR analysis showed that LY294002 and Rapamycin increased NIS mRNA steady-state levels by 1.7- and 1.8-fold respectively (n = 2; average and the extent of deviation from average are shown). (B) A representative western blot analysis showed that both LY294002 and Rapamycin increased NIS protein levels. The amount of protein loaded for western blot analysis was normalized by β-actin. (C) Densitometry analyses of multiple experiments showed that NIS protein levels were increased 2.0±0.8-fold by LY294002 (n = 4) and 1.9±0.5-fold by Rapamycin (n = 7). *P value < 0.05.
(n = 7) respectively (Fig. 1C). Taken together, NIS expression was increased by LY294002 and Rapamycin in both chronic and acute TSH-treated PCCl3 cells.

Rapamycin failed to increase NIS-mediated RAIU most likely due to its activation of Akt and ERK signaling

Since increased NIS protein levels do not always contribute to an increase in NIS-mediated RAIU (Vadysirisack et al. 2007b), we further examined NIS-mediated RAIU in LY294002 or Rapamycin-treated PCCl3 cells under chronic TSH stimulation. Similar to studies performed in thyroid cells acutely treated with TSH after prolonged TSH deprivation (Kogai et al. 2008b), an increase in NIS protein levels (Fig. 2B, left panel) by LY294002 was accompanied with an increase in NIS-mediated RAIU (Fig. 2A, 1.8 ± 0.4-fold; n = 4). In contrast, the increase in NIS protein levels (Fig. 2B, right panel) by Rapamycin did not result in an increase in NIS-mediated RAIU (Fig. 2A, 0.69 ± 0.07-fold; n = 4). This is different from a recent report that NIS-mediated RAIU was increased by Rapamycin in PCCl3 cells acutely treated with TSH (de Souza et al. 2010). Akt and ERK signaling nodes appeared to be differentially modulated by LY294002 vs Rapamycin in PCCl3 cells under chronic TSH stimulation. As shown in a representative western blot analysis, pAkt and pERK levels were decreased by LY294002 (Fig. 2B, left panel) but not by Rapamycin (Fig. 2B, right panel). For cells treated with LY294002, pAkt and pERK were decreased by 0.37- and 0.51-fold respectively (Fig. 2C, top panel; n = 2). For cells treated with Rapamycin, pAkt and pERK levels remained unchanged or increased (Fig. 2C, bottom panel, pAkt: 0.99- to 2.66-fold; pERK: 1.02- to 1.62-fold; n = 7). Indeed, Rapamycin has been shown to downregulate S6K1, which alleviates negative regulation of S6K1 on PI3K-mediated Akt or ERK activation (Wan et al. 2007, Carraceno et al. 2008).

To investigate whether Akt and/or ERK activation leads to repression of NIS activity in Rapamycin-treated cells, we examined whether co-treatment with MEK inhibitor (PD98059) or Akt inhibitor (Akti-1/2) increased NIS-mediated RAIU in Rapamycin-treated cells. As shown in Fig. 2D, NIS-mediated RAIU was decreased 0.77 ± 0.01-fold by Rapamycin alone, yet was comparable to vehicle-treated cells (1.01 ± 0.08-fold; n = 2) by co-treatment with Rapamycin and

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Figure 2: Rapamycin failed to increase NIS-mediated RAIU most likely due to its activation of ERK and Akt signaling. (A) LY294002 increased, yet Rapamycin decreased, NIS-mediated RAIU (n = 4). *P value < 0.05. **Cell surface NIS was normalized with Na+/K+ - ATPase; Total NIS was normalized with β-actin. (B) A representative western blot analysis showed that LY294002 and Rapamycin differentially modulate pAkt and pERK levels. (C) On average, LY294002 decreased pAkt and pERK levels by 0.37- and 0.51-fold respectively (top panel; n = 2; average and the extent of deviation from average are shown). For cells treated with Rapamycin, pAkt, and pERK levels remained unchanged or increased (bottom panel, pAkt: 0.99- to 2.66-fold; pERK: 1.02- to 1.62-fold; n = 7). *P value < 0.05. **Protein level was normalized with β-actin. (D) Co-treatment with PD98059 and Rapamycin for 24 h slightly increased NIS-mediated RAIU compared to Rapamycin alone, and was comparable to vehicle-treated cells (left panel; each experiment in triplicates; n = 2, average and the extent of deviation from average are shown). Co-treatment with Akti-1/2 and Rapamycin markedly increased NIS-mediated RAIU (right panel; each experiment in triplicates; n = 2, average and the extent of deviation from average are shown).
PD98059. In comparison, NIS-mediated RAIU was increased by co-treatment with Rapamycin and Akti-1/2 (2.96 ± 0.48-fold; n = 2). Taken together, these data suggest that the inability of Rapamycin to increase NIS-mediated RAIU, despite of the accompanied increase in NIS protein level, involves activation of ERK and Akt.

Akti-1/2 significantly increased NIS-mediated RAIU without increasing NIS protein levels

As the effect of MEK/ERK inhibition on NIS modulation has been extensively studied (Knauf et al. 2003, Vadysirisack et al. 2007), we focused on the effect of Akt inhibition on NIS expression and function in PCC13 cells. While NIS mRNA levels were reported to be slightly increased by 30 µM Akti-1/2 treatment in FRTL-5 cells acutely stimulated by TSH (Kogai et al. 2008b), our data indicate that NIS mRNA level was not affected by 10 µM Akti-1/2 in PCC13 cells under chronic TSH stimulation (Fig. 3A; n = 2). In fact, in our system, NIS protein levels were often decreased by Akti-1/2 (Fig. 3B, 0.52 ± 0.27-fold; n = 8). Since NIS is known to have a relatively long half-life (t1/2 = 3–5 days) in PCC13 cells under chronic TSH stimulation (Riedel et al. 2001), the decrease of NIS protein level in Akti-1/2-treated cells seems more likely to be due to an increase in degradation rather than a decrease in synthesis.

![Akti-1/2 significantly increased NIS-mediated RAIU without increasing NIS protein levels.](image)

Figure 3. Akti-1/2 significantly increased NIS-mediated RAIU without increasing NIS protein levels. (A) Akti-1/2 had little effect on NIS mRNA levels as shown by qRT-PCR analysis (n = 2; average and the extent of deviation from average are shown). (B) A representative western blot analysis showed that Akti-1/2 decreased NIS protein levels. On average, densitometry analysis of multiple experiments (n = 8) showed that NIS protein levels were decreased by 0.52 ± 0.27-fold. *P value < 0.01. Protein loading was normalized with β-actin. (C) Akti-1/2 increased NIS-mediated RAIU by 3.93 ± 1.58-fold (each experiment in triplicates; n = 4) as shown by RAIU assay. *P value < 0.05. (D) While NIS-mediated RAIU was decreased upon MEK/E217/E227 induction by doxycycline (bar 1 vs bar 3), Akti-1/2 increased NIS-mediated RAIU in cells with or without MEK/E217/E227 induction (bar 1 vs bar 2; bar 3 vs bar 4). Average and the extent of deviation from average of two independent experiments (n = 2), each performed in triplicates, are shown. RAIU not mediated by NIS was excluded by performing the assay in the presence of perchlorate (ClO4).
Surprisingly, the decrease in NIS protein levels by Akti-1/2 was accompanied by a substantial increase in RAIU (Fig. 3C, 3.93 ± 1.58-fold; n = 4). Of note, the extent of increase in NIS-mediated RAIU by Akti-1/2 seemed to correlate with a lesser reduction of NIS. For example, Akti-1/2 increased NIS-mediated RAIU by 5.7-, 5.2-, 3.8-, and 2.2-fold in PCCl3 cells where their corresponding NIS protein levels were reduced to 0.91, 0.87, 0.44, and 0.28 relative to the baseline respectively.

Treatment with an alternative Akt inhibitor, AktV, which shares little structural similarity with Akti-1/2, also decreased NIS protein levels to 0.23-fold (Fig. 4A), yet increased NIS-mediated RAIU by 3.2-fold in PCCl3 cells (Fig. 4B). In addition, molecular inhibition of Akt 1 and 2 using stable shRNA also reduced NIS protein levels (Fig. 4C) but increased NIS-mediated RAIU compared to vector-expressing controls (Fig. 4D). Taken together, our data indicate that NIS-mediated RAIU can be increased by Akt inhibition either by pharmacological or genetic approaches even though NIS protein levels are reduced.

The perturbation of pERK levels by Akti-1/2 was inconsistent among different experimental trials. The extent of increase in RAIU by Akti-1/2 did not appear to correlate with pERK levels, suggesting that NIS modulation by Akt inhibition may be dominant over ERK signaling in PCCl3 cells. To further examine the interaction between ERK and Akt signaling nodes

Figure 4 Akt inhibition by pharmacological reagent, AktV, or shRNA targeted knockdown of Akt1/2 decreased NIS protein levels yet increased NIS-mediated RAIU in PCCl3 cells. (A and B) Treatment with 200 μM AktV for 24 h decreased NIS protein levels yet increased NIS-mediated RAIU in PCCl3 cells. (C and D) shRNA targeted knockdown of Akt1/2 decreased NIS protein levels yet increased NIS-mediated RAIU in PCCl3 cells. PCCl3 cells stably expressing vector control or shRNA targeted to Akt1/2 knockdown were established and maintained in PCCl3 media with 200 μg/ml G418. For western blot analysis, average and the extent of deviation from the average of two independent experiments (n = 2), are shown. The amount of protein loaded for western blot analysis was normalized by β-actin. For RAIU assay, average and the extent of deviation from average of two independent experiments (n = 2), each performed in triplicates, are shown.
on NIS modulation, PCCl3 cells with doxycycline-induced expression of constitutively active MEK\(^{E217/E221}\) were treated with Akti-1/2. As expected, induction of MEK\(^{E217/E221}\) decreased NIS-mediated RAIU in the absence of Akti-1/2 (bar 1 vs bar 3 in Fig. 3D). However, the extent of NIS-mediated RAIU enhanced by Akti-1/2 treatment was not affected by MEK\(^{E217/E221}\) induction (Fig. 3D: bar 3 vs bar 4 compared to bar 1 vs bar 2).

**Akti-1/2 increased RAIU by decreasing iodide efflux rate and increasing NIS-mediated iodide transport rate and iodide affinity**

To understand the discordance between NIS protein levels and NIS-mediated RAIU in Akti-1/2-treated cells, we first examined whether Akti-1/2 increased cell surface NIS level as cell surface but not total NIS protein levels confer NIS-mediated RAIU. As shown in Fig. 5A, cell surface biotinylation followed by avidin pull down and subsequent NIS probing by western blot analysis demonstrated that cell surface NIS level was also decreased by Akti-1/2. Consequently, the ratio of cell surface NIS levels vs total NIS protein levels was comparable between DMSO (0.25) and Akti-1/2-treated cells (0.26). Immunofluorescent labeling of NIS showed that both cell surface and intracellular NIS were decreased in Akti-1/2-treated cells compared to DMSO-treated cells (data not shown). This indicates that the increase in RAIU by Akti-1/2 was not due to an increase in cell surface NIS levels.

As steady-state iodide accumulation in thyroid cells is the net outcome of iodide uptake and iodide efflux, we next examined the effect of Akti-1/2 on iodide efflux rate in PCCl3 cells. The effect of 17-AAG on decreasing iodide efflux rate (Marsee et al. 2004) was performed in parallel for comparison. As shown in Fig. 5B, the percentage of iodide remaining in the cells was significantly increased by Akti-1/2 (dotted line with solid triangles; \(P < 0.0001\)), albeit at a lesser degree than that by 17-AAG (dotted line with open circles; \(P < 0.0001\)), compared to DMSO (solid line with solid diamonds). In comparison, LY294002 (dotted line with solid squares) had little effect on the percentage of iodide remaining in the cells. Taken together, our data indicate that iodide...
efflux rate was significantly decreased by Akti-1/2 and 17-AAG. However, while the extent of decrease in iodide efflux rate by Akti-1/2 was less than that by 17-AAG (P value < 0.0001), the extent of increase in RAIU by Akti-1/2 was greater than that by 17-AAG (Fig. 5C). Thus, the decrease in iodide efflux rate does not fully account for the extent of increase in RAIU in Akti-1/2-treated cells.

We next examined whether Akti-1/2 affects iodide transport rate and/or iodide affinity of NIS. While cell surface NIS level was decreased by Akti-1/2, the maximal velocity of iodide uptake (V_{max}-I^-) was increased 1.3-fold by Akti-1/2 (supplementary data 1) suggesting that the increase in V_{max}-I^- by Akti-1/2 is due to increased velocity of iodide transport. Furthermore, K_{m-I^-} was decreased 0.71-fold by Akti-1/2 (supplementary data 1) suggesting that Akti-1/2 slightly increased iodide affinity of NIS. Taken together, Akti-1/2 increased NIS-mediated RAIU by decreasing iodide efflux rate, increasing iodide transport rate, and increasing iodide affinity of NIS.

**NIS modulation by Akti-1/2 prevailed over LY294002, Rapamycin and PD98059**

Although LY294002, Rapamycin, and PD98059 all increased NIS protein levels to different extents, the extent of increase in NIS protein level appeared to be lessened by co-treatment with Akti-1/2 (Fig. 6A). This finding is in agreement with the conclusion that the effects of LY294002, Rapamycin, and PD98059 mainly reflected at NIS mRNA level yet Akti-1/2’s action on NIS protein level occurs at posttranscriptional level. In addition, while treatment with LY294002, PD98059, or Rapamycin had differential effects on RAIU, RAIU were all increased when cells were co-treated with Akti-1/2 (Fig. 6B). However, it is interesting to note that RAIU in cells treated with Akti-1/2 alone was not further increased by co-treatment with LY294002 (Fig. 6B, top panel), but was further increased by co-treatment with Rapamycin (Fig. 6B, middle panel) and reduced by co-treatment with PD98059 (Fig. 6B, bottom panel). Taken together, NIS modulation by Akti-1/2 prevailed over LY294002,

![Figure 6](https://www.endocrinology-journals.org/)

**Figure 6** NIS modulation by Akti-1/2 prevailed over LY294002, Rapamycin, and PD98059 in PCC13 cells under chronic TSH stimulation. PCC13 cells were treated with LY294002 (10 μM), Rapamycin (20 nM), or PD98059 (40 μM) with or without Akti-1/2 (10 μM) for 24 h prior to western blot analysis or RAIU assay. (A) The extent of increase in NIS protein level by LY294002 (top panel), Rapamycin (middle panel), or PD98059 (bottom panel) was diminished by co-treatment with Akti-1/2 as shown by a representative western blot analysis. (n=2, average and the extent of deviation from average are shown). (B) Akti-1/2 co-treatment further increased RAIU in LY294002 (top panel), Rapamycin (middle panel), and PD98059 (bottom panel) treated PCC13 cells (n=2, each n in triplicates; average and the extent of deviation from average are shown). Of note, the extent of increase in RAIU in cells treated with Akti-1/2 alone was comparable to cells co-treated with LY294002, less than cells co-treated with Rapamycin, and greater than cells co-treated with PD98059. RAIU not mediated by NIS was excluded by performing the assay in the presence of perchlorate (ClO₄⁻).
Rapamycin, and PD98059, yet there were subtle differences among the interplay of Akti-1/2 with LY294002, Rapamycin, or PD98059.

**Akti-1/2 decreased NIS protein level yet had no effect on RAIU in PCCl3 cells acutely stimulated by TSH**

The dynamics of signaling network is expected to be different in thyroid cells under chronic vs acute TSH stimulation. We further examined the effects of LY294002, Akti-1/2, and Rapamycin on NIS expression/function in PCCl3 cells under acute TSH stimulation after 3 days of TSH deprivation. Similar to PCCl3 cells under chronic TSH stimulation, TSH-induced NIS expression and RAIU were both increased by LY294002 (Fig. 7A and B; lane/bar 1 vs lane/bar 3). Interestingly, while Akti-1/2 decreased NIS protein level in both chronic and acute TSH-treated PCCl3 cells (Fig. 7A; lane 1 vs lane 4), Akti-1/2 did not increase NIS-mediated RAIU in acute TSH-treated PCCl3 cells (Fig. 7B; bar 1 vs bar 4). Moreover, Rapamycin had little effect on NIS protein level and NIS-mediated RAIU (Fig. 7; lane 1 vs lane 5) in PCCl3 cells under acute TSH stimulation. Taken together, there was no difference in NIS modulation by PI3K inhibition between thyroid cells under chronic or acute TSH stimulation; yet the effect of Akt inhibition on NIS activity in thyroid cells under chronic TSH stimulation was not found in thyroid cells under acute TSH stimulation. The effect of NIS modulation by Rapamycin appeared to be highly dynamic and may depend on the doses and/or duration of treatment, as our data (Fig. 7) are different from data recently reported by de Souza et al. (2010). In this study, IGF1 was included to activate PI3K signaling, however, the effects of inhibitors for PI3K, Akt, and mTORC1 on NIS modulation were not affected by the presence of IGF1 (Fig. 7; lanes/bars 3, 4, 5 vs lanes/bars 6, 7, 8).

**LY294002 and Akti-1/2 decreased NIS-mediated RAIU in nonthyroid cells expressing exogenous NIS**

The effect of LY294002 and Akti-1/2 on NIS protein levels and RAIU in PCCl3 cells was also observed in another rat thyroid cell line, FRTL-5, as well as in RET/PTC1-transformed PCCl3 cells (supplementary data 2). In contrast to thyroid cell models with endogenous NIS, LY294002, and Akti-1/2 had little effect on cell surface (Fig. 8C) or total NIS protein levels (Fig. 8B) in HEK293 human kidney cells expressing exogenous Flag-tagged rat NIS or COS7 monkey kidney cells expressing exogenous NIS (data not shown). Please note that the glycosylation status of Flag-tagged rNIS proteins in HEK293 cells appears to be quite different from endogenous rNIS in PCCl3 cells, which have been reported in our previous studies.
Vadysirisack et al. 2007b). LY294002 and Akti-1/2 did not increase but instead decreased NIS-mediated RAIU in HEK293 cells (Fig. 8A) or COS7 cells expressing exogenous rat NIS (data not shown). Thus, NIS modulation by LY294002 and Akti-1/2 appears to be in a cell context-dependent manner.

Discussion

In this study, we showed that LY294002, Akti-1/2, and Rapamycin differentially modulate NIS expression and function at multiple levels in thyroid cells under chronic TSH stimulation. Inhibition of PI3K by LY294002 mainly increased NIS-mediated RAIU through upregulation of NIS expression. In comparison, inhibition of mTORC1 by Rapamycin did not increase NIS-mediated RAIU despite increasing NIS protein levels. This was most likely due to accompanied activation of Akt and ERK by Rapamycin as co-treatment with PD98059 or Akti-1/2 did increase NIS-mediated RAIU. Akti-1/2 treatment alone markedly increased NIS-mediated RAIU by decreasing iodide efflux rate and increasing iodide transport rate and iodide affinity of NIS. Furthermore, NIS modulation by Akti-1/2 appeared to be dominant over LY294002, Rapamycin, and PD98059. While both Akti-1/2 and LY294002 increased NIS-mediated RAIU in thyroid cells through different mechanisms, both of them decreased RAIU in HEK293 or COS7 nonthyroid cells expressing exogenous NIS. Together
with previous reports showing that PI3K/Akt inhibition decreases tRA-induced NIS expression/function in MCF-7 human breast cancer cells (Kogai et al. 2008a, Ohashi et al. 2009), our data suggested that LY294002 and Akti-1/2 may selectively increase thyroidal radioiodine accumulation, raising PI3K/Akt inhibition as a potential strategy to improve the efficacy of radioiodine therapy on thyroid cells without increasing off-target effects.

Akti-1/2 appears to decrease NIS protein stability independently from its effects on increasing NIS-mediated RAIU in thyroid cells under chronic TSH stimulation. Akti-1/2 has a similar effect on NIS protein stability in thyroid cells under acute TSH stimulation (Fig. 7A). Akti-1/2 modulation of NIS protein stability is further supported by our observation that the extent of the increases in NIS protein levels induced by LY2942002, Rapamycin, or PD98059 was lessened by co-treatment with Akti-1/2 (Fig. 6A). Indeed, Akt activation has been reported to prevent protein breakdown in muscle cells by decreasing expression of an E3 ubiquitin ligase (Yoshida et al. 2010). Interestingly, we identified ubiquitin and Nedd4-2, an E3 ubiquitin ligase, as potential NIS-associated proteins (Vadysirisack DD & Jhiang SM 2007, unpublished observations), and Nedd4-2 have been reported by others to be directly inhibited by Akt activation in FRT rat thyroid cells (Lee et al. 2007).

It will be interesting to further investigate the possible role of Nedd4-2 in Akti-1/2-mediated NIS protein degradation.

Since the increased RAIU did not take place until more than 8 h of treatment with Akti-1/2 (data not shown), NIS is most likely not the direct target of Akti-1/2 in increasing NIS-mediated RAIU. Akti-1/2 may elicit de novo synthesis of proteins that could modulate NIS directly at the posttranslational level and/or act indirectly on other non-NIS factors that contribute to the observed increase in NIS-mediated RAIU. In addition, our lab has previously reported that the status of NIS phosphorylation at Serine 43 and Serine 581 modulates iodide transport rate of NIS and that these two phosphorylation sites are not modulated by MEK signaling (Vadysirisack et al. 2007a). Based on KinasePhos, NetPosK1.0, GPS, and PPSP consensus motif programs, Serine 43 and Serine 581 are predicted to be phosphorylated by PI3K/Akt down-stream effectors S6K, IKK, and PAK (Vadysirisack et al. manuscript in preparation). Further study on NIS phosphorylation status and/or NIS complex formation in thyroid cells upon Akti-1/2 treatment may help to identify candidate factors that modulate NIS activity, as well as NIS protein stability.

Multiple studies have demonstrated that the interplay among TSH, PI3K, Akt, mTOR, and MAPK is highly dynamic in acute TSH-treated rat thyroid cell lines (Tsygankova et al. 2000, 2001, Ciullo et al. 2001, Iacovelli et al. 2001, Lou et al. 2002), such that conflicting results were acquired under various experimental conditions. Nevertheless, increased NIS expression/function by PI3K inhibition was consistently reported among different studies in thyroid cells under acute TSH stimulation (Garcia & Santisteban 2002, Kogai et al. 2008b, Zaballos et al. 2008). Our study showed a similar effect of LY294002 on NIS modulation in thyroid cells under chronic TSH stimulation. Conversely, Akti-1/2 only increased NIS activity in thyroid cells under chronic but not acute TSH stimulation. Resolving the effect of Akti-1/2 on the dynamics of Akt signaling in thyroid cells under chronic vs acute TSH stimulation may enable identification of the molecular targets that mediate Akti-1/2 action on NIS-mediated RAIU.

Among LY294002, Rapamycin, 17-AAG, and PD98059, Akti-1/2 showed the greatest extent of increase in NIS-mediated RAIU in thyroid cells under chronic TSH stimulation. The fact that this effect is limited to thyroid cells implies the involvement of a yet to be identified thyroid specific co-factor(s) that modulate NIS-mediated RAIU. Taken together, Akti-1/2 or its derivatives may represent interesting pharmacological reagents to explore as potential adjuncts for improving NIS-mediated radioiodine therapy for thyroid cancer patients.

Declaration 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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Author contribution statement

Dr Ringel has previously served on an advisory board for Veracyte and has been on an advisory panel for Astra Zeneca. Neither was involved in the experiments presented in this manuscript.
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