Phosphoinositide-3-kinase inhibition induces sodium/iodide symporter expression in rat thyroid cells and human papillary thyroid cancer cells

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Abstract

TSH stimulation of sodium iodide symporter (NIS) expression in thyroid cancer promotes radioiodine uptake and is required to deliver an effective treatment dose. Activation of the insulin/phosphoinositide-3-kinase (PI3K) signaling pathway in TSH-stimulated thyroid cells reduces *NIS* expression at the transcriptional level. We, therefore, investigated the effects of PI3K pathway inhibition on iodide uptake and *NIS* expression in rat thyroid cell lines and human papillary thyroid cancer cells. A PI3K inhibitor, LY294002, significantly enhanced iodide uptake in two rat thyroid cell lines, FRTL-5 and PCCL3. The induction of *Nis* mRNA by LY294002 occurred 6 h after treatment, and was abolished by a translation inhibitor, cycloheximide. Expression of the transcription factor, Pax8, which stimulates *NIS* expression, was significantly increased in PCCL3 cells after LY294002 treatment. Removal of insulin abrogated the stimulatory

effects of LY294002 on *NIS* mRNA and protein expression, but not on iodide uptake. These findings suggest that PI3K pathway inhibition results in post-translational stimulation of *NIS*. Inhibition of the PI3K pathway also significantly increased iodide uptake (~3.5-fold) in BHP 2-7 papillary thyroid cancer cells (Ret/PTC1 positive), engineered to constitutively express *NIS*. Pharmacological inhibition of Akt, a factor stimulated by the PI3K pathway, increased exogenous *NIS* expression in BHP 2-7 as was seen with LY294002, but not increase the endogenous *NIS* expression in FRTL-5 cells. PI3K pathway inhibition increases functional *NIS* expression in rat thyroid cells and some papillary thyroid cancer cells by several mechanisms. PI3K inhibitors have the potential to increase radioiodide accumulation in some differentiated thyroid cancer.

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Introduction

The β -emitting isotope, radioiodide-131 (¹³¹I), is commonly used in the treatment of differentiated thyroid cancer after thyroidectomy. The sodium/iodide symporter (NIS), expressed in more than 70% of differentiated thyroid cancer, mediates ¹³¹I accumulation. Functional *NIS* expression, however, is reduced in tumor tissue compared with the normal thyroid gland, as evidenced by the usual finding of a 'cold' area, which does not concentrate iodide on a standard radioiodine scan. Reduced *NIS* mRNA expression, as well as reduced *NIS* trafficking to the plasma membrane, has been reported in differentiated thyroid cancer (Kogai *et al.* 2006).

TSH induces *NIS* expression in thyroid follicular cells (Kogai *et al.* 1997, Saito *et al.* 1997) and differentiated thyroid cancer cells (Saito *et al.* 1998). The thyrotrophin (TSH) signaling pathways upregulate *NIS* expression in thyroid cells at the transcriptional and post-transcriptional levels (Kogai *et al.* 1997, Riedel *et al.* 2001, Taki *et al.* 2002). TSH receptor activation stimulates adenylyl cyclase through the stimulatory $G\alpha$ protein, followed by intracellular cAMP accumulation. The TSH/cAMP pathway stimulates the *NIS* proximal promoter (Endo *et al.* 1997,

Ohmori *et al.* 1998) and the *NIS* upstream enhancer (NUE; Ohno *et al.* 1999, Taki *et al.* 2002), stabilizes the *NIS* protein, and stimulates trafficking of *NIS* to the cell surface membrane (Riedel *et al.* 2001). ¹³¹I uptake in thyroid cancer is enhanced by an increase in endogenous TSH, accomplished by cessation of thyroid hormone supplementation in athyreotic patients post-thyroidectomy, or administration of recombinant TSH.

Expression of *NIS* alone in mammalian cells enables them to accumulate iodide (Kosugi *et al.* 1996, Saito *et al.* 1997, Shimura *et al.* 1997, Kogai *et al.* 2001). *Nis* mRNA expression level is correlated with iodide uptake in TSH-stimulated FRTL-5 cells (Kogai *et al.* 1997). Abundant expression of *NIS* mRNA and protein, however, do not always confer increased iodide uptake in thyroid cancer tissues, likely due to the failure of *NIS* translocation to the membrane (Dohan *et al.* 2001, Riesco-Eizaguirre *et al.* 2006).

Insulin, as well as insulin-like growth factor-I (IGF-I), reduces TSH-induced iodide uptake in FRTL-5 rat thyroid cells (Saji & Kohn 1991). Phosphoinositide-3-kinase (PI3K) is one of the major mediators of insulin/IGF signaling. Selective inhibitors of PI3K, such as LY294002 and Wortmannin, significantly increase *Nis* mRNA expression in rat thyroid

cells by stimulating its transcription (Garcia & Santisteban 2002, Giuliani *et al.* 2007). Although enhancement of iodide uptake with LY294002 has been incidentally observed in TSH-stimulated FRTL-5 cells (Kogai *et al.* 2008), the effects of PI3K inhibition on the iodide uptake in thyroid cells have not been well characterized.

Optimizing iodide uptake and prolonging the period of radioiodine tumor residence are important for effective ^{131}I treatment of thyroid cancer. We, therefore, investigated the effects of PI3K inhibitors on iodide uptake and efflux, as well as NIS expression, in papillary thyroid cancer cells. We further investigated whether PI3K inhibition influences the post-transcriptional regulation of NIS by using papillary thyroid cancer cells and anaplastic thyroid cancer cells engineered to constitutively express NIS. The *in vitro* response of NIS to stimulators, such as TSH and retinoic acid, often differs between normal thyroid cells and thyroid cancer cell lines (Schmutzler *et al.* 1997, Kogai *et al.* 2001). We, therefore, compared the response in thyroid cancer cells to that in FRTL-5 and PCCL3 rat thyroid cells.

Materials and Methods

Cell culture

FRTL-5 cells (a fresh sub-clone F1, passage number 6–12) were maintained in Coon's modified Ham's F-12 medium (Sigma) supplemented with 5% calf serum (Invitrogen) and a six-hormone (6H) mixture containing bovine insulin (10 $\mu\text{g}/\text{ml}$), hydrocortisone (1 nM), transferrin (5 $\mu\text{g}/\text{ml}$), L-glycyl-histidyl-lysine (10 ng/ml), somatostatin (10 ng/ml), and bovine TSH (1 mU/ml; 6H5% medium), as previously described (Kogai *et al.* 1997). PCCL3 cells (passage number 8–11) were also maintained in the 6H5% medium. To study the effects of TSH, forskolin (FSK), and/or inhibitors, 50–60% confluent cells were pre-treated without TSH in the presence of 5% calf serum (5H5% medium) for 7 days. To study the effects of FSK, insulin, and/or inhibitors in FRTL-5 cells, cells were pre-treated in the 5H5% medium for 5 days, followed by 4H medium (without TSH or insulin) with reduced serum (0.2%; 4H0.2% medium) for 2 days. Thyroid cancer cells, BHP 2–7 cells, NPA cells, and ARO cells, were maintained in RPMI1640 medium (Sigma) with 10% fetal bovine serum (Invitrogen), as described (Kogai *et al.* 2001). To study the effects of insulin, FSK, and/or inhibitors in thyroid cancer cells, 40–50% confluent cells were pre-treated in the 4H0.2% medium for 2 days.

Chemicals

Signal transduction inhibitors, purchased from EMD Biosciences (La Jolla, CA, USA), were aliquoted in dimethyl sulfoxide (DMSO) at 30 mM (or 1 mM for Wortmannin), stored at -20°C , and used within 2 months. Bovine TSH, bovine insulin, and other chemicals were purchased from Sigma, unless otherwise noted.

Iodide uptake assay

Iodide uptake assay was performed with 20 mCi/mmol Na^{125}I , as described (Kogai *et al.* 2005), with minor modifications. Briefly, cells grown in 12-well dishes were incubated for 1 h at 37°C with 500 μl Hank's balanced salt solution (HBSS) containing $\sim 0.1 \mu\text{Ci}$ carrier-free Na^{125}I (GE Healthcare, Piscataway, NJ, USA) and 10 μM NaI. After the incubation, the cells were washed twice with ice-cold HBSS, scraped from each well, and radioactivity measured in a γ -counter. Cell number was determined by counting in a hemocytometer. The radioactivity was normalized to the cell number at the time of the assay. For cells transiently expressing NIS, cells were lysed with 200 μl passive lysis buffer (Promega) after the incubation with Na^{125}I . Radioactivity of the cell lysates was measured in a γ -counter, and normalized with *Renilla* luciferase (RLuc) activity derived from a co-transfected RLuc reporter vector, pRL-CMV (Promega).

Iodide efflux assay

The procedure was performed as has been described previously (Kogai *et al.* 2000). Briefly, cells in 12-well dishes were incubated with HBSS containing 10 μM NaI and 20 mCi/mmol Na^{125}I for 1 h, and the medium was replaced every 5 min with fresh HBSS without NaI. The content of ^{125}I in the collected supernatant was measured by γ -counter. After the last time point (60 min), the cells were extracted with 400 μl ethanol to count residual radioactivity.

Reverse transcription and quantitative real-time PCR (RT-qPCR) analysis

Two-step quantitative RT-PCR was carried out, as described (Kogai *et al.* 2005), with minor modifications. Briefly, total RNA was isolated by RNeasy mini kit (Qiagen) from cells grown in six-well plates. On-column DNase I digestion was performed as recommended by Qiagen. Three micrograms of purified total RNA were reverse-transcribed by using 50 units of Superscript III reverse transcriptase (Invitrogen) with oligo(dT) $_{12-18}$ primer (1 μg). Quantitative PCR of human NIS and human GAPDH was performed with DNA Engine Opticon System (MJ Research, Waltham, MA, USA), as described (Kogai *et al.* 2005). PCR of rat Nis and rat Gapdh was performed with the following primers; rat Nis forward, GATGTGTTCCAGGTTGTGGTAATG; rat Nis reverse, CAGGGTCAAAGTCCACTAGGTT; rat Gapdh forward, AGTCAAGGCTGAGAATGGGAAG; rat Gapdh reverse, GGTGGTGAAGACGCCAGTAGA. Cycle parameters for rat Nis and Gapdh were the same as that for human NIS (Kogai *et al.* 2005). PCR of rat Pax8 was performed with QuantiTect Primer Assay (Qiagen), as recommended. Standard curves representing six-point serial dilution of mixed cDNA of the control group, BHP 2–7 cells constitutively expressing NIS for human NIS and GAPDH, or FRTL-5 cells maintained in the 6H5% medium for rat Nis and Gapdh, were analyzed in each

assay and used for calculation of relative expression values. The sample quantifications of *NIS* mRNA were normalized by the internal control *GAPDH* mRNA.

Transfection

A human *NIS* expression vector, pcDNA3-human *NIS* cDNA (Saito *et al.* 1997), was transfected to thyroid cancer cells with Nucleofector system (Amaxa, Gaithersburg, MD, USA). For transient expression study, pRL-CMV was co-transfected, and RLuc assay was performed with a commercial kit (Promega). For stable expression, the transfected cells were selected with G418 (Invitrogen) for 3–4 weeks. Constitutive expression of exogenous gene was confirmed with RT-qPCR.

Western blot analysis

Whole cell lysates were prepared in 10 mM Hepes–KOH (pH 7.5) and 1 mM EGTA with a protease inhibitor cocktail (Sigma), and quantified with the Bio-Rad Protein Assay. Thirty micrograms of protein were denatured with 2% SDS and 50 mM dithiothreitol, loaded on a SDS-PAGE (7.5%), transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and immunoblotted with anti-rat *NIS* antibody (generously provided by Dr Nancy Carrasco, Albert Einstein College of Medicine, Bronx, NY, USA) at 1:3000. The filter membrane was stripped with restore western blot stripping buffer (Pierce Biotechnology, Rockford, IL, USA) and reprobed with anti- β actin antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:2000. Blots were exposed to X-ray films, and intensities of signals were scanned and quantitated by using the ImageJ program version 1.40 (Abramoff *et al.* 2004).

Statistical analysis

Statistical comparison was performed using StatView 5.0 software (Abacus Concept, Berkeley, CA, USA) with significance at $P < 0.05$. Comparison between groups was determined by conducting a paired Student's *t*-test.

Results

Effects of PI3K pathway inhibition by LY294002 on iodide uptake in rat thyroid cells

To study the effects of PI3K inhibition on iodide uptake in thyroid cells, we treated TSH/cAMP-stimulated FRTL-5 cells with a PI3K inhibitor (LY294002). The cells, maintained in the 5H5% medium (in the presence of 5% serum but without TSH) for 7 days, were treated for 48 h with or without LY294002, TSH, and/or an adenylyl cyclase stimulator FSK. PI3K inhibition (LY294002, 30 μ M) significantly increased the iodide uptake in TSH-stimulated FRTL-5 cells (~ 1.9 -fold), as well as FSK-stimulated cells (~ 1.8 -fold; Fig. 1A).

Figure 1 Effects of PI3K inhibition by LY294002 on iodide uptake in FRTL-5 cells. (A) Cells maintained in the 5H5% medium (without TSH) for 7 days were treated with 1 mU/ml bovine TSH, 10 μ M FSK, and/or 30 μ M LY294002 for 48 h, and iodide uptake assay was performed with ¹²⁵I (20 mCi/mmol) and 10 μ M NaI. Non-specific uptake of iodide was measured with 30 μ M of KClO₄, the specific inhibitor of NIS, in duplicate wells. Values are expressed as means $\pm$ s.d. ($n=3$). * $P < 0.02$; ** $P < 0.01$. (B) Time course of induction of iodide uptake by LY294002 in FRTL-5 cells. Cells continuously maintained in the 6H5% medium (with 1 mU/ml of TSH) were treated with 30 μ M of LY294002 for the indicated time, and iodide uptake assay was performed. Values are expressed as means $\pm$ s.d. ($n=3$). ** $P < 0.01$, when compared with the cells before the treatment (0 h). (C) Iodide efflux in FRTL-5 cells treated with or without LY294002. Cells, continuously maintained in the 6H5% medium, were treated with 30 μ M of LY294002 for 48 h, and iodide efflux assay was performed. Values are expressed as means $\pm$ s.d. ($n=3$).

A specific inhibitor of NIS-mediated iodide uptake, KClO₄, abolished the uptake induced by TSH, FSK, and/or LY294002 (Fig. 1A), indicating that the increased iodide uptake is mediated by NIS. Since non-specific iodide accumulation in the presence of KClO₄ was not significantly changed with LY294002, the induction of iodide uptake by PI3K pathway inhibition may be dependent on the induction of functional NIS expression.

Modulation of the TSH/G $\beta\gamma$ signaling by LY294002 has been proposed as the mechanism for *NIS* induction (Zaballos *et al.* 2008). LY294002 treatment, however, significantly increased iodide uptake (~ 2.5 -fold), even in the absence of TSH stimulation (Fig. 1A). Significant induction of iodide uptake (~ 2.8 -fold) was also observed in FRTL-5 cells continuously treated with the 6H5% medium (Fig. 1B), in which the cells were chronically stimulated by TSH. These data indicate that the induction of iodide uptake by PI3K pathway inhibition (LY294002) is demonstrated in the presence and absence of TSH/cAMP pathway activation.

A time course study of the induction of iodide uptake by PI3K inhibition (LY294002) in FRTL-5 cells maintained in the 6H5% medium was performed. Significant induction of iodide uptake was seen at 48 h and reached a maximum at 72 h (Fig. 1B). The stimulation of iodide uptake was also observed in another rat thyroid cell line, PCCL3, maintained with 6H5% media (data not shown).

An increase in the net iodide uptake is predominantly the result of an increase in iodide influx, but is also influenced by iodide efflux. We, therefore, measured iodide efflux in FRTL-5 cells treated with PI3K pathway inhibition (LY294002) and TSH for 48 h, and compared with cells treated with TSH alone. The time point at which 50% of iodide remained in the TSH-stimulated cells was ~ 3.4 min (Fig. 1C), consistent with previous data (Kogai *et al.* 2000). PI3K pathway inhibition (LY294002) did not significantly influence this time point (~ 3.8 min; Fig. 1C). These data indicate that PI3K pathway inhibition (LY294002) in FRTL5 cells increases iodide uptake by increasing influx mediated by NIS, but does not influence efflux.

Effects of PI3K pathway inhibition by LY294002 on Nis mRNA expression in rat thyroid cells

TSH induces iodide uptake in TSH-starved FRTL-5 cells 12–24 h after the initiation of treatment (Kogai *et al.* 1997). Since the induction of *Nis* mRNA by TSH is relatively rapid (within 6 h), the delayed induction of iodide uptake has been thought to involve post-transcriptional regulation (Kogai *et al.* 1997, Riedel *et al.* 2001). The stimulatory effect of PI3K inhibition (LY294002) on iodide uptake was not seen until 48 h (Fig. 1B). We therefore performed a time course study of *Nis* mRNA induction by LY294002 in FRTL-5 cells. In contrast with the rapid effect of TSH on *Nis* mRNA stimulation, significant induction of *Nis* mRNA by LY294002 was observed in cells continuously maintained with TSH (6H5% medium) at 24 h (Fig. 2A). The translation inhibitor, cycloheximide, abolished the LY294002-induced *Nis* mRNA expression (Fig. 2B). The delayed induction of *Nis* mRNA by LY294002, therefore, is likely due to *de novo* protein synthesis for the *NIS* induction.

Effects of PI3K pathway inhibition by LY294002 on Pax8 expression in rat thyroid cells

NIS expression is transcriptionally regulated by TSH/cAMP signaling through a thyroid-specific *NIS* upstream enhancer, NUE (Ohno *et al.* 1999, Taki *et al.* 2002). The NUE recruits a paired domain-containing transcription factor PAX8, and cAMP-response element-binding proteins, in response to the TSH/cAMP signal (Taki *et al.* 2002). PI3K inhibition by LY294002 increases the binding of PAX8 to the NUE in PCCL3 rat thyroid cells (Zaballos *et al.* 2008). Since the *NIS* mRNA induction by LY294002 likely requires newly synthesized protein, we hypothesized that the induction of *Pax8* is required for the full induction of *NIS*. We therefore

Figure 2 Induction of *Nis* mRNA after PI3K inhibition by LY294002 in FRTL-5 cells. (A) Time course of induction of *Nis* mRNA by LY294002. Cells continuously maintained in the 6H5% medium were treated with 30 μ M LY294002 for the indicated time, and RT-qPCR of *Nis* was performed. Values are expressed as means $\pm$ s.d. ($n=3$). * $P<0.01$, when compared with the cells before the treatment (0 h). (B) Effect of cycloheximide on the *Nis* mRNA expression in FRTL-5 cells. Cells continuously maintained in the 6H5% medium were treated with 5 μ g/ml cycloheximide (CHX) and/or 30 μ M LY294002 (LY294) for 24 h, and RT-qPCR of *Nis* was performed. Values are expressed as means $\pm$ s.d. ($n=4$). * $P<0.01$, when compared with the cells treated with only LY294002.

performed RT-qPCR of *Pax8* in FRTL-5 cells as well as PCCL3 cells, both of which were treated with LY294002 for 24 h. PI3K inhibition by LY294002 significantly increased the *Pax8* mRNA expression in PCCL3 cells (3.26-fold, Table 1), consistent with the upregulation of NUE with *Pax8* previously reported in these cells (Zaballos *et al.* 2008). Unexpectedly, LY294002 did not significantly induce the *Pax8* mRNA in FRTL-5 cells (Table 1), suggesting induction of other protein(s) required for the full induction of *Nis* after PI3K inhibition by LY294002 in FRTL-5 cells. Comparison of *Nis* mRNA expression in both cell lines induced in response to PI3K inhibition (Table 1) indicate that the magnitude of *Nis* induction in PCCL3 (~ 9.0 -fold) was significantly greater than that in FRTL-5 cells (~ 6.8 -fold). The *Pax8* induction by LY294002 may produce an additive effect on the *NIS* induction in PCCL3 cells.

Comparison of effects of various PI3K inhibitors on iodide uptake and *Nis* mRNA expression in rat thyroid cells

LY294002 is a potent selective inhibitor of PI3K with broad spectrum for PI3K isoforms. LY294002, however, also interacts many cellular proteins and inhibits some other kinases, such as casein kinase II, Pim-1 kinase, glycogen synthase kinase 3, and the 97 kDa valosin-containing protein (Gharbi *et al.* 2007). To investigate whether the effect of LY294002 on iodide uptake is dependent on PI3K inhibition, we characterized the effect of LY294002 on iodide uptake in FRTL-5 cell compared with LY303511, an inactive analogue of LY294002, as well as another pan-PI3K inhibitor Wortmannin and a class 1 PI3K p110 α (PI3KCA)-selective inhibitor PI-103 (Knight *et al.* 2006). The effect of LY294002 on the iodide uptake stimulated by TSH was concentration-dependent with an EC_{50} of 3.57 μ M (Fig. 3A), in the range of previously reported values for *in vivo* inhibition of PI3K (El-Kholy *et al.* 2003). The EC_{50} for iodide uptake in PCCL3

Table 1 Differential effects of PI3K inhibition by LY294002 on *Nis* mRNA and *Pax8* mRNA expression in FRTL-5 cells and PCCL3 cells. Cells were pre-treated with the 5H5% medium for 7 days, and stimulated with 10 µM of FSK for 24 h in the presence or absence of LY294002 (30 µM)

	LY294002	Nis/Gapdh		Pax8/Gapdh	
		Fold induction ^a	+LY/−LY	Fold induction ^a	+LY/−LY
FRTL5	—	23.8±3.1		0.33±0.03	
	+	160.4±11.8	6.75±1.09	0.24±0.08	0.79±0.10
PCCL3	—	69.9±11.8		0.51±0.06	
	+	629.0±86.4	9.05±1.65*	1.69±0.26	3.26±0.27 [†]

**P*<0.05; [†]*P*<0.01, when compared with FRTL-5 cells (*n*=6).
^aThe cells without FSK or bovine insulin (data not shown) were set at 1.

cells was 6.74 µM, consistent with the range for PI3K inhibition in FRTL-5 cells. The inactive analogue, LY303511, did not significantly influence iodide uptake in FRTL-5 cells (Fig. 3B). Wortmannin, which inhibits PI3K at nano-molar concentrations, enhanced the iodide uptake in a concentration-dependent manner, with an EC₅₀ of 12.4 nM (Fig. 3C). Interestingly, the PI3KCA-selective inhibitor, PI-103, did not significantly increase the iodide uptake in FRTL-5 cells (Fig. 3D).

To address the discrepancy between the effects of LY294002 and PI-103 on iodide uptake, we performed RT-qPCR of *Nis* mRNA in FRTL-5 cells treated with these inhibitors. Twenty-four hour treatment with TSH significantly increased the *Nis* mRNA expression in FRTL-5 cells pretreated in the 5H5% medium (without TSH) for 5 days (Fig. 4), as reported previously (Kogai *et al.* 1997). The addition of LY294002, as well as PI-103, significantly enhanced the induction of *Nis* mRNA (~8.4- and ~6.2-fold respectively; Fig. 4), consistent with the previous observations with LY294002 (Garcia & Santisteban 2002,

Giuliani *et al.* 2007). These data indicate that PI-103 induces the expression of *Nis* mRNA, but not iodide uptake. PI-103 may inhibit the translation of *Nis* mRNA or translocation of *Nis* protein to the plasma membrane.

Although LY294002 possesses an ‘off-target’ effect on casein kinase II (Gharbi *et al.* 2007), 4,5,6,7-tetrabromobenzo-triazole, a selective casein kinase II inhibitor, did not increase the *Nis* mRNA in FRTL-5 cells (Fig. 4). The inactive analogue LY303511 also did not significantly affect the *Nis* mRNA expression in FRTL5 cells (Fig. 4). These observations validate the association of PI3K pathway inhibition and *Nis* mRNA induction.

PI3K activates its downstream effector, Akt, through phosphatidylinositol (3,4,5)-trisphosphate. Although the PI3K inhibition significantly increased the iodide uptake, an Akt1/Akt2 selective inhibitor, Akti-1/2, did not significantly increase the iodide uptake in FRTL-5 cells in the micro-molar range (Fig. 3E). The Akt inhibitor modestly increased the *Nis* mRNA in TSH-stimulated FRTL-5 cells, but the magnitude was not as significant as that seen with PI3K inhibitors (Fig. 4). These data suggest that unknown downstream effector(s) of PI3K, rather than Akt, are associated with the *Nis* induction in FRTL-5 cells.

Figure 3 Effects of various inhibitor of PI3K pathway on iodide uptake in FRTL-5 cells. Cells maintained in the 5H5% medium (without TSH) for 7 days were treated with 1 mU/ml of bovine TSH and the indicated inhibitor for 48 h at the indicated concentration. During the treatment, cells were fed with fresh medium and each inhibitor every 24 h. Iodide uptake was performed with ¹²⁵I (20 mCi/mmol) and 10 µM NaI. Values are expressed as means ± s.d. (*n*=3). **P*<0.01, when compared with the cells treated with only TSH (not shown).

Figure 4 Effects of various kinase inhibitors on *Nis* mRNA expression in FRTL-5 cells. Cells maintained in the 5H5% medium (without TSH) for 7 days were treated with or without TSH (1 mU/ml), 30 µM LY294002 (LY294), 30 µM LY303511 (LY303), 3 µM PI-103, 30 µM of a casein kinase II inhibitor (CK2i), and/or 30 µM of an Akt1/Akt2 selective inhibitor (Akti-1/2), for 24 h, and RT-qPCR of *Nis* was performed. Values are expressed as means ± s.d. (*n*=3–4). **P*<0.01.

Requirement of insulin and TSH/cAMP stimulation for the NIS induction by LY294002

The stimulatory effect of PI3K inhibition by LY294002 on *Nis* mRNA expression is associated with the suppression of insulin signaling (Garcia & Santisteban 2002). To investigate whether treatment with insulin is necessary for the induction of iodide uptake by LY294002, we evaluated the effects of LY294002 on iodide uptake in serum-deprived FRTL-5 cells. The cells were maintained in the 5H5% medium (without TSH) for 5 days, the 4H0.2% medium (with 0.2% serum but without TSH or insulin) for 2 days, and then treated with FSK, insulin, and/or LY294002 for 48 h. The addition of insulin modestly decreased the iodide uptake in the cells without FSK (Fig. 5A; columns 1 and 5). The stimulatory effect of LY294002 was observed both in the presence (Fig. 5A; columns 5 and 6; 7 and 8) and absence (columns 1 and 2; 3 and 4) of insulin. The effects were not dependent on the FSK stimulation, consistent with the data in cells treated with the 5H5% medium (Fig. 1A). These results indicate that neither insulin nor cAMP stimulation is required for the induction of iodide uptake by PI3K inhibition.

Figure 5 Dependency of the NIS induction by LY294002 on insulin and the cAMP pathway in FRTL-5 cells. Cells were maintained in the 5H5% medium for 5 days, the 4H0.2% medium for 2 days, and then treated with 30 μ M of LY294002 (LY294), 10 μ M of FSK, and/or 10 μ g/ml of bovine insulin (Ins) in the 4H0.2% medium for 48 h for iodide uptake (A) or for 24 h for RT-qPCR of *Nis* (B). Values are expressed as means $\pm$ S.D. ($n=3-4$). * $P<0.01$.

We also performed RT-qPCR of *Nis* mRNA with the same batch of FRTL-5 cells pretreated with the 5H5% medium for 5 days, followed by the 4H0.2% medium for 2 days. The treatment with LY294002 (24 h) significantly increased the FSK-induced *Nis* mRNA expression in the presence of insulin (~ 2.5 -fold; Fig. 5B, columns 7 and 8), although the magnitude was smaller than that with 5% serum (~ 6.8 -fold; Table 1). In contrast to the induction of iodide uptake, removal of insulin abolished the stimulatory effect of LY294002 on the *Nis* mRNA expression (Fig. 5B, columns 3 and 4). In the insulin-treated cells, the stimulatory effect of LY294002 was observed in the presence of FSK (Fig. 5B, columns 7 and 8), but not in the absence of FSK (columns 5 and 6). These data indicated that *Nis* mRNA induction by LY294002 requires both insulin and cAMP pathway stimulation, although the induction of iodide uptake does not require either of those treatments.

The discrepancy in the effects of LY294002 between iodide uptake and *Nis* mRNA expression in the insulin-deprived FRTL-5 cells suggests a stimulatory effect(s) of the PI3K inhibitor on the NIS protein synthesis and/or its post-translational regulation. To investigate whether the PI3K inhibition affects the translation of NIS, we performed Western blot analysis of NIS with whole cell lysates from the serum-deprived FRTL-5 cells treated with FSK, insulin, and/or LY294002 for 48 h. The addition of FSK induced the glycosylated NIS, indicated as broad bands (distributed between 65 and 100 kDa), as well as a non-glycosylated NIS around 50 kDa (Levy *et al.* 1998), both in the presence and absence of insulin (Fig. 6, lanes 2 and 4). Removal of insulin significantly increased the NIS expression (Fig. 6, lanes 2 and 4), consistent with previous observations (Garcia & Santisteban 2002). PI3K inhibition by LY294002 significantly increased the expression of glycosylated NIS (~ 2.6 -fold), as well as the non-glycosylated NIS, in the presence of insulin (Fig. 6, lanes 4 and 5), but not in the absence of insulin (Fig. 6, lanes 2 and 3). These data indicate that the effects of LY294002 as well as the insulin starvation on the NIS protein expression (Fig. 6) are correlated with those on the *Nis* mRNA expression in FRTL-5 cells (Fig. 5B). LY294002, therefore, does not likely affect the translation of NIS, but possibly upregulates processing of NIS and/or translocation of NIS to the plasma membrane. In the insulin-deprived cells, LY294002 abolished the expression of non-glycosylated NIS (Fig. 6, lane 3). Since the non-glycosylated NIS produces a less iodide uptake (Levy *et al.* 1998), the inhibition of PI3K may upregulate the NIS by modulating its glycosylation in the insulin-deprived cells.

PI3K inhibition by LY294002 upregulates iodide uptake in Ret-PTC expressing papillary thyroid cancer cells constitutively expressing NIS

We next applied the PI3K inhibitor to two papillary thyroid cancer cell lines, BHP 2-7, RET/PTC1-expressing cells (Vitagliano *et al.* 2004) and NPA, BRAF mutant (V600E)-expressing cells. Although the 24 h treatment with FSK, as well as LY294002, tended to increase the endogenous NIS mRNA

Figure 6 Effects of LY294002 on the NIS protein expression in FRTL-5 cells treated with or without insulin. Cells were maintained in the 5H5% medium for 5 days, the 4H0·2% medium for 2 days, and then treated with 30 μ M LY294002 (LY294), 10 μ M of FSK, and/or 10 μ g/ml bovine insulin (Ins) in the 4H0·2% medium for 48 h. Whole cell lysates were prepared and western blot analysis was performed with anti-rat NIS antibody, as well as anti β -actin antibody. *glycosylated NIS; **non-glycosylated NIS. Lower panel. Quantitative analysis of the Western blotting of NIS. The broad bands of glycosylated NIS were quantitated and normalized with β -actin. The cells without FSK, insulin, or LY294002 were set at 1. Values are means $\pm$ s.d. ($n=2$). * $P<0\cdot01$.

expression in BHP 2–7 cells, the expression levels were still quite low, and reproducible quantitation was not obtained (data not shown).

NIS is regulated by TSH both at the transcriptional and post-transcriptional levels in rat thyroid cells (Ohno *et al.* 1999, Riedel *et al.* 2001, Taki *et al.* 2002). To investigate whether the PI3K inhibitor up-regulates NIS at the post-transcriptional level in thyroid cancer cells, we introduced a mammalian expression vector carrying human NIS cDNA (Saito *et al.* 1997) into BHP 2–7 cells, NPA cells, and ARO anaplastic thyroid cancer cells. The transient expression of NIS significantly increased iodide uptake in the tested cells ($\sim 5\cdot9$ -fold, compared with background, in BHP 2–7 cells; $\sim 5\cdot1$ -fold in NPA cells; and $\sim 13\cdot5$ -fold in ARO cells). LY294002 significantly enhanced the iodide uptake in BHP 2–7 cells (up to 3·5-fold; Fig. 7A), but not in NPA cells or ARO cells (data not shown). The effect in BHP 2–7 cells was concentration-dependent with an EC_{50} of 3·54 μ M (Fig. 7A). The specific inhibitor of NIS, $KClO_4$, abolished the

Figure 7 Effects of LY294002 on iodide uptake in BHP2-7 papillary thyroid cancer cells constitutively expressing NIS. (A). Cells were transiently transfected with pcDNA3-human NIS cDNA and pRL-CMV and treated with the indicated concentration of LY294002 for 48 h. Iodide uptake assay and RLuc assay were performed with cell lysate after the incubation with ^{125}I (20 mCi/mmol) and 10 μ M NaI for 1 h. The value of iodide uptake was normalized with the RLuc activity. (B) Cells, stably transfected with pcDNA3-human NIS cDNA by using G-418 as a selection marker, were pre-treated with the 5H5% medium for 2 days, treated with the indicated concentration of LY294002 for 48 h, and iodide uptake assay was performed. Values are expressed as means $\pm$ s.d. ($n=3$). * $P<0\cdot01$ when compared with the cells treated without LY294002.

iodide uptake induced by LY294002 (Fig. 7A), indicating that the induced uptake is mediated by NIS. Expression of RLuc, derived from co-transfected pRL-CMV vector, was not increased by LY294002 in the same batch. Since both exogenous NIS and RLuc are controlled by the CMV promoter, the stimulatory effect of LY294002 is selective for the NIS expression, likely with the post-transcriptional regulation on the NIS mRNA.

To confirm the results in the transient expression studies, we established stable transfectant of NIS in BHP 2–7 cells with G-418 as a selection marker. The transfected cells expressed abundant iodide uptake (66·1-fold, compared with background; Fig. 7B) after the selection with G-418, consistent with previous data (Kogai *et al.* 2001). LY294002 significantly enhanced the iodide uptake in those cells in a concentration-dependent manner with the EC_{50} of 8·52 μ M (Fig. 7B).

We further confirmed the effects of LY294002 on the NIS mRNA expression in the NIS-constitutively expressing BHP 2–7 cells. LY294002 (30 μ M) significantly increased the NIS mRNA expression in the serum-deprived cells (Fig. 8A). The effect was observed both with and without insulin (Fig. 8A), although the magnitude with insulin (3·71-fold) was slightly higher than that without insulin (2·63-fold). A time course

Figure 8 Effects of PI3K/Akt pathway inhibitors on the *NIS* mRNA expression in BHP 2–7 cells constitutively expressing *NIS*. (A) Induction of the *NIS* mRNA by LY294002 in *NIS*-stably transfected BHP 2–7 cells. Geneticin-selected cells, pre-treated with the 4H0.2% medium for 2 day, were treated with 30 μ M LY294002 and/or 10 μ g/ml bovine insulin for 24 h, and RT-qPCR of *NIS* was performed. (B) Time course of the *NIS* mRNA induction by LY294002 in *NIS*-stably transfected BHP cells. Cells, maintained with 10% fetal bovine serum, were treated with 30 μ M LY294002 for the indicated time. (C) Effects of an Akt inhibitor on the *NIS* mRNA expression in *NIS*-stably transfected BHP 2–7 cells. Cells, maintained with 10% fetal bovine serum, were treated with 30 μ M LY294002 (LY294) or 30 μ M Akti-1/2 for 24 h, and RT-qPCR of *NIS* was performed. Since the expression of endogenous *NIS* was estimated more than 1000-fold less than that of the exogenous *NIS* (data not shown), the indicated values represent the exogenous *NIS* expression. Values are expressed as means $\pm$ s.d. ($n=2-4$). * $P<0.01$, when compared with the cells treated without LY294002.

study indicated a more rapid induction of *NIS* mRNA within 8 h (Fig. 8B) than that of endogenous *NIS* induction in FRTL-5 cells (Fig. 2). The effect of LY294002 was not significantly inhibited by a translation inhibitor cycloheximide (data not shown), suggesting no requirement of *de novo* protein synthesis for the post-transcriptional induction of *NIS*. The Akt1/Akt2 inhibitor, Akti-1/2 (30 μ M), mimicked the effect of LY294002 on the exogenous *NIS* expression in BHP 2–7 cells (Fig. 8C). Those data suggest that the inhibition of the PI3K/Akt pathway likely upregulates the *NIS* expression at the post-transcriptional level in Ret/PTC1-expressing papillary thyroid cancer cells.

Discussion

In this study, we demonstrated that inhibition of PI3K significantly induced iodide uptake, mediated by NIS, in rat

thyroid cells, at the transcriptional and post-translational level. Stimulation of iodide uptake was also shown in papillary thyroid cancer cells with Ret/PTC1 expression engineered to constitutively express NIS, although the regulatory mechanism was distinct in those cells (summarized in Table 2). In rat thyroid cells, the induction of *Nis* mRNA after treatment with a PI3K inhibitor required insulin treatment and the stimulation of the cAMP pathway. Inhibition of the PI3K effector, Akt, did not influence *Nis* mRNA. Newly synthesized proteins, such as Pax8 and/or other protein(s), were required for the full induction of *Nis*. In contrast, the induction of iodide uptake did not require insulin treatment or the cAMP pathway stimulation. In the insulin-treated cells, the induction of iodide uptake is dependent on the upregulation of *Nis* mRNA and protein, while post-translational regulation is likely important in the insulin-deprived cells. PI3K inhibition also increased exogenous *NIS* expressed in Ret/PTC1-expressing papillary thyroid cancer cells. This post-transcriptional effect required Akt inhibition, but not the cAMP pathway stimulation, insulin, or newly synthesized protein.

Some differentiated thyroid cancer cells express abundant *NIS* mRNA and protein (Saito *et al.* 1998), but the radioiodide accumulation is reduced, likely due to the failure of *NIS* translocation to the plasma membrane (Dohan *et al.* 2001, Riesco-Eizaguirre *et al.* 2006). *NIS* can be regulated both at the transcriptional and post-translational levels in thyroid cells (Ohno *et al.* 1999, Riedel *et al.* 2001, Taki *et al.* 2002). In FRTL-5 cells, treatment with insulin and stimulation of cAMP pathway were both necessary for the induction of *NIS* mRNA and protein by LY294002, while the induction of iodide uptake did not require those treatments. LY294002 may increase the translocation of *NIS* to the plasma membrane in the absence of insulin or the cAMP stimulation. Post-translational regulation of *NIS* by PI3K has also been proposed in breast cancer cells (Knostman *et al.* 2007).

TSH stimulates *Nis* expression at the transcriptional level through the *NIS* far-upstream enhancer, NUE (Ohno *et al.* 1999, Taki *et al.* 2002), and the proximal promoter (Endo *et al.* 1997, Ohmori *et al.* 1998) in rat thyroid cells. A paired

Table 2 Summary of conditions for *NIS* induction by LY294002

	FRTL-5		NIS-expressing BHP 2–7	
	Iodide uptake	NIS mRNA	Iodide uptake	NIS mRNA
TSH treatment	No	No ^a		
Stimulation of AC ^b	No	Yes ^a	No	No
Insulin treatment	No	Yes	No	No
Inhibition of Akt	No	No		Yes
De novo protein synthesis		Yes		No

^aYes' in column indicates that the factor is required for the induction.
^aEither FSK or TSH is required for the induction.
^bAC, adenyllyl cyclase.

box-containing transcription factor, PAX8, is required for the activation of the NUE (Taki *et al.* 2002). In FRTL-5 cells, LY294002 has been reported to target putative Nis regulatory sequence(s) between the proximal promoter and the NUE (Garcia & Santisteban 2002), while PI3K inhibition stimulates the NUE through the upregulation of PAX8 in PCCL3 in other rat thyroid cells (Zaballos *et al.* 2008). Our RT-qPCR study indicated the significant induction of *Pax8* mRNA in PCCL3 cells, but not in FRTL-5 cells. The selective induction of PAX8 in PCCL3 cells may confer the higher NIS expression in those cells. Indeed, we observed a greater induction of Nis by LY294002 in PCCL3 cells, compared with FRTL-5 cells.

One of the TSH receptor signaling pathways, TSH receptor/G $\beta\gamma$ /PI3K, downregulates NIS expression in cAMP-independent manner in PCCL3 rat thyroid cells (Zaballos *et al.* 2008). Downregulation of G $\beta\gamma$, as well as PI3K inhibition, increases NIS expression through the upregulation of Pax8 binding to the NUE (Zaballos *et al.* 2008). LY294002 abolishes the inhibitory effect of G $\beta\gamma$ on the Pax8 binding to NUE, resulting in activation of the NUE in TSH-stimulated PCCL3 cells. Since FSK targets adenyl cyclase, downstream of another G protein, G α , and does not stimulate the G $\beta\gamma$ production, the up-regulation of PAX8 binding has not been observed in FSK-stimulated PCCL3 cells (Zaballos *et al.* 2008). By contrast, our data with FRTL-5 cells indicated that the inhibition of PI3K induced *Nis* mRNA in both TSH and FSK stimulation. *Pax8* mRNA was induced by LY294002 in PCCL3 cells, but not in FRTL-5 cells. On the other hand, our experiments with a translation inhibitor cycloheximide in FRTL-5 cells indicate that another newly synthesized protein is required for the *Nis* induction by LY294002. The effects of PI3K inhibitor, therefore, are potentially mediated by at least two pathways, Pax8 dependent and independent. Since the induction of *Nis* mRNA by LY294002 in PCCL3 cells was significantly greater than that in FRTL-5 cells, activation of both pathways likely produces greater induction of *Nis* in PCCL3 cells.

Two broad-spectrum PI3K inhibitors, LY294002 and Wortmannin, but not a PI3KCA-selective inhibitor PI-103, significantly induced the iodide uptake in FRTL-5 cells. The present study and some previous reports (Garcia & Santisteban 2002, Giuliani *et al.* 2007) indicated that these PI3K inhibitors, including PI-103, significantly induced the *Nis* mRNA expression. PI-103 likely inhibits the translation and/or post-translational regulation of NIS. Each inhibitor possibly has different 'off-target' effects, which may influence NIS expression at the different levels. A potent PI3K inhibitor, Quercetin, paradoxically reduces the *Nis* mRNA expression in FRTL-5 cells through inhibition of phospholipase-A₂ pathway (Giuliani *et al.* 2007), although the NIS-inducing LY294002 is a derivative of Quercetin.

The effects of PI3K inhibitor on the endogenous NIS mRNA expression varied among tested cell lines. PCCL3 cells were the most sensitive cells, while papillary thyroid cancer cells, BHP 2-7 or NPA, did not significantly respond. Less response of NIS expression in thyroid cancer cells may be due to the reduced

activity of NIS proximal promoter (Kogai *et al.* 2001), as well as epigenetic modifications of *NIS* (Venkataraman *et al.* 1999, Puppini *et al.* 2005). Our preliminary experiments with primary porcine thyroid cells indicated reduction of *NIS* mRNA by LY294002 in cells maintained with the 6H5% medium (Kogai & Brent, unpublished observation, 2008). The differential response among species is possibly dependent on the difference of regulatory sequence of *NIS* gene; the similarity of the 5'-flanking region (between *NIS* and the next gene *Rpl18a*) between rat (GenBank accession number NT_039500) and pig (CU463003) is only 7.15%.

Insulin, a major regulator of Akt, was not required for the exogenous NIS induction by LY294002 in Ret/PTC1-expressing BHP 2-7 cells, although the induction was mimicked by pharmacological inhibition of Akt. Expression of Ret/PTC stimulates the PI3K/Akt pathway in thyroid cells even in the absence of insulin or serum (Miyagi *et al.* 2004). In Ret/PTC1-expressing BHP 2-7 cells, the PI3K/Akt pathway may be still active, even without insulin or serum. A recent preliminary study has indicated that targeting of Akt in combination with MAPK inhibition significantly induced the *NIS* mRNA expression in several thyroid cancer cell lines (Hou *et al.* 2007).

The PI3K inhibitors have a potential to increase the radioiodide accumulation, as well as produce inhibitory effects on cell proliferation (Furuya *et al.* 2007), in some differentiated thyroid cancer tissue. Such inhibitors may promote uptake in some thyroid cancer metastases that are unresponsive to TSH stimulation, although these tumors may also be resistant to PI3K inhibition. High cumulative doses of radioiodine are associated with adverse effects, including infertility, salivary, and lacrimal gland dysfunction, and an increased risk of secondary cancer and leukemia (Schlumberger 1998). PI3K inhibition may permit effective treatment of thyroid cancer with lower dose of radioiodide. Variable selectivity of PI3K inhibitors was seen in cell lines as well as species differences. Further investigations with human cells and models are needed to optimize PI3K inhibition for NIS induction and iodide uptake in human thyroid tissues.

Declaration of interest

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

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