MEK signaling modulates sodium iodide symporter at multiple levels and in a paradoxical manner

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Abstract

The Na⁺/I[−] symporter (NIS)-mediated iodide uptake is the basis for targeted radioiodine ablation of thyroid cancers. However, NIS-mediated radioiodide uptake (RAIU) activity is often reduced in thyroid cancers. As mitogen activated protein kinase (MAPK) signaling pathway is activated in about 70% of papillary thyroid carcinoma, we investigated whether MEK (MAPK kinase) inhibition will restore NIS protein levels and NIS-mediated RAIU activity in RET/PTC oncogene-transformed thyroid cells. We found that MEK inhibitor PD98059 increased NIS protein levels within 30 min of treatment. However, the increase of NIS protein level was not accompanied with an increase in NIS-mediated RAIU activity, particularly at early time points of PD98059 treatment. PD98059 also decreased RAIU activity mediated by exogenous NIS in non-thyroid cells. The transient decrease of RAIU activity by PD98059 in thyroid cells was not due to decreased NIS cell surface level, decreased NIS binding affinity for I[−], or increased iodide efflux. While PD98059 moderately decreased Na⁺/K⁺-ATPase activity, ouabain titration indicates that the extent of decrease in Na⁺/K⁺-ATPase activity is much greater than the extent of decrease in RAIU activity. Additionally, a decrease of Na⁺/K⁺-ATPase activity was not accompanied with a decrease of biotin uptake activity mediated by Na⁺-dependent multivitamin transporter. Since PD98059 reduced V_{max}–I[−] without decreasing NIS cell surface levels, it is most likely that PD98059 decreases the turnover rate of iodide transport with an yet to be identified mechanism.

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Introduction

The Na⁺/I[−] symporter (NIS) is an intrinsic membrane glycoprotein that mediates active iodide uptake into thyroid follicular cells for synthesis of iodide-containing thyroid hormones. NIS co-transporters one I[−] ion against its concentration gradient along with two Na⁺ ions down their concentration gradient into cells (Eskandari *et al.* 1997). The Na⁺ electrochemical gradient, which is maintained by Na⁺/K⁺-ATPase activity, provides the driving force for NIS to transport iodide against its concentration gradient. NIS-mediated iodide concentrating activity is the molecular basis for the post-operative use of radioiodine in the detection and targeted ablation of residual, recurrent,

and metastatic thyroid cancers. However, NIS expression, as well as NIS-mediated radioiodide uptake (RAIU) activity, is lost in some patients with advanced thyroid cancers, such that these patients cannot benefit from radioiodine imaging and therapy (Arturi *et al.* 1998, Lazar *et al.* 1999, Arturi *et al.* 2000). It is therefore clinically significant to devise strategies to increase NIS expression and activity in the thyroid tumors of these patients.

The *RET/PTC1* chimeric oncogene, formed by the intrachromosomal rearrangement of the *RET* receptor-type tyrosine kinase proto-oncogene with the *H4* gene, is frequently found in papillary thyroid carcinomas (for review, see Jhiang 2000). *RET/PTC1* has been shown

to reduce NIS expression and radioiodine concentrating activity in thyroid tumors of transgenic mouse model (Jhiang *et al.* 1998, Cho *et al.* 1999, Buckwalter *et al.* 2002) as well as in cultured thyroid cells (Trapasso *et al.* 1999, Venkateswaran *et al.* 2004). Recently, it has been reported that RET/PTC oncoprotein reduces NIS mRNA levels in PCCl3 rat thyroid cells through the Shc/Ras/MAPK signaling pathway (Knauf *et al.* 2003). The authors also showed that MAP/ERK kinase (MEK) (MAPK kinase) inhibitors restored NIS mRNA levels in PCCl3 cells expressing RET/PTC. Thus, we hypothesized that MEK inhibition will restore NIS protein levels and facilitate NIS-mediated RAIU activity in RET/PTC-transformed thyroid cells and potentially in other papillary thyroid tumors harboring Ras or B-Raf genetic alterations.

In this study, we investigated the effects of MEK inhibition on both NIS protein levels and NIS-mediated RAIU activity in PCCl3 rat thyroid cells, PCCl3 cells stably expressing RET/PTC1, PCCl3 cells with doxycycline-inducible expression of constitutively active MEK1, as well as in COS7 cells expressing exogenous NIS. We showed that treatment of PD98059 increased endogenous NIS protein levels in thyroid cells, yet decreased RAIU activity mediated by either endogenous or exogenous NIS. Efforts made to identify the mechanism(s) underlying the decrease of RAIU activity by MEK inhibition excluded the possible effects of MEK inhibition on NIS cell surface trafficking, NIS binding affinity for I^- , or iodide efflux. Taken together, our study uncovers an yet to be identified mechanism that modulates NIS-mediated RAIU activity by MEK signaling and MEK signaling appears to regulate NIS at multiple levels and in a paradoxical manner.

Materials and methods

Cell culture

PCCl3 rat thyroid cells were maintained in Kaighn's medium (Sigma), 5% calf serum, 2 mM glutamine, 1% penicillin–streptomycin, 10 mM $NaHCO_3$, and 6H hormone (1 mU/ml bovine thyrotropin, 10 μ g/ml bovine insulin, 10 nM hydrocortisone, 5 μ g/ml transferrin, 10 ng/ml somatostatin, and 2 ng/ml L-glycyl–histidyl–lysine). PCCl3 cells stably expressing RET/PTC1 were generated as described (Venkateswaran *et al.* 2004). PCCl3 cells with doxycycline-inducible MEK1 (E217/E221) expression (kindly provided by Drs Jeffrey A Knauf and James A Fagin, University of Cincinnati) were induced with 2 μ g/ml doxycycline for 48 h. COS7 monkey kidney cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin–streptomycin,

and were transfected with rat NIS cDNA or human SMVT cDNA (kindly provided by Dr Puttur D Prasad, Medical College of Georgia) using FuGENE 6 (Roche).

RAIU assay

Steady-state radioiodide accumulation was determined as follows. Cells were treated with DMSO or PD98059 (Promega) for 4 h, and then incubated with 2 μ Ci $Na^{125}I$ in 5 μ M non-radioactive NaI for 30 min at 37 °C with 5% CO_2 . Subsequently, cells were washed twice with cold Hank's balanced salt solution (HBSS) and lysed with 95% ethanol for 20 min. The cell lysate was collected and radioactivity was counted by a gamma-counter. Experiments were performed in triplicate.

Iodide-dependent kinetic analysis

Initial rate of iodide uptake and binding affinity of substrate iodide was determined as described (Dohan *et al.* 2002). Cells were treated with DMSO or PD98059 for 4 h, and then incubated for 2 min with varying concentration of NaI (eight concentrations ranging from 1 to 400 μ M) containing $Na^{125}I$ of a specific activity of 80 mCi/mmol. The amounts of accumulated iodide were measured as described for RAIU assay. The K_m and V_{max} values for I^- were derived from fitted Michaelis–Menten equation according to the Eadie–Hofstee plot. Experiments were performed in triplicate.

Iodide efflux assay

Efflux of ^{125}I was performed as described (Marsee *et al.* 2004). Cells were treated with DMSO or PD98059 for 4 h and then incubated in media containing $Na^{125}I$ as described for RAIU assay. Cells were washed twice and incubated with 1 ml HBSS. After 2 min, the medium was collected and replaced with HBSS. 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 of each well was calculated as the sum of the efflux washes and the lysates. Experiments were performed in triplicate.

Cell surface biotinylation

Cells were treated with DMSO or PD98059 for 4 h and then washed with cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS-Ca/Mg). Cells were incubated with 1 mg/ml sulfo-NHS-LC-biotin (Pierce) in PBS-Ca/Mg for 1 h at 4 °C. The reaction was quenched with PBS-Ca/Mg containing 100 mM glycine 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 μ g/ml aprotinin, and 10 μ g/ml

leupeptin) for 30 min at 4 °C. An 18 ½ gage needle was used to homogenize the cells. Lysates were centrifuged at 14 000 *g* for 20 min at 4 °C, and supernatants (whole cell lysates) were collected. Protein concentrations were determined by Bradford assay (Bio-Rad). To separate biotinylated surface proteins from non-biotinylated proteins, whole cell lysates were incubated with avidin-coated agarose beads (Pierce) overnight at 4 °C. The beads were washed with lysis buffer, and biotin-labeled proteins were eluted with 2× Laemmli sample buffer for 5 min at 95 °C.

Western blot analysis

Proteins were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% dry milk, then incubated with one of the following primary antibodies: PA716 polyclonal rabbit anti-rat NIS (kindly provided by Dr Bernard Rousset, Institut National de la Santé et de la Recherche Médicale; diluted 1:1500), monoclonal mouse anti- Na^+/K^+ -ATPase α_1 (Santa Cruz Biotechnology Inc., Santa Cruz CA, USA; diluted 1:1000), polyclonal rabbit anti-phosphorylated extracellular signal-regulated kinase (ERK) (Santa Cruz; diluted 1:250), polyclonal rabbit anti-ERK (Santa Cruz; diluted 1:250), or monoclonal mouse anti- β -actin (Abcam, Cambridge, MA, USA; diluted 1:2000). Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Amersham Pharmacia; diluted 1:4000) or HRP-conjugated anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA; diluted 1:2500). The signal was detected by enhanced chemiluminescence detection reagents. The signal intensities were measured densitometrically using NIH Image software. To determine equal protein loading of total and cell surface NIS protein levels, as well as total and cell surface Na^+/K^+ -ATPase α_1 protein levels, the membrane was probed with a polyclonal rabbit antibody against V-ATPase E subunit (kindly provided by Dr Beth Lee, Ohio State University; diluted 1:1000).

Ouabain-sensitive ^{86}Rb uptake assay

Na^+/K^+ -ATPase-mediated ion transport activity was measured as ouabain-sensitive uptake of ^{86}Rb . Cells were treated with DMSO or PD98059 for 4 h, and then treated in the presence or absence of the specific Na^+/K^+ -ATPase inhibitor ouabain (1 mM final concentration) for 15 min at room temperature. Subsequently, cells were incubated with 0.5 μCi $^{86}\text{RbCl}$ in DMEM for 15 min at 37 °C with 5% CO_2 . Cells were washed twice with cold HBSS and then lysed with 95%

ethanol for 20 min at room temperature. The cell lysate was collected and radioactivity was counted by a liquid scintillation counter. Ouabain-sensitive ^{86}Rb was calculated as the difference between the ^{86}Rb uptake measured in the presence and the absence of ouabain. Experiments were performed in triplicate.

[^3H]biotin uptake assay

Cells were incubated with 1.5 μCi [^3H]biotin in uptake medium containing 25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , and 5 mM glucose for 30 min at 37 °C with 5% CO_2 . Cells were washed twice with cold uptake medium and then lysed with cold 95% ethanol for 20 min at room temperature. The cell lysate was collected and radioactivity was counted by a liquid scintillation counter. Experiments were performed in triplicate.

Adenovirus-mediated dominant negative MEK1 transduction

Cells were seeded for RAIU assay or western blot analysis 24 h prior to infection with recombinant adenovirus carrying LacZ or dominant negative MEK1 (A217/A221) (Cell Biolabs, Inc.). Cells were washed with PBS, incubated with 2% FBS, and infected with adenoviruses for 3 h. Subsequently, cells were washed with PBS and cultured in regular medium. Forty-eight hours post-infection, RAIU assay and western blot analysis were performed.

Statistical analysis

Comparisons between groups were performed using paired *t*-test. A $P < 0.05$ was considered to be statistically significant.

Results

MEK inhibition increases NIS protein levels, yet decreases NIS-mediated RAIU activity in RET/PTC1-expressing thyroid cells

To investigate the role of MEK signaling in RET/PTC1 effects on NIS expression and functional activity, we examined the effects of the pharmacological MEK inhibitor PD98059 (Alessi *et al.* 1995) on NIS protein levels and NIS-mediated RAIU activity in PCC13 rat thyroid cells stably expressing RET/PTC1. We found that treatment of 40 μM PD98059 for 4 h increased total NIS protein levels about twofold in RET/PTC1-expressing cells (lane 1 versus lane 2 in Fig. 1A), but not evident in parental PCC13 cells at 4h treatment (lane 3 versus lane 4 in Fig. 1A). Unexpectedly,

Figure 1 MEK inhibition increases NIS protein levels, yet decreases RAIU activity without decreasing NIS cell surface levels in RET/PTC1-expressing cells and parental PCCl3 cells. **A:** Western blot analysis showed that treatment of 40 μM PD98059 for 4 h increased total NIS protein levels in RET/PTC1-expressing cells, but not evident in parental PCCl3 cells. Densitometry analysis was performed to determine the fold increase of total NIS normalized with V-ATPase. The results are representative of three independent experiments. **B:** PD98059 decreased NIS-mediated RAIU activity at 4 h in a dose-dependent manner in both RET/PTC1-expressing cells and parental PCCl3 cells. Treatment of 40 μM PD98059 for 4 h decreased RAIU activity in RET/PTC1-expressing cells and parental PCCl3 cells by 35 and 27% respectively. The results represent the mean ± s.d. of three independent experiments performed in triplicate. Asterisks denote statistically significant difference in comparison with DMSO-treated cells ($P < 0.05$). **C:** Treatment of 40 μM PD98059 for 4 h did not decrease cell surface NIS levels in RET/PTC1-expressing cells or parental PCCl3 cells. Cell surface proteins were biotinylated and later isolated by avidin-coated beads and subjected to western blot analysis to detect cell surface NIS levels. Densitometry analysis was performed to determine the fold increase of cell surface NIS normalized with V-ATPase. The results are representative of two independent experiments.

PD98059 failed to increase NIS-mediated RAIU activity in RET/PTC1-expressing cells. Instead, PD98059 decreased RAIU activity in a dose-dependent manner in RET/PTC1-expressing cells as well as parental PCCl3 cells (Fig. 1B). Treatment of 40 μM PD98059 for 4 h decreased RAIU activity in RET/PTC1-expressing cells and parental PCCl3 cells by 35 and 27% respectively. Similar effects on RAIU activity were observed using an alternative pharmacological MEK inhibitor, U0126 at 20–60 μM, in RET/PTC1-expressing cells and parental PCCl3 cells (data not shown).

Since it is the NIS protein localized at the cell surface, but not the total NIS protein level, that confers NIS-mediated RAIU activity, we examined the effects of PD98059 on NIS cell surface levels. Cell surface biotinylation studies showed that 4 h treatment of

40 μM PD98059 did not decrease NIS cell surface levels in RET/PTC1-expressing cells or parental PCCl3 cells (Fig. 1C). Immunofluorescence studies using confocal microscopy confirmed that PD98059 had no apparent effect on NIS cell surface expression (data not shown). Taken together, the decrease in RAIU activity by PD98059 was not contributed by a decrease in NIS cell surface levels.

PD98059 increases NIS protein levels, but not NIS-mediated RAIU activity, in PCCl3 cells expressing constitutively active MEK

The direct effects of MEK activation on NIS protein levels and NIS-mediated RAIU activity were investigated in PCCl3 cells with doxycycline-inducible MEK1 (E217/E221) expression. As expected, acute expression of constitutively active MEK1 decreased

Figure 2 PD98059 increases NIS protein levels, but decreases RAIU activity, in doxycycline-inducible MEK1 (E217/E221) expressing PCC13 cells. **A:** Western blot analysis showed that NIS protein levels were decreased upon doxycycline induction and that treatment of 40 μ M PD98059 for 4 h increased total NIS protein levels in doxycycline-induced MEK1 expressing cells. Densitometry analysis was performed to determine the fold increase of total NIS normalized with V-ATPase. The results are representative of two independent experiments. **B:** Treatment of 40 μ M PD98059 for 4 h decreased RAIU activity in non-induced and doxycycline-induced cells by 30 and 40% respectively. The results represent the average and range of two independent experiments performed in triplicate. Asterisks denote statistically significant difference in comparison with DMSO-treated cells ($P < 0.05$).

both total NIS protein levels (lane 1 versus lane 3 in Fig. 2A) and NIS-mediated RAIU activity (Fig. 2B). We found that treatment of 40 μ M PD98059 for 4 h increased NIS protein levels in doxycycline-induced MEK1 cells (lane 3 versus lane 4 in Fig. 2A), but not in non-induced MEK1 cells (lane 1 versus lane 2 in Fig. 2A). Consistent with the finding of the effects of PD98059 on RAIU activity in RET/PTC1-expressing cells and parental PCC13 cells, PD98059 also decreased RAIU activity in PCC13 cells with doxycycline-induced MEK1 cells as well as in non-induced MEK1 cells (Fig. 2B).

PD98059 increases NIS protein levels and decreases RAIU activity within 30 min in thyroid cells

The temporal profile of PD98059 on NIS modulation in RET/PTC1-expressing cells and parental PCC13 cells was investigated. PD98059 increased NIS protein levels

within 30 min of treatment, and the increase persisted after 48 h treatment, in RET/PTC1-expressing cells (Fig. 3A). In comparison, PD98059 also increased NIS protein levels in parental PCC13 cells but to a lesser extent (Fig. 3B). Thus, RET/PTC1-expressing cells appeared to be more sensitive to MEK inhibition on NIS increase than parental PCC13 cells. As shown in Fig. 3C and 3D, PD98059 decreased RAIU activity within 30 min and persisted after 4 h treatment. However, RAIU activity was increased about 22% after 48 h treatment in both cell lines. Thus, the decrease of RAIU activity by PD98059 was transient in nature for both cell lines. Nevertheless, the extent of increase on RAIU activity at 48-h treatment was not proportional to the extent of increase on NIS protein levels.

PD98059 also reduces RAIU activity in cells expressing exogenous NIS, without notable changes in total or cell surface NIS levels

To investigate whether PD98059 modulation of NIS expression and activity is restricted to thyroid cells, we examined the effects of PD98059 on NIS protein levels and RAIU activity in COS7 monkey kidney cells expressing exogenous NIS. As shown in Fig. 4A, PD98059 only slightly increased total NIS protein levels in COS7/NIS cells after 4h treatment. However, PD98059 also decreased RAIU activity in COS7/NIS cells after 4 h treatment (Fig. 4B). Cell surface biotinylation studies showed that 4 h treatment of PD98059 did not decrease, but modestly increased, NIS cell surface levels in COS7/NIS cells (Fig. 4C). Thus, the decrease in RAIU activity by PD98059 was not contributed by a decrease in NIS cell surface levels. The finding that PD98059 decreased NIS-mediated RAIU activity in COS7/NIS cells indicates that PD98059 effects on RAIU activity is not restricted to thyroid cells expressing endogenous NIS.

Adenovirus infection of dominant negative MEK1 increases NIS protein levels in thyroid cells and decreases RAIU activity in cells expressing exogenous NIS

To confirm that the effects of the pharmacological MEK inhibitor PD98059 on NIS expression in thyroid cells were due to MEK inhibition, we infected RET/PTC1-expressing cells, and parental PCC13 cells with recombinant adenovirus carrying dominant negative MEK1 (A217/A221). As shown in Fig. 5A, total NIS protein levels were increased about six- to sevenfold in parental PCC13 cells and RET/PTC1-expressing cells respectively after 48-h post-infection with dominant negative MEK1. Thus, the increase of

Figure 3 Temporal profile of PD98059 effect on NIS protein levels and RAIU activity in thyroid cells. RET/PTC1-expressing cells and parental PCCl3 cells were treated with 40 μM PD98059 for the indicated time. A: Western blot analysis showed the temporal kinetics of increased NIS protein levels by PD98059 in RET/PTC1-expressing cells, and B: parental PCCl3 cells. Densitometry analysis was performed to determine the fold increase of total NIS normalized with total ERK. The results are representative of two independent experiments. C: PD98059 decreased RAIU activity in RET/PTC1-expressing cells by 16, 19, and 25% at 30 min, 1 h, and 4 h time points respectively. However, RAIU activity was increased by 22% after 48 h in RET/PTC1-expressing cells. D: PD98059 decreased RAIU activity in parental PCCl3 cells by 13, 23 and 29% at 30-min, 1-h, and 4-h time points respectively. However, RAIU activity was also increased by 22% after 48 h in parental PCCl3 cells. The results represent the mean ± s.d. of three independent experiments performed in triplicate. Asterisks denote statistically significant difference in comparison with DMSO-treated cells ($P < 0.05$).

NIS protein levels by PD98059 in thyroid cells is at least in part mediated by MEK inhibition. Unfortunately, RAIU activity in both RET/PTC1-expressing cells and parental PCCl3 cells was reduced significantly following adenovirus infection (either with recombinant adenovirus carrying LacZ or dominant negative MEK1), which prevented us from examining the effects of rAdDNMEK1 on RAIU activity in thyroid cells. As PD98059 also decreased RAIU activity in COS7 cells expressing exogenous NIS, we infected COS7/NIS cells with rAdDNMEK1 to investigate the effects of rAdDNMEK1 on RAIU activity. We found that RAIU activity in COS7/NIS cells was reduced about 24% following infection with rAdDNMEK1 (Fig. 5B). This suggests that the decrease in RAIU activity by PD98059 in COS7/NIS is at least in part mediated by MEK inhibition.

PD98059 does not alter NIS binding affinity for iodide

We then examined the possible effects of 4 h PD98059 treatment on the kinetic properties of I^- uptake in RET/PTC1-expressing cells, parental PCCl3 cells, and COS7/NIS cells. We demonstrated that PD98059 had no apparent effect on K_m-I^- in all the three cell lines, indicating that the decrease in RAIU activity by PD98059 was not contributed by a decrease in NIS

binding affinity for substrate I^- (Table 1). Instead, PD98059 decreased the maximal rate of I^- uptake ($V_{max}-I^-$) about 26–35% in these cell lines (Table 2). Since PD98059 does not decrease cell surface NIS levels, it is most likely that PD98059 decreases RAIU activity by decreasing the velocity of iodide transport ($V_{max}-I^-$).

PD98059 does not alter the rate of iodide efflux

Since the equilibrium of active iodide uptake and iodide efflux contributes to steady-state radioiodide accumulation, we analyzed the effects of PD98059 on the rate of iodide efflux in RET/PTC1-expressing cells and parental PCCl3 cells. We showed that PD98059 had no apparent effect on the rate of iodide efflux in either RET/PTC1-expressing cells (Fig. 6A) or parental PCCl3 cells (Fig. 6B). Thus, the decrease in RAIU activity by PD98059 was not contributed by an increase in the rate of iodide efflux.

PD98059 reduces $Na^+/K^+-ATPase$ activity

NIS-mediated RAIU activity depends on the Na^+ electrochemical gradient maintained by the $Na^+/K^+-ATPase$. We therefore investigated the effects of PD98059 on total and cell surface $Na^+/K^+-ATPase$ protein levels, as well as $Na^+/K^+-ATPase$ activity in RET/PTC1-expressing cells, parental PCCl3 cells, and

Figure 4 PD98059 decreases RAIU activity in COS7 cells expressing exogenous NIS. Cells were treated with 40 μM PD98059 for the indicated time. **A:** Western blot analysis showed that PD98059 slightly increased total NIS protein levels in COS7/NIS cells. Densitometry analysis was performed to determine the fold increase of total NIS normalized with total ERK. The results are representative of two independent experiments. **B:** PD98059 decreased RAIU activity in COS7/NIS cells by 42% at the 4-h time point. The results represent the mean ± s.d. of three independent experiments performed in triplicate. Asterisks denote statistically significant difference in comparison with DMSO-treated cells ($P < 0.05$). **C:** Cell surface biotinylation showed that PD98059 treatment for 4 h did not decrease, but modestly increase, cell surface NIS levels in COS7/NIS cells. Densitometry analysis was performed to determine the fold increase of total or cell surface NIS normalized with V-ATPase. The results are representative of two independent experiments.

Figure 5 Adenovirus infection of dominant negative MEK1 (A217/A221) increases NIS protein levels in thyroid cells and decreases RAIU activity in COS7 cells expressing exogenous NIS. Cells were infected with either recombinant adenovirus carrying LacZ (rAdLacZ) or dominant negative MEK1 (rAdDNMEK1) at the indicated multiplicity of infection (MOI) for 48 h prior to western blot analysis or RAIU assays. **A:** Total NIS protein levels were increased by rAdDNMEK1 infection in both RET/PTC1-expressing cells and parental PCC13 cells. Densitometry analysis was performed to determine the fold increase of total NIS normalized with β-actin. The results are representative of two independent experiments. **B:** NIS-mediated RAIU activity was reduced by 22% in COS7/NIS cells infected with rAdDNMEK1. The results represent the average and range of two independent experiments performed in triplicate. Asterisks denote statistically significant difference in comparison with DMSO-treated cells ($P < 0.05$).

COS7/NIS cells. As shown in Fig. 7A, the change in total or cell surface Na^+/K^+ -ATPase levels by PD98059 is minimal in all the three cell lines. Nevertheless, PD98059 reduced Na^+/K^+ -ATPase activity (by 15–18%), as measured by ouabain-sensitive ^{86}Rb uptake activity, in all the three cell lines (Fig. 7B). The decreased Na^+/K^+ -ATPase activity may lead to decreased Na^+ gradient and consequently decreased NIS-mediated RAIU activity.

Table 1 PD98059 does not alter NIS $K_m - \text{I}^-$

Cell lines	Control	PD98059
RET/PTC1	88.5 ± 28.9	90.5 ± 41.7
PCC13	77.0 ± 1.4	75.5 ± 7.8
COS7/NIS	55.0 ± 2.8	59.0 ± 5.6

Table 2 PD98059 decreases NIS $V_{\text{max}} - \text{I}^-$

Cell lines	Decreased V_{max} (%)
RET/PTC1	74.0 ± 12.5
PCC13	74.0 ± 14.5
COS7/NIS	65.5 ± 6.4

Figure 6 PD98059 does not increase the rate of iodide efflux in either A: RET/PTC1-expressing cells or B: parental PCC13 cells. Cells were treated with 40 μ M PD98059 for 4 h prior to iodide efflux studies. Each data point was performed in triplicate and the mean $\pm$ s.d. are shown. The results are representative of three independent experiments.

Ouabain titration indicates that the extent of decrease in Na^+/K^+ -ATPase activity is much greater than the extent of decrease in RAIU activity

We performed ouabain titration to investigate whether a 15% reduction in Na^+/K^+ -ATPase activity is accompanied with a 25–38% reduction in RAIU activity in RET/PTC1-expressing cells. While ouabain decreased both Na^+/K^+ -ATPase and RAIU activities (Fig. 8), the extent of decrease in Na^+/K^+ -ATPase activity is much greater than the extent of decrease in RAIU activity. For example, at 50 μ M ouabain, Na^+/K^+ -ATPase activity was decreased by 16%, yet RAIU activity was only decreased by 7%. Thus, the effect of PD98059 on decreasing RAIU activity cannot be solely contributed by its effect on decreasing Na^+/K^+ -ATPase activity in RET/PTC1-expressing cells. It appears that MEK inhibition reduced both Na^+/K^+ -ATPase and RAIU activities, yet without one being the cause of the other. To further investigate whether a decrease in Na^+/K^+ -ATPase activity is always accompanied with a decrease in the functional activity of Na^+ -dependent co-transporter, we examined the

effects of PD98059 on the functional activity of Na^+ -dependent multivitamin transporter (SMVT). SMVT is also a member of the sodium-dependent solute family member and shares high homology with NIS. As shown in Fig. 9A, PD98059 increased, rather than decreasing, biotin uptake activity in SMVT-expressing COS7 cells despite decreasing Na^+/K^+ -ATPase activity (Fig. 9B). Thus, the decrease of NIS-mediated RAIU activity by PD98059 is unlikely due to the decrease of Na^+ gradient.

Discussion

In this study, we found that MEK inhibition increases NIS protein levels in RET/PTC1- and MEK-expressing PCC13 rat thyroid cells, as well as in parental PCC13 cells. Unexpectedly, the increase of NIS protein level was not accompanied with an increase in NIS-mediated RAIU activity, particularly at early time points of PD98059 treatment. The transient decrease of RAIU activity by PD98059 in thyroid cells was not due to decreased NIS cell surface level, decreased NIS binding affinity for I^- , increased iodide efflux, or decreased Na^+ gradient. Since PD98059 reduced $V_{\text{max}} - \text{I}^-$ without decreasing NIS cell surface levels, it is most likely that PD98059 decreases the turnover rate of iodide transport with an yet to be identified mechanism.

It is well established that NIS expression and function in thyroid cells are mainly stimulated by thyroid-stimulating hormone/thyrotropin (TSH), which acts primarily through cAMP (Weiss *et al.* 1984, Kaminsky *et al.* 1994, Kogai *et al.* 1997, Levy *et al.* 1997, Saito *et al.* 1997, Riedel & Carrasco 2001). Recent studies demonstrated that NIS expression, as well as the expression of thyroglobulin and thyroid peroxidase, is inhibited by Raf/MEK/ERK signaling pathway (Kupperman *et al.* 1996, Miller *et al.* 1998, Knauf *et al.* 2003). Indeed, in both PCC13 and WRT rat thyroid cells, TSH preferentially activates the Ras/PI3K signaling pathway instead of the Ras/Raf/MEK/ERK pathway (Al-Alawi *et al.* 1995, Miller *et al.* 1998, Ciullo *et al.* 2001). Moreover, TSH-stimulated NIS expression is blocked in cells transiently expressing Ras effector mutant (rasV12S35) that signals preferentially through Raf-1 (Cass & Meinkoth 2000). In Ras- or RET/PTC-transformed thyroid cells, NIS promoter activity (Missero *et al.* 2000) as well as NIS mRNA levels (Knauf *et al.* 2003) was decreased primarily through MEK activation. In this study, we further confirmed that RET/PTC1 reduces NIS protein levels primarily mediated by the MEK signaling pathway (Fig. 1), and

Figure 7 PD98059 decreases Na⁺/K⁺-ATPase activity in RET/PTC1-expressing cells, parental PCCl3 cells, and COS7 cells expressing exogenous NIS. Cells were treated with 40 μM PD98059 for 4 h prior to western blot analysis or ouabain-sensitive ⁸⁶Rb uptake assays. A: Western blot analysis showed that the change in total or cell surface Na⁺/K⁺-ATPase levels by PD98059 was minimal in all the three cell lines. Densitometry analysis was performed to determine the fold increases of cell surface and total Na⁺/K⁺-ATPase α₁ normalized with V-ATPase. The results are representative of two independent experiments. B: Treatment of PD98059 for 4 h decreased ouabain-sensitive ⁸⁶Rb uptake in all the three cell lines. PD98059 decreased Na⁺/K⁺-ATPase activity in RET/PTC1-expressing cells and parental PCCl3 cells by 15%, and in COS7/NIS cells by 18%. Each data point was performed in triplicate and the mean ± s.d. are shown. The results are representative of two independent experiments. Asterisks denote statistically significant difference in comparison with DMSO-treated cells (*P* < 0.05).

that MEK activation is sufficient to reduce NIS protein levels (Fig. 2) in PCCl3 rat thyroid cells. Interestingly, PD98059 appears to increase NIS protein levels to a greater extent in RET/PTC1-expressing cells (where MEK signaling is constitutively activated) than in parental PCCl3 cells (where MEK is not constitutively activated).

Paradoxically, while NIS protein levels were increased readily by PD98059, NIS-mediated RAIU activity was transiently decreased in RET/PTC1-expressing cells as well as in parental PCCl3 cells. While we could not exclude the possible off-target effects of PD98059, we believe that the effect of PD98059 on NIS expression/activity was mainly mediated by MEK inhibition. Not only were similar results obtained using a structurally unrelated MEK inhibitor U0126 (data not shown), but cells infected with rAdDNMEK1 also had increased NIS expression and decreased RAIU activity. The decrease of RAIU activity by MEK inhibition was not restricted to thyroid cells, as MEK inhibition also decreased RAIU activity in non-thyroid cells expressing exogenous NIS.

We showed that the decrease in RAIU activity by PD98059 was not contributed by decrease in NIS cell

Figure 8 Ouabain titration decreases Na⁺/K⁺-ATPase activity to a much greater extent than RAIU activity in RET/PTC1-expressing cells. Cells were treated with varying concentrations of ouabain as indicated prior to ouabain-sensitive ⁸⁶Rb uptake or RAIU assays. Ouabain at 50, 100, 300, and 500 μM decreased Na⁺/K⁺-ATPase activity by 16, 34, 60, and 84% respectively. In comparison, ouabain at 50, 100, 300, and 500 μM only decreased RAIU activity by 7, 13, 22, and 35% respectively. Each data point was performed in triplicate and the mean ± s.d. are shown. The results are representative of two independent experiments. Asterisks and dagger denotes statistically significant difference in comparison with DMSO-treated cells (*P* < 0.05), ouabain-sensitive ⁸⁶Rb uptake (*), or RAIU assays (†).

Figure 9 PD98059 increases SMVT-mediated biotin uptake activity, despite decreasing Na^+/K^+ -ATPase activity, in COS7 cells expressing exogenous SMVT. Cells were treated with 40 μM PD98059 for 4 h prior to [^3H]biotin uptake assay or ouabain-sensitive ^{86}Rb uptake assay. (A) PD98059 increased biotin uptake activity in COS7/SMVT cells. The results represent the mean $\pm$ s.d. of three independent experiments performed in triplicate. Asterisks denote statistically significant difference in comparison with DMSO-treated cells ($P < 0.05$). (B) PD98059 decreased ouabain-sensitive ^{86}Rb uptake in COS7/SMVT cells about 22%. The results represent the mean $\pm$ s.d. of three independent experiments performed in triplicate. Asterisks denote statistically significant difference in comparison with DMSO-treated cells ($P < 0.05$).

surface levels, decrease in NIS binding affinity for I^- , or increase in the rate of iodide efflux. During the preparation of this manuscript, [Riesco-Eizaguirre et al. \(2006\)](#) reported that $\text{BRAF}^{\text{V600E}}$ expression decreased NIS protein levels and impaired NIS targeting to the cell surface, and that MEK inhibitor U0126 readily increased NIS protein levels, but was not able to completely restore NIS cell surface levels in $\text{BRAF}^{\text{V600E}}$ -expressing PCC13 cells in the time period examined. Together with our study, it is clear that NIS protein levels are readily increased by MEK inhibition in thyroid cells expressing RET/PTC1, B-RAF $^{\text{V600E}}$, or MEK1 (E217/E221). However, NIS cell surface trafficking as well as NIS-mediated RAIU activity appears to lag behind the increase of NIS protein levels.

Several studies have indicated that MEK/ERK signaling is involved in modulation of Na^+/K^+ -ATPase

activity by various hormones ([Isenovic et al. 2004](#), [Khundmiri et al. 2004](#), [Zhong et al. 2004](#), [Khundmiri et al. 2005](#)). Our study confirmed that MEK/ERK signaling is essential for optimal Na^+/K^+ -ATPase activity in many cell types, as PD98059 decreased ouabain-sensitive ^{86}Rb uptake activity in all cell lines we investigated. However, the findings that ouabain decreased Na^+/K^+ -ATPase activity to a much greater extent than RAIU activity and that PD98059 increased SMVT-mediated biotin uptake activity despite Na^+/K^+ -ATPase activity indicate that the decrease in RAIU activity by PD98059 was not contributed by a decrease in Na^+ gradient. Thus, it is most likely that PD98059 modulates NIS activity by post-translational modifications, such as changes in protein phosphorylation or recruitment of the NIS protein to lipid rafts. We are currently in the process of examining this possibility.

In summary, our study showed that MEK inhibition increases NIS protein levels, but transiently decreases NIS-mediated RAIU activity, in RET/PTC1-transformed thyroid cells as well as in parental PCC13 cells. This finding is of clinical significance, as it indicates that MEK inhibition is sufficient to increase NIS protein levels, but may not be effective in restoring NIS-mediated RAIU activity in papillary thyroid carcinomas harboring RET/PTC, Ras, or B-Raf mutations. Most importantly, our study uncovers that MEK signaling modulates not only NIS expression, but also NIS-mediated RAIU activity. Further study identifying MEK downstream signaling that differentially modulates NIS expression from functional activity is warranted. In doing so, novel strategies can be developed to increase NIS expression as well as NIS-mediated RAIU activity in advanced thyroid carcinomas.

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