Apigenin in Combination with Akt Inhibition Significantly Enhances Thyrotropin-Stimulated Radioiodide Accumulation in Thyroid Cells

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Background: Selectively increased radioiodine accumulation in thyroid cells by thyrotropin (TSH) allows targeted treatment of thyroid cancer. However, the extent of TSH-stimulated radioiodine accumulation in some thyroid tumors is not sufficient to confer therapeutic efficacy. Hence, it is of clinical importance to identify novel strategies to selectively further enhance TSH-stimulated thyroidal radioiodine accumulation.

Methods: PCC13 rat thyroid cells, PCC13 cells overexpressing BRAF^{V600E}, or primary cultured tumor cells from a thyroid cancer mouse model, under TSH stimulation were treated with various reagents for 24 hours. Cells were then subjected to radioactive iodide uptake, kinetics, efflux assays, and protein extraction followed by Western blotting against selected antibodies.

Results: We previously reported that Akt inhibition increased radioiodine accumulation in thyroid cells under chronic TSH stimulation. Here, we identified Apigenin, a plant-derived flavonoid, as a reagent to further enhance the iodide influx rate increased by Akt inhibition in thyroid cells under acute TSH stimulation. Akt inhibition is permissive for Apigenin's action, as Apigenin alone had little effect. This action of Apigenin requires p38 MAPK activity but not PKC- δ . The increase in radioiodide accumulation by Apigenin with Akt inhibition was also observed in thyroid cells expressing BRAF^{V600E} and in primary cultured thyroid tumor cells from *TR β ^{PV/PV}* mice.

Conclusion: Taken together, Apigenin may serve as a dietary supplement in combination with Akt inhibitors to enhance therapeutic efficacy of radioiodine for thyroid cancer.

Introduction

THE Na⁺/I⁻ SYMPORTER (NIS) is a glycoprotein expressed on the basolateral membrane of thyroid follicular cells that facilitates active uptake of iodide from circulating blood. The iodide is further retained in the thyroid follicle by organification, where it is incorporated into the tyrosine amino acid residues of thyroglobulin, the precursor of thyroxine (T4) and triiodothyronine (T3) thyroid hormones. Thyroidal radioiodine accumulation serves as the basis for targeted ablation of post-thyroidectomy remnants. Since radioiodine accumulation in most thyroid tumors can be further enhanced by elevation of serum thyrotropin (TSH) levels, many patients with recurrent and metastatic thyroid cancers can benefit from radioiodine therapy upon administration of recombinant human TSH or T4 withdrawal (1,2). However, in a substantial number of patients, the extent of

TSH-stimulated radioiodine accumulation is not sufficient to confer therapeutic efficacy. Thus, it is of clinical importance to identify novel strategies to selectively further enhance TSH-stimulated thyroidal radioiodine accumulation.

Pharmacological inhibitors targeting signaling pathways activated in thyroid cancers, such as MEK/ERK (3), Hsp90 (4), and PI3K/Akt (5), have been shown to increase radioactive iodide uptake (RAIU) in PCC13 rat thyroid cells. To date, the effect of MEK and BRAF inhibition (6,7) and 17-AAG (4) on increasing RAI accumulation in cultured thyroid cells have been validated in mouse models of thyroid cancer (7,8), and promising results were recently reported in a clinical trial for patients treated with a MEK inhibitor as pretreatment for ¹³¹I therapy (9).

We examined the effects of various inhibitors on RAIU in PCC13 cells, which had undergone TSH withdrawal for five days followed by acute TSH stimulation for 24 hours prior to

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treatment with inhibitors. In this experimental setting, we show that Akt inhibitor (Akti1/2) had the greatest extent of increase in RAIU, and Apigenin further increased thyroidal RAIU in combination with Akti1/2. The action of Apigenin to further increase Akti1/2-induced RAIU in thyroid cells is dependent on p38 MAPK activity. Taken together, Apigenin has the potential to serve as a dietary supplement along with Akt inhibitors to increase the efficacy of radioiodine therapy for patients with advanced thyroid cancer.

Methods

Cell culture, reagents, and $TR\beta^{PV/PV}$ mouse model

PCC13 rat thyroid cells were maintained in 6H media with 5% bovine serum as described by Liu *et al.* (5), unless specified otherwise. Experiments were performed under acute TSH stimulation, where cells were withdrawn from TSH for five days (5H media) and then TSH was added back for 24 hours prior to treatment with various reagents for an additional 24 hours, unless specified otherwise. PCC13/TetOn-BRAF^{V600E}, a generous gift from Dr. James Fagin, Memorial Sloan Kettering Cancer Center, New York, NY (10), and PCC13/TetOn-PTC1 cells (4) were genetically modified from PCC13 cells to allow doxycycline-inducible expression of BRAF^{V600E} or PTC1 oncogenes respectively. Experiments were performed under acute TSH stimulation with or without 2 μ g/mL of doxycycline for 48 hours, followed by treatment with reagents for an additional 24 hours. Primary cultured cells from mouse thyroid tumors were isolated using a tumor dissociation kit (Miltenyi Biotec, Inc., Bergisch Gladbach, Germany), according to the manufacturer's protocol, and were cultured in 6H media. Reagents used in this study are listed as follows: Akti1/2 also known as Akt inhibitor VIII, 17-AAG, and SB203580 (EMD Millipore, Billerica, MA), LY294002 (Cayman Chemical Company, Ann Arbor, MI), PD98059 (Cell Signaling Technology, Inc., Beverly, MA), Apigenin and DMSO (Sigma-Aldrich, St. Louis, MO), BIRB-796, MK-2206 (Selleck Chemicals, Houston, TX), and Silencer select scrambled and PKC- δ siRNAs (Ambion, Austin, TX). Control vector and shAkt1/2 plasmids were generous gifts from Dr. Mingzhao Xing at The Johns Hopkins University School of Medicine. $TR\beta^{PV/PV}$ genetically engineered mice were obtained from Dr. Sheue-yann Cheng, National Cancer Institute, Bethesda, MD (11).

RT² profiler polymerase chain reaction array and Ingenuity Pathway Analysis

A rat epithelial to mesenchymal transition (EMT) RT² profiler polymerase chain reaction (PCR) array that profiles the expression of 84 key genes was purchased from SABiosciences (Valencia, CA). Total RNA isolated from PCC13 cells treated with DMSO, Akti1/2, and TGF- β was reversed transcribed to cDNA, and real-time PCR was performed per the manufacturer's instructions. Genes with expression levels of Ct value <30 in either the experimental or the control group along with their fold changes were submitted for Ingenuity Pathway Analysis (IPA) to predict upstream transcription factors and their activation status. Additional downstream targets of these transcription factors were identified by IPA. Among targets identified, we searched for ones that are oppositely modulated by Akti1/2 and TGF- β .

Transfections

Plasmid transfections were conducted using FuGENE[®]6 (Promega, Madison, WI) for 24 hours followed by replacement with fresh media for 24 hours. Stably transfected cells were selected under media containing 800 μ g/mL of G418 (Life Technologies, Carlsbad, CA) for two weeks with media replacement every other day. Mixed stable clones were then maintained in 400 μ g/mL of G418. Transfections with siRNAs were facilitated by incubating with Lipofectamine RNAiMAX (Life Technologies) for 24 hours followed by replacement with fresh media with or without drug treatments for 24 hours.

RAIU, kinetics, and efflux assays

These assays were performed as previously described by Vadysirisack *et al.* (3) with ¹²⁵I in NaI (80 mCi/mmol). Note that RAIU in all figures, except efflux assay, represents NIS-mediated RAIU acquired by subtracting the background RAIU value from parallel experiments in the presence of 100 μ M perchlorate. The magnitude of RAIU in the presence of perchlorate varies between 10² and 10³ cpm among different trials. However, the background values did not vary much among various experimental groups within the same trial. Accordingly, the background measurement for each experimental group within each trial was not always replicated. The background value (or the average of replicates) was subtracted from the corresponding experimental group before statistical analysis.

Western blot analysis

Cells were lysed and subjected to gel electrophoresis and Western blot analysis as previously described (5). In this study, 4–20% gradient Tris-Glycine or Tris-HEPES SDS-PAGE gels (NuSep, Inc., or Bio-Rad Laboratories, Inc.) were used, and the NIS protein was detected with the PA716 rNIS polyclonal antibody (provided by B.R.) at a dilution of 1:1500. Phospho-Akt, p38 MAPK, MAPKAPK2, and PKC- δ were detected using 1:1000 dilution of antibodies from Cell Signaling Technology, Inc. (Cat. 9271, 9212, 3042) and Santa Cruz Biotechnology, Inc. (Dallas, TX; Cat. sc-213) respectively. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies were used accordingly. Equivalent protein loading among samples was monitored by probing for β -actin (Abcam plc, Cambridge, MA; Cat. 8226) or GAPDH (Cell Signaling Technology, Inc.; Cat. 2118). Densitometry analysis was performed using ImageJ software.

Cell surface biotinylation

Cell surface biotinylation was performed as previously described (3). Equivalent loading of cell surface proteins was monitored by probing for Na⁺/K⁺ ATPase using mouse anti-Na⁺/K⁺ ATPase monoclonal antibody (Santa Cruz Biotechnology, Inc.; Cat. sc-21712) and enrichment of cell surface proteins was confirmed by the absence of β -actin in the cell surface eluate. In this study, phosphatase inhibitors were not added to the cell lysis buffer.

Statistical analysis

All experiments had at least two independent trials with three replicates for each experimental group within each trial.

For RAIU assay, all the data values were log10 transformed to reduce variance and skewness, and then linear mixed effects models were used to take account of the correlations among observations from the same trial. From the model, all the prespecified comparisons for each experiment were obtained and adjusted for multiple comparisons using a sequentially rejective Bonferroni test (12,13) to control for type I error at 0.05. SAS v9.2 software was used for analysis (SAS Institute, Inc., Cary, NC). If no significance was found, we interpreted this as no significant difference between the compared conditions instead of using the included comparisons to exclude the outcome for another comparison. GraphPad Prism v6.0 was used to perform statistical analyses for efflux assay and to determine Km and Vmax values for kinetics assay.

Results

Akti1/2 increases TSH-stimulated RAIU to the greatest extent among inhibitors examined in PCCl3 cells

Small molecule inhibitors, such as Akti1/2 (Akt1/2 inhibitor), LY294002 (PI3K inhibitor), 17-AAG (Hsp90 inhibitor), and PD98059 (MEK/ERK inhibitor), were examined for their effect on RAIU in PCCl3 cells under acute TSH-stimulated conditions. Cells deprived of TSH for five days have little RAIU activity, and cells regain RAIU activity after the addition of TSH for 24 hours. As shown in Figure 1, all inhibitors examined significantly further increased TSH-stimulated RAIU activity. Among them, Akti1/2 increased TSH-stimulated RAIU to the greatest extent. Hence, we fo-

FIG. 1. Thyrotropin (TSH)-stimulated radioactive iodide uptake (RAIU) in PCCl3 cells can be further increased by various reagents. Cells were deprived of TSH (–TSH) for five days and then stimulated with TSH (+TSH) for 24 hours followed by treatment with 10 μ L DMSO (vehicle control), 10 μ M Akti1/2, 10 μ M LY294002, 3 μ M 17-AAG, or 40 μ M PD98059 for 24 hours before RAIU analysis. RAIU in +TSH/DMSO-treated cells was significantly higher than in –TSH/DMSO-treated cells. RAIU in TSH-stimulated cells treated with Akti1/2, LY294002, 17-AAG, and PD98059 was significantly higher than in DMSO-treated cells. Data are expressed as mean $\pm$ standard deviation (SD; $n=3$).

cused on Akti1/2 and searched for reagents that could further increase RAIU in Akti1/2-treated cells.

Among all inhibitors shown to increase RAIU, none of them can further increase RAIU in Akti1/2-treated PCCl3 cells. In FRTL-5 rat thyroid cells, TGF- β decreases RAIU (14,15), and we have confirmed the same in PCCl3 cells (data not shown). TGF- β induces EMT (16–18), yet Akt inhibition decreases EMT (19–23). Taken together, Akti1/2 and TGF- β

FIG. 2. Apigenin further increases RAIU activity in Akti1/2-treated PCCl3 rat thyroid cells. (A) RAIU in Apigenin (AP)-treated cells was not significantly higher than in DMSO-treated cells, yet RAIU in AP + Akti1/2-treated cells was significantly higher than in Akti1/2-treated cells. RAIU in Akti1/2-treated cells was significantly higher than in DMSO-treated cells. (B) RAIU in cells treated with a combination of 20 μ M AP and 10 μ M or 30 μ M Akti1/2 is significantly higher than RAIU in cells treated with 10 μ M or 30 μ M Akti1/2 alone. At 50 μ M, AP did not significantly further increase RAIU compared to 20 μ M AP in the presence or absence of Akti1/2 treatment. RAIU in cells treated with Akti1/2 at 10 μ M and 30 μ M were significantly higher than in DMSO-treated control. (C) Western blots show that phospho-Akt levels are decreased in cells treated with AP, Akti1/2, or AP + Akti1/2. Total Akt levels remained the same (data not shown). β -actin served as a loading control. Data are expressed as mean $\pm$ SD ($n=3$). All cells were acutely stimulated with TSH for 24 hours prior to treatments.

modulate both RAIU and EMT in an opposite manner. We thus hypothesized that EMT-associated genes may modulate RAIU in thyroid cells. We examined expression levels of 84 EMT-associated genes using EMT PCR array in cells treated with DMSO, Akt1/2, or TGF- β . The results were subjected to IPA analysis, and involucrin, a transglutaminase substrate protein, was predicted to be downregulated by Akt1/2 yet upregulated by TGF- β . Since involucrin expression appears to be inversely correlated with RAIU, we speculated that Apigenin, a plant-derived flavonoid known to inhibit involucrin expression in keratinocytes, may further increase Akt1/2-induced RAIU.

Apigenin further increases RAIU in Akt1/2-treated PCC13 cells

Apigenin (20 μ M) alone had little effect on RAIU in PCC13 cells. However, in combination with Akt1/2, Apigenin further increased RAIU in TSH-stimulated PCC13 cells to a greater extent than Akt1/2 alone (Fig. 2A). While Akt1/2 at 30 μ M further increased RAIU compared to 10 μ M (Fig. 2B), cells had evident morphological changes. Apigenin at 50 μ M did not significantly increase RAIU to a greater extent than 20 μ M in the presence of either 10 μ M or 30 μ M Akt1/2. Accordingly, 10 μ M Akt1/2 and 20 μ M Apigenin were chosen for further studies. As shown in Figure 2C, 20 μ M Apigenin decreased the phospho-Akt level in PCC13 cells as reported in many other cell types (24–28), and the combination of Apigenin and Akt1/2 further decreased phospho-

Akt levels. The effect of Apigenin to further increase RAIU in cells treated with Akt inhibitor was confirmed in cells treated with 5 μ M MK-2206 (data not shown). MK-2206 is another allosteric Akt inhibitor that is currently in clinical trials (29). Taken together, Apigenin alone had little effect on RAIU, yet it consistently further enhanced RAIU in PCC13 cells in combination with Akt inhibitors.

Akt inhibition via shRNA knockdown or via hormonal modulation allows Apigenin to further increase RAIU in PCC13 cells

We further investigated whether Akt inhibition by other means would also allow Apigenin to increase RAIU in PCC13 cells. RAIU was increased by ~1.7-fold in PCC13 cells stably transfected with shAkt1/2 (Fig. 3A), in which the phospho-Akt level was evidently decreased (Fig. 3B), compared to cells stably transfected with the control plasmid vector. Note that the relative molecular mass (M_r) of NIS protein was increased in cells treated with Apigenin in combination with Akt inhibition. As expected, Apigenin further increased RAIU in PCC13-shAkt1/2 cells, yet had little effect on RAIU in PCC13-vector control cells.

It has been shown that phospho-Akt level is decreased in the absence of insulin in FRTL-5 rat thyroid cells under low-serum culture media (30). To recapitulate this culture condition, PCC13 cells were deprived of TSH and insulin (4H) with 0.2% serum for five days, and then 4H culture media was replaced with 4H + TSH or 6H (4H + TSH + insulin) culture

FIG. 3. Akt inhibition appears to be permissive for Apigenin to enhance RAIU in PCC13 cells. (A) RAIU is significantly increased by AP treatment in shAkt1/2 stably transfected cells but not in vector-transfected cells. RAIU in shAkt1/2-stably transfected cells is significantly higher than RAIU in vector-transfected cells. (B) Western blots show that phospho-Akt levels are decreased in shAkt1/2 stably transfected cells due to effective knockdown of total Akt (data not shown). Note an increase in relative molecular mass M_r of Na^+/I^- Symporter (NIS) in shAkt1/2-transfected cells treated with AP. (C) RAIU in AP-treated cells is greater than in DMSO-treated cells under 4H + TSH conditions but not under 4H or 6H conditions. Cells were deprived of TSH and insulin (4H) with 0.2% serum for five days, and then 4H culture media was replaced with 4H + TSH or 6H (4H + TSH + insulin) culture media for 24 hours before adding either DMSO vehicle or AP for an additional 24 hours. (D) Western blots show that phospho-Akt level in cells with 4H + TSH is lower than that in cells treated with 4H or 6H media, and AP suppressed phospho-Akt levels. NIS is undetectable in cells under 4H media but was evidently increased in cells under 4H + TSH and 6H. Total Akt levels remained the same in all lanes (data not shown). For panels (A) and (C), data are expressed as mean $\pm$ SD ($n=3$). In panels (B) and (D), GAPDH served as a loading control. Arrowheads indicate hyperglycosylated ($\blacktriangleright$) and hypoglycosylated ($\triangleright$) NIS.

media for 24 hours before adding either DMSO vehicle or Apigenin for an additional 24 hours. As shown in Figure 3C, Apigenin did not increase RAIU in cells cultured in the 4H or 6H condition, yet it did further increase RAIU in cells with 4H+TSH culture media. Note that the phospho-Akt level in cells with 4H+TSH was lower than that in cells with 4H or 6H culture media (Fig. 3D). Apigenin suppressed phospho-Akt levels in all three experimental groups, and the extent of suppression was more pronounced in 0.2% serum than 5% serum conditions (Fig. 3D vs. Fig. 2B).

Since TSH is required for NIS expression, protein stability, and function, NIS protein is absent in cells under 4H conditions. Accordingly, Apigenin was not able to induce RAIU in the absence of TSH. The inability of Apigenin to increase RAIU under 6H conditions may be attributed to other effects of insulin rather than a higher phospho-Akt level prior to Apigenin addition. Taken together, Akt inhibition appears to be permissive for Apigenin to further increase RAIU, yet Apigenin's action to further enhance RAIU does not correlate with its effect on suppressing phospho-Akt levels.

Apigenin further increases iodide influx rate in Akti1/2-treated PCCl3 cells

Since iodide uptake is mediated by NIS protein localized on the cell surface, we examined if Apigenin increases cell surface NIS protein levels in Akti1/2-treated PCCl3 cells. As shown in Figure 4A, Apigenin did not increase total or cell surface NIS protein levels in Akti1/2-treated cells. The absence of β -actin in cell surface fractions confirms the absence of cytosolic proteins. Because RAIU activity represents a steady state equilibrium between iodide influx and iodide efflux, we investigated whether Apigenin decreases iodide efflux rate in Akti1/2-treated cells. As shown in Figure 4B, Apigenin had little effect on the iodide efflux rate, which is different from the mechanism of 17-AAG (4).

Effects of Apigenin on maximum velocity of iodide influx (V_{max}) and iodide affinity ($\propto 1/K_m$) were evaluated. Similar to previous studies conducted on cells under chronic TSH stimulation (5), Akti1/2 alone increases V_{max} 1.44-fold compared to DMSO-treated cells. In combination with Akti1/2, Apigenin further increased V_{max} 1.24-fold compared to Akti1/2 treatment alone with little change in the K_m value. Since Apigenin did not increase cell surface NIS protein levels, the increase in V_{max} suggests that Apigenin increases the rate of iodide uptake by each NIS molecule on the cell surface.

Apigenin's effect to further increase RAIU in Akti1/2-treated cells requires p38 MAPK activity

Since p38 MAPK is a well-known downstream effector of Apigenin (31–33), we examined if p38 MAPK mediates Apigenin's effect on RAIU in Akti1/2-treated PCCl3 cells. Pretreatment with an ATP-pocket binding inhibitor of p38MAPK, SB203580, for one hour and its continuous presence for 24 hours diminished Apigenin's effect to further increase RAIU in Akti1/2-treated cells. The fact that SB203580 treatment decreased RAIU in cells of all experimental groups indicates that p38MAPK activity plays a functional role to maintain optimal RAIU activity in PCCl3 cells. Apigenin's effect to further increase RAIU in Akti1/2-treated cells appears to be dependent, at least in part, upon p38 MAPK activity (Fig. 5A).

FIG. 4. Apigenin increases iodide influx rate in Akti1/2-treated PCCl3 cells. **(A)** Western blots show that total or cell surface NIS protein levels are not changed by AP in Akti1/2-treated cells. Exposure times for total and cell surface NIS were 2 and 10 minutes respectively. Arrowheads indicate hyperglycosylated ($\blacktriangleright$) and hypoglycosylated ($\triangleright$) NIS. Na⁺/K⁺ATPase served as a loading control for cell surface proteins and β -actin for total cell lysates. Enrichment of cell surface proteins was confirmed by the absence of β -actin. **(B)** Efflux assays show that neither Akti1/2 nor AP + Akti1/2 treatment significantly decrease the iodide efflux rate compared to DMSO treatment. 17-AAG, known to significantly decrease the iodide efflux rate, was used as the positive control. The iodide efflux rate is shown as the percentage of iodide remaining in the cells plotted at two-minute intervals. **(C)** Iodide kinetics assay show that the maximum velocity of iodide influx (V_{max}) was increased by AP + Akti1/2 compared to Akti1/2 alone, yet there is little change in the K_m value. V_{max} and K_m from two independent experiments are expressed as average $\pm$ range. All cells were acutely stimulated with TSH for 24 hours prior to treatments.

SB203580 does not decrease the phosphorylation levels of p38 MAPK but inhibits its catalytic activity to phosphorylate its downstream substrates, such as MAPKAPK2. Using antibodies against MAPKAPK2, the nonphosphorylated form (lower band) can be resolved from the phosphorylated form (upper band). As shown in Figure 5B, MAPKAPK2 was predominantly in the phosphorylated form in acute TSH-stimulated PCCl3 cells, and the relative abundance of the nonphosphorylated form was increased by SB203580 treatment. The decrease of RAIU in SB203580-treated cells was not accompanied with a decrease in total NIS protein levels. Note that the relative molecular mass (M_r) of NIS protein was

FIG. 5. Apigenin's effect to further increase RAIU in Akti1/2-treated PCC13 cells requires p38 MAPK activity. **(A)** In the presence of SB203580, RAIU in cells treated with DMSO, AP, Akti1/2, and AP + Akti1/2 is significantly decreased, and AP does not significantly increase RAIU in Akti1/2-treated cells. **(B)** Western blots show an increase in the relative abundance of nonphosphorylated MAPKAPK2, an immediate downstream effector of p38 MAPK, thereby confirming the inhibition of p38 MAPK by SB203580. The NIS protein level is not decreased by SB203580. Note an increase in M_r of NIS in cells treated with AP + Akti1/2 in the presence or absence of SB203580. **(C, D)** Similar results were obtained using an allosteric p38 MAPK inhibitor, BIRB-796. Data are expressed as mean $\pm$ SD ($n=3$). In **(B, D)**, GAPDH served as a loading control. Arrows indicate phosphorylated ($\leftarrow$) and nonphosphorylated ($\leftarrow$) MAPKAPK2. Arrowheads indicate hyperglycosylated ($\blacktriangleright$) and hypoglycosylated ($\blacktriangleright$) NIS. All cells were acutely stimulated with TSH for 24 hours prior to treatments.

increased in cells treated with Apigenin in combination with Akt inhibition. Similar results were obtained using an allosteric p38 MAPK inhibitor, BIRB-796 (Fig. 5C and D). Using siRNA to knock down either each individual or all four isoforms, p38MAPK was knocked down up to 60%, and this was not sufficient to decrease p38 MAPK activity. Accordingly, neither an increase in nonphosphorylated form of MAPKAPK2 nor a decrease in RAIU was observed (data not shown).

Apigenin's action to increase RAIU in Akti1/2-treated cells is independent of PKC- δ

Another well-known downstream effector of Apigenin is PKC- δ (32,33). To test if Apigenin modulates RAIU via PKC- δ , genetic knockdown of PKC- δ was performed using siRNAs. As shown in Figure 6A, neither RAIU in cells of all experimental groups nor the further increase of RAIU by Apigenin in Akti1/2-treated cells was affected by PKC- δ knockdown. Western blot analysis confirmed that PKC- δ protein levels were considerably decreased by siRNA knockdown (Fig. 6B). The total NIS protein level was modestly decreased by PKC- δ knockdown in acute TSH-stimulated PCC13 cells, yet without accompanied decrease in RAIU activity.

Apigenin in combination with Akti1/2 further increases TSH-stimulated RAIU in BRAF^{V600E}-expressing thyroid cells and thyroid tumor cells from TR $\beta^{PV/PV}$ mice

The BRAF^{V600E} oncogene is the most commonly found oncogene in papillary thyroid carcinomas that is associated

with aggressive clinical features (34) and reduced radioiodine uptake (35). To ensure the clinical relevance of this finding, we investigated whether Apigenin's effect on RAIU is reproducible in PCC13 cells overexpressing the BRAF^{V600E} oncogene and in primary cultured cells derived from thyroid tumors of a preclinical mouse model.

As expected, BRAF^{V600E} induction via doxycycline resulted in decreased RAIU (Fig. 7A) and decreased NIS protein levels (Fig. 7B) in PCC13/Tet-On BRAF^{V600E} cells. Akti1/2 alone increased RAIU in both uninduced and doxycycline-induced cells to a much lesser extent (Fig. 7A) compared to PCC13 parental cells. Apigenin alone had little effect on RAIU. However, Apigenin was able to further increase RAIU in Akti1/2-treated cells (Fig. 7A). Similar results were obtained in doxycycline-induced PCC13/TetOn-PTC1 cells (data not shown). These results indicate that increased RAIU by combination treatment of Apigenin and Akti1/2 is not limited to nontransformed PCC13 cells but also present in PCC13 cells expressing oncogenes that are relevant to thyroid cancer patients.

TR $\beta^{PV/PV}$ genetic-engineered mice develop thyroid tumors (36) that maintain NIS expression (37). It is of interest to note that combination treatment of Apigenin with Akti1/2 significantly increased RAIU in primary cultured cells derived from thyroid tumors developed in TR $\beta^{PV/PV}$ mice (Fig. 7C). Taken together, Apigenin in combination with Akti1/2 increases RAIU in established rat thyroid cell lines, as well as primary cultured cells of thyroid tumors from a preclinical thyroid cancer mouse model.

FIG. 6. Apigenin's effect to further increase RAIU in Akt1/2-treated PCC13 cells is independent of PKC- δ . Cells were transfected with 10 nM of scrambled (Scr) or PKC- δ siRNA for 24 hours followed by treatment with reagents for additional 24 hours. **(A)** RAIU in Scr siRNA-transfected cells was not significantly different from PKC- δ siRNA-transfected cells. Data are expressed as mean $\pm$ SD ($n=3$). **(B)** PKC- δ knockdown was confirmed by evident decrease in PKC- δ protein level in Western blots. NIS protein level was moderately decreased by PKC- δ knockdown. Arrowheads indicate hyperglycosylated (▶) and hypoglycosylated (▷) NIS. GAPDH served as a loading control.

Discussion

In PCC13 rat thyroid cells, we show that acute TSH-stimulated RAIU is enhanced by various inhibitors targeting signaling nodes overactivated in thyroid tumors. Among them, Akt1/2 increased RAIU to the greatest extent, and Apigenin further increased RAIU in Akt1/2-treated thyroid cells. This action of Apigenin was not mediated by an increase in cell surface NIS protein levels or a decrease in iodide efflux rate. Instead, Apigenin increased the iodide influx rate in Akt1/2-treated cells, and this effect required p38MAPK activity. Most importantly, the increase in TSH-stimulated RAIU activity by Apigenin in combination with Akt1/2 was confirmed in BRAF^{V600E}-expressing PCC13 cells, as well as in primary cultured tumor cells from *TRβ^{PV/PV}* genetic-engineered mice.

Akti1/2 alone increased the iodide influx rate, and Apigenin in combination with Akt1/2 further increased the iodide influx rate (Fig. 4C). To date, molecular mechanisms underlying the modulation of the iodide influx rate mediated by NIS remain unknown. Phosphorylation has been shown to modulate the activity of many transporters, such as glucose transporter (38) and Na⁺/H⁺ Exchanger isoform-1 (39). NIS is a phosphorylated protein (40), and several phosphorylation

FIG. 7. AP in combination with Akt1/2 increases RAIU in BRAF^{V600E}-expressing PCC13 cells and in thyroid tumor cells from *TRβ^{PV/PV}* mice. **(A)** RAIU in doxycycline-induced cells is significantly decreased compared to uninduced cells. RAIU in AP+Akt1/2-treated cells is significantly higher than Akt1/2-treated cells in both uninduced and induced cells. **(B)** Western blots show that total NIS protein levels are decreased nearly by half in BRAF^{V600E}-expressing cells. Arrowheads indicate hyperglycosylated (▶) and hypoglycosylated (▷) NIS. Note an increase in M_r of NIS by AP+Akt1/2 in both uninduced and induced cells. GAPDH served as a loading control. **(C)** Primary cultured thyroid tumor cells from *TRβ^{PV/PV}* mice were treated with DMSO or AP+Akt1/2, one day post-seeding. RAIU in AP+Akt1/2-treated cells was significantly greater than RAIU in DMSO-treated cells. Data are expressed as mean $\pm$ SD ($n=3$).

sites have been identified (41). An increase of M_r in NIS protein was noted in cells treated with Apigenin in combination with either Akt inhibitor or Akt knockdown in parental and BRAF^{V600E}-expressing PCC13 cells. In these experimental settings, the increase of M_r in NIS was accompanied with an increase in RAIU. It will be of interest to determine whether the increase of M_r reflects NIS phosphorylation and whether NIS phosphorylation leads to an increased iodide influx rate in thyroid cells.

However, an increase of M_r in NIS did not always translate into increased RAIU. As shown in Figure 5, one hour

pretreatment of p38 MAPK inhibitor followed by its continuous presence for 24 hours diminished Apigenin's effect to further increase RAIU in Akti1/2-treated cells despite the increase of M_r in NIS remaining unchanged. Conversely, the iodide influx rate can be upregulated without an increase of M_r in NIS, as in the case of cells treated with Akti1/2 alone. Taken together, NIS-mediated iodide influx rate may be modulated by multiple mechanisms.

Akt inhibition alone, via inhibitor or shRNA knockdown, further increased TSH-stimulated RAIU in PCC13 cells. Apigenin alone also decreased phospho-Akt levels, yet with little effect on thyroidal RAIU activity. This discrepancy may be explained by a smaller decrease in phospho-Akt levels by Apigenin than Akti1/2 or shAkti1/2. Accordingly, one may speculate that a threshold of Akt inhibition needs to be achieved to increase RAIU, and that a further increase in RAIU by Apigenin in Akti1/2-treated cells was mediated by further Akt inhibition. However, Akt inhibition is not always accompanied with increased RAIU. For example, the evident decrease in phospho-Akt levels by Apigenin treatment was not accompanied by increased RAIU in PCC13 cells with 0.2% serum culture media. Furthermore, SB203580 diminished Apigenin's effect to further increase RAIU in Akti1/2-treated cells without a change in phospho-Akt levels. Finally, it is of interest to note that an increase of M_r in NIS only occurred in cells treated with Apigenin + Akt inhibition. This suggests that Apigenin's action to further increase RAIU in Akti1/2-treated cells may not be fully explained by its effect on further Akt inhibition.

SB203580 decreased TSH-induced NIS mRNA levels, when SB203580 and TSH were added at the same time to TSH-deprived FRTL-5 cells (42,43). In this study, a p38 MAPK inhibitor was added 24 hours after acute TSH stimulation, when NIS mRNA and protein were already synthesized. Neither SB203580 nor BIRB-796 altered steady state NIS protein levels, yet both resulted in a drastic decrease in RAIU. Accordingly, p38 MAPK activity is required not only for TSH-stimulated NIS expression but also for optimal NIS-mediated RAIU activity. However, the action of Akti1/2 on RAIU is independent of p38 MAPK activity, as neither SB203580 nor BIRB-796 affected the fold of increase in RAIU by Akti1/2 alone.

Matowe *et al.* reported that PKC- δ disappeared within 24 hours of TSH withdrawal and reappeared 24 hours after TSH addition in FRTL-5 rat thyroid cells (44). Our study shows that steady state NIS protein levels were modestly decreased upon PKC- δ knockdown, yet without an accompanied change in RAIU. This discrepancy may be explained by an absent change in cell surface NIS protein levels or by increased NIS activity that compensates the decreased NIS protein levels. Neither Akti1/2-induced RAIU nor Apigenin's ability to further increase Akti1/2-induced RAIU was dependent upon PKC- δ .

The increase in RAIU by Apigenin + Akti1/2 was reproduced in BRAF^{V600E}- or RET/PTC1-expressing thyroid cells. Apigenin + Akti1/2 also increased RAIU in thyroid tumor cells from *TR β ^{PV/PV}* mice. Accordingly, Apigenin + Akti1/2 may increase TSH-stimulated RAIU in various thyroid tumors that carry the BRAF^{V600E} mutation, RET/PTC1 rearrangement, or PI3K overactivation. A recent study reported a synergistic effect between Apigenin and an Akt inhibitor on cytotoxic effects of PLX4032-treated anaplastic

thyroid carcinoma cells (45). It would also be of interest to examine further whether other flavonoids with a similar structure to Apigenin would have the same effect.

In summary, our studies suggest that Apigenin as a dietary supplement, along with Akt inhibitors, can further enhance the efficacy of radioiodine therapy for thyroid cancer patients. Several Akt inhibitors, such as MK-2206 or GSK2110183, are currently in oncological pipelines to treat other types of cancer. Our finding has the potential for translation into clinical trials upon confirmation in preclinical thyroid cancer mouse models.

Acknowledgments

This work was supported by NIH grant PO1-CA-124570. We appreciate M.P. Brandt for dissecting thyroid tumors, and Dr. M. Ostrowski, Dr. B. Kaur, and A. Suresh for scientific input.

Author Disclosure Statement

No competing financial interests exist.

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