Activation of the PI3 Kinase Pathway By Retinoic Acid Mediates Sodium/Iodide Symporter Induction and Iodide Transport in MCF-7 Breast Cancer Cells

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Abstract

Iodide uptake in the thyroid and breast is mediated by the sodium/iodide symporter (NIS). NIS activation is used for radioiodide imaging and therapeutic ablative of thyroid carcinoma. NIS is expressed in >70% of breast cancers but at a level insufficient for radioiodine treatment. All-trans retinoic acid (tRA) induces NIS gene expression and functional iodide uptake in human breast cancer cell lines and mouse breast cancer models. tRA usually regulates gene expression by direct interaction of RA receptor (RAR) with a target gene, but it can also act through nongenomic pathways. We report a direct influence of tRA treatment on the phosphoinositide 3-kinase (PI3K) signaling pathway that mediates tRA-induced NIS expression in MCF-7 breast cancer cells. MCF-7 cells express all three RAR isoforms, α, β, and γ, and RXRα. We previously identified RARα and RXRα as important for NIS induction by tRA. Treatment with LY294002, the PI3K inhibitor, or p85α knockdown with siRNA reduced NIS expression. Immunoprecipitation experiments and glutathione S-transferase pull-down assay showed a direct interaction between RARβ2, RXRα, and p85α. RA also induced rapid activation of Akt in MCF-7 cells. Treatment with an Akt inhibitor or Akt knockdown with siRNA abolished tRA-induced NIS expression. These findings indicate that RA induction of NIS in MCF-7 cells is mediated by rapid activation of the PI3K pathway and involves direct interaction with RAR and retinoid X receptor. Defining these mechanisms should lead to methods to further enhance NIS expression, as well as retinoid targets that influence growth and differentiation of breast cancer. [Cancer Res 2009;69(8):3443–50]

Introduction

The sodium/iodide symporter (NIS) is a transmembrane glycoprotein that transports iodide into the thyroid to maintain adequate thyroid hormone production (1). NIS is expressed on the basolateral membrane of alveolar cells in lactating mammary gland, concentrating iodide in milk, but is not expressed in the nonlactating breast (2–4). It has been reported that >70% of breast cancer tissue expresses NIS, although the level of basal iodide transport is very low (3, 4). A variety of approaches have been used to enhance functional NIS expression in breast cancer, with the goal of using radioiodide to image and treat some differentiated breast cancer (5). Our previous studies have shown that all-trans retinoic acid (tRA) significantly induces NIS gene expression and iodide uptake in human breast cancer cell line and breast cancer mouse models, but the precise mechanism has not been established (6, 7).

Retinoids, active metabolites of vitamin A, comprise both naturally occurring and synthetic compounds that have been used in animal models and humans as differentiation agents for various types of cancers, including breast cancer and promyelocytic leukemia (8, 9). The classic retinoid pathway involves the ligand-activated nuclear receptors, RA receptors (RAR) and retinoid X receptors (RXR; ref. 9). Both RAR and RXR have three isoforms, α, β, and γ. RAR-RXR heterodimers bind to RA or retinoid X-response element and activate transcription (9, 10). Many hormones classically acting through nuclear receptors, however, have been shown to also act through nongenomic pathways (11, 12). It has been reported that nuclear receptors can initiate second messenger production and interact with other cellular systems (11–13). The mechanism of rapid nuclear receptor signaling, however, is not established and a variety of models have been described (12, 13). We have recently studied the human NIS gene and directly characterized 44 kb of upstream and downstream sequence but could not identify a functional RA response element (14). We used signal transduction inhibitors to show that RA induction of NIS is likely to be through a nongenomic pathway (14). Signal transduction pathways have been implicated by several studies of NIS expression in breast cancer (15, 16).

Phosphoinositide 3-kinase (PI3K)/Akt signaling pathway influences cell growth, cell survival, and cell movement, and signaling defects have been identified in a wide spectrum of human cancers (17, 18). PI3Ks are a family of enzymes and can be subdivided into three classes—class I, II, and III (19). Class IA PI3Ks are heterodimers of regulatory and catalytic subunits (18, 19). A regulatory subunit of class IA PI3Ks, p85α, is a phosphoprotein substrate of many cytoplasmic and receptor tyrosine kinases (17, 19). Members of the nuclear receptor superfamily, such as the estrogen, thyroid hormone, and RARs, have been shown to interact with p85α, and activate the PI3K/Akt signaling pathway (20–23).

We have investigated the mechanism of tRA induction of NIS. We show that rapid activation of PI3K/Akt by RA induces NIS expression in MCF-7 cells. We also show that this rapid activation of PI3K/Akt pathway is initiated by a direct interaction between p85α and RARα, in association with RXRα.

Materials and Methods

Cells and culture conditions. MCF-7 cells and COS-7 cells were obtained from the American Type Culture Collection and maintained
according to the recommended conditions. In the perofmance of immunoprecipitation, Akt phosphorylation, and cell fractionation studies, cells were serum-starved in phenol red-free DMEM for at least 24 h, and then treated with 1 μmol/L 1,25-dihydroxyvitamin D3 (1,25-dihydroxyvitamin D3) for the indicated time.

Reagents and antibodies. IRA and 9cRA were purchased from Sigma. LTR294002 and Akt inhibitor VIII were purchased from EMD Biosciences. Antibodies for phosphorylated 5473 Akt, total Akt, β-actin, and PARP were purchased from Cell Signaling Technology. RXRα antibody was purchased from Santa Cruz Biotechnology. p85α antibody was purchased from Millipore. FLAG antibody was purchased from Sigma. NIS antibody was a gift from Dr. Nancy Carrasco (Albert Einstein College of Medicine, Bronx, NY).

Western blot analysis. Whole cell lysates were prepared as described previously (21) with minor modifications. Briefly, cells were washed with ice-cold PBS and lysed with cell lysis buffer (Tris-HCl [20 mmol/L (pH 7.4)], EDTA [10 mmol/L], NaCl [100 mmol/L], Nonidet P-40 [1%], sodium deoxycholate [5%], Na3VO4 [1 mmol/L], sodium PPI [2.5 mmol/L], β-Glycerophosphate [1 mmol/L], and the protease inhibitor cocktail [Sigma]). Cells were sonicated for 20 s, centrifuged for 10 min at 14,000 × g at 4°C. Supernatant was collected and 2× sample buffer was added, then incubated at 95°C for 5 min. Membrane fraction was prepared as previously described (6). Protein concentrations of the samples were measured using the Bio-Rad protein assay (Bio-Rad). Western blot was performed as described previously (24), except that the protein was visualized by ECL Western blotting detection reagents (GE Healthcare Bio-Sciences). For the same experiment, membranes were stripped with a stripping buffer (Ferm) and immunoblotted with another antibody.

Immunoprecipitations. Cells were washed with ice-cold PBS and lysed with cell lysis buffer. Immunoprecipitating antibody was added to equal amounts of cell lysates (3–5 mg) in 1 mL of lysis buffer for 12 h at 4°C with cell lysate buffer. Immunoprecipitating antibody was added to equal amounts of cell lysates (3–5 mg) in 1 mL of lysis buffer for 12 h at 4°C rotating. Then, 40 μL of 1:1 Protein-A agarose was added and rotated for another 3 h at 4°C. The immunoprecipitates were washed with an ice-cold lysis buffer for 5 times, then resuspended in a sample buffer and incubated at 95°C for 5 min. The bound proteins were analyzed by Western blot analysis. The FLAG-IP, anti-FLAG M2 affinity gel (Sigma) was used and immunoprecipitation was performed according to the manufacturer’s protocol.

Glutathione S-transferase pull-down assay. Glutathione S-transferase (GST) fusion protein was expressed in BL21 bacteria cells and purified using standard techniques. For the pull-down assays, bovine recombinant GST-p85α protein or GST bound to glutathione-agarose beads was suspended in 500 μL of AM-1 Buffer [20 mmol/L Tris-HCl (pH 8.0), 20% Glycerol, 100 mmol/L KCl, 1 mmol/L DTT, and 0.2 mmol/L EDTA] and incubated with 1 μg human recombinant RXR (Active Motif) for 1 h at 4°C. The samples were washed 5 times with ice-cold PBS, then resuspended in a sample buffer and incubated at 95°C for 5 min. The bound proteins were analyzed by Western blotting.

Reverse transcription-PCR and quantitative real-time PCR analysis. Total RNA isolation, reverse transcription-PCR (RT-PCR), and real-time PCR analysis were performed using total RNA isolated from cells previously described (5, 7). Sequences of PCR primer pairs for RARα, p85α, and Akts are shown in Supplementary Data. Primers for NIS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been described previously (5).

Plasmid constructs. pcDNA3.0-RARβ2 and FLAG-RARβ2 were subcloned from pcCMX-RARβ2 into HindIII-BamHI site of pcDNA3.0 (Invitrogen) and p5XFLAG-I4 vector (Sigma), respectively. For RARβ4, inserts were created by PCR amplification of cDNA obtained from the breast cancer cell line, Hs578T. cDNA was obtained as described above. Primers used for cloning are as follows: 5′-CAGCCTATAGGCTCTGGATTGAGGAGCTGTAAG-3′ (forward primer), 5′-ACTCGAGAAGGTACTGGGGAATGTTTGAAGTAG-3′ (reverse primer). TOPO-Cloning kit was used and performed according to the manufacturer’s manual. RARβ4 construct was then subcloned into HindIII-Xhol site of pcDNA3.0. Because there was another ATG site at nucleotide 411.37 bp of the RARβ4 transcription start site at nucleotide 448, the ATG site at 411 was modified to TTG by in vitro site-directed mutagenesis using PCR. Primer used for mutagenesis was 5′-GAGTCAGGGCTTTACACCTTCAACAGGGCTGCT-3′, pcCMX-RARβ2 was a gift of Dr. David Mangelsdorf (Howard Hughes Medical Institute, Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX), pGEX-p85α used to produce GST-p85α fusion protein was a gift from Dr. Masato Kasuga (Kobe University, Kobe, Japan). pSG5-RXRα was a gift from Dr. Ronald Evans (Salk Institute, La Jolla, CA).

Figure 1. Influence of retinoids on NIS mRNA expression and iodide uptake in MCF-7 cells. A, response to treatment with natural and synthetic RAR/RXR ligands. MCF7 cells were incubated with each ligand at a concentration of 1 μmol/L for 24 h and iodide uptake was measured. Perchlorate (ClO4−; 30 μmol/L) is a specific inhibitor of NIS function, and was used to determine NIS-independent 125I− transport. *statistical significance at a P value of <0.05; NS, not significant. B, pan-RAR and pan-RXR antagonists block the iodide uptake induced by tRA and 9cRA. MCF7 cells were incubated with a combination of RA (0.1 μmol/L) and an antagonist (5 μmol/L) for 24 h and iodide uptake was measured. C, effect of RAR/RXR ligands on NIS mRNA expression. MCF7 cells were incubated with each ligand at a concentration of 1 μmol/L for 12 h, and RT-qPCR of NIS and GAPDH mRNA was performed. The average ratio of NIS/GAPDH in untreated cells was set as 1.
Small interfering RNA preparation. Small interfering RNAs (siRNA) targeting RARβ2 gene were designed and were chemically synthesized by Dharmacon RNA Technologies. The sequences for siRNAs are as follows: GTATGGATGTCTGTGACTG and GGATTCTACACTGCGAGT. Nontargeting control siRNA (SMARTpool control) and predesigned siRNAs against RARα, p53α, Akt1, and Akt2 (siGENOME siRNA) were also purchased from Dharmacon.

Transfection. Plasmids were transfected into MCF-7 cells and COS-7 cells with Effectene and Polyfect (Qiagen), respectively. Transfection was performed according to the manufacturer's protocol. siRNAs were transfected into cells at a final concentration of 100 nmol/L by using transfection reagent, DharmaFECT 1 (Dharmacon). Transfection was performed according to the manufacturer's protocol.

Iodide uptake. The iodide uptake assay was performed as described previously (5). The uptake was normalized to the cellular protein concentration measured with Bio-Rad protein assay.

Statistical analysis. For statistical analysis, one-factor ANOVA followed by the Scheffe's F test was used. Values were expressed as means ± SD, and P value of <0.01 was considered significant. All the experiments were performed with at least three replicates.

Results

Response to natural and synthetic retinoids. We used isofrom-specific retinoid receptor ligands to characterize the isoform specificity of tRA induction of NIS expression. We have reported previously that an RARβ/γ agonist was the most potent retinoid stimulator of NIS expression (5). We extended these studies and performed iodide uptake and NIS mRNA expression with the RAR α/β agonist, Am80, a pan-RAR agonist, Re80, and pan-RXR agonists, PA024 (Fig. 1A and C). Although a natural pan-RAR agonist, tRA, and a natural pan-RAR/RXR agonist, 9cRA, induced iodide uptake and NIS mRNA expression to the greatest level of expression, Am80 induced NIS to a similar level as Re80. This result, along with those from our previous study (5), indicates that RARβ plays the major role in NIS induction by tRA. In addition, we performed iodide uptake after treatment with an RAR antagonist, LE540, and an RXR antagonist, HX531. Interestingly, both LE531 and HX531 blocked tRA-stimulated iodide uptake (Fig. 1B). The RXR antagonist, HX531, is known to selectively inhibit the activity of RAR-RXR heterodimers (25). Inhibition of NIS induction by HX531 indicates that heterodimerization of RAR and RXR is required for the tRA-induction of iodide uptake.

Nuclear receptor RARβ2 and RXRα are required for the NIS induction by tRA in MCF-7 cells. The pharmacologic studies showed that RARβ and RXR are important for tRA-mediated NIS induction. We further evaluated the role of RARβ and RXR by determining the influence of loss- and gain-of-function experiments on NIS induction. MCF-7 breast cancer cells express RARα, β, γ, and RXRα (26, 27). RARβ generates multiple variants by using two promoters and alternative splicing (26). Variants of RARβ have been identified in human breast cancer cells: β2, β2S, β4, and β5 (Fig. 2A; refs. 26, 28, 29). RARα encodes the full-length isoform, whereas RARβ2S, β4, and β5 are truncated variants of RARβ2, and the corresponding sequences are indicated by gray boxes.
(26, 28, 29). Two kinds of protein are translated from these variants. One is the product of RARγ2 and β2S, which include the receptor domains A to F, and the other is the product from β4 and β5, which lack the A, B, and C domains (Fig. 2A). MCF-7 cells express β2, β2S, β4, and β5 mRNA as determined by RT-PCR (data not shown). We stably transfected MCF7 cells with vectors expressing one of the two RARγ protein product variants, either RARγ2 or β4, and performed RT-qPCR of NIS mRNA and iodide uptake (28, 29). Overexpression of RARγ2 significantly increased tRA-induction of NIS mRNA and iodide uptake (data not shown), whereas RARγ4 expression reduced both (Fig. 2A). Selective knockdown of RARγ2, as well as RXRα, significantly decreased RA-induced NIS mRNA expression in MCF-7 cells (Fig. 2C). These data indicate that tRA-induced NIS expression uses RARγ2 and RXRα, likely as heterodimers, whereas the truncated RARγ4 functions as an inhibitor.

**Activation of PI3K is required for NIS induction by tRA.** The specific inhibitor of PI3K, LY294002, blocked the induction of NIS mRNA expression by tRA in a time-dependent manner (Fig. 3A). LY294002 treatment also inhibited NIS protein expression on the membrane in a dose-dependent fashion (Fig. 3B). This is consistent with our recent study showing that LY294002 blocked the RA-induction of iodide uptake at an IC50 of 7.87 ± 1.1 μmol/L (14).

Furthermore, tRA-induction of NIS mRNA did not require *de novo* protein synthesis because cotreatment of tRA and cycloheximide did not influence NIS mRNA expression (data not shown). These pharmacologic data, as well as our previous studies in which a functional RA response element was not identified in the NIS gene locus or adjacent sequences (14), suggest that tRA treatment induces NIS expression in MCF-7 cells by modulating the PI3K signal transduction pathway.

We further investigated the involvement of PI3K in NIS induction by tRA, by using siRNA targeting p85α, one of the regulatory subunit of class IA PI3Ks. Knockdown of p85α significantly decreased the NIS mRNA induced by tRA compared with the control cells (Fig. 3C).

**RARγ2 and RXRα forms a complex with p85α.** To determine the mechanism of PI3K activation by tRA, we investigated whether RARγ2 and RXRα directly interact with p85α, by performing coimmunoprecipitation and pull-down assays. Immunoprecipitation experiments using COS-7 cells transfected with RARγ2, p85α, and RXRα showed a direct interaction between RARγ2 and p85α in a tRA ligand-independent manner (Fig. 4A). Conversely, a similar stable interaction between RARγ2 and p85α was observed with immunoprecipitation performed with anti-p85α antibody (Fig. 4B). These finding show direct ligand-independent binding of RARγ2
and p85α. We then examined the interaction between RXRα and p85α in a cell-free system by performing a pull-down assay using GST-p85α fusion protein. GST-p85α fusion protein bound to the human recombinant RXRα but GST alone did not. Interestingly, both pan-RAR and -RXR antagonist inhibited the binding of RXRα to p85α (Fig. 4D). This is consistent with findings from the functional studies using pharmacologic antagonists that blocked tRA-induced NIS expression (Fig. 1B). Furthermore, using the coimmunoprecipitation assay with endogenous p85α and RXRα from cell lysates of MCF-7 cells, we found that tRA increases the association of p85α and RXRα (Fig. 4C).

Akt, downstream of PI3K pathway, is rapidly activated after treatment with tRA in MCF-7 cells. Several nuclear receptors have been shown to rapidly activate Akt after PI3K activation. We determined whether tRA activates Akt in MCF-7 cells. The cells were serum starved for 24 to 48 hours before tRA treatment. Treatment with 1 μmol/L tRA rapidly increased Ser-473 phosphorylation of Akt at 5 to 10 minutes and then decreased to the basal level at 20 minutes (Fig. 5A). Treatment with an Akt inhibitor VIII, an isozyme-specific inhibitor that inhibits Akt1 and Akt2 selectively, reduced NIS mRNA induced by tRA (Fig. 5B). Combining Akt inhibition with PI3K inhibition did not further reduce NIS mRNA (Fig. 5B). High concentrations of Akt inhibitor VIII (IC50, 4.5 μmol/L) inhibits tRA-induced iodide uptake in MCF-7 cells (Fig. 5C). Combination treatment of Akt1 and Akt2 siRNAs in MCF-7 cells also decreased tRA-induced NIS mRNA expression ~25%, but it was not a significant reduction (Fig. 5D). We also performed a selective knockdown of Akt1 or Akt2 separately, but there was a trend in these studies toward increased NIS mRNA induced by tRA (data not shown).

**Discussion**

A high fraction, ~70%, of breast cancers express NIS, but functional iodide concentration is rare (3, 4). Selective induction of NIS provides the potential for developing NIS-mediated radionuclide therapy as a safe and effective treatment for breast cancer. Baseline iodide transport in breast cancer, however, is insufficient and stimulation of expression is necessary. Identification of the mechanism(s) of RA induction of NIS expression is essential to determine approaches to augment functional uptake. The involvement of signal transduction pathways, including PI3K, in NIS induction in breast cancer has been suggested by several previous studies (15, 16). The current study shows that liganded RAR directly
induces the rapid activation of PI3K/Akt pathway, leading to the activation of a yet unknown transcription factor, which then induces NIS mRNA expression (Fig. 6).

The activation of PI3K/Akt has been reported by other nuclear receptors, including estrogen, thyroid hormone, and glucocorticoid receptors (20–23). Recently, it has been reported that RA can activate PI3K/Akt pathway through interaction between RARα and p85α (22, 32). In this study, we have identified a novel ligand-dependent interaction between RARβ2, RXRα, and p85α. Ligand binding in MCF7 cells facilitates the association of RXRα, subsequently activating PI3K and Akt. Although we were able to observe ligand-dependent interaction of RXRα and p85α by immunoprecipitation assay, we were not able to observe the same result in GST-p85α pull-down assay. This may be due to the differences between the in vitro and in vivo conditions. The interaction between RARβ2 and p85α, however, was ligand-independent. This finding is consistent with previous reports showing the interaction of thyroid hormone receptor and RARα with p85α (20, 22). Inhibitor for PI3K and Akt, as well as siRNAs for p85α and Akt1/Akt2, decreased tRA-induced NIS mRNA expression. We have also found that Akt was rapidly phosphorylated within 5 to 10 minutes after tRA stimulation. These results indicate that the rapid activation of the PI3K/Akt pathway by tRA led to an increase in NIS mRNA expression and iodide uptake. Several studies have shown that tRA has a negative effect on PI3K activity in MCF-7 cells (33, 34). One group showed that long-term treatment with tRA induced growth inhibition of MCF-7 cells, mediated by down-regulation of IRS-1/PI3K/Akt pathway (33). The different influences of tRA on PI3K may be due to short-term rather than long-term treatment.

Figure 5. Rapid activation of Akt is involved in tRA induction of NIS. A, time-dependent effects of treatment with 1 μmol/L tRA on Ser-473 phosphorylation of Akt. MCF-7 cells were serum starved and treated with 1 μmol/L tRA for the indicated time period. Whole cell lysates (20 μg) were used for Western blot analysis. Band density was measured by ImageJ software. B, Akt inhibitor VIII or PI3K inhibitor LY294002 reduces RA induction of NIS. MCF-7 cells were incubated with 1 μmol/L tRA and the addition of Akt and/or PI3K inhibitor (10 μmol/L) for 12 h, and then RT-qPCR for NIS and GAPDH was performed. The average ratio of NIS/GAPDH in untreated cells was set as 1. *, statistical significance compared with tRA-treated cells (P < 0.01). C, influence of the Akt inhibitor VIII at increasing concentration on iodide uptake in MCF7 cells (IC50 4.5 μmol/L). D, MCF-7 cells transfected with siRNAs targeted for Akt1 and Akt2 were treated with/without 1 μmol/L tRA for 12 h and RT-qPCR for NIS and GAPDH was performed (right). The average ratio of NIS/GAPDH in untreated cells was set as 1. Control siRNA, nontargeting siRNAs. Efficiency of the knockdown is shown by RT-PCR of Akt 1/Akt2 and β-actin (right).
Activated kinase, Rac, and p38 inhibitors also partially inhibited the induction of NIS by tRA (14). Akt inhibition also reduced iodide uptake, suggesting actions of Akt activation on NIS protein processing or membrane insertion. The combination knockdown of Akt1 and Akt2 decreased the NIS expression, but knockdown of each Akt1 or Akt2 did not decrease but rather increased the tRA-induced NIS mRNA expression. Blocking either Akt1 or Akt2 pathway may activate the other pathway and increase the overall Akt activation, resulting in an increase in NIS expression.

Growing evidence shows that rapid nongenomic actions of nuclear receptors are initiated outside the nucleus; however, considerable controversy about these actions remain (11, 13, 36). Transfection of a chimeric RARα/c-Src myristylation domain construct, which was targeted to plasma membrane, in mouse embryonic fibroblast-RAR(αL/C0/C0)-null cells and COS-7 cells resulted in strong activation of PI3K/Akt signaling, indicating that interaction of RARα and p85α can occur at the plasma membrane (22). The interaction among RARβ2, RXRα, and p85α might also take place in the cytoplasm or near the plasma membrane, although p85α is localized in both cytoplasm and nucleus. Phosphorylation of RARα in promyelocytic leukemia cells reduces transcriptional activation, and RAR/RXR phosphorylation may influence signal transduction (37). Further studies are required to determine the underlying mechanism.

Due to their role in the regulation of cell growth and differentiation in preclinical models, retinoids are being extensively evaluated in clinical trials of cancer prevention and therapy, including breast cancer (8). Retinoid receptors are expressed in normal and malignant epithelial breast cells and are critical for normal development. It has been suggested that biological effects of tRA are partially mediated through multiple signal transduction pathways. These nongenomic actions of RARα may play an important role in indirectly regulating gene expression, not only for NIS induction but also for other tRA-regulated influences on cancer growth and differentiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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