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Farnesol induces thyroid hormone receptor (THR) β1 but inhibits THR-mediated signaling in MCF-7 human breast cancer cells

Robin E. Duncan [a,1], Michael C. Archer [a,b,*]

[a] Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto, Ont., Canada M5S 3E2
[b] Department of Medical Biophysics, Faculty of Medicine, University of Toronto, Toronto, Ont., Canada M5S 3E2

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Abstract

Anti-cancer effects of farnesol are well established, although mechanisms mediating these effects are not fully understood. Since farnesol has been shown to regulate gene transcription through activation of the farnesoid X receptor and the peroxisome proliferator-activated receptors-α and -γ, we hypothesized that farnesol may also mediate some of its effects through other nuclear hormone receptors. Here we showed that in MCF-7 human breast cancer cells, farnesol induced the expression of thyroid hormone receptor (THR) β1 mRNA and protein at concentrations that inhibited cell growth. Changes in the expression of THR responsive genes, however, suggested that farnesol inhibits THR-mediated signaling. Protein extracts from cells treated with farnesol displayed decreased binding to oligodeoxynucleotides containing a consensus sequence for the THR response element, despite the higher THRβ1 content, providing a mechanism to explain the decreased transcriptional activity of cellular THRs.

Keywords: Farnesol; MCF-7 human breast cancer cells; Thyroid hormone receptor β1; Proliferation; Spot 14; Sodium iodide symporter; Malic enzyme; Glycerol-3-phosphate dehydrogenase 1

Farnesol is an isoprenyl alcohol found in fruits and vegetables [1] that is also synthesized in mammalian cells as a metabolite of the mevalonate/cholesterol biosynthetic pathway [2]. Anti-cancer effects of farnesol have been demonstrated in a number of studies that show suppression of tumor cell proliferation [3,4] and induction of tumor cell apoptosis in vitro [5,6], and inhibition of tumor growth and development in vivo [5,7]. Mechanisms mediating these effects are not fully understood. Elucidation of the mode of action of farnesol may, therefore, have significance for the prevention and/or treatment of malignant disease.

Transcriptional control of genes required for cellular growth and metabolism may be one mechanism contributing to the anti-cancer effects of farnesol. Previous studies have shown that farnesol regulates gene transcription through activation of nuclear hormone receptors, including the farnesoid X receptor (FXR) [8] and peroxisome proliferator-activated receptors-α (PPARα) and -γ (PPARγ) [9]. FXR, PPARα, and PPARγ are members of the steroid/thyroid nuclear receptor superfamily of ligand-activated transcription factors [10]. These nuclear hormone receptors form heterodimers with retinoid X receptors (RXRs) to bind DNA and modulate gene transcription [10]. We hypothesized that farnesol may regulate other members of this nuclear hormone receptor superfamily to mediate some of its cellular and anti-cancer effects.

Here we examine the effect of farnesol on thyroid hormone receptor (THR) β1 expression in MCF-7 human breast cancer cells. We also assessed DNA binding activity and gene regulation by THRs in these cells after treatment with farnesol.

* Corresponding author. Fax: +1 416 971 2366.
E-mail address: m.archer@utoronto.ca (M.C. Archer).
[1] Present address: Department of Nutritional Sciences and Toxicology, University of California, Berkeley 94720, USA.

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Materials and methods

Materials. trans,trans-Farnesol, and all other chemicals, unless otherwise indicated, were purchased from Sigma (Oakville, Ont., Canada). Fetal bovine serum was from Hyclone (Fisher Scientific, Ottawa, Ont., Canada). Culture medium was prepared by the University of Toronto tissue culture facility. Antibodies against THRβ1 were from Upstate Biotechnology Inc. (Charlottesville, VA, USA), and the antibody against GAPDH was from Abcam (Cambridge, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell culture. MCF-7 cells were purchased from the American Type Cell Culture collection and routinely cultured in 1:1 DME/F12 in 75 cm^2 flasks at 37 °C and 5% CO_2 in the presence of 1% penicillin/streptomycin with 10% fetal bovine serum. Farnesol was dissolved in dimethyl sulfoxide (DMSO) immediately prior to use. Final concentration of DMSO in control and treatment media was 0.05%.

Cell growth. Cells grown in 24-well plates were treated with farnesol or DMSO (control) for 24 or 48 h. Cells were washed with PBS, fixed with methanol for 20 min, stained with crystal violet for an additional 20 min, washed with water, and air-dried [11]. Dye was eluted overnight at room temperature from cells with 1 ml of 1% sodium dodecyl sulfate, and absorbances were determined at 595 nm.

Preparation of protein extracts and immunoblotting. Total cell lysates were prepared as follows. Cells were washed repeatedly with ice-cold PBS (minus Ca/Mg) and then scraped into RIPA lysis buffer (1× PBS, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing 1% protease inhibitor cocktail. Cells were incubated on ice for 30 min with frequent vortexing, and then centrifuged at 4 °C for 20 min at 12,000*g*, after which supernatants containing total cellular extracts were stored at −80 °C. Five micrograms of protein were mixed with 2× Laemmli buffer, heated for 5 min at 95 °C, electrophoresed in 10% SDS–polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 h then probed overnight at 4 °C first with antibodies to THRβ1, and then with horseradish peroxidase-conjugated anti-rabbit antibodies. Signals were detected by enhanced chemiluminescence. Band densities were quantified in arbitrary units using an Alpha Innotech Fluorochem (San Leandro, CA, USA) and then expressed as relative density compared with untreated controls that were taken as 100%. GAPDH was used as a loading control.

RNA isolation, cDNA synthesis, and PCR. Total RNA was harvested from cells using Trizol (Invitrogen, Burlington, Ont., Canada) according to the manufacturer's protocol. cDNA was synthesized from 5 μg of total RNA by oligo(dT) priming using the SuperScript™ First-Strand Synthesis System for RT-PCR from Invitrogen (Burlington, Ont., Canada). The cDNA (1 μl) was amplified by PCR under the following conditions: denaturation at 94 °C for 5 min, followed by a pre-determined number of cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, with the final extension at 72 °C for 10 min. The optimum cycle number for each primer set was determined so that the product was obtained during the exponential phase of the amplification (data not shown). Based on these preliminary studies the following cycle numbers, product size, and primer sets, respectively, were used: 36B4 (20 cycles; 422 bp; forward primer 5′-TGGGCTCCAAGCAGATGC-3′; reverse primer 5′-GGCTTCGCTGGCTCCCAC-3′); THRβ1 (25 cycles; 324 bp; forward primer 5′-GGCTGCAAGGGTTTCTTTAG-3′; reverse primer 5′-CGTTGGTCGCCACATGGGCT-3′); Spot 14 (S14) (35 cycles; 339 bp; forward primer 5′-CTGCTGCCGCGGGAGGAGTG-3′; reverse primer 5′-CTTCTGGCTGCAGGTCTAGG-3′); sodium iodide symporter (NIS) (38 cycles; 319 bp; forward primer 5′-GTTCTACACTGACTGCGACC-3′; reverse primer 5′-GGACACCTCCTCCGAGCAGT-3′); malic enzyme (ME) (25 cycles; 440 bp; forward primer 5′-ATGGAATGGGCATCCCTGTG-3′; reverse primer 5′-GTGTGCAATCCCCTAGGGCAG-3′); glycerol-3-phosphate dehydrogenase 1 (GPD1) (35 cycles; 499 bp; forward primer 5′-TGGAGAGCTGTGGTGTTGCT-3′; reverse primer 5′-CAAATGTGGTGGCATGAGGC-3′). PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. Band densities were imaged and quantified by densitometry in arbitrary units under UV light using an Alpha Innotech Fluorochem (San Leandro, CA). Densities of amplified cDNA were normalized to the density of the corresponding 36B4 band, and relative densities were expressed as a percentage of control values that were taken as 100%.

DNA binding and gel shift assay. Complementary oligodeoxynucleotides containing a consensus sequence for the THR-response element (TRE) were synthesized by ACGT Corporation (Toronto, Ont., Canada) (forward strand: 5′-GCAGCTTCAGGTCACAGGAGGTCAGACAGCT-3′; reverse strand: 5′-ATAGCTCTCTGACCTCCTGTGACCTGAAGCT-3′), annealed, and 5′-overhangs were filled in using dNTPs with [α-^{32}P]CTP by Klenow large fragment. The DNA binding reaction was performed essentially as described previously [12], with minor modifications. Cellular extracts containing 25 μg of protein were incubated with 10 ng of ^{32}P-labeled DNA oligonucleotide for 15 min at room temperature in binding buffer (20 mM Hepes, pH 7.6, 1 mmol/L EDTA, 10 mmol/L $(NH_4)_2SO_4$, 1 mmol/L DTT, 0.2% (v/v) Tween 20, and 30 mmol/L KCl). DNA–protein complexes were resolved on a non-denaturing 4% polyacrylamide gel in Tris–glycine electrophoresis buffer. Gels were imaged using a Packard Instant Imager (Canberra Packard Canada, Mississauga, Ont., Canada).

Statistical analysis. All values shown are means ± SEM. Values reported for immunoblots and PCR are means from at least four individual samples from at least two separate experiments. Differences between treated groups and controls were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test.

Results and discussion

Farnesol induces THRβ1 mRNA and protein expression in MCF-7 cells at concentrations that significantly decrease cell growth

Treatment of MCF-7 human breast cancer cells with farnesol significantly decreased cell growth in a concentration- and time-dependent manner (Fig. 1). Interestingly, this decrease was associated with an increase in the expression of THRβ1 protein levels. Western blotting showed that after 48 h, 20 μM farnesol significantly induced

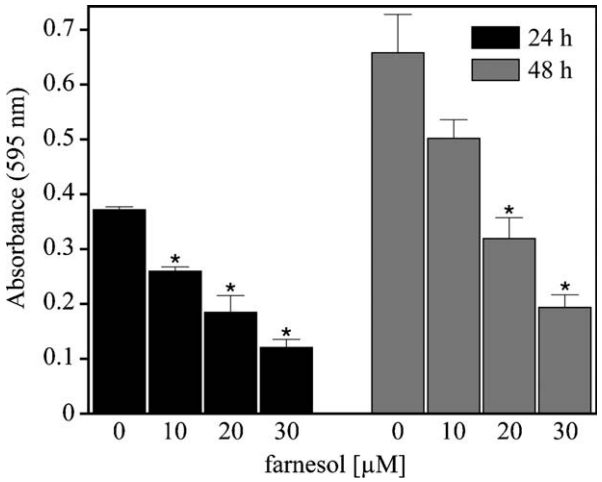

Fig. 1. Farnesol decreases MCF-7 cell growth. Cells were seeded in complete medium in 24-well plates one day prior to treatment with farnesol ($n = 5$). At the indicated times, cells were fixed, stained with crystal violet, and the absorbance of eluted dye was measured at 595 nm. *$P < 0.001$ versus untreated control cells measured at the same time-point.

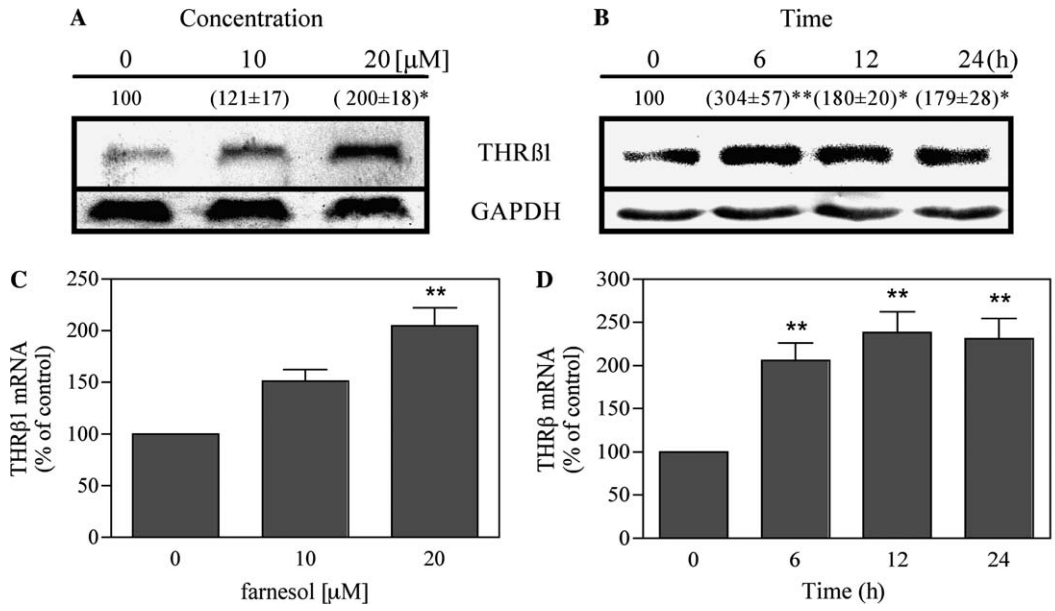

Fig. 2. Farnesol induces THRβ1 expression in MCF-7 cells in a concentration- and time-dependent manner. (A) Immunoblot of THRβ1 expression in cells treated for 48 h with increasing concentrations of farnesol ($n = 6$). (B) Immunoblot of THRβ1 expression in cells treated with 20 μM farnesol for the indicated time periods ($n = 4$). Numbers in parentheses are means ± SEM. (C) RT-PCR analysis of THRβ1 mRNA expression in cells treated for 48 h with increasing concentrations of farnesol ($n = 4$). (D) RT-PCR analysis of THRβ1 expression in cells treated with 20 μM farnesol for the indicated time periods ($n = 6$). All PCR data were normalized to mRNA expression of 36B4 and then expressed as a percentage of control values. *$P < 0.05$; **$P < 0.01$.

THRβ1 by approximately 2-fold (Fig. 2A). Furthermore, time-dependence measurements showed that this up-regulation was already evident by as early as 6 h after treatment, when THRβ1 protein levels were elevated by ~3-fold (Fig. 2B). Changes in THRβ1 protein levels were paralleled by increases in mRNA expression (Figs. 2C and D), indicating that regulation of THRβ1 by farnesol occurred at the level of transcription. These findings identify THRβ1 as another member of the steroid/thyroid superfamily of nuclear hormone receptors, in addition to FXR [8], PPARα, and PPARγ [9], that is regulated by farnesol.

Farnesol alters expression of THR-regulated genes in MCF-7 cells

We next investigated the effect of farnesol on the expression of genes that are controlled by THRs. Spot 14 (S14), also known as thyroid hormone responsive protein, is rapidly induced in response to ligand activation of THRs [13], and its expression is inhibited in the hypothyroid state [14]. Thus, S14 is an excellent marker of thyroid hormone receptor signaling. Surprisingly, we found a rapid and significant decrease in S14 mRNA levels following farnesol treatment (Table 1), suggesting that farnesol inhibited THR-mediated nuclear signaling in MCF-7 cells. Glycerol-3-phosphate dehydrogenase (GPD1) and malic enzyme (ME) that are known to be induced by THR activation [15,16] were also down-regulated following farnesol treatment, supporting this conclusion. Furthermore, expression of the sodium iodide symporter gene (NIS) that has been shown previously to be potently down-regulated by THR activation [17,18] was increased following farnesol treatment (Table 1). Taken together, these data indicate that farnesol impairs the regulatory action of THRs on transcription in MCF-7 cells.

Table 1
mRNA expression of THR-responsive genes in MCF-7 cells treated with 20 μM farnesol for 6 h[a]

Gene	% of control	*P* value
S14	63 ± 3	<0.01
GPD1	83 ± 4	<0.01
ME	79 ± 3	<0.01
NIS	160 ± 3	<0.01

[a] mRNA expression was assessed by densitometry. All values were normalized to expression of 36B4 and are expressed as a percentage of untreated control values.

Farnesol decreases DNA binding of THRs in MCF-7 cells

To determine whether changes in binding of THRs may have mediated the observed effects of farnesol on gene expression, we incubated lysates from farnesol-treated MCF-7 cells with radiolabeled oligodeoxynucleotides containing a consensus sequence for the TRE. Protein-bound oligonucleotides were separated from free DNA by electrophoresis in non-denaturing polyacrylamide gels. Results from this experiment shown in Fig. 3 indicate that although farnesol increased cellular THRβ1 content, it led to a time-dependent decrease in binding of THRs to DNA. Since DNA binding is required for transcriptional activation or repression by THRs, this finding provides an explanation

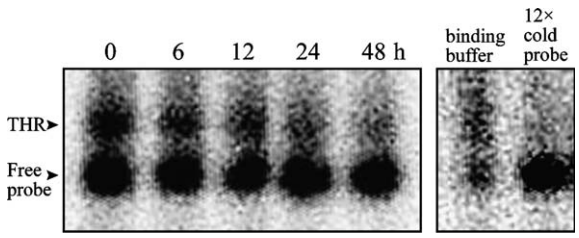

Fig. 3. Farnesol decreases binding activity of THRs. Extracts from MCF-7 cells treated with 20 μM farnesol were incubated with oligodeoxynucleotides containing a consensus sequence for the THR-response element and then resolved in non-denaturing gels. Addition of excess unlabeled probe decreased protein–DNA binding.

for the loss of THR-mediated transcription regulation observed in Table 1, including the induction of NIS that is normally suppressed by activated THRs.

Determination of the complete mechanism whereby farnesol disrupts DNA binding by THRs will require considerable additional study. Active THRs are heterodimeric complexes consisting of the THR subunit bound to its ligand as well as to an RXR subunit that is liganded to 9-cis retinoic acid [10]. Decreased DNA binding may result from impaired assembly of any one or multiple components of the receptor complex. Interestingly, a recent study of rats fed soy protein also reported finding in liver extracts an induction of THRβ1 that displayed decreased DNA binding ability [12]. Farnesol and soy protein may share a common mechanism mediating the induction of transcriptionally inactive THRβ1.

Findings from the present study may have implications for the treatment of breast cancer. Thyroid hormone receptor activation is reported to stimulate the growth of breast cancer cells in culture [19–22]. We have found in the present study that farnesol inhibits DNA binding of THRs, resulting in a decrease in the transcriptional activity of these nuclear hormone receptors that is associated with a decrease in breast cancer cell growth. Furthermore, we report that expression of the THR target gene S14 is decreased by farnesol in MCF-7 cells. S14 regulates the expression of a number of key lipogenic genes involved in malignant cell growth and is known to be expressed in breast tumors and breast cancer cells, but not in normal, non-lactating mammary glands [23]. Our findings suggest that farnesol and foods rich in farnesol may find a role in adjuvant therapy for breast cancer by inhibiting the transcriptional activity of THRs and the expression of key THR-regulated genes. Our results also suggest a novel use for farnesol in the treatment of thyroid and breast cancer. NIS, which mediates active iodide transport, is responsible for the efficacy of radioactive iodide treatment on thyroid tumor growth and for selective ablation of thyroid tumor metastases [17]. Breast cancer is the only other cancer that has been demonstrated to express endogenous, functional NIS, and recently breast cancer metastases have also been shown to express this symporter [17,18]. Our finding that farnesol induces NIS mRNA suggests that this compound may enhance the efficacy of radioactive iodide therapy for the treatment of breast and thyroid cancers.

In summary, we found that farnesol induces THRβ1 mRNA and protein expression in MCF-7 cells in a time- and concentration-dependent manner at concentrations that inhibit cell growth. Changes in the expression of genes that are regulated by THR activation suggested, however, that THR-mediated transcriptional activity is decreased following farnesol treatment. We observed a reduction in binding of cellular extracts to the TRE, despite the induction of THRβ1, providing a mechanism to explain impaired transcriptional regulation by THRs in farnesol-treated cells.

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