Influence of estradiol and triiodothyronine on breast cancer cell lines proliferation and expression of estrogen and thyroid hormone receptors

Efeito do estradiol e da triiodotironina na proliferação de linhagens celulares de câncer de mama e na expressão de receptores de estrógeno e hormônio tireoidiano

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ABSTRACT

Objective: To better understand the estrogen (E2) agonist action of triiodothyronine (T3) the effects of these hormones on ER negative MDA-MB-231 breast cancer cells were compared with those on S30, a clone of MDA-MB-231 stably transfected with ERα cDNA, in terms of proliferation and modulation of hormone receptors. **Results:** Growth experiments showed that MDA-MB-231 was not modulated by any hormone or tamoxifen (TAM). Treatment with E2, 10^{-8}M or 10^{-9}M had little effect on S30 proliferation. T3 at 10^{-8}M significantly inhibited proliferation. This effect was not reverted by TAM. Treatments with 10^{-8}M concentration of E2 or T3 reduced ERα gene expression in S30, an effect partially blocked by association with TAM, with no effect on TR expression. These results suggest that, in S30, 10^{-8}M T3 has a similar action to E2 relative to ERα gene modulation. **Conclusions:** Such results emphasize the need of determining T3 levels, before the introduction of antiestrogenic forms of treatment in breast cancer patients. Arq Bras Endocrinol Metab. 2009;53(7):859-64.

Keywords
Triiodothyronine; estradiol; breast neoplasms; cell line

RESUMO

Objetivo: Para compreender melhor a ação da triiodotironina (T3) agonista de estrógeno (E2), foram comparados os efeitos destes hormônios em células de câncer de mama MDA-MB-231 ER negativas com um clone de MDA-MB-231, transfectado estavelmente com o cDNA de ERα (S30), em termos de proliferação e modulação dos receptores hormonais. **Resultados:** Experimentos de crescimento mostraram que MDA-MB-231 não foi modulada por qualquer hormônio ou pelo tamoxifeno (TAM). O crescimento de S30 foi essencialmente inalterado por tratamento com E2 10^{-9}M ou 10^{-8}M, mas T3 10^{-8}M inibiu significativamente a proliferação quando comparada a ambas concentrações de E2. Esse efeito não foi revertido pelo TAM, sugerindo um resultado não genômico, independente de ERE. Tratamentos com 10^{-8}M de E2 ou de T3 reduziram a expressão do gene ERα em S30, efeito parcialmente impedido pela associação com TAM, sem efeito na expressão de TR. Os resultados sugerem que, em S30, T3 10^{-8}M tem ação semelhante ao E2 com relação à modulação do gene ERα. **Conclusões:** Esses resultados enfatizam a necessidade de dosagem de T3 circulante antes da introdução do tratamento antiestrogênico no câncer de mama. Arq Bras Endocrinol Metab. 2009;53(7):859-64.

Descritores
Triiodotironina; estradiol; neoplasias da mama; linhagem celular

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INTRODUCTION

Estrogens are pivotal in the growth of both normal and neoplastic mammary tissues, and mediate most of their actions via ligand-dependent transcription factors, known as estrogen receptors (ER) (1,2). The involvement of thyroid hormone (TH) in the development and differentiation of normal breast tissue has been established (3-5). Although epidemiologic and experimental studies have associated TH pathologies with an increase in the risk of breast cancer, the role of these hormones remains controversial. Kapdy and Wolfe (6) and Mustacchi and Greenspan (7) found an association between TH ingestion and increase in the risk of mammary cancer. On the other hand, Vorherr (8) described an increase in the survival of hyperthyroid patients with breast cancer. Saraiva and cols. (9) suggest the existence of a biological link between breast cancer in post-menopausal woman and subclinical hyperthyroidism. Turken and cols. (10) and Gogas and cols. (11) reported an increase in the prevalence of thyroid autoimmune disease in patients with breast cancer. Cristofanilli and cols. (12) showed that spontaneous clinical hypothyroidism may decrease the aggressiveness of breast cancer and reduce tumor incidence.

Most, if not all, major triiodothyronine (T3) actions are mediated by specific high affinity nuclear receptors (TR), which are encoded by two genes, TRα and TRβ (13). Recent results reveal substantial changes in the expression profile of TH receptors, suggesting a possible deregulation that could trigger breast cancer development (14).

In a previous work (15), we demonstrated in MCF-7 cells, an ER-positive breast cancer cell line, that T3 mimicked the effects of estradiol (E2), stimulating growth, modulating mRNA transcription of growth factors and inducing the expression and activity of E2-inducible proteins. In addition, these T3 effects were antagonized by the simultaneous addition of tamoxifen (TAM), which is a competitive inhibitor of E2 binding to ER. Similar effects, however, were not observed in the ER-negative MDA-MB-231 breast cancer cells, in spite of the presence of high TR amounts, suggesting that, in MCF-7 cells, both ligands share a common signaling pathway via ER. In addition, Dinda and cols. (16) indicated that in T47D breast cancer cells also ER positive, T_3 regulates T47D cell cycle progression and proliferation raising the p53 level and causing hyperphosphorylation of pRb by a common mechanism involving ER and T3 receptor (T3R)-mediated pathways. In line with these results, a physiological concentration of L-thyroxine (T_4) has been recently reported to induce MCF-7 cells proliferation measured by tritiated thymidine or appearance of proliferating cell nuclear antigen (PCNA) (17).

Jeng and cols. (18) demonstrated previously that after the stable transfection of ERα in MDA-MB-231 cells (devoid of ERα and ERβ) with an ERα vector (S30), these ER negative cells regained hormonal responsiveness.

With the objective of clarifying the E2 agonist action of T3, we herein compared the effects of E2 and T3 in terms of proliferation and modulation of ER and TR in two breast cancer cell lines: MDA-MB-231, which is estrogen-insensitive, but possesses TRα and TRβ, and S30 cell, that is a MDA-MB-231 transfected with wild ERα (19).

METHODS

Cell culture and growth experiments

Cell growth was assessed by using two methods: WST-1 (Roche Diagnostics, Mannheim, Germany) – indirect method, a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial deshydrogenases in viable cells; and cell count – direct method, using a Neubauer chamber and trypan blue staining (Sigma, St Louis, MO, USA). Both experiments were performed in parallel and made in quadruplicates.

MDA-MB-231 and MCF-7 cells were grown in RPMI 1640 medium supplemented with 1.2 g/L NaHCO3, 10 nM Hepes pH 7.4, and 10% fetal bovine serum (FBS). S30 cells were kindly provided by Dr. VC Jordan (Northwestern University, Chicago, IL) and were grown in minimal essential medium (MEM) without phenol red, with Earle's Salts and 25 mM Hepes, supplemented with 1% antibiotic antimycotic solution, 200 nM L-glutamine, 10 nM non-essential amino acids (NEAA), 5% FBS, 6 ng/mL bovine insulin, and 500 μg/mL G418. Both cells were kept at 37 °C in humidified 5% CO_2 and air. The medium was changed every two days. Before starting hormone treatments, the medium was replaced by phenol red-free medium to eliminate all known sources of estrogen, and FBS was replaced by 5% charcoal-stripped FBS. After 24 hours (day 0), media were changed and hormones added in absolute ethanol, with daily media changes. The free hormone levels were further measured directly in the

media by radioimmunoassay and the expected hormone concentrations were detected.

WST-1

The media were changed and hormones added dissolved in absolute ethanol after 24 hours (day 0), with daily media changes. Two hours before the readings of absorbance Spectra Max 190 (Molecular Devices) at a wavelength of 450 nm, 10 µL WST-1 was added to each well to determine proliferation and cell viability. The same procedure was followed for seven days.

Cell count

The media were changed and hormones added dissolved in absolute ethanol after 24 hours (day 0), with daily media changes. Cells were harvested in triplicate at the indicated times and cell numbers were counted. Cell numbers were plotted as a logarithmic function against time and cell population doubling time (DT) was estimated at the exponential phase.

Semi-quantitative reverse transcription-polymerase chain reaction analyses of mRNA for ERα, PR, TRα and TRβ

Total RNA was extracted from cells by TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) which is based on the guanidine thiocyanate method. Frozen cells were mechanically homogenized on ice in 1 mL of ice-cold TRIzol. Total RNA was solubilized in Rnase-free H_2O and quantified by measuring the optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio of approximately 2.0. Two micrograms of RNA was reverse-transcribed with oligo dT primers and Superscript™ reverse transcriptase (RT) in a total volume of 21 µL, according to standard methods (Invitrogen Life Technologies, Carlsbad, CA, USA). Control "No RT" reactions were performed by omitting the RT enzyme. These reactions were then polymerase chain reaction (PCR)-amplified to ensure that DNA did not contaminate RNA. Primer pairs for ERα, TRα, TRβ and PR were designed from a sequence published in GenBank. PCR conditions were as follows: cycles of 94 °C for 30 seconds, annealing of specific primers (Table 1) 1 minute to ERα; 30 seconds to PR, TRα and TRβ, 30 seconds at 72 °C for extension (except ERα, 1 minute). The bands corresponding to each gene were quantified by densitometry as integrated optical density. PCR products were run in duplicate on a different gel for each gene, and results were averaged. The size (then number of base pairs) of each band corresponded to the size of processed mRNA. The PCR signals were normalized by co-amplification (multiplex) of a human cyclophilin transcript with 0.5 µL of each primer, except for ERα PCR, which was performed in simplex. Final numeric values are expressed as a ratio between each and the internal (housekeeping) gene, human cyclophilin.

Statistical analysis

Data are expressed as mean ± sd. Two-way ANOVA was used to analyze cellular proliferation and receptor amplification. Multiple comparisons were performed using the Tukey's test (20). The level of significance was $p < 0.05$.

RESULTS

T3, E2 and steroidal antagonist (TAM) action on cell proliferation

MDA-MB-231 growth was not modulated by the addition of T3, E2 or TAM. The concentrations of E2 (10^{-7}M and 10^{-8}M) were equally effective in stimulating MCF-7 cell proliferation. The simultaneous addition of TAM (10^{-6}M) inhibited the proliferative effect of E2 (data not shown).

Table 1. Oligonucleotide primers used for polymerase chain reaction (PCR) amplification of reverse transcribed RNA

Product	Sequence		T_A (ºC)	Cycles
	Forward primer	Reverse primer		
ERα	5'- AAGACCGAAGAGGAGGGAGA-3'	5'- GATGTGGGAGAGGATGAGGA - 3'	59,8	35
PR	5'- AGCAGTTCTAGTCCCCCAGA -3'	5'- CCTCCTCCTCAACCTCCAC - 3'	56	35
TRα	5'- GACCCAGAGGAGAACAGTGC-3'	5'- CACCCGCTTCGAGTCATCTA - 3'	56	32
TRβ	5'- GAGGAGAACCGGGAGAAAAG -3'	5'- TCTGGGTCATAGCGCACAG - 3'	56	35

T_A: annealing temperature.

S30 growth was not modulated by E2 or TAM. Neither the treatment with two T3 concentrations, 10^{-10}M and 10^{-9}M nor the concomitant addition of TAM affected DT of S30 cells in comparison with controls. The concentration of T3 (10^{-8}M) significantly increased DT and, therefore, significantly reduced cell proliferation when compared to the concentrations of E2, but this effect was not reversed by TAM (Figure 1).

The results of the cell proliferation experiments performed in parallel were similar. Figures 1 and 2 show the data obtained by the cell count method.
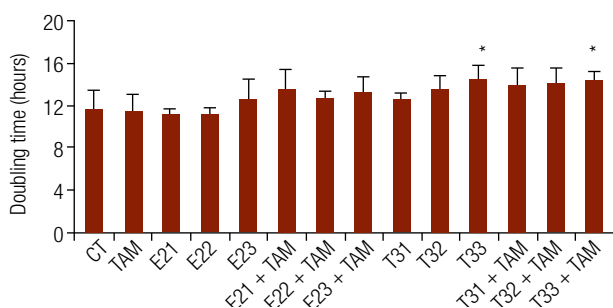

**Figure 1.** Growth experiments in S30 breast cancer cells.

S30 breast cancer cells were cultivated with MEM without phenol red, with Earle's Salts and 25 mM Hepes, supplemented with 1% antibiotic antimycotic solution, 200 nM L-glutamine, 10 nM non-essential amino acids (NEAA), 5% FBS, 6 ng/mL bovine insulin, and 500 µg/mL G418. Cells were seeded (2.5 x 10^3 cells per well) and treated for three days with estradiol 10^{-9}M (E2 1), 10^{-8}M (E2 2), 10^{-7}M (E2 3), triiodothyronine 10^{-10}M (T3 1), 10^{-9}M (T3 2), 10^{-8}M (T3 3), and tamoxifen 10^{-6}M (TAM). Results are mean of 4 simultaneous experiments ± sd with * p < 0.01 (p values determined in relation to E2 1 and E2 2).

Figure 2. Expression of ERα, PR, TRα, TRβ with co-amplification of a human cyclophilin transcript in MCF-7, MDA-MB-231 (MDA) and S30 breast cancer cell lines.

ER, progesterone and thyroid receptors determination in S30 cell

RT-PCR was used to detect ER, progesterone (PR) and thyroid receptors (TR) transcripts in order to better characterize our model. Both MDA-MB-231 and S30 expressed TRα and TRβ, but not PR. Only S30 cell expressed ERα. MCF-7 cells, which express ER, PR and TR, were used as a positive control (Figure 2).

Effects of E2 and T3 in TRα, TRβ and ERα expression

Treatment of either MDA-MB-231 (data not shown) or S30 cells with T3 (10^{-8}M) or E2 (10^{-8}M), both in the absence or presence of TAM (10^{-6}M), did not affect TRα and TRβ levels (data not shown). In S30 cells, the presence of E2 or T3 significantly decreased ERα expression. Association of TAM (10^{-6}M) with either E2 (10^{-8}M) or T3 (10^{-8}M) reverse partially the reduction of ERα expression induced by the presence of the hormones alone (Figure 3).
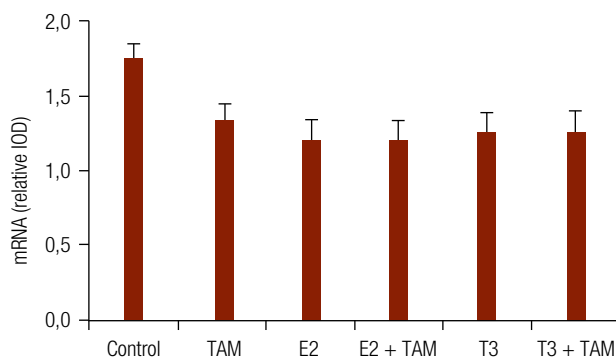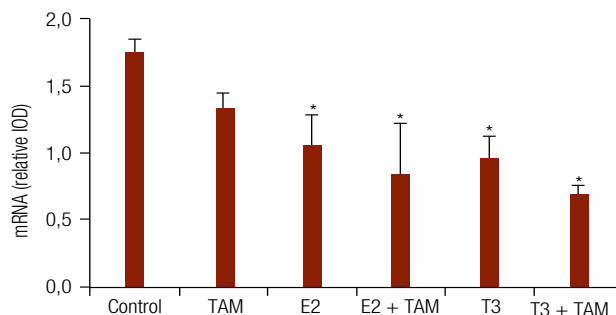
* p < 0.05.

Figure 3. Expression of ERα (A) and TRα (B) transcript in S30 breast cancer cell line estimated by reverse transcription-polymerase chain reaction (RT-PCR) in each treated groups.

Data were run in duplicate on different gels for each gene, and the results were averaged. Quantification of the PCR signal was obtained by densitometric analysis of the product in integrated optical density (IOD). Gene expressions were normalized to the cyclophilin signal from the same RT product. Normalized data are expressed as means ± sd.
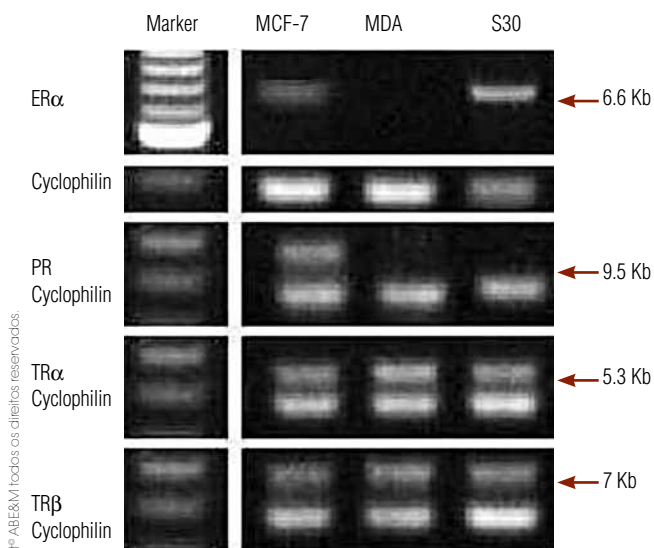
DISCUSSION

The aim of this paper was to expand our previous investigations (Nogueira and Brentani, 1996) suggesting that TH elicits estrogenic effects via ER. The effects of E2 and T3 on the proliferation and the modulation of ER and TR were examined in MDA-MB-231 stably transfected with the wild type ERα (S30) and compared to the parental ER-negative MDA-MB-231 breast cancer cells.

In agreement with our previous results, MDA-MB-231 growth was independent from the addition of E2, T3, and TAM (15). In S30 transfected cells, ER-mRNA levels were similar to those shown by ER responsive MCF-7 cells (21). However, ER expression in MDA-MB-231 did not lead to significant effects on cell proliferation upon E2 treatment. Contrariwise several authors have reported that restoration of ERα expression in MDAMB-231 leads to a ligand dependent inhibition of growth (22-24) an effect blocked by antiestrogens. Moreover, Moggs and cols. (25) demonstrated that this antiproliferative effect of E2 in MDAMB-231 transduced with ER is mediated by down regulation of many genes involved in cell cycle progression as revealed by a microarray approach.

We have no clear explanation for our divergent results. This abnormal behavior may be explained by the specific conditions of our S30 cells. According to Vignon and cols. (26) quiescent cells maintained in low serum concentrations (2%) were insensitive or slightly stimulated by E2 treatment. As late passages of S30 cells were used here, it is possible that ER had lost efficiency, even though its concentrations remained high, as in ER positive hormone-independent tumors. According to Garcia and cols. (22), E2 inhibitory effects are small and reduced the total cell content by only 30-40% after four to six days of treatment. They also observed that the maximal inhibitory effect of E2 (2 nM) was compatible with the occupancy of ER sites and did not increase further with concentrations up to 1 μM. One explanation of our results may be related to the evident reduction of ERα mRNA levels after E2 treatment of S30 cells, leading to no significant effects on cell growth. T3 at 10^{-8}M concentration also reduced ERα gene expression in those cells as compared to controls, suggesting that E2 and T3 actions were similar. As the down regulation is partly inhibited by TAM, it seems that T3 may interact with ERE or influence pathways controlled by ERE. Down regulation ERα protein by E2 was previously reported in MCF-7 and T47D (27), and it has been suggested to be due to the degradation of ER induced by E2. However, unlike E2, 10^{-8}M T3 treatment inhibited S30 proliferation which is the classical effect of E2 in the same cells. As TAM could not reverse the proliferation rate of S30 cells in combination with T3, these data that suggested that this inhibitory effect of T3 is probably not dependent on ERE, pointing to a non-genomic pathway or resulting from different interactions with other promoter elements, including AP1 or SP1 (28,29). According to Zhou-Li and cols. (30), antiestrogens are non competitive inhibitors of T3 only in the cells whose growth is responsive to E2.

In conclusion, our results showed that the effects of T3 in S30 growth differ from those of E2, but both hormones down regulated ERα gene expression. Thus, in absence of E2, clinically important changes in TH levels could influence pathways controlled by ER.

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