Sarcoplasmic reticulum Ca^{2+} ATPases genes differential expression in breast cancer cells

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Abstract

Introduction: Sarcoplasmic reticulum Ca^{2+} ATPases (SERCA) enzymes are essential for intracellular Ca^{2+} homeostasis. SERCA genes (ATP2A1-3) encode for different functional isoforms of the protein, whose expression or function is altered in several types of cancer, such as gastric and oral, as well as colon, breast, lung, thyroid, liver, and prostate cancer, among others. However, the role played by SERCA pumps in carcinogenesis is unknown. **Methods:** Techniques such as real-time polymerase chain reaction, optical microscopy, proliferation and cell death assays, as well as bioinformatic analyses were used. **Objectives:** To evaluate the expression levels of the ATP2A2 and ATP2A3 genes in cell lines representative of different subtypes of breast cancer. **Results:** The results show that the MDA-MB-231 cell line expresses lower mRNA levels for the ATP2A3 gene in comparison with MCF-7 cells. The use of the phytoestrogen resveratrol induces ATP2A3 expression, decreases proliferation, and induces apoptosis in both cell types. **Conclusions:** SERCA expression might function as a tool to differentiate breast cancer subtypes, which have different treatment requirements.

KEY WORDS: SERCA. Cancer. Gene expression. Calcium.

Expresión diferencial de los genes SERCA en células de cáncer de mama

Resumen

Introducción: Las enzimas SERCA son esenciales para la homeostasis intracelular de Ca^{2+} . Los genes SERCA (ATP2A1-3) codifican para distintas isoformas funcionales de la proteína, cuya expresión o función se encuentra alterada en diversos tipos de cáncer, como el gástrico y el oral, así como de colon, mama, pulmón, tiroides, hígado y próstata, entre otros. Sin embargo, se desconoce el papel de las bombas SERCA en la carcinogénesis. **Métodos:** Se utilizaron estudios como reacción en cadena de la polimerasa en tiempo real, microscopía óptica, ensayos de proliferación y muerte celular, así como análisis bioinformáticos. **Objetivos:** Evaluar los niveles de expresión de los genes ATP2A2 y ATP2A3 en líneas celulares que representan diferentes subtipos de cáncer de mama. **Resultados:** La línea celular MDA-MB-231 expresa niveles más bajos del ARNm para el gen ATP2A3, en comparación con las células MCF-7. El uso del fitoestrógeno resveratrol induce la expresión de ATP2A3, disminuye la proliferación e induce apoptosis en ambos tipos de células. **Conclusiones:** La expresión de SERCA puede funcionar como una herramienta para diferenciar los subtipos de cáncer de mama, los cuales tienen distintas necesidades de tratamiento.

PALABRAS CLAVE: SERCA. Cáncer. Expresión génica. Calcio.

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Introduction

Ca^{2+} ion is a second messenger that transmits signals for the regulation of cellular processes such as proliferation, cell death, transcription and malignant cells metabolism¹⁻³. Sarcoplasmic reticulum Ca^{2+} ATPases (SERCA) are among the most important proteins in the maintenance of homeostasis and Ca^{2+} signaling, given that they pump cytosolic Ca^{2+} into the lumen of the endoplasmic reticulum to restore resting state. The *ATP2A1*, 2 and 3 genes encode for different isoforms of the SERCA1, 2 and 3 enzymes, respectively⁴. Expression of these Ca^{2+} pumps is regulated by the type of tissue and stage of development⁵. Alterations in Ca^{2+} signaling are involved in the development of cardiac and neurological pathologies and cancer⁶.

Breast cancer (BC) is the most common neoplasm in women, both in Mexico and worldwide^{7,8}, and despite multiple screening strategies, its incidence continues to increase⁹. The first evidence of SERCA Ca^{2+} pumps expression alteration in BC was provided by Brouland's group, who analyzed SERCA3 expression by immunohistochemistry in patient samples and observed that it was inversely correlated with tumor histological grade, and that it was abundant in normal tissue¹⁰. These findings led to propose SERCA3 as a differentiation marker, since its expression decreases even in precancerous lesions¹⁰.

However, the role of SERCAs is not yet clear, given that SERCA3 expression has been observed to be decreased in certain cancer lineages, such as gastric, colon, breast, liver and lung cancer¹⁰⁻¹⁴. SERCA2 overexpression has also been observed to promote proliferation in colon and prostate cancer^{15,16}.

In this work, we quantify and compare the expression of the *ATP2A2* and *ATP2A3* genes in MCF-7 and MDA-MB-231 BC cell lines, which represent the luminal A and triple-negative subtypes, respectively, and reflect varying degrees of differentiation; MCF-7 is representative of primary cancer, and MDA-MB-231, of metastatic cancer. In addition, we evaluated the effect of resveratrol (RSV), a phytoestrogen that modulates SERCA3 expression on cell proliferation and death, which are crucial cellular processes of cancer².

Methods

Cell cultures and drug treatments

The MCF-7 and MDA-MB-231 BC cell lines were acquired at ATCC®, and their identity was verified by the Genomic Diagnostic Laboratory of the National Institute of Genomic Medicine. MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium with glucose, 10% fetal bovine serum, 0.5% penicillin/streptomycin and 70 mg/L of kanamycin. They were incubated for culture at 37 °C, with 95% air and 5% CO_2 . Non-malignant MCF-10A cells were cultured with DMEM/F12 medium, with 10% fetal bovine serum, insulin, hydrocortisone and human epidermal growth factor. The cells in exponential growth phase were trypsinized and seeded in different culture plates. On reaching 60% confluence, increasing concentrations of RSV (0-200 μM) were added over 72 h, with the culture medium being replaced every 24 h.

Cell proliferation assays

The cells that were ready to carry out the experiment were removed from the culture medium and washed with phosphate buffered saline, lysed with a lysis buffer added with the CyQUANT dye at a 1:80 ratio, and fluorescence was then measured (480 nm excitation and 520 nm emission), directly proportional to the amount of DNA.

Apoptosis assays

Cell death was determined using an assay based on peptide Ac-DEVD-pNA substrate hydrolysis by various caspases and in which pNA was released. The results are presented as the caspase activation index compared to that of the control; the control cells, treated only with the vehicle (ethanol), were regarded as having an activation index of 1.

RNA extraction and real-time polymerase chain reaction (PCR)

RNA concentration was determined by spectrophotometry and its integrity was evaluated by agarose gel electrophoresis with 1% formaldehyde. The reverse transcription reaction was carried out with 1 μg of total RNA with M-MLV reverse transcriptase. Real-time

Table 1. *ATP2A2* and *ATP2A3* genes expression in MCF-10A, MCF-7 and MDA-MB-231 cell lines

Cell line	<i>ATP2A2</i> threshold cycle	<i>ATP2A2</i> normalized expression	<i>ATP2A3</i> threshold cycle	<i>ATP2A3</i> normalized expression	β -actin threshold cycle
MCF-10A	14.79 $\pm$ 0.23	0.68 $\pm$ 0.11	27.25 $\pm$ 0.44	0.0013 $\pm$ 0.0001	8.26 $\pm$ 0.05
MCF-7	14.64 $\pm$ 0.64	1.15 $\pm$ 0.21	16.43 $\pm$ 0.78	1.16 $\pm$ 0.23	9.03 $\pm$ 0.36
MDA-MB-231	13.46 $\pm$ 0.14	1.06 $\pm$ 0.0005	22.89 $\pm$ 0.12	0.0015 $\pm$ 0.0003	7.68 $\pm$ 0.11

Threshold cycle values obtained by qRT-PCR for each gene are shown. Also shown, quantitatively, in the columns highlighted in green, the comparison of *ATP2A2* and *ATP2A3* expression between the three cell lines normalized with β -actin expression and based on expression abundance of these genes in MCF-7 cells. Data are expressed as the average of three independent experiments (n = 3) $\pm$ standard deviation.

PCR (qPCR) reactions were performed in triplicate with 25 ng of complementary DNA as template, which was obtained in the previous step. β -actin expression was used to normalize the results.

Single-cell RNA sequencing public data analysis

To analyze *ATP2A2* and *ATP2A3* genes expression in different BC subtypes, the database with access number GSE75688 was used, which comes from a study where the gene expression profile of 515 cells obtained from 11 patients with BC was analyzed¹⁷. The results of *ATP2A2* and *ATP2A3* expression were analyzed and visualized in the Single Cell Expression Atlas and, for the purposes of this study, the 515 BC cells were divided into four subtypes: ER+ and HER2+; HER2+; ER+; and PR-, ER- and HER2-¹⁸.

Statistical analysis

Data were analyzed using the GraphPad Prism program. The results are expressed as the mean of three independent experiments $\pm$ standard deviation. The comparisons between experimental groups were carried out using Dunnett's method (single-factor ANOVA). Differences were considered to be significant with a p-value < 0.05.

Results

MCF-7 and MDA-MB-231 cells express different levels of *ATP2A2* and *ATP2A3* genes

Using quantitative real-time PCR, we evaluated the abundance of *ATP2A2* and *ATP2A3* genes in MCF-7, MDA-MB-231 and MCF-10A BC cell lines, with the

latter being used as a model of normal breast epithelium. Table 1 shows that the threshold cycle value for mRNA amplification for both genes is similar between the three cell lines for the *ATP2A2* gene, which is why it is concluded that it is expressed at similar levels between these lineages. However, the threshold cycle of the *ATP2A3* gene is lower (Ct1 6.43) in MCF-7 cells than in MDA-MB-231 cells (Ct 22.89), which indicates lower expression of this gene (Table 1). Surprisingly, the MCF-10A cell line showed a lower threshold cycle for *ATP2A3* than the MDA-MB-231 cells (Ct 27.25). The table also shows that *ATP2A2* relative expression with regard to β -actin was similar in all three cell lines. However, *ATP2A3* relative expression was different in the three cell lines and was lowest in the MCF-10A cell line, followed by MDA-MB-231 triple-negative cell line, and was highest in the MCF-7 cell line.

RSV affects cell morphology and number in MCF-7 and MDA-MB-231 cell lines

In order to analyze the effect of RSV on the different cell lines, different concentrations (10, 50, 100 and 200 μ M) were added and cultures were carried out with 12, 24, 48 and 72 hours of treatment. Figures 1A and B show that ethanol (vehicle) has no effect on MCF-7 and MDA-MB-231 cells, respectively. MCF-7 cells show a significant decrease in their number depending on dose-exposure time, especially at high concentrations (> 50 μ M RSV) at 72 h (Fig. 1A). MDA-MB-231 cells were shown to be more resistant than MCF-7 cells (Fig. 1B). In the case of MCF-10A cell line, it is more accentuated with high RSV concentrations (200 μ M), even at short times (24 hours), and shows signs of senescence or stress¹⁹ (Fig. 1C). Low RSV concentrations (between 10 and 50 μ M) do not appear to significantly prevent proliferation, since they behave similar to the control.

Figure 1. Effect of RSV on MCF-7 (panel A), MDA-MB-231 (panel B) and MCF-10A cells (panel C). Upon reaching 60 to 70 % confluence, the cells were exposed to RSV increasing concentrations (10, 50, 100 and 200 μ M) for 12, 24, 48 and 72 hours of treatment, with the medium being changed every 24 hours. The photomicrographs were taken at 10x magnification.

RSV decreases cell proliferation and induces apoptosis in MCF-7 and MDA-MB-231 malignant cells, without affecting non-malignant MCF-10A cells

To quantify cell proliferation, we used a dye (CyQUANT), which binding to DNA, increases its fluorescence, thereby allowing DNA to be quantified by directly measuring the number of cells and their proliferation. Figure 2A shows how RSV decreases the proliferation of MCF-7 and MDA-MB-231 cells after 72 h in a dose-dependent manner. MDA-MB-231 cells are more resistant at low doses, but when RSV concentration is increased, they behave similarly to MCF-7 cells. It can also be observed that MCF-10A cells show no decrease, but their proliferation rate is slow. Ethanol had no effect on the proliferation of the three lineages.

Cell death was also determined by changes in the pNA product absorbance. While in non-malignant MCF-10A cells no significant changes were observed in pNA absorbance, absorbance increases starting at the lower concentrations were detected in malignant

MCF-7 and MDA-MB-231 cells, which indicates that RSV selectively affects malignant cells (Fig. 2B).

ATP2A2 and ATP2A3 expression in BC subtypes

Figure 3A shows the distribution of cells according to the four subtypes: ER+ HER2+ (yellow), ER+ (light blue), HER2+ (blue) and PR-, ER- and HER2- (red). Single-cell RNA sequencing data indicate that *ATP2A2* expression (blue) is homogeneous across all four BC subtypes (Fig. 3B), whereas *ATP2A3* expression is heterogeneous (blue), which shows that the highest levels of expression occur in cells corresponding to the ER+ HER2+ subtype, followed by the PR-, ER-, and HER2- subtype and, finally, the lowest levels were found in ER+ and HER2+ phenotypes (Fig. 3C).

Discussion

SERCA expression or function is altered in pathologies such as cancer⁶, which is why studying its role in carcinogenesis and maintenance of tumor phenotype is important in order to understand its etiology

Figure 2. Proliferation decrease and apoptosis increase in MCF-7 and MDA-MB-231 cells treated with RSV. The cells were treated for 72 hours with increasing concentrations of RSV (0 to 200 μ M); subsequently, the cells were lysed and total DNA was then quantified in order to determine cell proliferation (panel A), and total extracts were incubated with a substrate susceptible to hydrolysis by several caspases as indicative of apoptosis (panel B). Data are expressed as the average of three independent experiments ($n = 3$) $\pm$ standard deviation. Each cell line was compared against its respective control: cells under normal culture conditions. In panel B, absorbance values (A) were directly plotted.

and for possible development of new therapeutic and prognostic strategies. Papp and Brouland reported that precancerous and cancerous lesions derived from patients with BC show almost non-existent levels of SERCA3 expression in comparison with non-neoplastic tissue¹⁰, which suggests that SERCA3 expression loss is an early event within the carcinogenic process. These and other data obtained in gastric, colon and lung cancer^{11,12,20} have led to proposing SERCA3 as a differentiation marker, given that its expression is proportionally inversely correlated with tumor histological grade.

In this work, we used two cell lines that represent different BC subtypes to quantify *ATP2A2* and *ATP2A3* genes mRNA expression. On one hand, the MCF-7 cell line is representative of luminal-type BC, which preserves some epithelial characteristics, while MDA-MB-231 cells represent a triple-negative type, highly aggressive and less differentiated²¹⁻²³.

Sustained proliferative signaling and resistance to apoptosis are distinctive characteristics of cancer²,

which is why the effect of RSV (which selectively modulates *ATP2A3* expression without altering *ATP2A2* expression)²⁴ on these two cellular processes was evaluated. A dose-time-dependent selective decrease in the number of cells in both malignant cell lines was observed to exist (Fig. 1). Figure 2 quantitatively shows that RSV arrests cell proliferation and induces apoptosis.

In the laboratory, we have identified transcriptional and epigenetic mechanisms that control SERCA3 expression in cancer. For example, in gastric and colon cancer, transcription factors Sp1, Sp3 and KL4 are important for *ATP2A3* gene basal transcription¹²; in addition, its expression is also controlled at the epigenetic level, since promoter methylation decreases as the cells differentiate by confluence, which promotes changes in the chromatin structure through histone marks²⁰. Furthermore, in BC cell lines, we observed that RSV induces *ATP2A3* expression through chromatin structure remodeling²⁵, and that *ATP2A3* silencing by RNAi partially prevents RSV-induced cell death²⁴, which is why SERCA3 might be a good target for treating proliferative tumors with high resistance to apoptosis.

The results obtained in our laboratory agree with available evidence, since MDA-MB-231 cells showed lower SERCA3 expression than MCF-7 cells (Table 1). Interestingly, when looking at the MCF-10A cell line, mRNA quantification showed that it expressed less SERCA3 than MDA-MB-231 cells. However, MCF-10A cells are of the basal type and are characterized by an absence of ER-, PR-, and HER2- receptors expression²³. This questions the true usefulness of MCF-10A cells as epithelial breast tissue model^{26,27}. Of note, we found that *ATP2A3* expression varies in the different BC subtypes (Fig. 3C), and that its highest expression levels occur in the ER+ HER2+ subtype, related to MCF-7 cells, followed by the triple-negative subtype (PR-, ER-, and HER2-), related to MDA-MB-231 cells, whereas the lowest levels were found in the HER2+ subtype, which, together with the triple-negative subtype, represent the BC subtypes with the worst prognosis.

All this is important because, although screening strategies to detect BC at earlier stages have been improved, many cases are still detected at advanced stages, where therapy is limited and less efficient, thus discouraging patient prognosis.

Figure 3. ATP2A2 and ATP2A3 expression in four breast cancer subtypes analyzed by single-cell RNA sequencing. Panel A shows the distribution of cells according to the four subtypes: ER+ HER2+ (yellow), ER+ (green), HER2+ (blue) and PR-, ER-, HER2- (red). Panels B and C show the results of ATP2A2 and ATP2A3 expression levels (gradient in blue), respectively. ATP2A2 and ATP2A3 expression was analyzed and visualized in the Single Cell Expression Atlas (EMBL-EBI).

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Conflict of interests

The authors declare that they have no conflicts of interest.

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Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this research.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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