Biochimica et Biophysica Acta 1853 (2015) 2621–2628

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells

Cátia V. Vaz [1], Daniel B. Rodrigues [1], Sílvia Socorro *, Cláudio J. Maia *

CICS-UBI — Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal

ARTICLE INFO

Article history:
Received 14 February 2015
Received in revised form 6 July 2015
Accepted 10 July 2015
Available online 11 July 2015

Keywords:
PNT1A
LNCaP
Prostate cancer
Regucalcin
Calcium
CaSR

ABSTRACT

Extracellular calcium (Ca^{2+}_o) and its receptor, the Ca^{2+}-sensing receptor (CaSR), play an important role in prostate physiology, and it has been shown that the deregulation of Ca^{2+} homeostasis and the overexpression of CaSR are involved in prostate cancer (PCa). Regucalcin (RGN), a Ca^{2+}-binding protein that plays a relevant role in intracellular Ca^{2+} homeostasis, was identified as an under-expressed protein in human PCa. Moreover, RGN was associated with suppression of cell proliferation, suggesting that the loss of RGN may favor development and progression of PCa. This work aims to unveil the role of Ca^{2+}_o on RGN expression and viability of non-neoplastic (PNT1A) and neoplastic (LNCaP) prostate cell lines. It was demonstrated that Ca^{2+}_o up-regulates RGN expression in both cell lines, but important differences were found between cells for dose- and time-responses to Ca^{2+}_o treatment. It was also shown that high $[Ca^{2+}]_o$ triggers different effects on cell proliferation of neoplastic and non-neoplastic PCa cells, which seems to be related with RGN expression levels. This suggests the involvement of RGN in the regulation of cell proliferation in response to Ca^{2+}_o treatment. Also, the effect of Ca^{2+}_o on CaSR expression seems to be dependent of RGN expression, which is strengthened by the fact that RGN-knockdown in PNT1A cells increases the CaSR expression, whereas transgenic rats overexpressing RGN exhibit low levels of CaSR. Overall, our results highlighted the importance of RGN as a regulatory protein in Ca^{2+}-dependent signaling pathways and its deregulation of RGN expression by Ca^{2+}_o may contribute for onset and progression of PCa.

1. Introduction

Prostate cancer (PCa) is the most common male cancer concerning the number of new cases diagnosed, and represents one of the major causes of cancer death in men worldwide [1,2]. Apart from androgens, it is well known the role of calcium (Ca^{2+}) in the physiology of prostate, and the deregulation of Ca^{2+} homeostasis has been intimately associated with development and progression of PCa [3]. Over recent years, a growing body of evidence has indicated that extracellular Ca^{2+} (Ca^{2+}_o), mainly through the activity of extracellular calcium-sensing receptor (CaSR), regulates cell proliferation, differentiation, and apoptosis [4]. In addition, several studies have pointed out that the activation of CaSR is associated with the onset of PCa metastasis [5,6], which strongly highlights the importance of Ca^{2+} signaling and homeostasis in the pathophysiology of prostate gland.

Regucalcin (RGN) is a Ca^{2+}-binding protein that plays an important role in maintaining intracellular Ca^{2+} homeostasis by regulating the activity of Ca^{2+} pumps in plasma membrane and endoplasmic reticulum, and the uptake of Ca^{2+} by mitochondria [7,8]. Also, it was reported that overexpression of RGN suppresses the expression of CaSR [9]. Noteworthy RGN was described as an underexpressed protein in different types of human cancer cell lines and tissues, namely, in the hepatocellular carcinoma (HCC), and in breast and prostate cancers [10–13]. Our previous work also demonstrated that the loss of RGN expression in human PCa cases is negatively correlated with the cellular differentiation of adenocarcinoma [12]. Moreover, it was recently shown that RGN decreases cell proliferation in rat prostate through regulating the expression of cell cycle regulators [14], which supports that reduced levels of RGN may be implicated in development and progression of prostate tumors. Regarding the regulation of RGN expression, it was demonstrated that DHT down-regulates RGN levels in rat prostate and in human androgen-sensitive LNCaP PCa cells. However, the myriad of factors that maintain RGN expression levels in human prostate cells is not entirely known. Considering the importance of Ca^{2+} in prostate pathophysiology, and the role of RGN in Ca^{2+} homeostasis and cell proliferation, this work aims to determine the effect of $[Ca^{2+}]_o$ on the

Abbreviations: Ca^{2+}, Calcium; PCa, Prostate cancer; RGN, Regucalcin; HCC, hepatocelular carcinoma; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; CaSR, calcium-sensing receptor; Tg-RGN, Sprague Dawley transgenic rats overexpressing RGN; qPCR, Quantitative Real-Time PCR; PBS, phosphate buffer saline.

* Corresponding authors at: Faculdade de Ciências da Saúde, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal.
E-mail addresses: ssocorro@fcsaude.ubi.pt (S. Socorro), cmaia@fcsaude.ubi.pt (C.J. Maia).

[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.bbamcr.2015.07.006
0167-4889/

expression of RGN, and on the viability of human non-neoplastic and neoplastic prostate cells. In addition, the expression of CaSR in human non-neoplastic and neoplastic prostate cells, as well as, in the prostate of transgenic animals overexpressing RGN (Tg-RGN), was also investigated.

2. Materials and methods

2.1. Cell lines and experimental layout

The human PCa cell lines, LNCaP and PC3, and the immortalized non-neoplastic human prostate epithelial cell line, PNT1A, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). All prostate cell lines were cultured in DMEM 21068 (Gibco, Paisley, UK), supplemented with 1.8 mM $CaCl_2$ (VWR, Leuven, Belgium), 10% FBS (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in an incubator equilibrated with 5% CO_2.

Cells were grown up to 60% confluence, and 24 h before stimulation, culture medium was changed to a Ca^{2+} free medium, DMEM 21068 (Gibco) containing 10% FBS (Biochrom) and 1% penicillin/streptomycin (Invitrogen). Cultured cells were maintained for additional 24 h and then exposed to different concentrations of $CaCl_2$ (0, 1, 1.8, 3, 5 and 10 mM) for 0, 1.5, 3, 6, 12, 24, 48 and/or 72 h. During the time-course experiment for different Ca^{2+} stimuli, cells were harvested and stored at −80 °C until RNA and protein extraction, or cell viability assays were performed.

For RGN gene knockdown in PNT1A and LNCaP cells, several transfection conditions were previously optimized. Cells were seeded in six plate multiwells and at 40% confluence were transfected with 10 nM of a small interfering RNA (siRNA) targeting the RGN (s17374) (Ambion, USA) and 5 μL of lipofectamine 2000 (Invitrogen, USA) for 24 h in Opti-MEM medium (Invitrogen), following manufacturer's instructions. As control for RGN specific targeting, a Scramble siRNA sequence (AM4635) (Ambion, USA) was used. The medium was replaced to DMEM 21068 (Gibco) supplemented with 1.8 mM $CaCl_2$ (VWR), 10% FBS (Biochrom) and 1% penicillin/streptomycin (Invitrogen), and the RGN gene knockdown was confirmed by Western blot after 36 h of incubation with the siRNA.

2.2. Animals

Wild-type male rats (*Rattus norvegicus*) of Sprague Dawley strain were obtained from Charles River (Barcelona, Spain). Sprague Dawley transgenic rats overexpressing RGN (Tg-RGN) were originally generated by Yamaguchi M by oocyte transgene pronuclear injection [15] and were purchased from Japan SLC (Hamamatsu, Japan) that commercializes the strain. All rats were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) and whole prostates were removed and frozen in liquid nitrogen for protein extraction.

2.3. Total RNA extraction and cDNA synthesis

Total RNA from human prostate cell lines was extracted using the TRI reagent (Sigma-Aldrich, Saint Louis, Missouri, USA) according to the manufacturer's instructions. The quantity and quality of extracted RNA were assessed by spectrophotometry (Pharmacia Biotech, Ultrospec 3000, Cambridge, England) and agarose gel electrophoresis, respectively. For cDNA synthesis, 1 μg of total RNA was denatured for 5 min at 70 °C together with 250 ng of random hexamer primers (Invitrogen), and reverse transcription was carried out at 37 °C for 60 min in a 20 μl reaction containing reverse transcriptase buffer, 1 μl of dNTP Mix (10 mM each; GE Healthcare, Buckinghamshire, UK) and 200 U of M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). The reaction was stopped at 75 °C for 15 min and synthesized cDNA was stored at −20 °C until further use.

2.4. Quantitative Real-Time PCR (qPCR)

Quantitative Real-Time (qPCR) was carried out to evaluate the mRNA expression of RGN in human prostate cell lines (PNT1A and LNCaP), in response to Ca^{2+} stimuli. Specific primers for human RGN (Sense: 5′-GCAAGTACAGCGAGTGACC-3′; antisense: 5′-TTCCCATCAT TGAAGCGATTG-3′) amplified a fragment of 177 bp. Human beta-2-microglobulin (β_2M) (Sense: 5′-ATGAGTATGCCTGCCGTGTG-3′; antisense: 5′-CAAACCTCCATGATGCTGCTTAC-3′) and GAPDH (sense: 5′-CGCCAGCCGAGCCACATC-3′; antisense: 5′-CGCCCAATACGACCAAAT CCG-3′) primers were used as internal controls to normalize RGN expression. qPCR reactions were carried out in IQ5 system (Bio-Rad, Hercules, USA) and the efficiency of amplifications was determined for all primer sets using serial dilutions of cDNA (1, 1:10 and 1:100). PCR conditions and reagents concentrations were previously optimized and the specificity of the amplicons was determined by melting curves analysis. For qPCR reactions, 1 μl of synthesized cDNA was used in a 20 μl reaction containing 10 μl Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas, Burlington, Canada) and sense and antisense primers (200 nM for RGN and 300 nM for β_2M and GAPDH). Reaction conditions comprised 5 min denaturation at 95 °C, followed by 35 cycles of 95 °C for 10 s, a specific annealing temperature of 60 °C for 30 s and 72 °C for 10 s. Samples were run in triplicate in each PCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ [16].

2.5. Western blot

Total proteins were extracted from human prostate cell lines (PNT1A and LNCaP) and rat prostate using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8 and 1 mM EDTA) supplemented with protease inhibitors cocktail. Protein concentration was determined by the Bradford assay (Bio-Rad) and approximately 60 μg of cell and tissue protein extracts was resolved by SDS-PAGE on 12% gels and electrotransferred to a PVDF membrane (GE Healthcare). Membranes were incubated overnight at 4 °C with mouse anti-RGN (1:1000, ab81721, Abcam, Cambridge, United Kingdom) or mouse anti-calcium sensing receptor clone HL1499 (1:1000, C0493, Sigma-Aldrich) primary antibodies. A mouse anti-β-actin antibody (1:5000, A5441, Sigma-Aldrich) was used for normalization of protein expression. Membranes were incubated for 1 h with goat anti-mouse IgG + IgM-AP (1:5000, NIF1316; GE Healthcare) used as secondary antibody. Finally, membranes were incubated with ECF substrate (GE Healthcare) for 3 min, and visualized on the Molecular Imager FX Pro plus MultiImager (Bio-Rad). Band densities were obtained according to standard methods using the Quantity One Software (Bio-Rad) and normalized by division with the respective β-actin band density.

2.6. Immunofluorescence microscopy

PNT1A and LNCaP cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 during 5 min. A blocking step was performed by incubating cells with 20% FBS in phosphate buffer saline (PBS) containing 0.1% tween®-20 (PBST) for 1 h at room temperature, and then, cells were incubated with anti-RGN antibody (1:50, ab81721, Abcam) for 1 h at room temperature. The Alexa fluor 488 and 546 conjugated goat anti-mouse IgG (Invitrogen) were used as secondary antibodies. The specificity of the staining was accessed by omission of the primary antibody. Cell nuclei were stained with Hoechst 33342 (10 mg/ml, Invitrogen) for 10 min. Lamellae were washed and mounted onto microscope slides with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany).