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Investigating the interplay between sarcosine and Ca^{2+}-dependent signaling in prostate cells

Vladislav Strmiska[1,2], Hana Buchtelova[1], Petr Michalek[1,2], Sona Krizkova[1,2], Vojtech Adam[1,2], Zbynek Heger[1,2]
[1]Department of Chemistry and Biochemistry
Mendel University in Brno
Zemedelska 1, 613 00 Brno
[2]Central European Institute of Technology
University of Technology in Brno
Technicka 3058/10, 615 00 Brno
CZECH REPUBLIC

vladislav.strmiska@mendelu.cz

Abstract: It has been shown that sarcosine supplementation stimulates proliferation and invassivenes of prostate cells. Nevertheless, the exact molecular mechanism responsible for this phenomenon is not known. In the present study we demonstrate that sarcosine increases expression of calmodulin (CaM), an important intracellular signaling molecule. Through this, sarcosine activates calmodulin-dependent protein kinases signaling. Pathway of activation CaM-dependent protein kinases can activate regulation of mitosis, proliferation, cell death, gene transcription and phosphorylation/ dephosphorlation of proteins. This is done through CaM binding of four Ca^{2+} ions. Interestingly, in this study, we identified decrese in free Ca^{2+} correlating with sarcosine-induced up-regulation of CaM. The influence of CaM to cell cycle changes was further verified using post transcritrional gene silencing using CaM-siRNA complex. Co-treatment of prostate cells with CaM-siRNA and sarcosine showed decrease in CaM-dependent kinases and cell invasiveness compared to sarcosine treatment only.

Key Words: sarcosine, calmodulin, prostate, prostate cancer, human cells

INTRODUCTION

Sarcosine is an imino acid and a potential biomarker of prostate cancer (PCa). Concentration of sarcosine is substantially increased during PCa progression to its metastasis (Sreekumar et al. 2009).

Calmodulin (CaM) is a ubiquitous calcium-binding protein (Zayzafoon 2006). It is responsible for intracellular interactions connected with regulation of proliferation and malignity (Rasmussen and Means 1989). CaM acts primarily through CaM-dependent signalling pathways connected into regulatory system important for cellular pathophysiology (Zayzafoon 2006). CaM-dependent protein kinases are activated after presence of CaM in neighbouring subunits activated by Ca^{2+} ions (Rokhlin et al. 2007). If CaM is accumulated in sufficient amount, autophosphorylation occurs, leading to persistent activation of the enzyme (Wang et al. 2015). The major mediator system affecting cellular proliferation driven by Ca^{2+}/CaM is CaM-dependent protein kinase II (CaMKII). CaMKII phosphorylate over 40 different proteins, including enzymes, kinases and transcription factors (Erickson 2014).

Our results demonstrate a unique connection between sarcosine and CaM-dependent signaling through up-regulation of CaM-dependent kinases. We also show that sarcosine affects Ca^{2+} homeostasis. Moreover, functional siRNA analyses revealed an importance of CaM-sarcosine interplay in proliferation and clonogenicity of prostate cells.

MATERIAL AND METHODS

Prostatic cell lines

Three human prostatic cell lines were used for an experiment, representing benign and malignant cells: *i*) the PNT1A human cell line established by immortalization of normal adult prostatic

epithelial cells by transfection with a plasmid containing SV40 genome with a defective replication origin *ii*) LNCaP human cell line estabished from an androgen-sensitive metastasis located in the left supralavicular lymph node, *iii*) PC3 human prostate adenocarcionoma cell line established from a grade 4 prostatic adenocarcinoma. All cell lines used for experiments were purchased from Health Protection Agency Culture Collections (Salisbury, UK).

Culture conditions and treatment protocols

All cell lines were culture in RPMI-1640 medium with 10% fetal bovine serum and supplemented by penicillin (100 U/mL) and streptomycin (0.1 mg/mL). The cells were maintained at 37 °C in humidified incubator with 5% CO_2. The exogenous supplementation with sarcosine (10 μM) and siRNA (200 pM) was initiated after cells reached ~80% confluence. The cells were harvested after 24; 48 and 72 h for western blotting, after 24 h for immunofluorescence and FURA-2. All experiments were designed as five biological replicates (n = 5) measured three times at each time point.

2D-DIGE proteome alanysis

Total cellular protein were isolated form cells stimulated by sarcosine (10 μM) after 24 h and un-treated group as a control. Compared group of proteins were labeled with fluorescen dyes directly and each 50 μg of proteins were separate by isoelectric point (IPG: 7 cm, pH 3 to 10). After that for second dimension were used 12.5% SDS-PAGE. Evaluated were different spots of protein expression between sarcosine stimulated and un-stimulated group of cells by Azure c600imager (Azure Biosystems, Dublin, CA, USA).

Imunocytometry (ICC) of CaM

For ICC, the cells were seded into eight-well chamber slides and after 24 h of adherence were treated by sarcosine (10 μM). As a control were used cells without treating. Cells were fixated after 24 h incubation by 4% formaldehyde, permeabilizated by 0.25% Triton X-100, blocked in 5% bovine serum albumin in phosphate buffered saline (PBS) and imunostained with primary antibody overnight in 4 °C. Detection was accomplished using CruzFluor™ 645 (CFL 645) labeled secondary antibody. DNA staining by Hoechst were used for counter. ICC was evaulated by confocal laser scanning microscope (CLSM) Carl Zeiss LSM 880 (Carl Zeiss, Jena, Germany).

Western blot of CaM and CaM-dependent kinases

Total cellular proteins were extracted with 100 μL of thiourea (2 M) buffer containing protease inhibitor cocktail. After electrophoresis, the proteins were electrotransferred onto a polyvinylidene fluoride membrane, with the rest of membrane surface blocked in 5% (w/v) bovine serum albumin in PBS for 1 h at 37 °C to avert non-specific binding. Membranes were incubated with primary rabbit anti-CaM (dilution 1:1000), rabbit anti-CaMKII (dilution 1:1000), rabbit anti-CaMKIV (dilution 1:200), rabbit anti CaMKK (dilution 1:100), rabbit anti-CaMKK2 (dilution 1:1000) or mouse anti-GAPDH (dilution 1:700) antibodys, overnight at 4 °C. After washing, membranes were incubated with goat anti-rabbit or goat anti-mouse secondary antibodies HRP-linked (Cell Signalling, Leiden, Netherlands)for 1 h at 20 °C. The chemiluminiscence was indicated by Bio-Rad Immun-Star HRP Luminol/Enhancer and detecton by Azure c600imager (Azure Biosystems, Dublin, CA, USA).

Free cytosolic Ca^{2+}

Intracellular free Ca^{2+} ions were evaluated by high affinity fluorescent selective indicator. Cells were seded into six-well plate and treated by transfection medium with control-siRNA and CaMI-siRNA (200 pM), sarcosine (10 μM)for 24 h and un-treated cells as a control group. Cells were subsequently labeled with Fura 2 acetoxymethyl ester (10 μg/mL) (Fura-2 AM, Abcam). The ratio of the emission correlated to the free amount of intracellular Ca^{2+} concentration. DNA staining by elipticine were used for nuclei conterstaining. Imaging was performed using EVOS FL Auto Cell Imaging System (Thermo-Fisher, Waltham, MA, USA).

Wound-healing assay (Scratch test)

The cells were seeded into 6-well plate to reach confluence ~80%. After seeding a pin was used to a scratch and remove cells from a discrete area of the confluent monolayer to form a cell-free zone. After that, cells were treated with sarcosine (10 μM), transfection medium with control-siRNA, CaMI-siRNA and untreated group as a control. After 6; 12, 24 and 48 h, the micrographs of cells were taken

using EVOS FL Auto Cell Imaging System and compared with micrographs obtained in 0 h, using TScratch software.

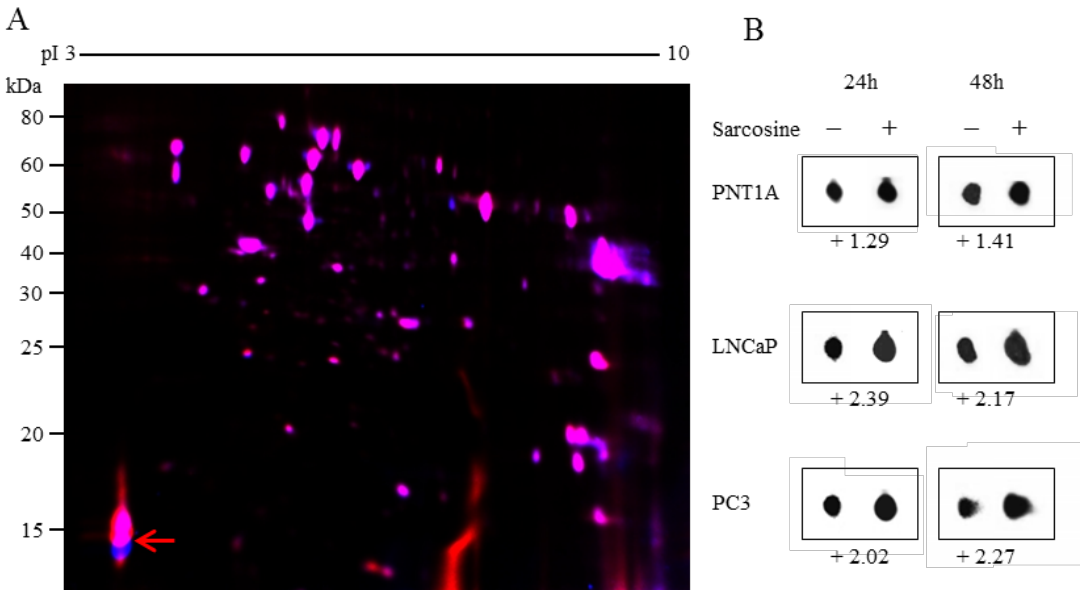
Descriptive statistics

For the statistical evaluation of the results, the mean was taken as the measurement of the main tendency, while standard deviation was taken as the dispersion measurement. Differences between groups were analyzed using paired t-test. Unless noted otherwise, the threshold for significance was $p < 0.05$. For analyses Software Statistica 12 (StatSoft, Tulsa, OK, USA) was employed.

RESULTS AND DISCUSSION

First, we compared differentially expressed proteins sarcosine treated cells and control non-treated group. Figure 1 illustratse representative 2D-DIGE proteomic signature with a quantitation of expression of spots annotated as CaM (IP 3.9, M_w 17 kDa). It clearly follows from the data that sarcosine influences proteome of prostate cells and up-regulates CaM. CaM can be further used in cells as an signaling molecule for CaM-dependent pathways, which can be connected with cancer progression and invasiveness.

Figure 1 (A) Representative 2D-DIGE of PCa cells (LNCaP) proteome. Red arrow shows spot identified as CaM. (B) Densitometry of CaM spots from 2D-DIGE for tested cell lines in two times of treatment (24 and 48 h).



CaM expression was also evaluated by CLSM (Figure 2) that indicates intracellular localization of CaM and also a significant up-regulation due to sarcosine treatment. All three tested cell lines show increased expression of intracellular CaM after sarcosine supplementation.

We further investigated CaM expression upon 24 and 48 h of sarcosine stimulation compared control. Figure 3 depicts increased expression for CaM in time. Noteworthy, expression of CaM-dependent kinases decreased after siRNA-mediated knock-down of CaM. This fact demonstrates that sarcosine can plausibly affect cell cycle directly through the intracellular messenger CaM.

Intracellular Ca^{2+} activates CaM. This means that intracellular level of Ca^{2+} could be bound and homeostasis should be altered. Indeed, different intracellular concentration of Ca^{2+} (Figure 4) correlated with expression of CaM. Lower free Ca^{2+} concentration shows higher occurrence upon up-regulation of CaM by sarcosine. Upon silencing of CaM, increased pool of Ca^{2+} occurs. Upon CaM silencing combined with sarocisne exposure, free Ca^{2+} increases, confirming a direct effect of sarcosine to CaM.

Figure 2 Representative ICC of CaM in tested cells after 24 h treatment with sarcosine (10 μM) and control un-treated group.

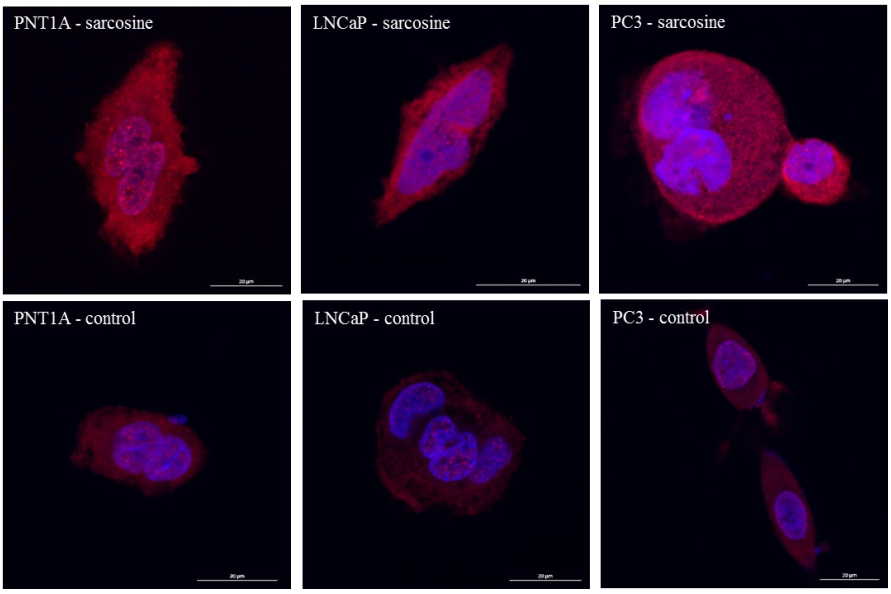


Figure 3 (A) Western blot validation of CaM expression. (B) Expression of CaM-dependent kinases upon CaM silencing and parallel administration to sarcosine.

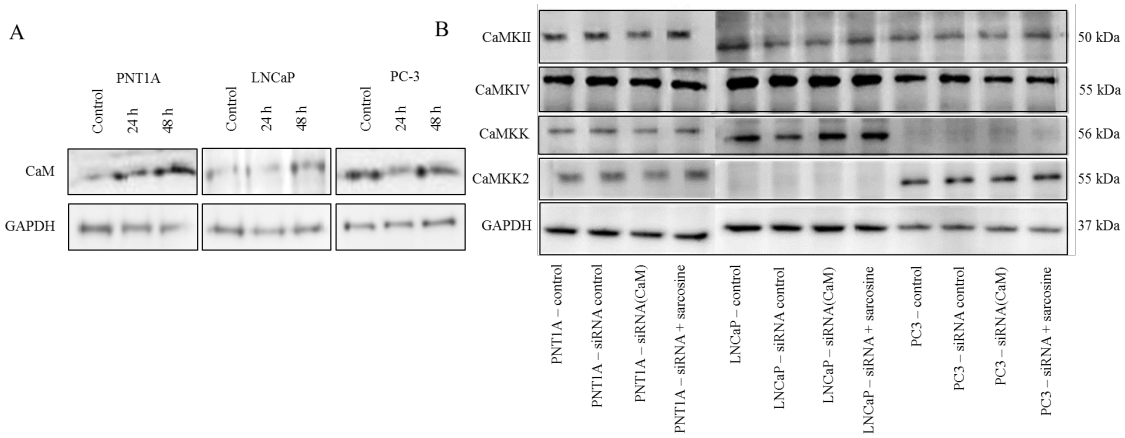
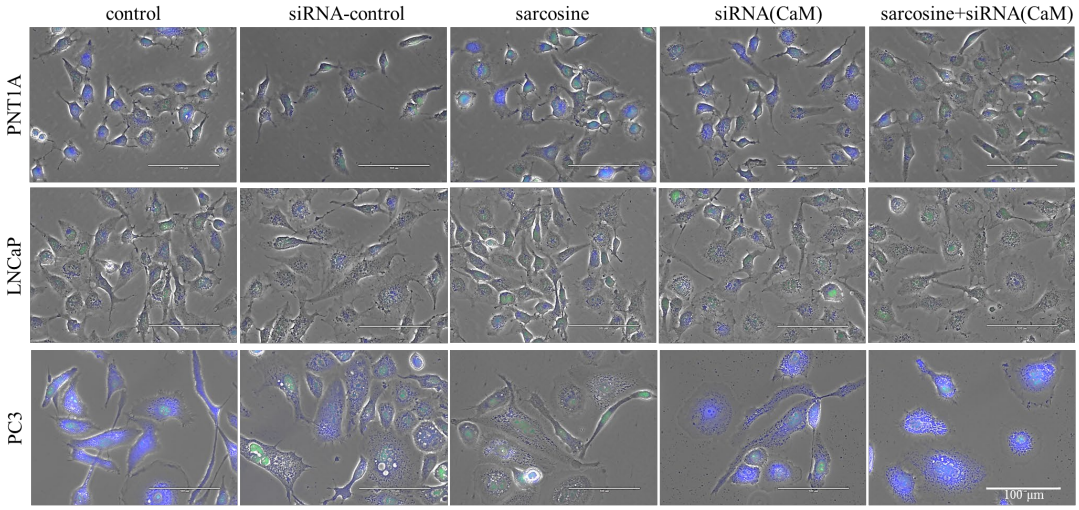


Figure 4 Concentraion of free intracellular Ca^{2+}in PCa cell after sarcosine treatment and CaM silencing. Ca^{2+} is shown in blue and nuclei in green. The scale bar is 100 μm.



CONCLUSION

Sarcosine up-regulates CaM in prostate-derived cells, and therefore alters the Ca^{2+} homeostasis and pathways drived by CaM-dependend kinases. This signaling cascade can alter cell cycle and proliferation. The need of calcium in this processes has been demonstrated many times. Possibility of silencing CaM can be a new modality to decrease tumor progression, including frequently developing bone methastases.

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