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Cellular differentiation analysis using SERCA3 protein expression in cancer cells.

diploma thesis

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Acknowledgments

Declaration

I hereby declare I have worked on this project solely by my own with the use of referenced literature and I am presenting my own original data.

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CONTENT

ABBREVIATIONS

IID8   Primary anti-SERCA2 antibody

AP1   Activator protein 1

APL   Acute promyelocytic leukaemia

ATCC   American Type Culture Collection

ATP   Adenosine triphosphate

ATRA   All-*trans*-retinoic acid

But   Butyrate

cAMP   Cyclic adenosine monophosphate

cADPR   Cyclic adenosine diphosphate ribose

CaM   Calmodulin

CEA   Carcioembryonic antigen

CICR   Calcium-induced calcium release

CPA   Cyclopiazonic acid

DAG   Diacylglycerol

ECL   Enhanced chemiluminescence

ER   Endoplasmic Reticulum

FBS   Fetal bovine serum

HC-toxin   Helminthosporium carbonum-toxin

HDAC   Histone deacetylase

IgG   Imunoglobulin G

IL-2   Interleukin-2

InsP$_3$R   Inositol-1,4,5-triphosphate receptor

mM   Milimolar

NAADP   Nicotinic acid adenine dinucleotide phosphate

NCXs   Na$^+$/Ca^{2+} exchangers

NF-AT   Nuclear factor of activated T-cells

NF-κB   Nuclear factor kappa B

NSCLC   Non small cell lung cancer

PB   Phenylbutyrate

PIP$_2$	Phosphatidylinositol-4,5,-bisphosphate
PKC	Protein kinase C
PLB	Phospholamban
PLCβ	Phospolipase Cβ
PLCγ	Phospolipase Cγ
PLIM430	Primary anti-SERCA3 antibody
PMA	Phorbol myristate acetate
PMCA	Plasma membrane Ca^{2+}-ATPase
RARα	Retinoic acid receptor α
ROCs	Receptor-Operated Channels
rpm	revolution per minute
RYR	Ryanodine receptor
SAHA	Suberoylanilide hydroxamic acid
SCFA	Short-chain fatty acids
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SERCA	Sarco/Endoplasmic Reticulum Calcium ATPase
SOCs	Store-Operated Channels
S1P	Sphingosine-1-phosphate
SPCA	Secretory-pathway Ca^{2+}-ATPases
SR	Sarcoplasmic Reticulum
tBHQ	2,5,-di-tert-butyl-1,4,-benzohydroquinone
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	NN,N'N'-tetramethyl-ethylenediamine
TG	Thapsigargin
TKR	Tyrosine kinases
TRIS	Tris-hydroxymehtyl-aminomethane
TWEEN 20	Polyethylen sorbitan monolaurate
Val	Valerate
VOCs	Voltage-Operated Channels

1. INTRODUCTION

Classical chemotherapy of cancers is associated with serious risk for the patient, due to numerous adverse effects of the cytotoxic drugs currently used. In addition, resistance of tumour cells to treatment and consequent relapse occur very frequently. Classical cytotoxic chemotherapeutics do not target tumour cells selectively. Rather, these molecules damage dividing cells in general and have often dose limiting toxicities in actively proliferating normal tissues such as the bone marrow or the gastrointestinal epithelium. Considerable effort is being made to develop more targeted therapies for cancer, that attack tumour cells more selectively and therefore with less side effects.

One possible way to achieve this goal is the use of molecules that induce cell differentiation, because the loss of the ability to undergo terminal differentiation is a major characteristic of cancer cells. It has recently been discovered that histone deacetylase inhibitors can induce growth arrest and cell differentiation in various types of human malignancies, and several histone deacetylase inhibitors are currently in Phase I and II in clinical trials for cancer.

We have previously described that induction of cell differentiation in acute promyelocytic leukaemia or colon and gastric carcinoma leads to significant changes of cellular calcium homeostasis. In particular, we have shown that the expression of the Sarco/Endoplasmic Reticulum Calcium ATPase type 3 calcium pump enzyme (SERCA3) is strongly induced during differentiation. SERCA2 and SERCA3 enzymes that are simultaneously expressed in these cells accumulate calcium in the endoplasmic reticulum and therefore play an important role in the control of calcium-dependent cell activation. These observations show that 1) detection of SERCA3 expression may be a useful new tool for the study of the phenotype of human tumours, and that 2) the investigation of endoplasmic reticulum calcium homeostasis may help to better understand the mechanisms of cell differentiation defects observed in malignancies.

Based on the above observations we are currently investigating whether a correlation between SERCA3 expression and cell differentiation can also be observed in other types of human cancers. In the present work we studied the expression of SERCA2 and SERCA3 in various types of lung cancer and the modulation of SERCA expression during histone deacetylase-induced differentiation of lung cancer cells. Our results show that, similarly to colon and gastric cancer, SERCA3 expression is selectively induced also in lung carcinoma cells undergoing histone deacetylase-induced differentiation, whereas the expression of the simultaneously expressed SERCA2 isoform remained unmodified. Our results show that deficient SERCA3 expression in cancer is a more general phenomenon than thought previously, and that the analysis of SERCA3 expression may be useful for the analysis of the phenotype of lung tumours as well.

3. THEORETICAL PART

2.1. Lung cancer

Currently, lung cancer is the most common cancer in the world and is the leading cause of cancer death in industrial nations [17,18,19]. The majority of cases is directly attributed to tobacco smoking [19], and cases of lung cancer observed in non-smokers are frequently related to genetic cancer predisposition syndromes [20]. The prognosis of the disease is very poor with a five years survival rate of around 10% only [19]. Almost all lung cancers are carcinomas, malignancies that arise from epithelial cells. They are classified into two main types: small cell lung carcinomas (SCLC) and non-small cell lung carcinomas (NSCLC).

The majority of lung cancer is represented by NSCLC (aroud 80%) with different incidence of various histological types of NSCLC at men and women, as well as in different populations. SCLC comprise about 20% of cases. Besides these two main types of lung cancer, there are also less common forms of lung tumours, such as typical and atypical carcinoid and large cell neuroendocrine carcinoma.

NSCLC can be divided in the following main groups: adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, bronchioalveolar carcinoma, and large cell carcinoma. The incidence of adenocarcinoma has recently surpassed that of squamous cell carcinoma in many countries [19].

In addition to histological type, NSCLC can be characterized according to the state of morphological differentiation into well, moderately and poorly differentiated types. The state of differentiation is an important parameter in NSCLC that is included in the regular pathology report of specimens, and has significant prognostic value. Moreover, lung adenocarcinoma cell lines *in vitro* can be induced by various treatments to undergo cell differentiation, as detected by the induction of the expression of various differentiation markers, such as gelsolin, p21 and others [21].

In the present work we investigated SERCA expression in cell culture models of NSCLC differentiation. We observed a significant, previously unknown relationship between the expression of the SERCA3 protein and cellular differentiation in this pathology. Our observation may help to better

understand the role of cellular calcium homeostasis in lung carcinogenesis, and may help to propose SERCA3 as a new marker of differentiation in lung tumours.

2.2. Calcium homeostasis

The maintenance of Ca^{2+} homeostasis in cells is an important regulatory mechanism. Ca^{2+} is a ubiquitous second messenger that is involved in many cellular processes [1]. This ion controls various processes in the cell from the beginning of its life to its death. Fertilization, control of development and differentiation of cells, gene transcription and cellular proliferation belong to these processes [1,2]. In cells that have been already differentiated, Ca^{2+} participates in muscle contraction, exocytosis, energy metabolism, chemotaxis and synaptic plasticity during learning and formation of memory [3,4].

The concentration of intracellular (cytosolic) Ca^{2+} is held around 20-100nM. The rise of $[Ca^{2+}]_i$ is a signal for numerous functions in the cell, cells are activated when $[Ca^{2+}]_i$ reaches a concentration around 1000nM. There are two ways to elevate intracellular concentration of calcium: calcium comes to cytosol either from extracellular space through plasma membrane or it is released from endoplasmic reticulum (ER), where calcium is stocked.

Other important organelles participating in the regulation of Ca^{2+} homeostasis are mitochondria, the Golgi complex and the nucleus [5]. The control of Ca^{2+} homeostasis is thoroughly regulated by a large system of various proteins (for example: receptors, Ca^{2+} binding proteins, pumps, channels and exchangers) these proteins that regulate cellular calcium-homeostasis taken together can be called „the Ca^{2+} signaling toolkit" [1].

<u>Calcium signaling:</u>

The process of Ca^{2+} signaling can be divided into four consecutive steps:

1. Signaling is triggered by a stimulus that generates various Ca^{2+} - mobilizing signals

2. These signals activate ON mechanisms that feed Ca^{2+} into the cytoplasm

3. Ca^{2+} functions as a messenger to stimulate numerous Ca^{2+} -sensitive processes

4. The OFF mechanism removes Ca^{2+} from the cytoplasm to restore the resting state

<u>ON mechanism:</u>

Cells accept Ca^{2+} signals from two sources, from the outside and from internal stores. Ca^{2+} enters from the outside through a variety of channels such as voltage-operated channels (VOCs), receptor-operated channels (ROCs), that open on binding external stimuli, usually transmitters such as glutamate, ATP or acetylcholine, or stored-operated channels (SOCs), channels in the plasma membrane which are activated by depletion of internal stores. Ca^{2+} released from internal stores provides most of the signal Ca^{2+} in most cells. There are two main types of Ca^{2+} channels: the ryanodine receptor (RYR) family and the inositol-1,4,5-triphosphate family receptor ($InsP_3R$) channels. Ca^{2+} release from internal stores, located primarily in the endoplasmic reticulum (ER) or in the equivalent organelle sarcoplasmic reticulum (SR) of muscle cells, is controlled by Ca^{2+} itself or by messengers such as inositol-1,4,5-triphosphate ($Ins(1,4,5)P_3$), cyclic ADP ribose (cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP) and sphingosine-1-phosphate (S1P) [4].

<u>InsP$_3$ and ryanodine receptors:</u>

These two principal intracellular calcium channels responsible for mobilizing stored calcium share considerable structural and functional homologies. The RYR has three family members: RYR1 found in sceletal muscle and certain neurons (Purkinje cells), RYR2 found in cardiac muscle, brain, as well as in some other cells, and RYR3 is found in smooth muscle, brain and other cells. The plant alkaloid ryanodine opens the channels at low (nanomolar) concentrations but closes them at higher doses (>micromolar). Caffeine can also open the RYR channels. Second, the InsP$_3$R family has a number of members. There are four InsP$_3$R genes, and further diversity results from alternative splicing [6].

<u>CICR (Calcium-induced calcium release):</u>

CICR is a positive feedback process whereby calcium triggers its own release. It is caused by a small influx of Ca^{2+} through VOCs and this incites an explosive release of stored calcium from the SR of muscle. This process of CICR contributes to the rapid rise of Ca^{2+} levels during the ON reaction and the development of the regenerative Ca^{2+} waves.

Both InsP$_3$R and RYR are under the dual regulation of two agonists, so Ca^{2+} release is induced by Ca^{2+} itself or by other Ca^{2+} mobilizing messengers: InsP$_3$, sensitive to InsP$_3$R and cyclic ADP ribose (cADPR), binding to RYR.

The function of InsP$_3$R and RYRs is determined by the concentration of Ca^{2+}. They are inactive at low nM concentration of Ca^{2+}, active at low μM concentration of Ca^{2+} and inactivated by high concentration of Ca^{2+} in the mM range [1]. Calcium contained within ER is released to the cytosol when InsP$_3$ binds to its receptor. There are two important pathways to form InsP$_3$, one initiated by a family of G-protein-linked receptors and the other by receptors linked by tyrosine kinases. G protein coupled receptors activate phospoliphase Cβ (PLCβ) and TKR activate PLCγ to cleave phosphatidylinositol-4,5,-bisphosphate (PIP$_2$) into IP$_3$ and DAG [6].

<u>OFF mechanism:</u>

During the OFF reaction, Ca^{2+} is rapidly removed from the cytoplasm by various pumps and exchangers. The plasma membrane Ca^{2+}-ATPase (PMCA) pumps and Na^+/Ca^{2+} exchangers extrude Ca^{2+} to the outside, whereas the sarco-endoplasmic reticulum ATPase (SERCA) pumps return Ca^{2+} to the internal stores. Another type of Ca^{2+} pumps, discovered recently, is the SPCA pump. These secretory-pathway Ca^{2+}-ATPases are closely-linked with SERCA, but they are responsible for Ca^{2+} sequestration into Golgi compartments [4].

2.3. Calcium pumps and exchangers

2.3.1. Plasma membrane Ca^{2+} pumps

The PMCA belongs to the P-type ATPase pump family which transports Ca^{2+} from the cytosol to the external environment. Human PMCAs have four major isoforms (PMCA 1, 2, 3, 4) that are encoded by four genes (ATP2B1-4). The isoforms are differently expressed in tissues and cell types and have differential Ca^{2+} extruding properties. At very low Ca^{2+} concentration these pumps are nearly inactive. They must be activated by calmodulin, acid phospholipids, protein kinases or by other means, for example a dimerization process. The reaction cycle of the PMCA pumps comprises two conformational states of the phosphorylated PMCA, E1 and E2 that have a different affinity to Ca^{2+}. This process is similar to the catalytic cycle of SERCA pumps which will be described later. There is only one difference: PMCAs hydrolyze 1 ATP molecule per Ca^{2+} ion transported at variance with SERCAs, which transport 2 Ca^{2+} ions per ATP hydrolyzed. Their molecular masses is between 125 and 140kDa and their structure is formed by intracellular N- and T-terminal tails, 10 membrane-spanning segments and two major cytosolic loops. The N- and T-terminal tails are among the least conserved regions in PMCAs and they are involved in numerous protein-protein interactions of functional importance including binding of the major regulator calmodulin (CaM).

The diversity of the PMCA family is enhanced by alternative RNA splicing affecting two major sites called A and C. The splice variants differ in the legth of the first intracellular loop (site A splicing) and in the C-terminal tail (site C splicing). These PMCAs variants show developmental-, tissue- and cell-specific patterns of expression and functional adaptation to the specific Ca^{2+} handling requirements of the cell. The genes for PMCA1 and PMCA4 isoforms are expressed ubiquitously, whereas the genes for PMCA2 and PMCA3 are expressed in a tissue restricted way: in human PMCA2 is expressed in brain and also in the mammary gland, PMCA3 is expressed in the brain and in skeletal muscles. PMCA1 is ubiquitous, but the most is expressed in brain, lung

and intestine, PMCA4 in kidney, erythrocytes, skeletal muscle, heart, stomach, intestine, brain and spermatozoa [7,8].

2.3.2. Na$^+$/Ca^{2+} exchangers

Na$^+$/Ca^{2+} exchangers (NCXs) are situated in the plasma membrane and ensure the movement of intracellular Ca^{2+} to the outside and extracellular Na$^+$ to the cytoplasm. Five isoforms of NCXs have been identified. NCX1, NCX2 and NCX3 are mammalian isoforms. Others were cloned from squid axon- NCX-SQ1 and Drosophila- CALX. (7) During transport, influx of three ions of Na$^+$ is coupled to the efflux of one ion of Ca^{2+}. This process depends on Na$^+$, Ca^{2+} and K$^+$ gradients across the plasma membrane and the membrane potential [9].

2.3.3. SPCA pumps:

SPCA are the recently discovered pumps, first found in yeast. They deliver not only Ca^{2+} but also Mn^{2+} into the secretory pathways (the Golgi lumen). Two genes *ATP2C1* and *ATP2C2* provide two protein isoforms, SPCA1 and SPCA2. Like SERCA, they are members of the group of P-type ATPases. They are localized in the Golgi area. In contrast to SERCA pumps, SPCA posses only one Ca^{2+} binding site (the more „primitive" site II) and that is why it is considered as phylogenetically the oldest. Other difference is that, unlike SERCA-type pumps, they are not inhibited by thapsigargin. This allows to find out how much of the Ca^{2+} uptake in the Golgi apparatus depends on SPCA1 activity, because only SERCAs are blocked by thapsigargin. In some cells more than 80% Ca^{2+} uptake in Golgi comples depends on SPCA1[10].

2.3.4. Sarco (endo)plasmic reticulum Ca^{2+} -ATPases (SERCAs): SERCA 1-3

SERCAs enzymes are localized in the membrane of the endoplasmic reticulum and ensure the accumulation of Ca^{2+} in the ER by its transport from the cytosol. SERCAs belong to the family of P-type of ATPases like PMCAs. Three SERCA genes have been identified: SERCA1 (*ATP2A1*), SERCA2 (*ATP2A2*),and SERCA3 (*ATP2A3*) which provide by alternative splicing several isoforms [11,12,13].

2.3.4.1. Localization of SERCAs in tissue

SERCA1a and 1b are expressed in adult fast-twitch and neonatal sceletal muscle, SERCA2a is expressed in cardiac muscle cells, SERCA2b is expressed ubiquitously in non-muscle cells and it is the main isoform expressed in smooth muscle cells. A minor isoform, SERCA2c, has been found in various non-muscle cell types. SERCA3 has six alternatively spliced isoforms which have been identified in several non-muscle cell types such as vascular endothelial cells, pancreatic β cells, cells of hematopoetic origin and Purkinje neurons. Biochemical characteristics of SERCA isoforms differ substantially. In particular, all SERCA3 isoforms have a lower calcium affinity (~1,2μM) than the other SERCA enzymes (~0,2μM) [11,14].

SERCA1 was the first pump of the family to be studied in detail and it represents the most specialized isoform of Ca^{2+} pumps. An adult SERCA1a comprises 994 amino acids and neonatal SERCA1b 1001 amino acids in human. Dysfunction of this isoform is linked to Brody's disease.

SERCA2 pumps are the most widespread of all SERCA isoforms and within the SERCA family they appear to be phylogenetically the oldest. SERCA2a is composed of 997 amino acids and SERCA2b of 1042 amino acids.

SERCA3 pump was discovered the most recently from all SERCA types. Compared with the other isoforms they have about five-fold lower affinity for cytosolic Ca^{2+} and are insensitive to PLB (phospholamban). SERCA3 is almost always co-expressed with house-keeping SERCA2b. In several cell types the expression of SERCA3 behaves as a differentiation marker [10] .

2.3.4.2. The transport of calcium from the cytosol to the ER, the catalytic cycle

The transit of calcium by SERCAs is enabled by its catalytic cycle that consists of the transformation between two major conformational states of the enzyme, named E1 and E2.

In the E1 conformation, the two Ca^{2+} binding sites are of high affinity and are facing the cytoplasm, while in the E2 conformation the Ca^{2+} binding sites are of low affinity and are facing the luminal side. Cytosolic ATP and Ca^{2+} bind to the E1 state and form the 2 Ca^{2+}-E1-ATP complex that undergoes phosphorylation to form $2Ca^{2+}$-E1-P. In this form, Ca^{2+} is occluded. A major conformational change leads to the state $2Ca^{2+}$-E2-P, whereby the Ca^{2+} binding sites are converted to a low affinity state and reorient towards the luminal face. The cycle is completed by the luminal release of Ca^{2+} and a conformational change from the E2 to the E1 state. E2 conformation is stabilized by thapsigargin, an important, high affinity inhibitor of SERCA [10].
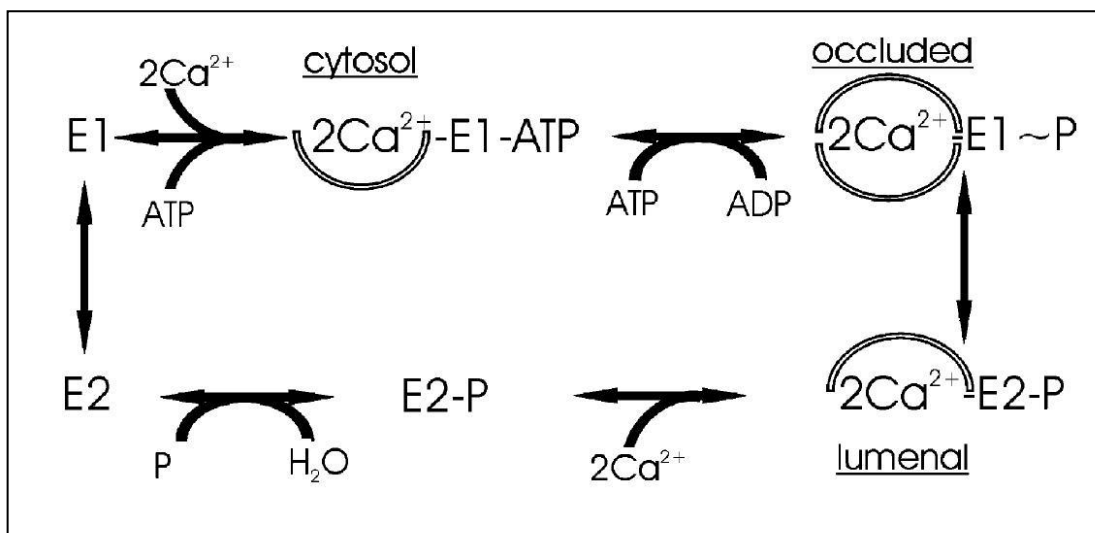
The catalytic cycle of the SERCA

2.3.4.3. Structure of the SERCAs

SERCAs are formed by a single polypeptide chain folded into four major domains: domains M, A, P and N. The transmembrane (M) domain is composed of ten transmembrane α-helices (M1-M10) and contains the important Ca^{2+} binding sites that are located in M4, M5, M6 and M8. The other three cytosolic domains are: the actuator (A) domain, phosphorylation (P) domain and the nucleotide-binding (N) domain. The domains A and P are connected to the M domain and N domain is connected to the P domain. P domain contains Asp351, the amino acid that is transiently phosphorylated by ATP during the catalytic cycle [10].
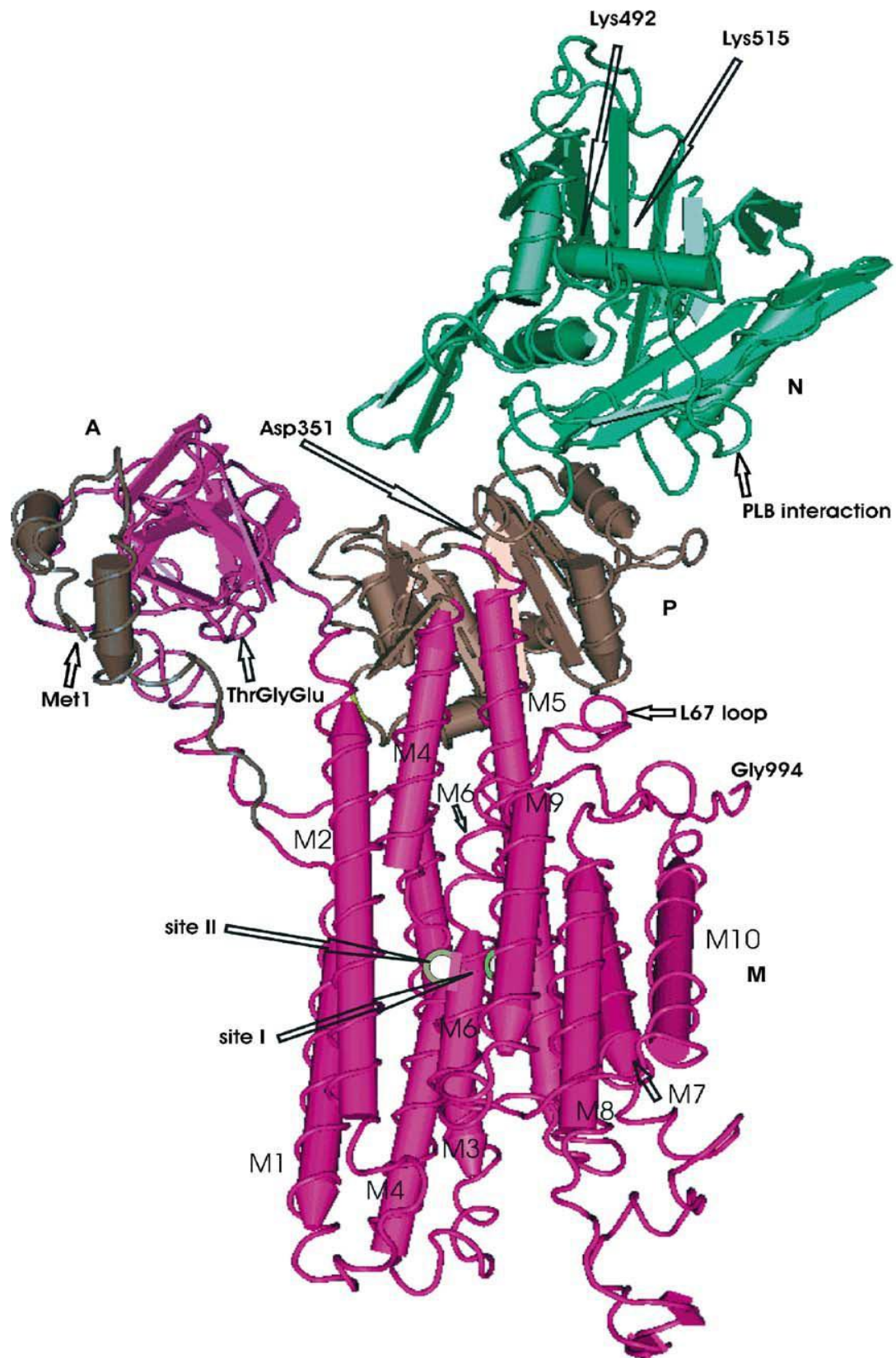
The structure of the SERCA

2.4. Previous work of the group concerned SERCA enzyme

Experimental data obtained by our group indicate that the SERCA composition of several carcinoma and leukaemia cell types undergoes significant changes during differentiation, and that this is accompanied by modifications of SERCA-dependent calcium accumulation in the ER. Our laboratory thus proposed that the modulation of the expression of various SERCA isoforms, in particular the induction of the expression of SERCA3, is an integral part of the differentiation program of several cancer and leukaemia cell types such as acute promyelocytic leukaemia or colon carcinoma (see below).

The expression of SERCA proteins is studied by immunoblotting with the utilization of the specific antibodies PLIM430 (anti-SERCA3) and IID8 (anti-SERCA2), that selectively recognize SERCA3 and SERCA2, respectively. In some studies cell-permeable SERCA-specific inhibitors such as thapsigargin (TG), 2,5,-di-*tert*-butyl-1,4,-benzohydroquinone (*t*BHQ) or cyclopiazonic acid (CPA) can be used.

In cell lines isolated from various carcinomas or sarcomas (colon, stomach, pancreas, lung, breast, thyroid, brain, skin, muscle, bone, and others) it was found that SERCA2 (mainly the ubiquitous SERCA2b isoform) is always present, while the expression of SERCA3 varies considerably. Depending on cell type, SERCA3 can be abundantly expressed or may be completely undetectable. The differences of SERCA expression levels could be observed even among closely related cell lines, obtained from similar pathologies (for example various colon adenocarcinomas). This prompted us to investigate SERCA expression in these cell types in greater detail, as described below [11].

2.4.1. SERCA expression during T-lymphocyte activation

Activation of the T-cell receptor complex by antigen presenting cells leads to the production DAG and IP_3 in T-lymphocytes. The formation of DAG activates protein kinase C (PKC) isoenzymes, IP_3 binding to its receptor provokes the release of Ca^{2+} from internal stores. The activation of transcription factors such as NF-κB, AP1 and NF-AT is consequently induced. *In vitro*, calcium mobilization and PKC activation can be pharmalogically induced by treating T-cell lines with PMA (phorbol myristate acetate) and the calcium ionophore ionomycin. This treatment modulates the expression of SERCA proteins, the expression of SERCA3 is decreased, whereas the expression of SERCA2b is two fold increased. Such a treatment induces also IL-2 synthesis and the expression of the α-chain of the IL-2 receptor, changes that indicate T cell activation. These processes are observed only when PMA and ionomycin are applied together, either drug when used alone is without any effect. SERCA3 down-modulation and IL-2 synthesis can be inhibited by the immunosuppressant, cyclosporin-A [10,15].

2.4.2. SERCA expression during differentiation of myeloid leukaemias

In APL (acute promyelocytic leukaemia) the t (15/17) chromosomal translocation fuses the RARα retinoic acid receptor gene to the PML gene giving rise to the PML/RARα fusion protein. At physiological all-*trans*-retinoic acid (ATRA) concentration (low nanomolar), this fusion protein acts as a transcriptional repressor of RARα-dependent gene expression, and consequently blocks neutrophil granulocytic maturation at the promyelotic stage leading to leukaemia. However, at ATRA concentrations around 0,1-1μM, the oncoprotein is converted into a transcriptional activator, leading to complete maturation of the leukaemic cells into nonproliferating granulocytes, which thereafter undergo apoptosis [11].

Our group observed in *ex vivo* experiments using primary APL cells or *in vitro* with HL-60 myeloblastic and NB4 promyelotic cells that the treatment of

the cells with ATRA that leads to terminal granulocytic differentiation is accompanied by the induction of the expression of the SERCA3 protein.

This differentiation promoted by ATRA is mediated by PML-RARα (in NB4 cells) and by RARα (in HL-60 cells).

Granulocytic differentiation of these cell lines can also be induced by cAMP analogy, and this is also accompanied by the induction of SERCA3 expression.

Induction of SERCA expression has been observed also during phorbol-ester induced monocytic differentiation of the bipotential HL-60 cell line and during the megakaryocytic differentiation of HEL and Meg-01 cells. Induction of SERCA3 expression during the differentiation of megakaryocytic cells further reinforces the idea that SERCA3 is a differentiation marker in these cells, because megakaryocytes are platelet precursors, and platelets express high levels of SERCA3 protein.

In other experiments the induction of differentiation of HL-60 and NB4 cells induced by ATRA was performed in the presence of SERCA inhibitors such as tBHQ. These experiments have shown that SERCA inhibition enhances the cell differentiation-inducing capacity of low concentrations (50 nM) of ATRA, allowing for cell differentiation similar to that observed at maximally efficient ATRA levels (0.1-1 μM). This shows an important role of SERCA inhibition in the ATRA induced differentiation of HL-60 cells. Other SERCA inhibitors such as TG and CPA gave similar results in combination with low doses of ATRA, whereas when cell lines were treated only by SERCA inhibitors in the absence of ATRA, the differentiation was not observed.

Treatment by SERCA inhibitors in combination with ATRA leads to the protection of RARα and PML-RARα proteins from degradation in ATRA-sensitive cells, suggesting that SERCA inhibition increases the stability of RARα and PML-RARα proteins in the presence of ATRA, leading to enhanced differentiation.

Ritodrine, a β-adrenergic agonist (in clinical practice used for the treatment of preterm labor) was also tested. It has also an inhibitory effect on SERCA proteins. Cell differentiation was observed when ritodrine was applied alone, and ritodrine also enhanced ATRA-induced differentiation of the HL-60

cell line [16]. This suggests that clinically available drugs such as ritodrine may be useful for the enhancement of ATRA-induced therapy of APL in the future.

2.4.3. SERCA proteins and colon cancer

The differentiation of normal colonic epithelium is physiologically enhanced by short-chain fatty acids (SCFA), principally *n*-butyrate, present in colonic lumen at milimolar concentrations, and produced by fermentation of dietary fibers by the colonic flora. Butyrate also induces the differentiation of colon cancer cells *in vitro*. It is thought that butyrate contributes to the prevention of colon carcinogenesis by enhancing the terminal differentiation of early, microscopical premalignant/malignant lesions. The effect of SCFAs is mediated mainly by inhibition of histone deacetylase (HDAC).

Butyrate and other histone deacetylase inhibitors are able to induce the differentiation, followed by apoptosis, of colon, and many other cancer cell types *in vitro*. Various butyrate-releasing prodrugs and other HDAC inhibitors are currently undergoing clinical trials for the treatment of cancer.

The SERCA expression pattern of various colon cancer cell lines is heterogeneous: SERCA2 is always present at roughly comparable levels, but SERCA3 expression is very variable, either undetectable in some cell lines, or clearly expressed in others. However, SERCA3 expression in colon carcinoma-derived cell lines is always inferior to that observed in normal colonic epithelial cells.

Our experiments show that SERCA3 expression is increased, when colon cancer cell lines are treated with butyrate. The induction of SERCA3 expression can also be obtained by treatment with other drugs such as valerate, propionate, caproate, phenylpropionate, phenylbutyrate, isovalerate and valproate, or butyrate-releasing pro-drugs such as tributyrylglycerol (tributyrin), or pivaloyloxymethylbutyrate.

SERCA3 expression can also be induced by the specific high affinity HDAC inhibitors HC-toxin, SAHA and apicidin. Induction is, however, weaker than when induced by butyrate or valerate. This suggests that SCFA-induced

modulation of SERCA expression may involve also mechanism unrelated to histone-deacetylation.

Caco-2 colon carcinoma cells undergo differentiation in postconfluent cultures. SERCA expression is induced postconfluently in the Caco-2 colon carcinoma cell line with a time course similar to other established differentiation markers such as carcioembryonic antigen (CEA), dipeptidyl-peptidase IV or the isoform-shift of the ZO-1 tight junction-associated protein. CEA is a well-established marker of differentiation for Caco-2 cells. Its expression was investigated to study the involvement of cellular calcium homeostasis in the differentiation process of colon cancer cells in the absence, and in the presence of specific SERCA inhibitors. Thapsigargin, tBHQ and cyclopiazonic acid, molecules that decrease ER calcium content enhanced cellular CEA production. This indicates that SERCA inhibition enhances cell differentiation in colon carcinoma, similarly to that observed in APL, as described above.

The expression of SERCA3 depends on the level of differentiation. When immunohistochemical staining was performed of surgical tumour specimens, it was observed that normal colonic epithelium expresses SERCA3 abundantly, whereas SERCA3 expression was barely detectable in moderately differentiated tumours and undetectable in poorly differentiated adenocarcinomas of the colon. Therefore SERCA3 can be considered as a useful new marker for the study of the state of differentiation of colon tumours.

Induction of SERCA protein expression could be also demonstrated on KATO-III gastric cancer cells. In untreated cells only SERCA2 is detected. However, after treatment for several days with butyrate, SERCA3 becomes expressed and SERCA2 expression decreases to approximately 40% of the untreated level.

All these observations taken together indicate that the study of the expression of SERCA type calcium pumps, and in particular that of SERCA3 constitutes an interesting new approach for the study of the differentiation of several types of human tumours and leukeamias.

Lung cancer is a major medical problem with poor survival rates that have not improved during the last 30 years. Therefore new concepts for the understanding of the physiopathology of the disease and of the behaviour of the

malignant cells are required. Based on our earlier data we therefore set out to study SERCA expression, and the modulation thereof, in lung carcinoma cells submitted to cell differentiation inducing agents.

3. AIM OF THE WORK

1. To study and compare the expression of SERCA2 and SERCA3 enzymes in various types of lung cancer

2. To study the modulation of SERCA expression during histone deacetylase inhibitor-induced differentiation of lung cancer cells

4. EXPERIMENTAL PROCEDURES

4.1. Materials and Methods

4.1.1. Instruments

Standard cell culture equipment:

Humidified cell culture incubator (37°C, 5%CO_2).

Laminar air flow hood

Centrifuges

Inverted microscope

freezing box -80°C

freezing and ice box -20°C

microscope

water bath

Stratagene cryobox for freezing of viable cells

Liquid nitrogen container for cryopreservation of viable cells

Equipment for Western immunblotting:

BioRad Mini-Protean 3 polyacrylamide electrophoresis and Western blot apparatus

BioRad power supply

Nitrocellulose membranes (0.2 μm pore size BioRad and Amersham)

Vortex shaker

pH-meter

Gilson precision pipettes (20, 200, 1000 μL)

Pipet-aid automatic pipette

digital and analytic balances

Roentgen films for chemiluminescent detection of Western blot signals

Photographic developer and fixing solutions (Kodak)

Enhanced chemiluminescence (ECL) substrate solution (Amersham, Chalfont Heights, UK

Coloured protein molecular weight marker solution (BioRad)

4.1.2. Chemicals

Cell growth media (obtained from Lonza, Verviers, Belgium):

RPMI 1640 medium

DMEM medium

Fetal bovine serum (FBS), heat inactivated (45°C 45 min).

Glutamine

Trypsine

Drugs (obtained from Sigma-Aldrich France, Saint-Quantin Fallavier, France):

Sodium valerate

Sodium butyrate

Sodium phenylbutyrate

Sodium chloride

Trichloroacetic acid

tris-hydroxymehtyl-aminomethane

sodium dodecyl sulfate

Acrylamide-bisacrylamide solution 40% (weight ratio 37.5/1)

Ammonium persulfate

TEMED (NN,N'N'-tetramethyl-ethylenediamine)

Methanol

Low-fat non-supplemented milk powder

Bromophenol blue

Dithiotreitol

Phenyl-methyl-sulfonyl-fluoride

TWEEN 20 (Polyethylen sorbitan monolaurate)

Ponceau red

Glycine

Hydrochloric acid

Primary anti-SERCA antibodies:

- PLIM 430 (obtained from hybridoma supernatant in the laboratory)
- IID8 (BioMol, Clinisciences, France)

Secondary antibody:

- Goat anti-mouse IgG-peroxydase conjugate (obtained from Jackson ImmunoResearch France)

4.2. Working procedures

4.2.1. Cell culture procedures

In this work the following lung cancer cells were used:

- A549 (lung carcinoma)
- Calu-3 (lung adenocarcinoma)
- ChaGok-1 (lung bronchogenic carcinoma)
- NCI-H292 (mucoepidermoid pulmonary carcinoma)
- NCI-H460 (large cell lung carcinoma)

Cells were obtained from the American Type Culture Collection (ATCC) through LGC Standards, France.

All cell lines grow as adherent cells and were cultured as follows:

Cells were cultivated in the DMEM or RPMI –based medium, according to the instructions of ATCC, supplemented with 10% heat-inactivated fetal calf serum and 2 mM glutamine, at 37°C and in a humidified cell culture incubator in an atmosphere of 95% air and 5% CO_2. Culture medium was changed every other day and cells were trypsinised at 70% confluence. Five days after plating cells were treated by various drugs as indicated in Results; growth medium containing drugs was renewed after two days. Cultures were terminated at confluency. Adherent cell monolayer was then washed twice with ice cold NaCl (150 mM) and precipitated with 5% trichloroacetic acid (TCA) overnight at 4°C.

Preparation of cell lysates:

Precipitated total cellular protein was resuspended in TCA by scraping, centrifuged for 8 minutes at rpm 1800, and pellet was transferred into pre-weighted 2 ml Eppendorf tubes. After centrifugation at 13000 rpm for 10 minutes at 4°C supernatant was aspirated and centrifugation was repeated once. After a second aspiration of the remaining supernatant, tubes containing

the TCA-precipitated cellular protein fraction were weighted. In order to homogenize protein loads of gels, TCA pellets were dissolved in SDS-PAGE sample lysis solution at a ratio of 30 mg TCA protein precipitate per ml lysis buffer.

4.2.2. SDS-polyacrylamide electrophoresis (SDS-PAGE) and Western blotting

Introduction.

Western immunoblotting after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used method for the study of proteins. Proteins are separated according to their molecular mass in the presence of SDS in polyacrylamide gels by electrophoresis. SDS is responsible for the denaturation of proteins and their negative charge whereby they move to anode. During migration proteins are separated according to their molecular weight, as smaller proteins move more easily across the gel. The migration of proteins is influenced by porosity of the gel that depends on the concentration of acrylamide and bis-acrylamide in the running gel.

Modern SDS polyacrylamide gels consist of two gel layers: the uppermost "top/stacking" gel is less concentrated and allows for the concentration during the earliest part of the migration, of the protein samples into a thin protein band, which is thereafter resolved in the "separating/resolving gel" during electrophoresis.

<u>4.2.2.1. SDS-PAGE</u>

1. Resolving gel
2. Stacking gel

Composition of gels:

Resolving gel (8%)	
TRIS (pH 8,8)	3,8ml
Acrylamide 40%	3ml
distilled water	8ml
TEMED	9µl
ammonium persulfate	150 µl
SDS 10%	150µl

Stacking gel (6%)	
TRIS (pH 6,8)	1,25ml
Acrylamide 40%	0,5ml
distilled water	3,2ml
TEMED	5µl
ammonium persulfate	50µl
SDS 10%	50µl

20 mL protein lysates/well was loaded and electrophoresis was performed at 120V constant Voltage for approximately two hours in 25 mM TRIS 190 mM glycine electrophoresis buffer containing 1% SDS. Electrophoresis was stopped when the bromophenol blue front arrived at the bottom of the gel.

4.2.2.2. Western Blotting

During Western blotting proteins are transferred from completed SDS-PAGE slab gels onto nitrocellulose membranes by electrophoretic migration. Transfer onto nitrocellulose permits consequent immunodetection of specific protein bands deposited on nitrocellulose using specific antibodies.

After migration the SDS-PAGE gel slab is placed together with the nitrocellulose membrane. The lateral transfer of proteins to the membrane is accomplished by migration at 400 mA (constant current) in 1/2xTRIS/glycine electrophoresis buffer supplemented with 20% methanol and 0.1% SDS.

4.2.3. Immunodetection

Following transfer, aspecific protein binding capacity of nitrocellulose was blocked with TBS-Tween buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 supplemented with 5% low-fat dry milk, for 1 hour. Then membranes were incubated with the various anti-SERCA primary antibodies (IID8 and PLIM430) for two hours. We used primary monoclonal antibodies at 0.2 μg/ml concentration.

After the incubation with the primary antibody, blots were washed in water and then incubated in TBS-Tween-milk buffer for 10 minutes twice.

Then, blots were incubated with peroxydase conjugated anti-mouse IgG secondary antibody for 1 hour (dilution 5000x), and blots were washed as outlined above.

For the detection of SERCA2 and SERCA3 proteins, blots were transferred into Enhanced Chemiluminescent Detection solution (Amersham) and exposed to autoradiographic photographic films in the dark. Luminograms were obtained by standard photographic developer and fixing solutions, and dried films were thereafter scanned for analysis.

5. RESULTS

In the present work we investigated the expression of SERCA2 and SERCA3 proteins in various non-small cell lung carinoma cells. The results show that, whereas the expression of the ubiquitous SERCA2 enzyme is constant that of SERCA3 varies considerably. In particular, we observed a marked and dose-dependent induction of SERCA3 expression in cells treated by short chain fatty acid-type histone deacetylase inhibitors. This effect was accompanied by a marked induction of expression of known markers of differentiation such as Mad and gelsolin, as well as that of the p21 protein, involved in the control of the cell cycle. The drugs used in our study are known to induce cell differentiation and cell cycle arrest, and some of them, such as phenylbutyrate, are in clinical trials for the therapy of various cancers. We can therefore propose the hypothesis that SERCA3 may be a phenotypic marker of NSCLC cell differentiation.

5.1. The pharmacological modulation of the expression of SERCA3 by phenylbutyrate in A549 lung cancer cells

Protein samples of treated, as well as untreated control A549 cells were submitted to SDS-PAGE and transferred to nitrocellulose membranes. After the incubation with the PLIM430 primary antibody and secondary anti-mouse IgG antibody, the expression of SERCA3 was detected by chemiluminescence. Basic expression of SERCA3 in untreated cells, as well as a marked induction of expression following phenylbutyrate treatment can be observed.
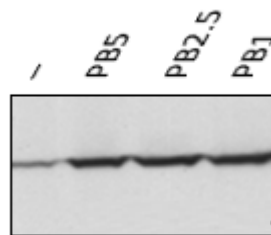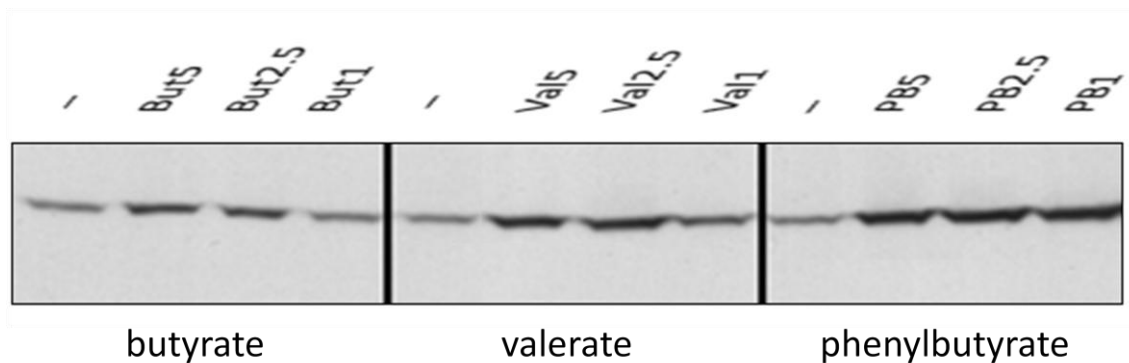

Figure 1

Immunodetection of SERCA3 expression in A549 cells treated by phenylbutyrate.

This Figure demonstrates the effect of phenylbutyrate at concentrations of 5 mM, 2,5 mM and 1 mM (lanes 2, 3 and 4, respectively) on the expression of SERCA3 protein as compared to untreated control (lane 1). We can see a clear induction of the expression of SERCA3 in comparison with untreated cells.

5.2. Comparative pharmacological modulation of the expression of SERCA3 in A549 lung cancer cells treated by butyrate, valerate and phenylbutyrate

      The samples were prepared and SERCA3 was detected in the same way as in Fig. 1, but in addition to phenylbutyrate, cells were treated also by butyrate and valerate in parallel. We used the concentration of 1 mM, 2,5 mM and 5 mM of the agents. The most intense response to treatment could be observed for phenylbuturate followed by valerate. On the other hand, butyrate induced SERCA3 expression from 2mM. Our observation shows different agents of histone deacetylase inhibitor group induce the expression of SERCA3 in A549 lung cancer cells with various potencies. This observation is in accordance with previous work of the group done on colon and gatric carcinoma cells.
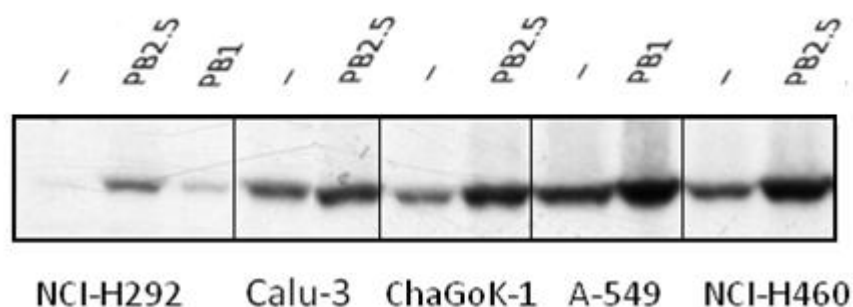

Figure 2

Immunodetection of SERCA3 protein expression in A549 cells treated by butyrate, valerate and phenylbutyrate at 5, 2,5 and 1 mM concentrations for 4 days.

5.3. Pharmacological modulation of the expression of SERCA3 in various lung cancer cell lines (H292, Calu-3, ChaGok-1, A549, H460)

In order to compare the induction of SERCA3 expression in various NSCLC cell lines treated by phenylbutyrate, NCI-H292, Calu-3, ChaGok-1 and NCI-H460 cells were treated for 4 days, and Western immunoblotting for SERCA3 was performed as in Materials and Methods. Untreated cells served as negative controls. As shown in Fig. 3, all studied cell lines responded to phenylbutyrate treatment. However, the intensity of SERCA3 induction varied according to the cell line. Whereas NCI-H292 cells express almost undetectable levels of SERCA3 at the untreated state, a strong induction of SERCA3 expression upon phenylbutyrate treatment is observed. By contrast, SERCA3 expression is intense in untreated A549 and H460 cells. However, phenylbutyrate treatment leads to a marked induction of SERCA3 expression also in these cells, and induction is evident also in Calu-3 as well as ChaGoK-1 cells.
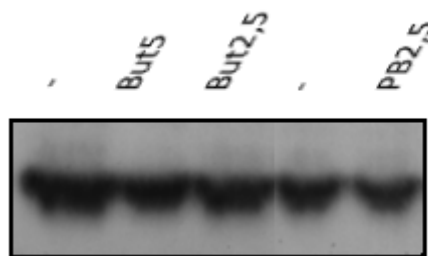

Figure 3

Immunodetection of SERCA3 expression in untreated, and phenylbutyrate treated NSCLC cell lines.

5.4. SERCA2 expression is not modified by histone deacetylase inhibitors in NSCLC

In order to investigate the specificity of SERCA3 induction in NSCLC by short chain fatty acid-type histone deacetylase inhibitors, the expression of the simultaneously expressed SERCA2 protein was also investigated.

As shown in Fig. 4, when A549 cells were treated with butyrate or phenylbutyrate at concentrations that induce SERCA3 expression (Figs. 1-3), in contrast to SERCA3, the expression of the SERCA2-type calcium transport ATPase was not modified. A similar result was also obtained on the other NSCLC cell lines as well (not shown).
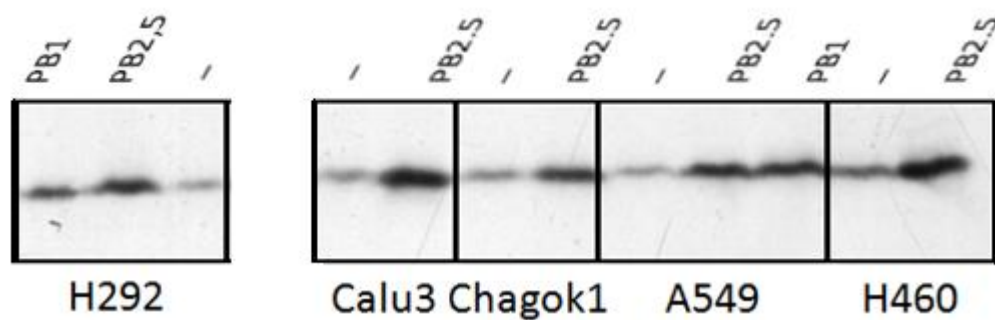

Figure 4

Immunodetection SERCA2 expression in A549 cells treated by short chain fatty acid-type histone deacetylase inhibitors. In contrast to SERCA3, the expression of SERCA2 is not modified by the treatments.

5.5. Investigation of the expression of established differentiation markers during short chain fatty acid-induced differentiation of NSCLC cell lines

In order to determine the effect of our treatments on the state of the differentiation of the NSCLC lines used in this study, as a control, we performed Western blot experiments for the p21 cell cycle inhibitor protein and for gelsolin. The expression of these proteins is known to be induced during NSCLC cell differentiation, and these proteins are widely used in the literature for the study of NSCLC cell differentiation.

As shown in Fig. 5, p21 expression was induced in all cell lines upon treatment by phenylbutyrate.

Figure 5

Expression of p21 protein in various lines of lung cancer treated with various concentrations of phenylbutyrate for 4 days as compared to untreated control.

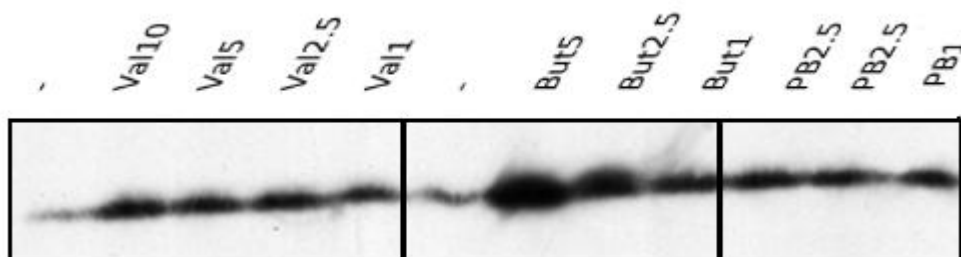Induction of p21 expression could also be observed upon treatment by valerate or n-butyrate (Fig. 6).

Figure 6

Induction of p21 expression in A549 cells treated with various concentrations of n-valerate, n-butyrate or phenylbutyrate for 4 days. Expression of p21 is induced by all three histone deacetylase inhibitors.

5.6. The expression of gelsolin in A549 lung cancer cells treated by butyrate, valerate and phenylbutyrate
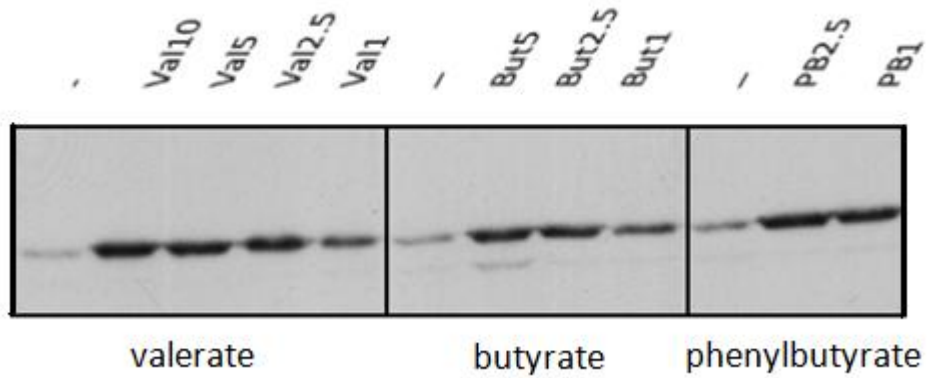
Figure 7

A549 cells were treated with 10, 5, 2.5 and 1 mM n-valerate, n-butyrate or 2.5 and 1 mM phenylbutyrate for 4 days in parallel with untreated cells, and gelsolin expression was determined by Western blotting. Treatments induced a marked over-expression of gelsolin in a concentration-dependent manner.

6. DISCUSSION AND CONCLUSIONS

Presently, malignant tumour diseases constitute one of the most serious problems of human health. The pharmacotherapy currently used is associated with a number of undesirable events such as drug resistance or adverse effects of the drugs, because the cytostatic activity of current drugs is not directed specifically against tumour cells, and therefore proliferating cells of normal tissue are also damaged. The problem of lack of selectivity of current cytostatic regimens often leads to limiting toxicities and overall therapeutic failure leading to death. Major scientific effort is thus being directed towards the discovery of more specific anticancer therapies. One such therapeutic possibility consists of the induction of the differentiation of tumour cells [22].

Various drugs such as all-*trans* retinoic acid or short chain fatty acid-derived and other types of histone desacetylase inhibitors can induce the terminal differentiation of various leukaemia and carcinoma types leading to growth arrest followed by apoptosis. Because of their distinct molecular action, these molecules are devoid of the general cytotoxic effects of classic anticancer drugs, and therefore they don't damage neighbouring normal tissue.

In the present work we investigated the process of cellular differentiation induced by histone deacetylase inhibitors in cell lines derived from various types of non-small cell lung cancer (NSCLC). On the basis of previous results obtained by this laboratory we explored the cross-talk between the process of cellular differentiation and calcium homeostasis. Our objective was to study the expression of Sarco-Endoplasmic Reticulum Calcium ATPases (SERCA proteins), enzymes that transport calcium from cytosol to the endoplasmic reticulum. Various lung cancer cell lines were grown *in vitro*, subjected to treatments by various short chain fatty acid-derived histone desacetylase inhibitors, and SERCA expression was determined in a semi-quantitative Western blot format using the IID8 and PLIM430 anti-SERCA monoclonal antibodies that recognise SERCA2 and SERCA3, respectively.

Our experiments show that the expression of SERCA proteins and cellular differentiation are interconnected in all lung studied cancer cell lines (A549, Calu-3, ChaGoK-1, NCI-H292 and NCI-H460). In particular, the induction of the expression of SERCA3 protein was observed after the induction of cellular differentiation by histone deacetylase inhibitors such as sodium butyrate, valerate and phenylbutyrate. Induction of SERCA3 expression was specific, because SERCA2 levels were not modified by the treatments.

Taken together, our results confirm and extend the original hypothesis regarding the existence of a relationship between the expression of SERCA proteins and cellular differentiation, observed in different leukaemia and gastric and colon carcinoma types by our laboratory. Based on our data we hypothesise that the modulation of SERCA expression may be a general phenomenon during the differentiation of various cell types. A better understanding of this phenomenon requires further research, and may open new avenues in the better understanding of the involvement of cellular calcium homeostasis and signalling in the process of carcinogenesis.

In conclusion, our work helps to better understand a cross-talk that exists between calcium homeostasis and cellular differentiation, extends previous work done on leukaemia and colon cancer also to NSCLC, and shows that SERCA3 protein may be a useful new immunohistochemical marker for the study of cell differentiation and lung cancer phenotype.

ABSTRAKT

V předložené práci jsme studovali proces buněčné diferenciace navozený inhibitory histon deacetyláz v buněčných liniích získaných z různých typů nemalobuněčného karcinomu plic.

Cílem práce bylo studovat vzájemné spoluovlivnění procesu buněčné diferenciace a exprese proteinu SERCA (ATPázy Sarko/Endoplazmatického retikula), který zajišťuje transport vápníku z cytozolu do endoplazmatického retikula. Buněčné linie různých plicních nádorů byly kultivovány *in vitro* za přidání různých inhibitorů histon deacetylázy ze skupiny mastných kyselin s krátkým řetězcem. Exprese SERCA proteinu byla hodnocena pomocí metody Western blot za použití primárních monoklonálních protilátek IID8 a PLIM430, které rozpoznávájí enzymy SERCA2 a SERCA3.

Naše experimenty ukazují, že exprese proteinů SERCA a proces buněčné diferenciace jsou vzájemně propojeny ve všech sledovaných buněčných liniích nádoru plic (A549, Calu-3, ChaGoK-1, NCI-H292 and NCI-H460). Po navození buněčné diferenciace inhibitory histon deacetylázy jako jsou butyrát, valerát či fenylbutyrát sodný, byla pozorována především zvýšená exprese proteinu SERCA3. Zvýšení exprese proteinu SERCA3 bylo specifické, neboť úroveň exprese proteinu SERCA2 i po přidání léčiv zůstala nezměněna.

Souhrnem lze říci, že naše výsledky potvrzují a rozšiřují původní hypotézu předpokládající vzájemný vztah mezi expresí proteinů SERCA a buněčnou diferenciací, již dříve pozorovaný naší laboratoří u různých typů kolorektálního karcinomu, karcinomu žaludku a leukémie.

ABSTRACT

In the present work we investigated the process of cellular differentiation induced by histone deacetylase inhibitors in cell lines derived from various types of non-small cell lung cancer (NSCLC).

Our objective was to study the cross-talk between the process of cellular differentiation and expression of Sarco-Endoplasmic Reticulum Calcium ATPases (SERCA proteins), enzymes that transport calcium from cytosol to the endoplasmic reticulum. Various lung cancer cell lines were grown *in vitro*, subjected to treatments by various short chain fatty acid-derived histone desacetylase inhibitors, and SERCA expression was determined in a semi-quantitative Western blot format using the IID8 and PLIM430 anti-SERCA monoclonal antibodies that recognise SERCA2 and SERCA3, respectively.

Our experiments show that the expression of SERCA proteins and cellular differentiation are interconnected in all lung studied cancer cell lines (A549, Calu-3, ChaGoK-1, NCI-H292 and NCI-H460). In particular, the induction of the expression of SERCA3 protein was observed after the induction of cellular differentiation by histone deacetylase inhibitors such as sodium butyrate, valerate and phenylbutyrate. Induction of SERCA3 expression was specific, because SERCA2 levels were not modified by the treatments.

Taken together, our results confirm and extend the original hypothesis regarding the existence of a relationship between the expression of SERCA proteins and cellular differentiation, observed in different leukaemia and gastric and colon carcinoma types.

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