

Altered Ca^{2+} -homeostasis of cisplatin-treated and low level resistant non-small-cell and small-cell lung cancer cells

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Abstract. *Background:* Chemotherapy often leads to encouraging responses in lung cancer. But, in the course of the treatment, resistance to chemotherapy ultimately limits the life expectancy of the patient. We aimed at investigating if treatment with cisplatin alters the intracellular Ca^{2+} -homeostasis of lung cancer cells and how this may be related to cisplatin resistance.

Methods: The squamous cell lung carcinoma cell line EPLC M1 and the small cell lung cancer cell line H1339 were exposed to cisplatin analogue to the *in vivo* pharmacokinetics. Changes in the cytoplasmic Ca^{2+} -concentration ($[\text{Ca}^{2+}]_c$) were recorded using fluorescence microscopy. Protein expression was quantified using immuno-fluorescence and Western Blot analysis. Changes in gene expression were accomplished by small-interfering (si) RNA techniques.

Results: Four “cycles” of cisplatin led to low level resistance in EPLC and H1339 cells. In the low level resistant cell clones, the Ca^{2+} -content of the endoplasmic reticulum (ER) was decreased. In low level resistant EPLC cells, this was correlated with an increased expression of the inositol-1,4,5-trisphosphate receptor (IP_3R). Inhibiting the increased expression of IP_3R using siRNA, the low level resistance could be reversed. In low level resistant H1339 cells, the decreased Ca^{2+} -content of the ER was correlated with a decreased expression of sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCA). Decreasing the expression of SERCA in naïve H1339 cells resulted in low level cisplatin resistance.

Conclusion: We conclude that cisplatin therapy leads to a decreased Ca^{2+} -content of the ER thereby inducing low level resistance. This is caused by upregulation of the IP_3R in EPLC and decreased expression of SERCA in H1339 cells.

Keywords: Lung cancer, cisplatin, calcium, SERCA, IP_3R

1. Introduction

In the industrial nations, lung cancer is the leading cause of cancer death in both men and women [1]. Despite recent advances, therapeutical regimens including chemotherapy support quality of life but frequently fail to increase long term survival. First-line chemotherapy often leads to encouraging responses, but, in the course of the treatment, resistance to chemotherapy ultimately limits the life expectancy of the patient. Platin-based therapy regimens have become the backbone of lung cancer chemotherapy with cisplatin being widely used. Cisplatin reacts with DNA resulting in intra- and interstrand cross-links, which has been proposed to be

the mechanism of cisplatin induced cell death [20]. However, several studies failed to show a correlation between DNA adducts and cisplatin cytotoxicity [9,47]. Mandic et al. even showed nucleus-independent cisplatin-induced apoptotic signaling [33]. The contradictory data, therefore, render the exact mechanisms of cisplatin-induced apoptosis in cancer cells still unclear. Possible mechanisms of resistance to cisplatin have been extensively studied and more than 100 proteins have been suggested to be involved [2,44]. However, a satisfying clarification of the underlying mechanisms is still lacking and none of the proposed proteins has ever been proven to be clinically relevant. Therefore, the problem of the development of cisplatin resistance in lung cancer cells still remains unresolved.

Calcium is a ubiquitous signal molecule that is involved in almost all cellular pathways [5,15]. This is in particular true for proliferation and apoptosis and the imbalance of cell growth and cell death finally leads to cancer. In apoptosis, calcium from the extra-

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cellular space or released from the endoplasmic reticulum (ER) leads to an increase in the mitochondrial Ca^{2+} -concentration, which in turn opens the permeability transition pore followed by an efflux of cytochrome C. Cytochrome C amplifies the Ca^{2+} -release from the ER and activates via caspase 9 the intrinsic pathway of apoptosis [18,34,36]. In proliferation, binding of growth factors leads to Ca^{2+} -release from the ER, which in turn activates store operated Ca^{2+} -influx from the extracellular space [31]. A sustained increase in cytoplasmic Ca^{2+} -concentration ($[Ca^{2+}]_c$) or sustained Ca^{2+} -oscillations activate the nuclear factor of activated T-cells (NFAT), which finally mediates proliferation.

So far, only few studies have investigated a possible role of the intracellular Ca^{2+} -homeostasis in cisplatin resistance. Liang et al. reported that in cisplatin resistant cells of the human lung adenocarcinoma cell line A549 the resting $[Ca^{2+}]_c$ was decreased [30]. The authors hypothesized that the lower $[Ca^{2+}]_c$ leads to increased expression of P-glycoproteins resulting in an increased rate of cisplatin-extrusion out of the cell. Tsunoda et al. postulated for the cisplatin resistance of bladder cancer cells a role for the inositol-1,4,5-trisphosphate receptor type 1 (IP₃R1) [46], which releases calcium from the ER. The authors showed that the IP₃R expression was down-regulated in resistant cells and after exposure of non-resistant cells to cisplatin. Recently, Spletstoeser and colleagues showed that in Hela-S3 but not in human osteoblastoma cells cisplatin induced Ca^{2+} -influx from the extracellular space involving IP₃R on the plasma membrane [43]. Taken together, these studies provide evidence that the intracellular Ca^{2+} -homeostasis may be involved in the resistance to cisplatin. But, the data are conflicting and the underlying mechanisms are not understood.

In our study, we show that cisplatin treatment of the non-small cell lung cancer (NSCLC) cell line EPLC M1 and the small cell lung cancer (SCLC) cell line H1339 leads to low level resistance. In both cell types, the low level resistance was correlated with a decreased Ca^{2+} -content of the ER. In EPLC cells, the low level resistance was mediated by upregulation of the IP₃R. But, in H1339 cells, the low level cisplatin resistance was a result of a decreased expression of SERCA. We, therefore, conclude that cisplatin therapy leads to a decreased Ca^{2+} -content of the ER thereby inducing low level resistance. The molecular mechanisms leading to the diminished ER Ca^{2+} -concentration ($[Ca^{2+}]_{ER}$) depend on the histological phenotype involving IP₃R in EPLC and SERCA in H1339 cells.

2. Materials and methods

2.1. Materials

Cell culture reagents were obtained from Life Technologies (Eggenstein, Germany). Other reagents were bought from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise. The human SCLC cell line H1339 was purchased from DSMZ, Braunschweig, Germany. The human lung squamous cell carcinoma cell line EPLC M1 was kindly provided by Dr. G. Jaques, Philipps-University, Marburg, Germany.

2.2. Ca^{2+} -imaging

For quantification of changes of the $[Ca^{2+}]_c$, cells were loaded for 30 min at 37°C with the calcium indicator dye Fluo-4 AM (10 µM, Molecular Probes, Eugene, OR) in supplemented Hanks Balanced Salt Solution (sHBSS) containing 0.2% Pluronic (Pluronic F-127, Calbiochem, La Jolla, CA). After loading, the cells were incubated for at least 30 min in sHBSS to allow for complete dye deesterification and examined with a fluorescence microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany). Images were recorded in time lapse (1 frame/s) using a digital CCD camera (AxioCam MRm, Carl Zeiss Vision, Munich, Germany). For each image, regions of interest (ROIs) were defined in single cells, and the average fluorescence intensity of each ROI was measured. Final fluorescence values were expressed as a fluorescence ratio (F/F_0) normalized to the initial fluorescence (F_0). Each analysis was performed using custom written macros in the image analysis software "Scion".

2.3. Western Blot analysis

EPLC and H1339 cells were washed twice with ice-cold phosphate-buffered saline (PBS; 10 mM, pH 7.4) and scraped in 0.02% EDTA solution. The cell suspensions were treated according to the manufacturer's protocols using the Mammalian Protein Preparation Kit for intracellular proteins (Quiagen GmbH, Germany) and Native Membrane Protein Extraction Kit for transmembrane proteins (Merck Biosciences, Germany). The extracts were collected, aliquoted and then stored at -20°C until use with Western Blot analysis. The protein concentrations of the extracts were determined with the Non-Interfering Protein Assay Kit according to the manufacturer's protocol (Merck Biosciences, Germany). The extracts were treated with Laemmli

sample buffer (at a final concentration of 32.5 mM Tris, 2.5% β -mercaptoethanol, 1% SDS and 12.5% glycerol) at 85°C for 10 min and separated by SDS-PAGE on 4–12% Bis-Tris (for calreticulin and SERCA1/2/3) or 3–8% Tris-Acetate mini-gels (for IP₃R1/2/3 and RyR) depending on the size of the target protein. Staining was performed using specific antibodies (goat anti-calreticulin, Santa Cruz Biotechnology, dilution 1:1000; rabbit anti-SERCA1/2/3, Santa Cruz Biotechnology, 1:50; rabbit anti-IP₃R, Santa Cruz Biotechnology, dilution 1:250) and secondary Antibodies (calreticulin: donkey anti-goat, Santa Cruz Biotechnology, dilution 1:10000; EPLC SERCA1/2/3: donkey anti-rabbit, Amersham Biosciences UK, dilution 1:10000; H1339 SERCA1/2/3: goat anti-rabbit, Biorad, dilution 1:10000; EPLC IP₃R: goat anti-rabbit, Biorad, dilution 1:10000; H1339 IP₃R: goat anti-rabbit, Biorad, dilution 1:5000). β -Actin staining served as loading control (mouse anti- β -actin (HRP), Abcam, 1:5000). Antibody complexes were visualized using Hyperfilm ECL chemiluminescence (Amersham Biosciences, UK) and evaluated using the “Image-J” analysis software.

2.4. Immuno-fluorescence

EPLC and H1339 cells were grown on microscope slides, fixed for 5 min in 100% acetone and washed in sHBSS containing 10% newborn calf serum (NCS). Antibodies were diluted 1:100 in sHBSS with 10% NCS. The cells were incubated for 1 h at room temperature with monoclonal antibodies (EPLC: rabbit anti-IP₃R1/2/3, Santa Cruz; H1339: rabbit anti-SERCA1/2/3, Santa Cruz). The cells were then washed in sHBSS with 10% NCS and incubated for 1 h at room temperature with FITC-conjugated secondary anti-rabbit antibodies (Santa Cruz). The slides were inspected on a fluorescence microscope and the fluorescence intensity was quantified using the image analysis software “Scion”.

2.5. Small interfering (si) RNA transfection

Cells were transfected according to the manufacturer's protocol (Dharmacon, Chicago, IL). The siRNA concentrations were adapted to ensure the desired decrease in protein expression without interfering with cell viability and growth as assessed by trypan blue staining and cell number. EPLC cells were transfected with 100 nM IP₃R type 1, type 2 and type 3 siRNA using the DharmaFECT 2 transfection reagent for 48 h. H1339 cells were transfected with 5 nM SERCA

type 1, type 2 and type 3 siRNA also using the DharmaFECT 2 transfection reagent for 48 h. Non-targeting siRNA served as control. After 48 h, protein expression was quantified using Western Blot analysis. Four days after transfection, protein expression was again quantified to ensure stable transfection for the duration of the experiments with cisplatin.

2.6. Statistics

One-way or two-way ANOVA or “ANOVA repeated measurements” (combined with pairwise multiple comparisons) were performed using the “Sigma Stat” software (Jandel Scientific, Chicago, IL). A *P* value of less than 0.05 was considered statistically significant.

3. Results

Analogue to the plasma concentration of unbound cisplatin in humans [17], EPLC and H1339 were exposed to 0.5, 1 and 2 μ g/ml cisplatin for 3 h and the survival fractions were determined for 4 days. In both cell lines, a concentration dependent effect of cisplatin could be observed (Fig. 1, upper panel). The cells that had survived 0.5 μ g/ml cisplatin were allowed to recover for 7 days and were again exposed to 0.5 μ g/ml cisplatin. After 4 “cycles”, the survival after a 5th exposure was compared to the survival of naïve cells. In EPLC and H1339 cells, the pretreated cells showed an increased survival fraction compared to naïve cells indicating a low level resistance to cisplatin (Fig. 1, middle panel). The cisplatin sensitivity was assessed for up to 5 passages but no change in cisplatin resistance in the low level resistant cells could be observed. To investigate the effect of a reduced $[Ca^{2+}]_c$ on the cisplatin-sensitivity, the Ca^{2+} -chelator BAPTA-AM was used. Buffering the intracellular calcium with 50 μ M BAPTA-AM, EPLC and H1339 cells showed an increased survival fraction (Fig. 1, lower panel).

Elevation of the $[Ca^{2+}]_c$ can result either from Ca^{2+} -influx from the extracellular space or from Ca^{2+} -release from internal Ca^{2+} -stores, primarily the ER. Proteins involved in the Ca^{2+} -release from the ER are the inositol-1,4,5-trisphosphate receptor (IP₃R) and the ryanodine receptor (RyR) (Fig. 2). Sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCA) force calcium against the concentration gradient from the cytoplasm into the ER. Within the ER, calcium is buffered by calreticulin [5,15]. To investigate the

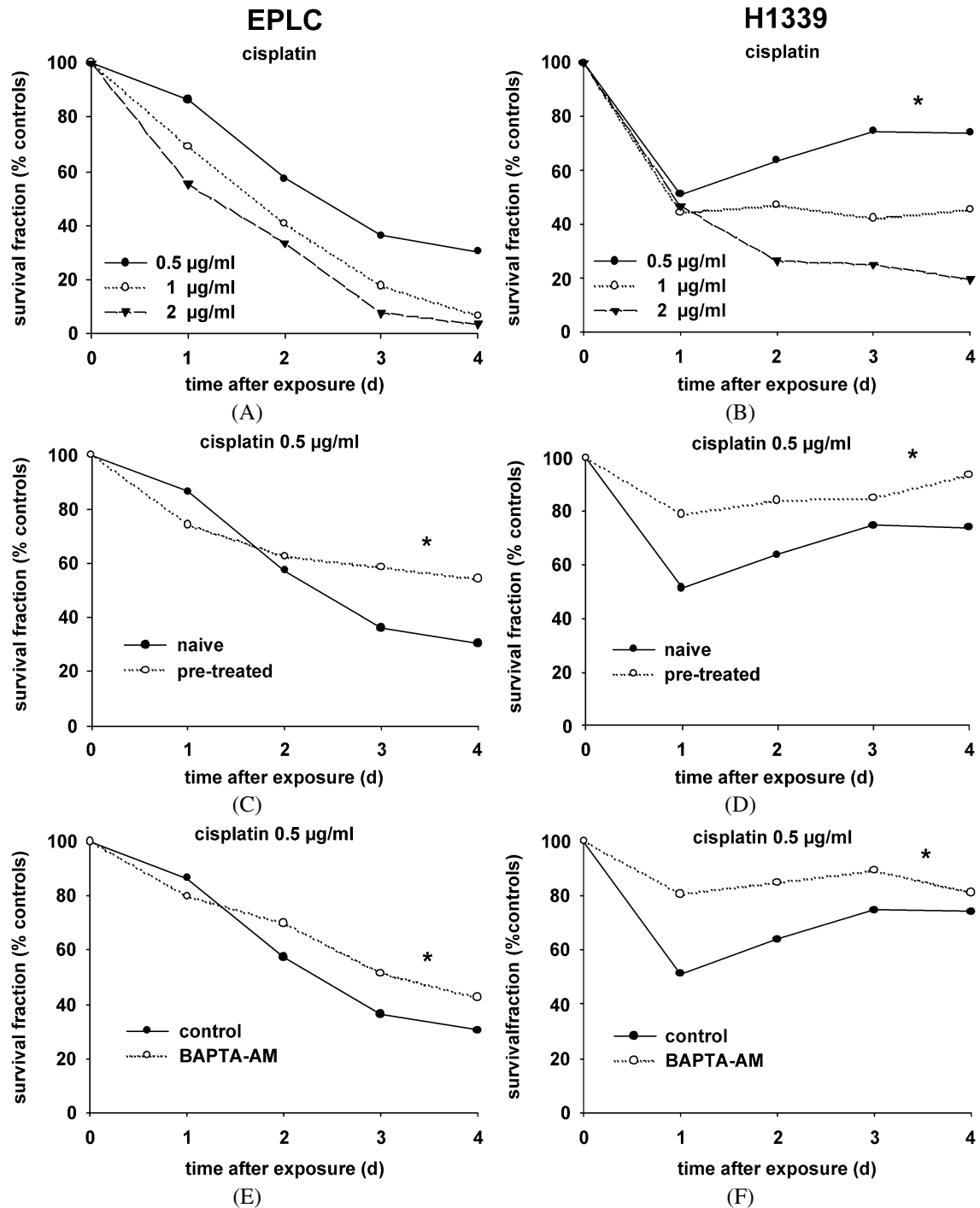


Fig. 1. (Upper panel) Analogue to the plasma concentration of unbound cisplatin in humans, (A) squamous lung carcinoma cells (EPLC) and (B) small cell lung carcinoma cells (H1339) were exposed to 0.5, 1 and 2 $\mu\text{g/ml}$ cisplatin for 3 h and the survival fraction was determined for 4 days. Cisplatin showed a concentration dependent effect. (Middle panel) (C) EPLC and (D) H1339 cells that had survived 0.5 $\mu\text{g/ml}$ cisplatin were allowed to recover for 7 days and were again exposed to 0.5 $\mu\text{g/ml}$ cisplatin. After 4 exposures the survival after a 5th exposure was compared to the survival of naïve cells. Pre-treated cells showed an increased survival fraction compared to naïve cell. (Lower panel) The effect of a reduced intracellular Ca^{2+} -concentration on the cisplatin-sensitivity was investigated using the cell permeable Ca^{2+} -buffer BAPTA-AM. After pre-treatment with 50 μM BAPTA-AM, (E) EPLC and (F) H1339 cells showed a higher survival fraction compared to cells without pretreatment (* $P < 0.01$, two way ANOVA).

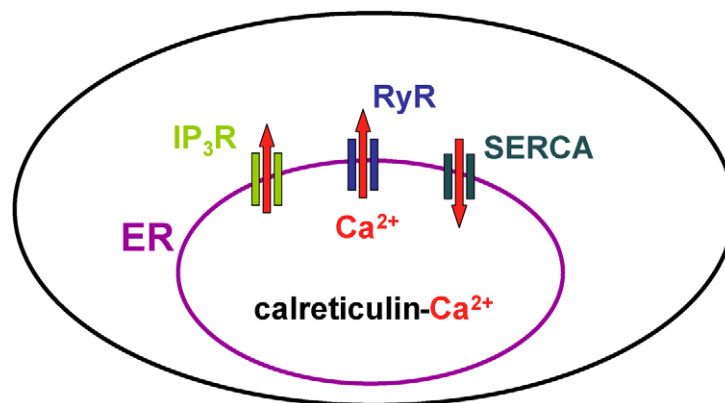


Fig. 2. The equilibrium of the Ca^{2+} -content of the endoplasmic reticulum (ER) is maintained by sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPases (SERCA) pumping calcium into the ER and inositol-1,4,5-phosphate- (IP_3R) and ryanodine-receptors (RyR) releasing calcium out of the ER. Within the ER, calcium is buffered by calreticulin.

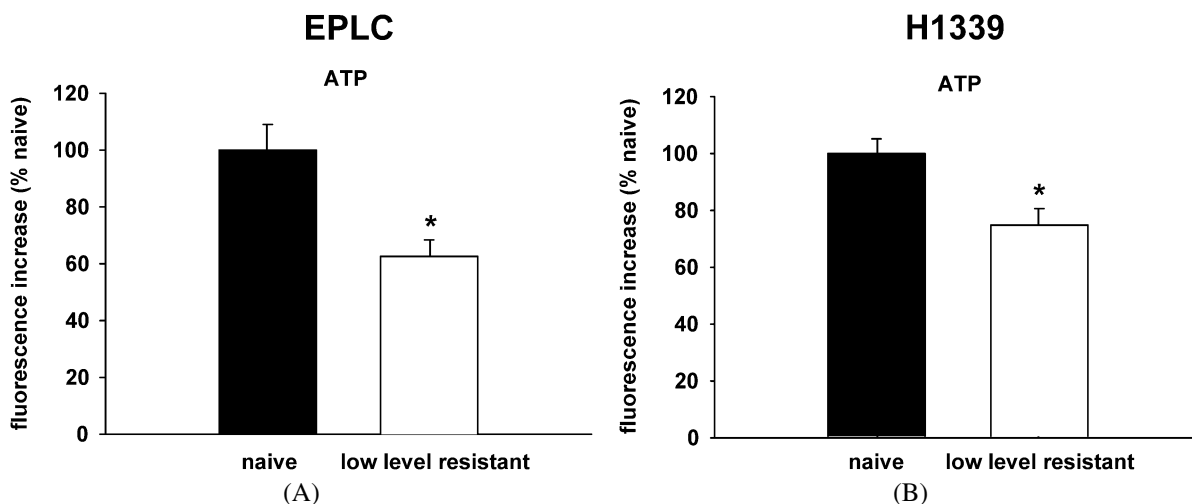


Fig. 3. (Upper panel: A and B) EPLC and H1339 cells were loaded with the Ca^{2+} -indicator Fluo-4, exposed to 1 mM ATP and the resulting increase in the cytoplasmic Ca^{2+} -concentration was quantified using fluorescence microscopy. In (A) EPLC and (B) H1339 cells, the increase was lower in the low level resistant clones ($n = 32-38$ cells, $*P < 0.001$). (Middle panel: C and D, next page) To address the influence of Ca^{2+} -influx from the extracellular space, the extracellular medium was replaced by phosphate buffered saline containing no calcium but 0.02% EGTA prior to the addition of 1 mM ATP. In (C) EPLC and (D) H1339, no significant differences in the ATP-induced Ca^{2+} -increase between the presence or absence of extracellular calcium could be observed ($n = 121-158$ cells). (Lower panel: E and F) To test if the Ca^{2+} -content of the ER was altered in the low level resistant cells, SERCA were inhibited using cyclopiazonic acid (CPA) in the absence of extracellular calcium leading to a net Ca^{2+} -efflux from the ER. The resulting increase in $[Ca^{2+}]_c$ was used as an estimate of the $[Ca^{2+}]_{ER}$. In the low level resistant clones of (E) EPLC and (F) H1339 cells, the resulting increase in $[Ca^{2+}]_c$ was lower compared to naïve cells indicating a lower $[Ca^{2+}]_{ER}$ ($n = 54-108$ cells, $*P < 0.001$).

Ca^{2+} -homeostasis in the normal and low level resistant clones, the cells were exposed to 1 mM ATP and the resulting increase in the cytoplasmic Ca^{2+} -concentration was quantified using fluorescence microscopy. The baseline fluorescence intensities reflecting the baseline Ca^{2+} -concentrations in the cells were identical in the normal and low level resistant clones (data not shown). In EPLC and H1339 cells, the increase in $[Ca^{2+}]_c$

was lower in the low level resistant clones (Fig. 3, upper panel). Fifty μM BAPTA-AM almost completely prevented the ATP-induced Ca^{2+} -increase in normal and low level resistant EPLC and H1339 cells (data not shown). To evaluate the underlying mechanisms of the altered Ca^{2+} -homeostasis, the contribution of Ca^{2+} -influx from the extracellular space was investigated. Prior to the exposure to ATP, the extracel-

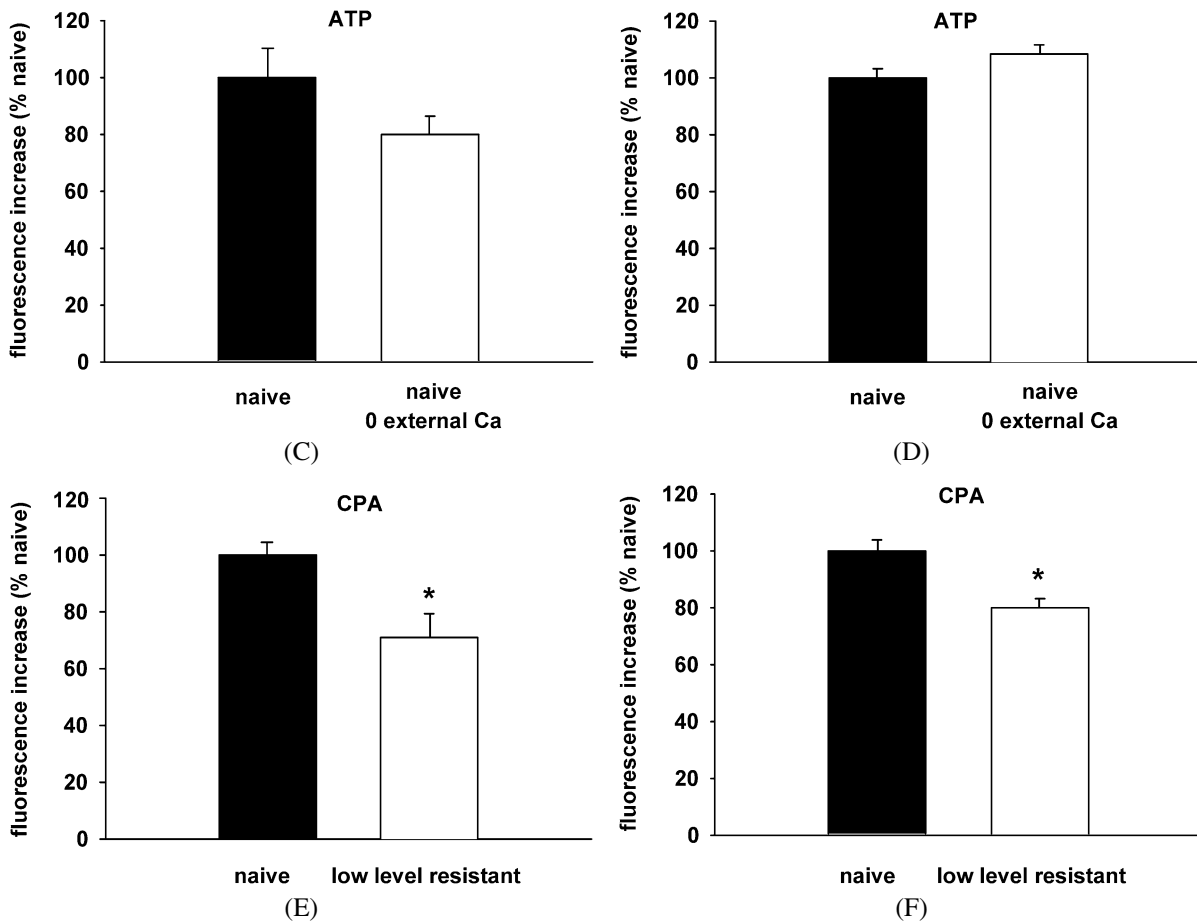


Fig. 3. (Continued).

lular medium was replaced by phosphate buffered saline containing no calcium but 0.02% EGTA. In EPLC and H1339 cells, no significant differences in the ATP-induced Ca^{2+} -increase between the presence or absence of extracellular calcium could be observed (Fig. 3, middle panel). Therefore, Ca^{2+} -influx from the extracellular space seemed to play a minor role. Unfortunately, reliable measurement of the $[Ca^{2+}]_{ER}$ using fluorescent Ca^{2+} -indicators is not feasible. Therefore, we used an indirect approach. SERCA were inhibited using 1 μ M cyclopiazonic acid (CPA) leading to a net Ca^{2+} -efflux out of the ER. The resulting increase in $[Ca^{2+}]_c$ was used as an estimate of the $[Ca^{2+}]_{ER}$ [4]. These experiments were performed in the absence of extracellular calcium to prevent store operated Ca^{2+} -influx. In EPLC and H1339 cells, the increase in $[Ca^{2+}]_c$ was lower in the low level resistant clones indicating a lower $[Ca^{2+}]_{ER}$ (Fig. 3, lower panel).

To investigate the underlying mechanisms of the decreased ER Ca^{2+} -content, the expression of proteins

regulating the $[Ca^{2+}]_{ER}$ was studied using Western Blot analysis and immuno-fluorescence. In EPLC and H1339 cells, the expression of RyR was found to be too low for quantitative analysis but showed no apparent differences (data not shown). The expression of the ER Ca^{2+} -buffer calreticulin was similar in the naïve and low level resistant clones (Fig. 4). However, in EPLC cells, the expression of IP₃R was higher in the low level resistant clone using an IP₃R antibody recognizing all 3 isoforms (Figs 4(A) and 5(A)). Conversely, in H1339 cells, no differences regarding the IP₃R could be found. But, in H1339 cells, the expression of SERCA was reduced in the resistant clone using an SERCA antibody recognizing all 3 isoforms (Figs 4(B) and 5(B)). In EPLC cells, the expression of SERCA was similar in the naïve and the low level resistant clone.

Using siRNA techniques we tested if the cisplatin-sensitivity could be manipulated in EPLC and H1339 cells by modifying the expression of IP₃R and SERCA,

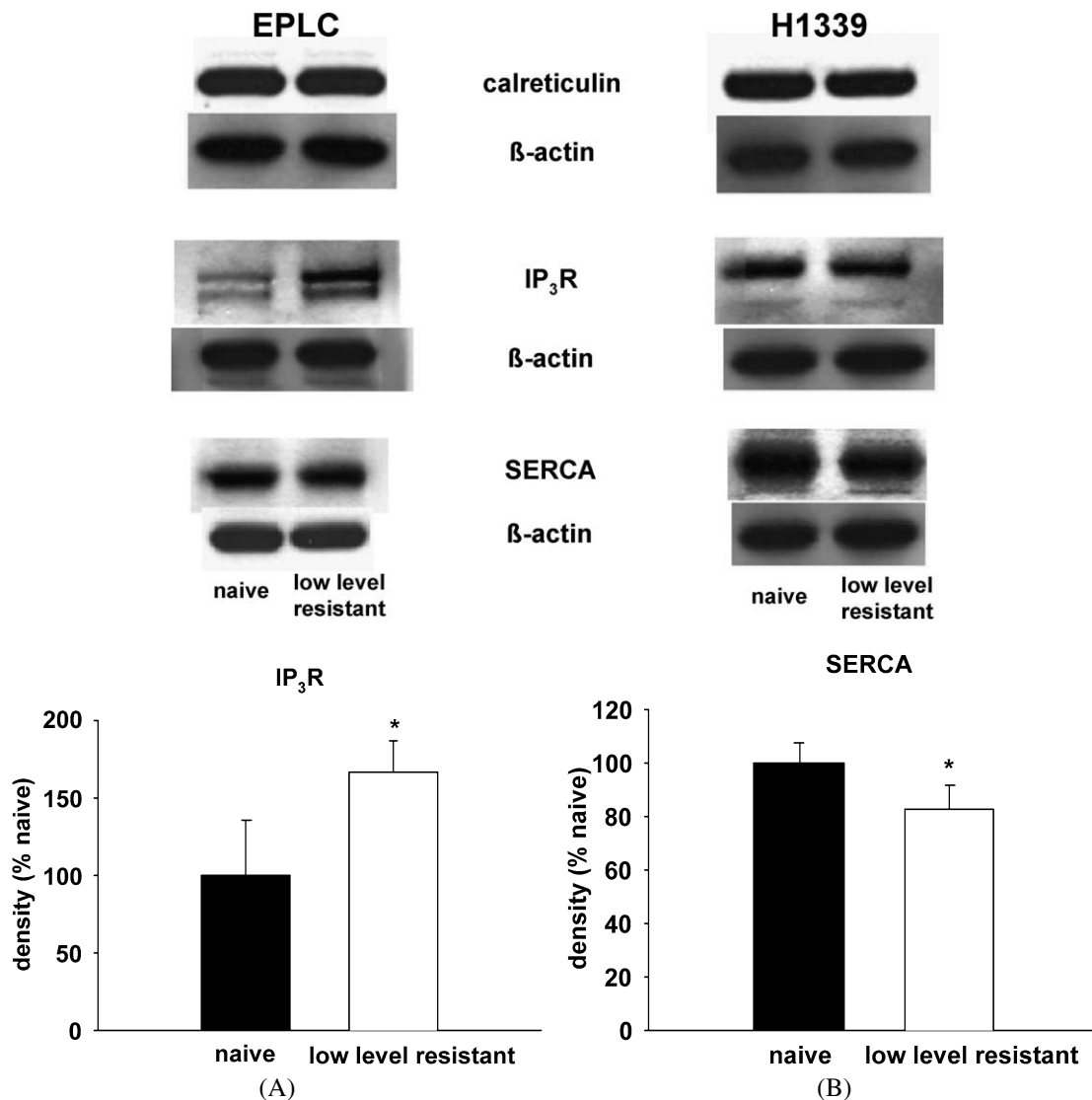


Fig. 4. The expression of proteins involved in the regulation of the ER Ca^{2+} -content was studied using Western Blot. (A) In EPLC cells, the expression of calreticulin and SERCA was similar in the naïve and the low level resistant clone. However, the expression of the IP₃R was higher in the low level resistant clone (double bands due to isoforms, $n = 5$, $*P < 0.05$). (B) In H1339 cells, the expression of calreticulin and IP₃R was similar in the naïve and low level resistant clone. But, the expression of SERCA was lower in the low level resistant clone ($n = 4$, $*P < 0.05$).

respectively. The siRNA concentrations used were chosen to ensure the desired decrease in protein expression without interfering with cell viability and growth. In low level resistant EPLC cells, the increased expression of the IP₃R could be reversed using 100 nM IP₃R-siRNA. Moreover, the decrease in IP₃R expression in low level resistant cells resulted in a cisplatin-sensitivity similar to naïve cells (Fig. 6(A)). In H1339 cells, the expression of SERCA was altered in naïve cells to mimic the changes observed in the low level re-

sistant clone. Treatment with 100 nM SERCA-siRNA resulted in a marked reduction in SERCA expression but also in cell viability (data not shown). After treatment with 5 nM SERCA-siRNA the SERCA expression was reduced to a lesser extent but no change in cell viability could be observed. Therefore, this concentration was used to suppress SERCA expression. Reduction in SERCA expression in naïve cells resulted in a cisplatin-sensitivity similar to low level resistant cells (Fig. 6(B)).

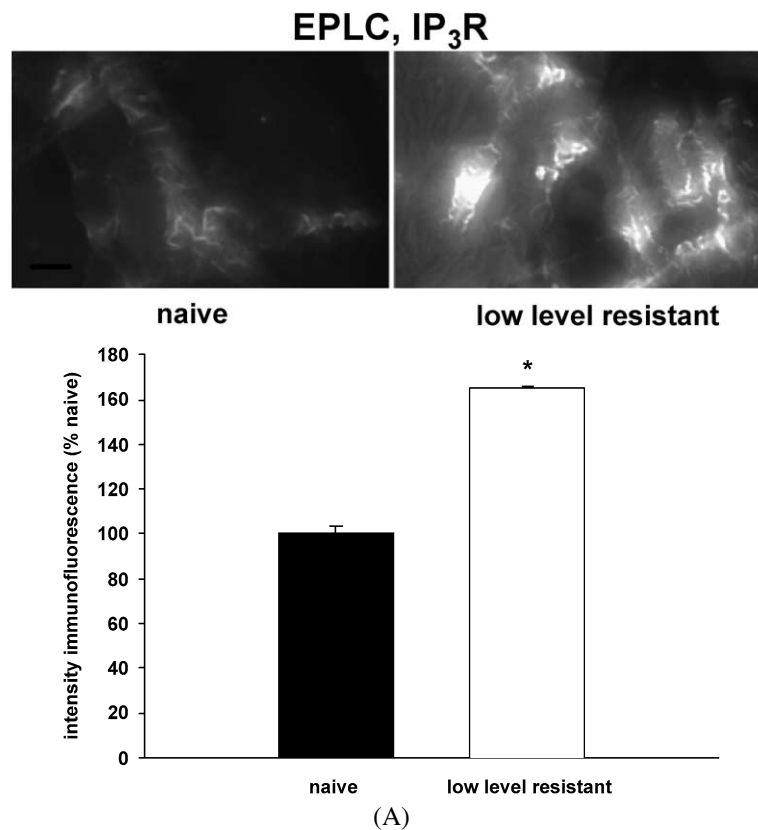


Fig. 5. To confirm the results obtained with Western Blot analysis, immuno-fluorescence stainings of the ER-membrane bound IP₃R and SERCA were performed. (A) The immuno-fluorescence in IP₃R-antibody labeled EPLC cells was significantly higher in the low level resistant clone ($n = 36$ cells, $*P < 0.001$). Bar = 10 μm . (B, next page) The immuno-fluorescence in SERCA-antibody labeled H1339 cells was significantly lower in the low level resistant clone ($n = 16$ cells, $*P < 0.001$). Bar = 2 μm .

4. Discussion

We showed that treatment of EPLC and H1339 cells with cisplatin according to the *in vivo* pharmacokinetics led to low level resistance. This could be mimicked by reducing the $[Ca^{2+}]_c$ during the treatment with cisplatin. In EPLC and H1339, the Ca^{2+} -content of the ER was found to be decreased in the low level resistant clones. In low level resistant EPLC cells, the reduced $[Ca^{2+}]_{ER}$ was correlated with an increased expression of the IP₃R. Inhibiting the increased expression of IP₃R using siRNA, the low level resistance could be reversed. In low level resistant H1339 cells, the decreased $[Ca^{2+}]_{ER}$ was correlated with a decreased expression of SERCA. Naïve H1339 cells in which the SERCA expression had been reduced with siRNA showed a cisplatin-sensitivity similar to the low level resistant clone.

In NSCLC and SCLC, cisplatin is one of the most effective and most frequently used chemotherapeutics.

De Jongh et al. analyzed the cisplatin pharmacokinetics in 268 adult patients and could show that the plasma concentration of unbound cisplatin peaked ~ 3 h after intravenous application at $\sim 1 \mu\text{g/ml}$ to rapidly decrease afterwards [17]. Rather than incubating for 24 h as performed in some studies, we, therefore, chose to expose cells for 3 h to 0.5, 1 and 2 $\mu\text{g/ml}$ cisplatin. In the clinical routine, patients typically receive 4 cycles of chemotherapy with the option of 2 more cycles. In our study, we also applied 4 “cycles” of cisplatin again trying to mimic the “*in vivo*” situation as closely as possible.

Calcium is a highly versatile intracellular signal molecule regulating numerous cellular functions. These functions include proliferation and apoptosis [5,11] and the imbalance between cell growth and cell death defines cancer. How can one signaling molecule regulate such different pathways? An extensive cellular Ca^{2+} -signaling toolkit enables very different spatial and temporal Ca^{2+} -dynamics within one cell [5].

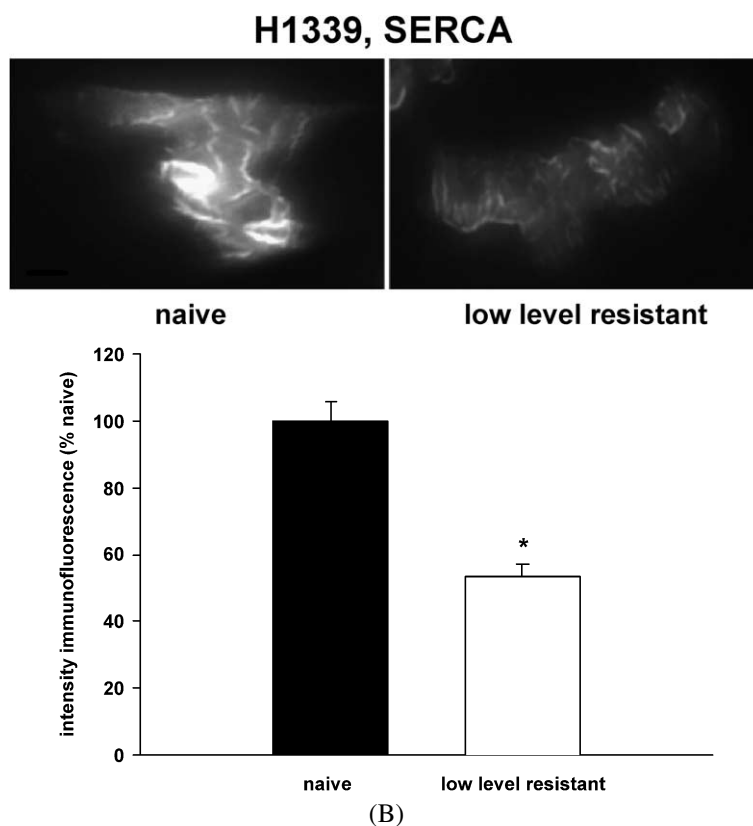


Fig. 5. (Continued).

As an example, prolonged increases in $[Ca^{2+}]_c$ or long-lasting Ca^{2+} -oscillations (hours) are believed to trigger proliferation via the calcineurin/NFAT signaling pathway [31]. On the other hand, short lasting, high amplitude elevations of the $[Ca^{2+}]_c$ can increase mitochondrial Ca^{2+} -concentrations finally promoting mitochondrial outer membrane permeabilization and cell death [19,34,36,40]. Therefore, the amplitude as well as the spatial and temporal aspects of the Ca^{2+} -signaling must precisely be regulated to avoid reduced apoptosis or uncontrolled proliferation. Accordingly, imbalances in the regulation of the Ca^{2+} -homeostasis have become the subject of recent cancer research, but the alterations of the Ca^{2+} -signaling machinery in malignant cells are just beginning to emerge [35].

Elevation of the $[Ca^{2+}]_c$ can be the result of Ca^{2+} -influx from the extracellular space or from Ca^{2+} -release from intracellular Ca^{2+} -stores. In EPLC and H1339 cells, removal of the extracellular calcium did not significantly alter the ATP-induced increase in the $[Ca^{2+}]_c$. We, therefore, focused on the ER as the cellular Ca^{2+} -source. We found the

Ca^{2+} -content of the ER of the low level resistant clones to be reduced in both EPLC and H1339 cells. Padar and colleagues showed that in a taxol resistant clone of the lung adenocarcinoma cell line A549 the Ca^{2+} -content of the Ca^{2+} -pools as well as Ca^{2+} -influx was diminished [38]. Vanoverbergh et al. found that in neuroendocrine differentiated prostate cancer cells apoptosis-resistance was associated with a decreased Ca^{2+} -filling of the ER [48]. In multidrug-resistant MCF-7 breast cancer cells, Chen et al. reported deficient Ca^{2+} -pools [12]. The authors hypothesized that free radicals induced by chemotherapeutics damage the Ca^{2+} -pools leading to increased $[Ca^{2+}]_c$ thereby inducing apoptosis. Consequently, deficient Ca^{2+} -pools would protect from apoptosis. Unfortunately, in these studies, the underlying changes in the components of the Ca^{2+} -regulating machinery were not investigated. However, evidence is being accumulated that a reduced Ca^{2+} -content of the intracellular Ca^{2+} -pool in fact might be a common mechanism in drug-resistance. This is underlined by the fact that in our study both NSCLC and SCLC cells, which represent lung cancer cells with clearly

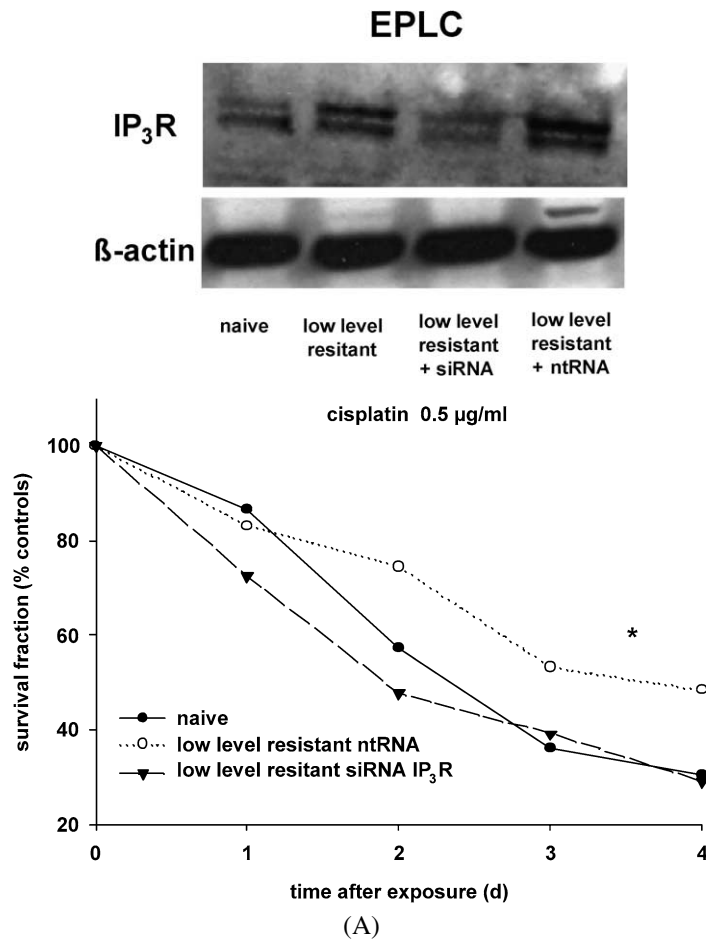


Fig. 6. (A, upper panel) In *low level resistant* EPLC cells, the expression of the IP₃R was reduced after treatment with 100 nM IP₃R-siRNA (IP₃R expression in “low level resistant + siRNA” ~64% of “low level resistant + ntRNA”). Non-targeting RNA had no effect on the expression of the IP₃R. (A, lower panel) In *low level resistant* cells, reduction in the expression of the IP₃R reversed the low level resistance to cisplatin and cells showed a cisplatin-sensitivity similar to naïve cells (**P* < 0.01, two way ANOVA). (B, upper panel, next page) In *naïve* H1339 cells treated with 100 nM SERCA-siRNA, the expression of SERCA was markedly reduced, but cell viability was also diminished (not shown). Therefore, cells were treated with 5 nM SERCA-siRNA, which resulted in smaller decrease in SERCA expression but did not affect viability. (B, lower panel) Reduction of the expression of SERCA in *naïve* cells (SERCA expression in “naïve + siRNA” ~75% of “naïve + ntRNA”) resulted in a cisplatin-sensitivity similar to resistant cells (**P* < 0.01 versus “naïve ntRNA”, two way ANOVA).

distinct clinical properties, showed this common aspect. In heart failure, reduced Ca^{2+} -pools lead to reduced contractility of heart muscle cells [50] and our group recently showed that in asthma bronchial hyper-reactivity is correlated with increased Ca^{2+} -contents of the Ca^{2+} -pools [4]. Thus, alterations of the ER Ca^{2+} -storage may be a common pathophysiological feature of rather different disease entities.

The IP₃R is a Ca^{2+} -channel that is composed of a tetramer of 4 subunits and that releases calcium upon the binding of IP₃ [6]. It is believed to be primarily located on the ER membrane. But, Spletstoeser and colleagues reported that cisplatin induced apoptosis in HeLa-S3 cells by Ca^{2+} -influx involving IP₃R

on the plasma membrane [43]. However, in our study, the IP₃R immuno-fluorescence showed the typical pattern of the ER and the IP₃R in EPLC cells were not located on the plasma membrane. Sakakura and colleagues showed that the IP₃R type 3 was overexpressed in a gastric cancer cell line established from peritoneal dissemination, but weakly expressed in a gastric cancer cell line established from a primary tumor as well as in normal gastric epithelial cells [41]. The authors, therefore, suggest a role for the IP₃R in the transition to a metastatic phenotype. In apoptosis, growing evidence is being accumulated that IP₃R-dependent pathways play a major role [27] because Ca^{2+} -release from the ER by the IP₃R is crucial for the Ca^{2+} -overload of the

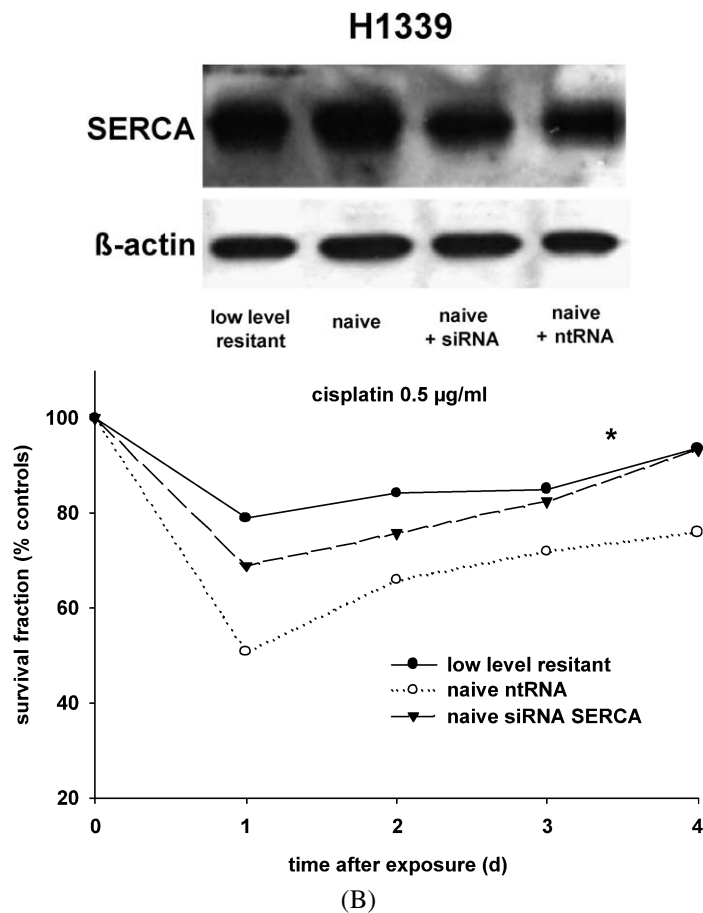


Fig. 6. (Continued).

mitochondria [19,23,26]. Caspase-3 has been reported to cleave the IP₃R and the resulting “channel-only” fragment was responsible for a sustained increase in $[Ca^{2+}]_c$ [26]. Cytochrome C was shown to bind to the IP₃R increasing Ca^{2+} -release from the ER thereby increasing cytochrome C release from the mitochondria forming an amplifying forward loop [7,34]. Further, Chen and colleagues reported that the anti-apoptotic protein Bcl-2 formed complexes with the IP₃R resulting in a reduced IP₃-induced Ca^{2+} -release from the ER [13]. Tsunoda et al. found the IP₃R type 1 to be down-regulated after exposure to cisplatin and in cisplatin resistant bladder cancer cells [46]. In all these studies, increased IP₃R expression implied increased and decreased IP₃R expression implied decreased apoptosis. In contrast, our data suggest that in EPLC cells upregulation of the IP₃R in fact protects from apoptosis. This is in agreement with *in vivo* data obtained from patients with resectable staged NSCLC, where Heighway et al. found amplification of the IP₃R type 2

gene in the tumor tissue compared to normal tissue. Interestingly, the IP₃R type 2 gene was coamplified with KRAS2 [24]. How can overexpression of the IP₃R protect from apoptosis? The Ca^{2+} -homeostasis of the ER is maintained by Ca^{2+} -uptake via SERCA and Ca^{2+} -release via IP₃R and RyR. The Ca^{2+} -release is triggered by binding of the respective second messenger to the receptor, which functions as a Ca^{2+} -channel. But, even in a resting cell, Ca^{2+} -release occurs due to the stochastic open probability of the channel and low levels of second messengers. This Ca^{2+} -release has been termed “ER Ca^{2+} -leak” and IP₃R have been suggested as possible mediators [10]. Similarly, Szlufcik et al. proposed the IP₃R to operate as a Ca^{2+} -leak channel [45]. Therefore, increased expression of IP₃R would result in a higher Ca^{2+} -leak leading to a lower Ca^{2+} -content of the ER. This, in turn, would protect from apoptosis and this is what we observed in our study. In this context, we presume the extrusion of the calcium that has leaked out of the ER to the extracel-

lular space by plasma membrane Ca^{2+} -ATPases to ensure the maintenance of a steady state $[Ca^{2+}]_c$. However, to our knowledge, this is the first report showing that upregulation of the IP_3R actually serves to protect from apoptosis. Further, we show for the first time that the IP_3R is involved in cisplatin-resistance in NSCL cells.

SERCA is an ER transmembrane protein, consists of a single polypeptide chain folded in four major domains and is encoded by the genes ATP2A1-3 [49]. Until now, the isoforms SERCA1a/b, SERCA2a/b and SERCA3a/b/c/d/e due to alternative splicing have been identified. As SERCA serve to maintain the concentration gradient between the cytoplasm and the ER by pumping calcium into the ER, SERCA has been regarded as a potential mediator of alterations of the ER Ca^{2+} -content. In heart failure, the ER Ca^{2+} -content of cardiac myocytes has been found to be reduced caused by an altered expression of SERCA [50]. Moreover, encouraging clinical phase I–II trials have been conducted using the SERCA2a activator istaroxime in patients with heart failure [22]. In our laboratory, bronchial hyperreactivity in an asthma model was correlated with an increased Ca^{2+} -content of the sarcoplasmic reticulum of airway smooth muscle cells [4]. Further, in an interleukin based asthma model, the increased Ca^{2+} -content was at least low level caused by an increased expression of SERCA (manuscript in revision).

The role of SERCA in carcinogenesis has been investigated by Prasad and colleagues. The authors used ATP2A2 knock-out mice – the ATP2A2 gene encodes for SERCA2 – and reported that haplo-insufficiency of this gene predisposed mice to squamous cell tumors of the forestomach [39]. This exciting report, therefore, constitutes a direct link between altered Ca^{2+} -signaling and carcinogenesis. Using high density gene expression arrays Spira and colleagues investigated the effects of cigarette smoke on the human airway epithelium cell transcriptome [3,42]. Comparing current smokers and never smokers, the authors found the expression of SERCA to be significantly upregulated and trends towards upregulation for IP_3R and RyR (online data bank: Smoking Induced Epithelial Gene Expression (SIEGE), Boston University). Therefore, changes in the Ca^{2+} -signaling and particularly in the ER Ca^{2+} -content regulating proteins may be involved in the early steps of carcinogenesis in lung cancer.

Several studies investigated the expression of SERCA in normal and tumor tissue. In patients with

colon or lung cancer, Korosec et al. found that changes in the ATP2A2 gene were more common suggesting a predisposition for these types of cancer in the case of ATP2A2 gene alterations [28]. Endo et al. showed that in human oral squamous cell carcinoma SERCA2 was downregulated probably by posttranslational modification or upregulated degradation [21]. Similarly, in a study by Pacifico et al. the authors showed that SERCA2b mRNA expression was dramatically decreased in tumorigenic thyroid cells [37]. Brouland et al. showed that the expression of SERCA3 was inversely related with the differentiation status of colon epithelial cells and was not detectable in poorly differentiated colon tumors [8]. But, in colorectal cancer, Chung et al. reported that SERCA2 mRNA was increased compared with normal tissue [14]. Moreover, increased SERCA2 protein levels were correlated with serosal invasion, lymph node metastasis, advanced tumor stage and poorer survival-rate. Hence, an altered Ca^{2+} -content of the ER might not only be involved in the early steps of carcinogenesis but may also cause further malignant transformation towards an invasive and aggressive phenotype.

Investigating the correlation of SERCA expression, ER Ca^{2+} -content and proliferation, Legrand et al. showed in prostate cancer cells that an increased growth rate was correlated with higher $[Ca^{2+}]_{ER}$ and increased SERCA2b expression. A decreased growth rate was correlated with decreased $[Ca^{2+}]_{ER}$ and decreased expression of SERCA2b [29]. Also in prostate cancer cells, the same group reported that the insulin-like-growth-factor induced cell growth and an increase in $[Ca^{2+}]_{ER}$ by overexpression of SERCA2b [25]. $TNF\alpha$, which reduced cell proliferation and induced apoptosis, decreased the $[Ca^{2+}]_{ER}$ by reducing the expression of SERCA2b. Further, a large scale, 15 min increase in $[Ca^{2+}]_c$ induced by thapsigargin led to apoptosis, while a minor 48 h increase in $[Ca^{2+}]_c$ also induced by thapsigargin led to proliferation. Neuroendocrine differentiation of prostate cancer cells is considered to mark increased aggressiveness of cancer growth. Vanverberghe et al. showed that neuroendocrine differentiation in these cells was associated with apoptosis resistance probably due a decreased filling of the ER Ca^{2+} -store caused by under-expression of SERCA2b and calreticulin [48]. But, Crepin et al. reported that prolactin stimulated proliferation in immortalized prostate cells by an increased $[Ca^{2+}]_{ER}$ due to increased SERCA2b expression [16]. In a comprehensive review, Lipskaia proposed that proliferation is associated with a sustained increase in $[Ca^{2+}]_c$ or sus-

tained Ca^{2+} -oscillations, decreased refilling of the ER because of SERCA inhibition and enhanced store operated Ca^{2+} -entry from the extracellular space [31]. The conflicting data of increased and decreased $[Ca^{2+}]_{ER}$ and SERCA expression in cancer may be due to the fact that calcium can promote malignant transformation by increased proliferation – induced by increased Ca^{2+} -signaling – or by reduced apoptosis – induced by reduced Ca^{2+} -signaling. However, in our study, we did not observe differences in proliferation between the naïve clones and the low level resistant clones regardless of the reduced $[Ca^{2+}]_{ER}$.

Although SERCA expression in cancer has been investigated with respect to carcinogenesis, proliferation and metastatic potential, the role of SERCA in therapy resistance is unknown. In fact, our study is the first report showing that a decreased expression of SERCA is involved in anti-cancer therapy resistance. This is of particular interest because our findings may lead to new therapeutical options. Over the last 2 decades, a number of drugs allowing the *in vitro* pharmaceutical alteration of the Ca^{2+} -content of the ER have been developed. To provide a few examples, thapsigargin and CPA are widely used inhibitors of SERCA. Many compounds have been shown to interact with the IP_3R with xestospongins and 2-aminoethoxy-diphenylborate (2-ABP) being the most common inhibitors. However, none of these drugs has ever found its way into clinical use. As mentioned before, a decreased $[Ca^{2+}]_{ER}$ is regarded as a pathophysiological mechanism in heart failure [50]. Istaroxime is a SERCA activator and has been successfully tested in a clinical phase 1–2 trial to be well tolerated and to improve cardiac function [22]. Gene therapy is another way to alter the Ca^{2+} -homeostasis of cardiac myocytes. Overexpression of SERCA or inhibition of the repressor effect of phospholamban on SERCA activity have been targeted in heart failure and are currently being tested for their potential use in humans [32]. In cancer, SERCA activating components may act as chemosensitizer and, therefore, may improve effectiveness of conventional therapeutical regimens. This may be the case if the reduced $[Ca^{2+}]_{ER}$ is the result of a decreased SERCA expression but also if other mechanisms led to the diminished $[Ca^{2+}]_{ER}$ as seen in EPLC cells. However, we believe that the emerging availability of therapeutics altering the intracellular Ca^{2+} -signaling could bring new hope to patients not only suffering from heart disease but also suffering from cancer.

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