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

Kathrin Schrödl, Hamza Oelmez, Martin Edelmann, Rudolf Maria Huber and Albrecht Bergner *

Pneumology, Medizinische Klinik-Innenstadt, Ludwig-Maximilians-University, Munich, Germany

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 $\text{Ca}^{2+}$-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 $\textit{in vivo}$ pharmacokinetics. Changes in the cytoplasmic $\text{Ca}^{2+}$-concentration ([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 $\text{Ca}^{2+}$-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$_3$R). Inhibiting the increased expression of IP$_3$R using siRNA, the low level resistance could be reversed. In low level resistant H1339 cells, the decreased $\text{Ca}^{2+}$-content of the ER was correlated with a decreased expression of sarco/endoplasmic reticulum $\text{Ca}^{2+}$-ATPases (SERCA). Decreasing the expression of SERCA in naive H1339 cells resulted in low level cisplatin resistance.

Conclusion: We conclude that cisplatin therapy leads to a decreased $\text{Ca}^{2+}$-content of the ER thereby inducing low level resistance. This is caused by upregulation of the IP$_3$R in EPLC and decreased expression of SERCA in H1339 cells.

Keywords: Lung cancer, cisplatin, calcium, SERCA, IP$_3$R

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-
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 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 (IP_{3}R1) [46], which releases calcium from the ER. The authors showed that the IP_{3}R expression was down-regulated in resistant cells and after exposure of non-resistant cells to cisplatin. Recently, Splettstoesser and colleagues showed that in Hela-S3 but not in human osteoblastoma cells cisplatin induced Ca^{2+}-influx from the extracellular space involving IP_{3}R 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 IP_{3}R. 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 IP_{3}R 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 manufactures 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 µg/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 µg/ml cisplatin were allowed to recover for 7 days and were again exposed to 0.5 µg/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 [Ca2+]c on the cisplatin-sensitivity, the Ca2+-chelator BAPTA-AM was used. Buffering the intracellular calcium with 50 µM BAPTA-AM, EPLC and H1339 cells showed an increased survival fraction (Fig. 1, lower panel).

Elevation of the [Ca2+]c can result either from Ca2+-influx from the extracellular space or from Ca2+-release from internal Ca2+-stores, primarily the ER. Proteins involved in the Ca2+-release from the ER are the inositol-1,4,5-trisphosphate receptor (IP3R) and the ryanodine receptor (RyR) (Fig. 2). Sarco/endoplasmic reticulum Ca2+-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 µg/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 µg/ml cisplatin were allowed to recover for 7 days and were again exposed to 0.5 µg/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\textsuperscript{2+}-concentration on the cisplatin-sensitivity was investigated using the cell permeable Ca\textsuperscript{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).
Ca\textsuperscript{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\textsuperscript{2+}-concentration was quantified using fluorescence microscopy. The baseline fluorescence intensities reflecting the baseline Ca\textsuperscript{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\textsuperscript{2+}]\textsubscript{c} was lower in the low level resistant clones (Fig. 3, upper panel). Fifty μM BAPTA-AM almost completely prevented the ATP-induced Ca\textsuperscript{2+}-increase in normal and low level resistant EPLC and H1339 cells (data not shown). To evaluate the underlying mechanisms of the altered Ca\textsuperscript{2+}-homeostasis, the contribution of Ca\textsuperscript{2+}-influx from the extracellular space was investigated. Prior to the exposure to ATP, the extracell-
Fig. 3. (Continued).

The cellular 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 \([\text{Ca}^{2+}]_{\text{ER}}\) using fluorescent Ca\(^{2+}\)-indicators is not feasible. Therefore, we used an indirect approach. SERCA were inhibited using 1 µM cyclopiazonic acid (CPA) leading to a net Ca\(^{2+}\)-efflux out of the ER. The resulting increase in \([\text{Ca}^{2+}]_c\) was used as an estimate of the \([\text{Ca}^{2+}]_{\text{ER}}\) [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 \([\text{Ca}^{2+}]_c\) was lower in the low level resistant clones indicating a lower \([\text{Ca}^{2+}]_{\text{ER}}\) (Fig. 3, lower panel).

To investigate the underlying mechanisms of the decreased ER Ca\(^{2+}\)-content, the expression of proteins regulating the \([\text{Ca}^{2+}]_{\text{ER}}\) 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 in the naïve and low level resistant clones (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 IP\(_3\)R was higher in the low level resistant clone using an IP\(_3\)R antibody recognizing all 3 isoforms (Figs 4(A) and 5(A)). Conversely, in H1339 cells, no differences regarding the IP\(_3\)R 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 IP\(_3\)R 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 IP\(_{3}\)R was higher in the low level resistant clone (double bands due to isoforms, \(n = 5\), \(\ast P < 0.05\)). (B) In H1339 cells, the expression of calreticulin and IP\(_{3}\)R was similar in the naïve and low level resistant clone. But, the expression of SERCA was lower in the low level resistant clone (\(n = 4\), \(\ast P < 0.05\)).

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 IP\(_{3}\)R could be reversed using 100 nM IP\(_{3}\)R-siRNA. Moreover, the decrease in IP\(_{3}\)R 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 resistant 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 [Ca²⁺]ᵢ during the treatment with cisplatin. In EPLC and H1339, the Ca²⁺-content of the ER was found to be decreased in the low level resistant clones. In low level resistant EPLC cells, the reduced [Ca²⁺]ᵢ 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 [Ca²⁺]ᵢ was correlated with a decreased expression of SERCA. Naive 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 ∼1 µg/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 µg/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 Ca²⁺-signaling toolkit enables very different spatial and temporal Ca²⁺-dynamics within one cell [5].
As an example, prolonged increases in \([\text{Ca}^{2+}]_{\text{c}}\) or long-lasting \(\text{Ca}^{2+}\)-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 \([\text{Ca}^{2+}]_{\text{c}}\) can increase mitochondrial \(\text{Ca}^{2+}\)-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 \(\text{Ca}^{2+}\)-signaling must precisely be regulated to avoid reduced apoptosis or uncontrolled proliferation. Accordingly, misbalances in the regulation of the \(\text{Ca}^{2+}\)-homeostasis have become the subject of recent cancer research, but the alterations of the \(\text{Ca}^{2+}\)-signaling machinery in malignant cells are just beginning to emerge [35].

Elevation of the \([\text{Ca}^{2+}]_{\text{c}}\) can be the result of \(\text{Ca}^{2+}\)-influx from the extracellular space or from \(\text{Ca}^{2+}\)-release from intracellular \(\text{Ca}^{2+}\)-stores. In EPLC and H1339 cells, removal of the extracellular calcium did not significantly alter the ATP-induced increase in the \([\text{Ca}^{2+}]_{\text{c}}\). We, therefore, focused on the ER as the cellular \(\text{Ca}^{2+}\)-source. We found the \(\text{Ca}^{2+}\)-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 \(\text{Ca}^{2+}\)-content of the \(\text{Ca}^{2+}\)-pools as well as \(\text{Ca}^{2+}\)-influx was diminished [38]. Vanoverberghe et al. found that in neuroendocrine differentiated prostate cancer cells apoptosis-resistance was associated with a decreased \(\text{Ca}^{2+}\)-filling of the ER [48]. In multidrug-resistant MCF-7 breast cancer cells, Chen et al. reported deficient \(\text{Ca}^{2+}\)-pools [12]. The authors hypothesized that free radicals induced by chemotherapeutics damage the \(\text{Ca}^{2+}\)-pools leading to increased \([\text{Ca}^{2+}]_{\text{c}}\) thereby inducing apoptosis. Consequently, deficient \(\text{Ca}^{2+}\)-pools would protect from apoptosis. Unfortunately, in these studies, the underlying changes in the components of the \(\text{Ca}^{2+}\)-regulating machinery were not investigated. However, evidence is being accumulated that a reduced \(\text{Ca}^{2+}\)-content of the intracellular \(\text{Ca}^{2+}\)-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
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 IP\(_3\)R is a Ca\(^{2+}\)-channel that is composed of a tetramer of 4 subunits and that releases calcium upon the binding of IP\(_3\) [6]. It is believed to be primarily located on the ER membrane. But, Splettstoesser and colleagues reported that cisplatin induced apoptosis in HeLa-S3 cells by Ca\(^{2+}\)-influx involving IP\(_3\)R on the plasma membrane [43]. However, in our study, the IP\(_3\)R immuno-fluorescence showed the typical pattern of the ER and the IP\(_3\)R in EPLC cells were not located on the plasma membrane. Sakakura and colleagues showed that the IP\(_3\)R 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 IP\(_3\)R in the transition to a metastatic phenotype. In apoptosis, growing evidence is being accumulated that IP\(_3\)R-dependent pathways play a major role [27] because Ca\(^{2+}\)-release from the ER by the IP\(_3\)R is crucial for the Ca\(^{2+}\)-overload of the
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 $[\text{Ca}^{2+}]_c$ [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 downregulated 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$^{2+}$-release 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 \([\text{Ca}^{2+}]_{\text{c}}\). However, to our knowledge, this is the first report showing that upregulation of the IP3R actually serves to protect from apoptosis. Further, we show for the first time that the IP3R 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 trails have investigated the effects of cigarette smoke on the human airway epithelium cell transcriptome [3,42]. Comparing current smokers and never smokers, the authors found 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 \([\text{Ca}^{2+}]_{\text{ER}}\) and increased SERCA2b expression. A decreased growth rate was correlated with decreased \([\text{Ca}^{2+}]_{\text{ER}}\) 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 \([\text{Ca}^{2+}]_{\text{ER}}\) by overexpression of SERCA2b [25]. TNFα, which reduced cell proliferation and induced apoptosis, decreased the \([\text{Ca}^{2+}]_{\text{ER}}\) by reducing the expression of SERCA2b. Further, a large scale, 15 min increase in \([\text{Ca}^{2+}]_{\text{c}}\) induced by thapsigargin led to apoptosis, while a minor 48 h increase in \([\text{Ca}^{2+}]_{\text{c}}\) also induced by thapsigargin led to proliferation. Neuroendocrine differentiation of prostate cancer cells is considered to mark increased aggressiveness of cancer growth. Vanverbergh 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 \([\text{Ca}^{2+}]_{\text{ER}}\) due to increased SERCA2b expression [16]. In a comprehensive review, Lipskaia proposed that proliferation is associated with a sustained increase in \([\text{Ca}^{2+}]_{\text{c}}\), 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 therapeutic 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\(_3\)R 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 therapeutic 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.

Acknowledgements

Supported by the Deutsche Forschungsgemeinschaft Grant BE 2356/2-3 and a Deutsche Gesellschaft für Pneumologie und Beatmungsmedizin Grant to A. Bergner. Data presented in this paper are part of the doctoral thesis of K. Schroedl.

References

[1] A.J. Alberg, J.G. Ford and J.M. Samet, Epidemiology of lung cancer: ACCP evidence-based clinical practice guidelines (2nd edition), Chest 132(Suppl. 3) (2007), 296–358.
[2] A. Alt, K. Lammens, C. Chiocchini, A. Lammens, J.C. Pieck, D. Kuic, K.P. Hopfner and T. Carell, Bypass of DNA lesions generated during anticancer treatment with cisplatin by DNA polymerase eta, Science 318(5852) (2007), 967–970.
[3] J. Beane, P. Sebastiani, G. Liu, J.S. Brody, M.E. Lenburg and A. Spira, Reversible and permanent effects of tobacco smoke exposure on airway epithelial gene expression, Genome Biol. 8(9) (2007), R201.
[4] A. Bergner, J. Kellner, A.K. Silva, F. Gamara and R.M. Huber, Ca\(^{2+}\)-signaling in airway smooth muscle cells is altered in T-bet knock-out mice, Respir. Res. 7 (2006), 33.
[5] M.J. Berridge, M.D. Bootman and H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, Nat. Rev. Mol. Cell Biol. 7(3) (2006), 517–529.
[6] I. Bezprozvanny, The inositol 1,4,5-trisphosphate receptors, Cell Calcium 38(3/4) (2005), 261–272.
[7] D. Boehning, R.L. Patterson, L. Sedaghat, N.O. Glebova, T. Kurosaki and S.H. Snyder, Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis, Nat. Cell Biol. 5(12) (2003), 1051–1061.
[8] J.P. Brouland, P. Gelebart, T. Kovacs, J. Enouf, J. Grossmann and B. Papp, The loss of sarco/endoplasmic reticulum calcium transport ATPase 3 expression is an early event during the multistep process of colon carcinogenesis, Am. J. Pathol. 167(1) (2005), 233–242.
[9] H. Burger, K. Nooter, A.W. Boersma, C.J. Kortland and G. Stoter, Lack of correlation between cisplatin-induced apoptosis, p53 status and expression of Bcl-2 family proteins in testicular germ cell tumour cell lines, Int. J. Cancer 73(4) (1997), 592–599.
[10] C. Camello, R. Lomax, O.H. Petersen and A.V. Tepikin, Calcium leak from intracellular stores – the enigma of calcium signalling, Cell Calcium 32(5/6) (2002), 355–361.
[11] C. Cerella, M. D’Alessio, M. De Nicola, A. Magrini, A. Bergamaschi and L. Ghiselli, Cytosolic and endoplasmic reticulum Ca\(^{2+}\) concentrations determine the extent and the morphological type of apoptosis, respectively, Ann. N.Y. Acad. Sci. 1010 (2003), 74–77.
[12] J.S. Chen, N. Agarwal and K. Mehta, Multidrug-resistant MCF-7 breast cancer cells contain deficient intracellular calcium pools, Breast Cancer Res. Treat. 71(3) (2002), 237–247.
Ca\(^{2+}\)-homeostasis of cisplatin-resistant lung cancer cells

[13] R. Chen, I. Valencia, F. Zhong, K.S. McColl, H.L. Roderick, M.D. Bootman, M.J. Berridge, S.J. Conway, A.B. Holmes, G.A. Mignery, P. Veleg and C.W. Distelhorst, Bel-2 functionally interacts with inositol 1,4,5-triphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5-triphosphate, J. Cell Biol. 166(2) (2004), 193–203.

[14] F.Y. Chung, S.R. Lin, C.S. Yeh, F.M. Chen, J.S. Hsieh, T.J. Huang and J.Y. Wang, Sarco/endoplasmic reticulum calcium-ATPase 2 expression as a tumor marker in colorectal cancer, Am. J. Surg. Pathol. 30(8) (2006), 969–974.

[15] D.E. Clapham, Calcium signaling, Cell 131(6) (2007), 1047–1058.

[16] A. Ceepin, G. Bidaux, F. Vanden Abeele, E. Dewailly, V. Goffin, N. Prevarskaya and C. Slomianny, Prolactin stimulates prostate cell proliferation by increasing endoplasmic reticulum content due to SERCA 2b over-expression, Biochem. J. 401(1) (2007), 49–55.

[17] F.E. de Jongh, J. Verweij, W.J. Loos, R. de Wit, M.J. de Jonge, A.S. Planting, K. Nooter, G. Stoter and A. Sarreboem, Body-surface area-based dosing does not increase accuracy of predicting cisplatin exposure, J. Clin. Oncol. 19(17) (2001), 3733–3739.

[18] N. Demaurex and C. Distelhorst, Cell biology. Apoptosis – the calcium connection, Science 306(5661) (2003), 65–67.

[19] A. Deniaud, E.D. Sharaf, E. Maillier, D. Poncet, G. Kroemer, S.K. Joseph, G. Hajnoczky, IP3 receptors in cell survival and apoptosis: Ca\(^{2+}\) pools and cell growth. Evidence for sarcoendoplasmic Ca\(^{2+}\)-ATPases 2B involvement in human prostate cancer cell growth control, J. Biol. Chem. 276(30) (2001), 47608–47614.

[20] X. Liang and Y. Huang, Intracellular free calcium concentration and cisplatin resistance in human lung adenocarcinoma A549 cells, Biosci. Rep. 20(3) (2000), 129–138.

[21] L. Lipskaia, J.S. Hulot and A.M. Lompre, Role of sarco/endoplasmic reticulum calcium content and calcium ATPase activity in the control of cell growth and proliferation, Pflugers Arch. 457(3) (2009), 673–685.

[22] L. Lipskaia, H. Ly, Y. Kawase, R. Hajjar and A.M. Lompre, Treatment of heart failure by calcium cycling gene therapy, Future Cardiol. 3 (2008), 413–423.

[23] A. Mandic, J. Hansson, S. Linder and M.C. Shoshan, Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling, J. Biol. Chem. 278(11) (2003), 9100–9106.

[24] M.P. Mattson and S.L. Chan, Calcium orchestrates apoptosis, Nat. Cell Biol. 5(12) (2003), 1041–1043.

[25] G.R. Monteith, D. McAndrew, H.M. Faddy and S.J. Roberts-Thomson, Calcium and cancer: targeting Ca\(^{2+}\) transport, Nat. Rev. Cancer 7(7) (2007), 519–530.

[26] S. Orrenius, B. Zhivotovsky and P. Nicotera, Regulation of cell death: the calcium-apoptosis link, Nat. Rev. Mol. Cell Biol. 4(7) (2003), 552–565.

[27] A.F. Kabore, J.B. Johnston and S.B. Gibson, Changes in the apoptotic and survival signaling in cancer cells and their potential therapeutic implications, Curr. Cancer Drug Targets 4(2) (2004), 147–163.

[28] B. Korosec, D. Glavac, T. Rott and M. Ravnik- Glavac, Alternations in the ATP2A2 gene in correlation with colon and lung cancer, Cancer Genet. Cytogen. 171(2) (2006), 105–111.

[29] G. Legrand, S. Humez, C. Slomianny, E. Dewailly, F. Vanden Abeele, P. Mariot, F. Wuytack and N. Prevarskaya, Ca\(^{2+}\) pool content and growth. Evidence for sarcoendoplasmic Ca\(^{2+}\)-ATPases 2B involvement in human prostate cancer cell growth control, J. Biol. Chem. 276(30) (2001), 47608–47614.

[30] X. Liang and Y. Huang, Intracellular free calcium concentration and cisplatin resistance in human lung adenocarcinoma A549 cells, Biosci. Rep. 20(3) (2000), 129–138.

[31] L. Lipskaia, J.S. Hulot and A.M. Lompre, Role of sarco/endoplasmic reticulum calcium content and calcium ATPase activity in the control of cell growth and proliferation, Pflugers Arch. 457(3) (2009), 673–685.

[32] L. Lipskaia, H. Ly, Y. Kawase, R. Hajjar and A.M. Lompre, Treatment of heart failure by calcium cycling gene therapy, Future Cardiol. 3 (2008), 413–423.

[33] A. Mandic, J. Hansson, S. Linder and M.C. Shoshan, Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling, J. Biol. Chem. 278(11) (2003), 9100–9106.

[34] M.P. Mattson and S.L. Chan, Calcium orchestrates apoptosis, Nat. Cell Biol. 5(12) (2003), 1041–1043.

[35] G.R. Monteith, D. McAndrew, H.M. Faddy and S.J. Roberts-Thomson, Calcium and cancer: targeting Ca\(^{2+}\) transport, Nat. Rev. Cancer 7(7) (2007), 519–530.

[36] S. Orrenius, B. Zhivotovsky and P. Nicotera, Regulation of cell death: the calcium-apoptosis link, Nat. Rev. Mol. Cell Biol. 4(7) (2003), 552–565.

[37] F. Pacifico, L. Ulianich, S. De Micheli, S. Treglia, A. Leonardi, P. Carminati and M. Gheorghiade, A phase 1-2 dose-escalating study evaluating the safety and tolerability of istaroxime and its concomitant effects on electrocardiographic and hemodynamic parameters in patients with chronic heart failure with reduced systolic function, Am. J. Cardiol. 99(2A) (2007), 47A–56A.

[38] G. Hajończyk, G. Corzas, M. Madesh and P. Pacher, Control of apoptosis by IP3(3) and ryanodine receptor driven calcium signals, Cell Calcium 28(5/6) (2000), 349–369.

[39] J. Heighway, D.C. Betticher, P.R. Hoban, H.J. Allmert and R. Cowen, Coamplification in tumors of KRAS2, type 2 inositol 1,4,5-trisphosphate receptor gene, and a novel gene, human Krag, Genomics 35(1) (1996), 207–214.

[40] S. Humez, G. Legrand, F. Vanden Abeele, M. Monet, P. Marchetti, G. Lepage, A. Crepin, E. Dewailly, F. Wuytack and N. Prevarskaya, Role of endoplasmic reticulum calcium content in prostate cancer cell growth regulation by IGF and TNFalpha, J. Cell. Physiol. 201(2) (2004), 201–213.

[41] S.K. Joseph, G. Hajończyk, IP3 receptors in cell survival and apoptosis: Ca\(^{2+}\) release and beyond, Apoptosis 12(5) (2007), 951–968.
A. Spira, J. Beane, V. Shah, G. Liu, F. Schembri, X. Yang, J. Palma and J.S. Brody, Effects of cigarette smoke on the human airway epithelial cell transcriptome, *Proc. Natl. Acad. Sci. USA* **101**(27) (2004), 10143–10148.

F. Splettstoesser, A.M. Florea and D. Busselberg, IP(3) receptor antagonist, 2-APB, attenuates cisplatin induced Ca$^{2+}$-influx in HeLa-S3 cells and prevents activation of calpain and induction of apoptosis, *B. J. Pharmacol.* **151**(8) (2007), 1176–1186.

D.J. Stewart, Mechanisms of resistance to cisplatin and carboplatin, *Crit. Rev. Oncol. Hematol.* **63**(1) (2007), 12–31.

K. Szlufcik, L. Missiaen, J.B. Parys, G. Callewaert and H. De Smedt, Uncoupled IP3 receptor can function as a Ca$^{2+}$-leak channel: cell biological and pathological consequences, *Biol. Cell* **98**(1) (2006), 1–14.

T. Tsunoda, H. Koga, A. Yokomizo, K. Tatsugami, M. Eto, J. Inokuchi, A. Hirata, K. Masuda, K. Okumura and S. Naito, Inositol 1,4,5-trisphosphate (IP3) receptor type 1 (IP3R1) modulates the acquisition of cisplatin resistance in bladder cancer cell lines, *Oncogene* **24**(8) (2005), 1396–1402.

K. Vanoverberghe, F. Vanden Abeele, P. Mariot, G. Lepage, M. Roudbaraki, J.L. Bonnal, B. Mauroy, Y. Shuba, R. Skryma and N. Prevarskaya, Ca$^{2+}$ homeostasis and apoptotic resistance of neuroendocrine-differentiated prostate cancer cells, *Cell Death Differ.* **11**(3) (2004), 321–330.

M. Yano, Y. Ikeda and M. Matsuzaki, Altered intracellular Ca$^{2+}$ handling in heart failure, *J. Clin. Invest.* **115**(3) (2005), 556–564.