Abstract. Metastatic non-small cell lung cancer (NSCLC) remains the most common cause of tumor mortality despite the introduction of novel agents. Female sex hormones play a role in NSCLC pathogenesis and negatively influence the course of this disease. Herein, we present data on possible underlying mechanisms. Both estrogen and progesterone pretreatment led to chemoresistance of A549 NSCLC cells in vitro by attenuating cisplatin-induced apoptosis. These effects were not antagonized by the estrogen or progesterone receptor antagonists ICI 182,780 and RU486 (mifepristone). Cisplatin induced apoptosis via activation of caspases -3/7, -8 and -9. Estrogen and progesterone attenuated levels of caspase activation. Interestingly, copper-transporter-1, which is responsible for the intracellular accumulation of cisplatin, was not modulated by sex hormones and the effects of estrogen and progesterone were neither additive nor synergistic. Our results suggest that estrogen and progesterone contribute to the development of chemotherapy resistance in NSCLC via non-classical sex hormone signaling pathways.

Lung cancer is the most common cause of cancer mortality worldwide for both men and women, and prognosis of patients with lung cancer remains unacceptably poor, with a median survival of approximately 12 months (1, 2). Smoking is the most important risk factor for lung cancer development. However, female never-smokers have a markedly higher incidence of non-small cell lung cancer (NSCLC), mainly adenocarcinoma, compared to male never-smokers and show remarkably distinct responses to therapy with the tyrosine kinase inhibitors (TKI) gefitinib and erlotinib (3). Furthermore, post-menopausal hormone replacement therapy increases mortality of patients suffering from lung cancer (4-9). Interestingly, Chlebowski and co-workers showed that this effect may depend on a combination therapy of estrogen and progesterone, since women treated with estrogen-alone showed no increase of lung cancer incidence and mortality (9). A crucial role of progesterone in this context is therefore very likely, although there is a scarcity of data on its influence on NSCLC behavior. Taken together, the clinical evidence implies a role for sex steroids in the development and progression this disease.

Estrogens are thought to play a major role in this scenario. Determination of local tissue estrogen content revealed significantly higher estrogen levels in neoplastic, compared to normal-appearing lung tissue (10). In line with these data, high aromatase expression levels were detected in malignant tissue samples (11-13). Aromatase inhibitors showed promising antiproliferative effects in cell culture and xenograft studies (12, 14). Abnormally high expression levels of estrogen receptors alpha and beta (ERα/β) have been demonstrated in lung cancer tissues of women and men (15-18). Moreover, expression of ERβ predicts for clinical response and longer progression-free survival after treatment with an epidermal growth factor receptor (EGFR) TKI (19). Furthermore, serum estrogen levels in combination with the ER status of the tumor tissue represent prognostic factors (20). Further clinical and pathological studies are required to establish the value of ER expression, as well as serum estrogen levels as prognostic and predictive biomarkers in lung cancer.

Several studies aimed at revealing potential underlying mechanisms of sex steroid-dependent modulation of lung cancer biology. The stimulation of cell proliferation by estrogen has been reported in vitro using different lung carcinoma cell lines and, compatible with these findings, estrogen promoted lung tumor growth and dissemination in xenograft mouse models. Tamoxifen, an estrogen receptor...
modulator and fulvestrant, a pure estrogen receptor antagonist, blocked estrogen-induced tumor progression in vivo (12, 14, 21-25). Fulvestrant has been found to influence tumor progression in combination with EGF TKIs in a xenograft model of NSCLC. Interestingly, fulvestrant significantly enhanced the antineoplastic activity of EGF TKIs (21).

Based on these data, post-menopausal women with NSCLC were recently treated in a pilot study with fulvestrant and gefitinib. The authors concluded that combination therapy with gefitinib and fulvestrant was well-tolerated and demonstrated therapeutic activity (26).

Progesterone has also been suspected to play a role in modulating lung cancer progression, but there is a paucity of both clinical data and in vitro results regarding this hypothesis. Progesterone receptors-A (PR-A) and -B (PR-B) are both expressed in lung cancer tissue and their expression levels are reported to be enhanced by estrogen (27, 28). Most recently, Check and colleagues showed that the PR antagonist mifepristone (RU486) improved survival of mice with spontaneous lung cancer (29). With regard to the finding that increased lung cancer mortality in post-menopausal women is associated with a combination therapy of estrogen and progesterone, interactions of both steroids appear to be crucial in mediating disease progression. The main objective of this study was to determine the effect of estrogen and progesterone in mediating chemoresistance of cultured A549 cells against cisplatin-induced cell death. Our results clearly show that not only estrogen, but also progesterone, independently inhibit cisplatin-induced cell death by counteracting cisplatin-induced apoptosis. This chemoresistance effect of both hormones appears to involve non-classical signaling pathways, such as the suppression of cisplatin-induced caspase activity.

Materials and Methods

Chemicals. 17β-Estradiol, progesterone and cisplatin were purchased from Sigma (St. Louis, MO, USA). The estrogen receptor antagonist ICI 182,780 was purchased from Tocris (Bristol, UK); mifepristone (RU486) was purchased from Biomol (Loerrach, Germany).

Cell lines and treatment. The A549 cell line was purchased (American Type Culture Collection, Manassas, VA, USA) and propagated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), supplemented with penicillin-streptomycin (Invitrogen, Karlsruhe, Germany). Experiments were performed in polyornithine-coated plastic chambers. Before treatment, cells were washed in 1×D-PBS to control media.

Semi-quantitative (sq) RT-PCR. RNA isolation, purity assessment, and reverse transcription were performed as described elsewhere (30, 31). sqRT-PCR reactions were carried out using a commercial PCR Kit (Bioline, Berlin, Germany), 2 μl cDNA and 0.25 μl of primer (10 pmol/μl). Reactions were conducted in standard tubes using the Mastercycler epgradientS (Eppendorf, Hamburg, Germany) under the following conditions: 3 min of initial denaturation at 95°C, followed by 35 cycles of each 40 s of denaturation at 95°C, 45 s of annealing at individual temperatures and 40 s of elongation at 72°C. For each experiment, negative controls were performed in which 2 μl of purified water were added to the PCR reaction instead of cDNA. Positive controls using cDNA samples which were affirmed to contain the template to be amplified were also performed. After PCR reaction, cDNA samples were applied to a 2% agarose gel containing ethidium bromide and separated by electrophoresis. Images of gels were taken using AlphaImager (formerly AlphaInnotech, ProteinSimple, Santa Clara, CA, USA). PCR reactions with an exon-intron spanning oligonucleotide primer sequence were routinely performed to exclude genomic DNA contamination of purified RNA.

Immunochemistry. For visualization of steroid receptors ERα and ERβ, A549 cells were cultured onto polyornithine-coated glass slides, and immunocytochemistry was performed as previously described (32) using appropriate antibodies (all from Abcam, Cambridge, UK). In brief, media were aspirated after treatment and cells were washed twice in D-PBS. Cells were subsequently fixed in methanol for 5 min and incubated with 1% bovine serum albumin/2% FCS in PBS for 20 min at room temperature prior to exposure to primary antibodies for 12 h at 4°C. After washing, sections were incubated with appropriate fluorescent secondary antibodies (AlexaFluor 568 1:500; Invitrogen). Slides were then incubated with Hoechst 33342 (Invitrogen; 1:1000) diluted in D-PBS for the staining of cell nuclei.

Cell viability assay. Lactate dehydrogenase (LDH) release was determined using the CytoTox 96 non-radioactive cytotoxicity assay (Promega, Mannheim, Germany) according to the manufacturer’s instructions. Three vials per experiment were treated for 1 h with a lysis solution containing Triton X-100 to obtain maximum LDH release. Results are given as percentage LDH release relative to maximum LDH release. Metabolic activity was determined using the CellTiter-Blue cell viability assay (Promega). Treatment with lysis solution served as negative control. The average fluorescence intensity of Triton X-100-treated cells was subtracted from fluorescence values obtained from cultured cells. Interference of Triton X-100 with fluorescence signals was excluded. Data are given as percentages of the control. The xCELLigence system was used according to the instructions of the supplier Roche Applied Science and ACEA Biosciences (33).

Flow cytometric analyses of cell death. The Annexin-V-Fluor Staining Kit (Roche Diagnostics, Mannheim, Germany) was used to discriminate between apoptosis and necrosis in the given culture system. The assay involves simultaneous staining with both annexin-
V and the DNA stain propidium iodide (PI). Three subpopulation of cells were discriminated: (a) PI-negative and (FITC)-negative viable cells (PI-/FITC-) that maintain the typical asymmetry of plasma membrane lipids; (b) PI-negative and FITC-positive early apoptotic cells (PI-/FITC+) capable of transporting PI outside the cell; and (c) PI-positive and (FITC)-positive late apoptotic or necrotic cells (PI+/FITC+) with a loss of plasma membrane integrity. For analysis, cells were prepared following the manufacturer’s protocol. Fluorescence intensity was measured by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

Caspase assays. Caspase-3/7, -8 and -9 activities were measured in A549 cells using a Caspase-Glo assay kit (Promega) according to the manufacturer’s protocol. Briefly, the pro-luminescent substrate containing the tetrapeptide sequences DEVD, LETD, or LEHD is cleaved by caspase-3, caspase-8, and caspase-9, respectively, after cell lysis proportional to the caspase activity present. After caspase cleavage, a substrate for luciferase is released; this results in the luciferase reaction and the production of a luminescent signal. Luminescence is proportional to the caspase activity present. The luminescence of each sample was measured in a plate-reading luminometer (Promega).

Statistics. For cell viability analysis, statistical analyses were performed using absolute data of all experiments. Differences between groups were tested by an analysis of variance (ANOVA), followed by an independent sample t-test using the SPSS software (SPSS Inc., Chicago, IL, USA). All data are given as arithmetic mean±standard error of the mean (SEM). A value of \( p < 0.05 \) was regarded as statistically significant. All experiments were conducted in triplicate and repeated at least three times.

Results

Cisplatin induces cell death in a dose-dependent manner. In a first set of experiments, we determined the optimal cell concentration for cell viability measurements. To this end, 40,000, 20,000, 10,000, and 5,000 A549 cells/well were seeded into an E-Plate 96 (Roche Applied Science, Penzberg, Germany) and impedance was determined during a 96-h culture period. If a cell attaches to the electrode surface and, thus, partially blocks the electrical current in the circuit, an increase in electrode impedance is induced (shown in the graphs as increase of the cell index) (33). Optimal cell concentrations were determined in order to reach 60% conflueney towards the end of the experiment (see time point 96 h in Figure 1A). Thus, cell proliferation and cell death could be determined in subsequent experiments. As shown in Figure 1B, cell index rapidly increases during the first 24 h after cell seeding. At the initiation of serum starvation at 0.1% FCS, cell indices still gradually increased, however, at a much slower rate as compared to the initial 24 h analysis period. Between 48 h to 72 h post-seeding (representing the hormone pre-treatment period), the cell index remained stable, indicating that proliferation had ceased. During the 24 h of cisplatin exposure (\( 10^{-4} \) M; 72 h to 96 h post-seeding), cell indices significantly declined, whereas the index remained stable in control cultures. As shown in Figure 1C, cell confluence was about 60% to 70% in control cultures at the end of the experiment if 10,000 cells were plated per well. This density of cells was used in all subsequent experiments.

Many different malignant cell types, including A549 cells, are vulnerable to cisplatin. However, dose response and temporal characteristics of in vitro responses to cisplatin differ among cancer cell lines. We, therefore, analyzed in a next set of experiments the susceptibility of A549 cells to a 24-h cisplatin challenge. Cell viability was assayed by LDH release in the culture supernatant and metabolic activity determination of attached cells. As shown in Figure 1D and 1E, cisplatin treatment induced a dose-dependent increase in LDH activity in the supernatant during the 24 h treatment period. This was closely paralleled by a decrease in the corresponding metabolic activity. After 24-h of cisplatin administration (\( 10^{-4} \) M), the viability of A549 cells declined by approximately 50%, as indicated by an increase in LDH activity (\( p < 0.01 \) versus control) and a decrease in metabolic activity (\( p < 0.01 \) versus control). Under control conditions, A549 cells stained with the LIVE/DEAD viability/ cytotoxicity assay and analyzed by cell sorting contained a high percentage of viable cells and only few necrotic or apoptotic cells (Figure 1F). Treatment with cisplatin at a concentration of \( 10^{-3} \) M or \( 10^{-4} \) M for 24 h induced significant cell death, thus confirming results obtained by LDH and metabolic activity determination. Specifically, FACS analysis revealed that the loss of cell viability was mainly due to necrosis in the \( 10^{-3} \) M treatment group and predominantly due to apoptosis in the \( 10^{-4} \) M group, respectively. Moderate (\( 10^{-5} \) M) or lack (\( 10^{-6} \) M) of cytotoxic effects were observed at lower concentrations of cisplatin.

A549 cells express ER and PR. In a next step, we analyzed the expression of steroid receptors in A549 cells in the context of the above-mentioned culture conditions. Analysis was performed 48 h post-seeding. RT-PCR analysis revealed that all three hormone receptors, namely ER\( \alpha \), ER\( \beta \), and PR are expressed in relevant amounts in untreated A549 cells (Figure 2A). Immunofluorescence microscopic imaging of ER\( \alpha \) and ER\( \beta \) confirmed our findings of mRNA expression analysis. ER\( \alpha \) was found to be expressed within the nuclear and cytoplasmic compartment, whereas ER\( \beta \) was found to be predominantly expressed within the cytoplasm (Figure 2B).

Estrogen and progesterone protect A549 cells from cisplatin-induced apoptosis. As demonstrated, a 24 h cisplatin (\( 10^{-4} \) M) treatment protocol yielding an approximately half-maximal decline in cell viability with predominant apoptosis and marginal necrosis was used for all further experiments. To identify the anti-apoptotic effects of 17β-estradiol and progesterone on A549 cisplatin-induced cell death, cells were
Figure 1. A: The experimental set-up. B: Dynamic monitoring of cell numbers by the xCELLigence system. C: Cell confluence after 96 h post-seeding (end of experiment). The effect of cisplatin administration (24 h) on lactate dehydrogenase (LDH) release and metabolic activity in cultured A549 is shown in (D) and (E), respectively. Determination of viable, necrotic, and apoptotic cells determined by fluorescence-activated cell sorting (FACS) analysis is shown in (F). Hatched columns in (D/E) show the cisplatin concentrations which produced semi-maximal cell destruction of A549 cells. This concentration was further applied in the subsequent experiments. Maximum LDH release was achieved by the application of Triton X-100 and set to 100% (expressed as arbitrary units). Effects of cisplatin are given in relation to these of Triton X-100. Metabolic activity was determined in untreated A549 cells (controls) and set to 100% (expressed as fluorescence intensity). Cisplatin data are given as percentiles. Data in (D/E) represent means±SEM. Changes of electronic impedance are shown as cell index values. If no cells are present on an electrode's surface, the sensor's electronic feature is not affected and the cell index is zero. *p<0.01, **p<0.001, ***p<0.0001 control vs. treatment. FCS: fetal calf serum.
treated with cisplatin ($10^{-4}$ M) and either 17β-estradiol and/or progesterone at concentrations from $10^{-10}$ M to $10^{-6}$ M. Higher concentrations were avoided because of the well-known antioxidative properties of estrogens (34). As shown in Figure 3A (results of FACS analysis; viable and apoptotic cells set to 100%, respectively), single applications of 17β-estradiol (concentrations of $10^{-6}$ M) only moderately reduced cisplatin-induced loss of cell viability (cisplatin, mean=100±3.0 versus estrogen=119±6; p<0.05). In contrast, progesterone strongly prevented cisplatin-induced A549 cell death (cisplatin=100±3 versus progesterone=142±7; p<0.001). The treatment with both steroids in combination was neither additive nor synergistic (cisplatin=100±3 versus estrogen plus progesterone=143±5; p<0.001). The treatment with both steroids in combination was neither additive nor synergistic (cisplatin=100±3 versus estrogen plus progesterone=143±5; p<0.001). As expected, similar results were observed when cisplatin-induced apoptosis was analyzed. As shown in Figure 3B, single applications of 17β-estradiol (concentrations of 10-6 M) only moderately reduced cisplatin-induced A549 apoptosis (cisplatin=100±1 versus estrogen=88±3; p<0.01). In contrast, progesterone prevented cisplatin-induced A549 apoptosis more distinctly (cisplatin=100±1 versus progesterone=78±2; p<0.001). Combination of both steroids did not further decrease cisplatin-induced A549 cell loss (cisplatin=100±1 versus estrogen and progesterone=74±5; p<0.01). A representative illustration of FACS analysis is given in Figure 3C. Anti-apoptotic effects of progesterone were paralleled by prevention of cisplatin-induced loss of metabolic activity (see Figure 3D) and reduced release of LDH in the cell culture supernatant (not shown). Lower concentrations of estrogen or progesterone were less effective ($10^{-8}$ M) or ineffective (<$10^{-8}$ M) in preventing cisplatin-induced A549 apoptosis (data not shown).

Interestingly, steroid effects were not abolished by the application of estrogen/progesterone nuclear receptor antagonists ICI 182,780 and mifepristone (Figure 3A and 3B). Both inhibitors ($10^{-6}$ M) were applied 30 min prior to hormone treatment to ensure for effective receptor binding. To our knowledge, this is the first demonstration of direct chemotherapy resistance mediated by progesterone.

**Estrogen and progesterone do not affect cisplatin uptake but attenuate cisplatin-induced caspase activation.** In mammalian cells, resistance to cisplatin is often accompanied by impaired uptake into the target cell by the copper transporter CTR1. To exclude prevention of cisplatin-induced A549 cell death being mediated by steroid-mediated reduction in CTR1 expression, we performed gene expression studies in control and hormone-treated cultures (35). No clear-cut regulation of CTR1 mRNA levels by any hormone applied (estrogen, progesterone, or a combination of both) was evident (data not shown).

After binding of cisplatin to DNA, DNA adducts are formed and various signal-transduction pathways are activated, such as those involved in DNA-damage
recognition and repair, cell-cycle arrest, and programmed cell death/apoptosis (36, 37). The final cellular outcome is generally apoptotic cell death, although the pathway(s) from platinum–DNA binding to apoptosis remain incompletely elucidated. Cisplatin-induced apoptosis is partially mediated by caspase activation. Therefore, we investigated time-dependent activity of caspases 3/7, -8 and -9 after cisplatin treatment (10⁻⁴ M) and the influence of estrogen and progesterone pre-treatment on caspase activity levels. In a first set of experiments, A549 cells were exposed to cisplatin (10⁻⁴ M) for up to 24 h, and caspase activity levels were determined after different exposure periods. As shown in Figure 4 (A-C), activity of all three apoptosis-related caspases was induced 16 h after initiation of the cisplatin
treatment. Therefore, A549 cells were pre-treated in another experiment with either estrogen or progesterone (both 10^{-6} M) before cisplatin (10^{-4} M) was added for another 16 h. As shown in Figure 4D, pre-treatment with steroid hormones significantly reduced activation of caspases-3/7, -8 and -9. Interestingly, estrogen appeared to be more potent in reducing cisplatin-induced activation of caspases as compared with progesterone.

**Discussion**

Emerging evidence indicates a role of estrogen as a disease-promoting stimulus in lung cancer. Biological responses to estrogen signaling involve growth stimulation, alteration of gene expression, and interaction with the epidermal growth factor receptor (EGFR) pathway. Furthermore, estrogen promotes tumor growth and dissemination in vitro and in xenograft mouse models in vivo. Consequently, ER modulators and ER antagonists, i.e. fulvestrant and tamoxifen, can partly abolish these effects (12, 14, 21). Analogous to the established role of local estrogen biosynthesis in breast cancer, aromatase is expressed in lung cancer tissue. Thus, local biosynthesis of estrogen is very likely in lung cancer (10, 12, 14). However, the pathophysiological importance of estrogen, and especially of progesterone, in this disease has still not been determined. In particular, the role of progesterone remains unclear. Moreover, data on potential combined effects of both female sex steroids with regard to treatment resistance are ambiguous. Interestingly, women with post-menopausal hormone replacement therapy display a higher incidence rate of lung cancer (9) and recent epidemiological data emphasize the role of progesterone in this context (38). Clinically, the main contributing factors to the poor prognosis of patients with NSCLC are poor response
to first-line treatment, early relapse, and development of metastasis, i.e., resistance to chemotherapeutic agents such as cisplatin [for review see (39)].

The contribution of gender and sex steroids to the development of drug resistance in lung cancer therapy is poorly understood. Hence, our study attempted to investigate the role of estrogen and progesterone on the development of therapy resistance. We provide clear evidence that the action of estrogen and progesterone contributes to the development of therapy resistance towards cisplatin. Cisplatin is an important compound in the treatment of NSCLC and induces apoptosis of tumor cells by binding to nuclear DNA, forming a variety of structural adducts, and triggering cellular responses leading to apoptosis (40-42). It is suggested that cisplatin causes tumor cells to be arrested in the G2 phase of the cell cycle, and that mitosis cannot be entered because of the inhibition of mRNA synthesis (43). Therefore, transcription inhibition is a critical determinant in the apoptosis pathway triggered by cisplatin. Since initial response rates of advanced NSCLC to cisplatin-containing chemotherapy are 20-50%, drug resistance is mainly regarded as acquired resistance, often with broad cross-resistance to other unrelated chemotherapy agents [for review see (44)]. A growing body of evidence suggests a role for active uptake of cisplatin by membrane proteins such as CTR1. Platinum resistance in lung cancer may, therefore, be associated with decreased CTR1 expression and consequently reduced intracellular accumulation of cisplatin (45-47). The role of sex steroids with regard to the regulation of CTR1 expression appears unclear. Our results show that estrogen and progesterone might not convey treatment resistance by down-regulating CTR1 expression, but rather by influencing apoptotic pathways.

One of the hallmarks of cancer cells is their ability to evade programmed cell death or apoptosis. Alterations in apoptotic pathways are common in cancer cells and, thus, alteration in the regulation of apoptosis has been implicated in both lung tumorigenesis and development of drug resistance (44, 48). Our data show that sex steroid action mediates drug resistance via attenuation of cisplatin-induced apoptosis. In NSCLC cell lines, chemotherapy resistance was, amongst others, associated with defects in expression of caspase-3, -8, and -9 (49). Our results point to the possibility that sex steroid-induced drug resistance is partly mediated by alteration of the caspase pathways. The regulation of caspase activation by estrogen and progesterone results in an attenuation of apoptosis. The detailed mechanisms of sex steroid-induced attenuation of apoptosis remain unclear, and further investigation is needed to gain a more accurate insight.

Interestingly, the effect of estrogen and progesterone were not antagonized by the classical ER and PR antagonists ICI 182,780 and mifepristone, respectively. Activation of extranuclear ERs/PRs activates downstream signaling pathways, such as calcium-dependent and mitogen-activated protein kinase pathways, by activation of receptor tyrosine kinases (50, 51). Most of the reported data concerning the potential of estrogen in promoting lung cancer show mediation via nuclear receptors. We cannot exclude the possibility that tumor cell growth and development of drug resistance by sex steroids arise mainly through separate pathways. Furthermore, there is emerging evidence that the EGFR pathway and the estrogen pathway interact intensively and inversely. An inhibition of EGFR action enhanced the antiproliferative potency of the estrogen receptor modulator tamoxifen and vice versa (21, 52). Hence, therapy with a TKI intended to reduce the impact of the EGFR pathway on tumor cell growth could theoretically be counterbalanced by increased activation of sex steroid-mediated drug resistance.

In summary, our data imply that both estrogen and, even more strongly progesterone attenuate cisplatin-induced apoptosis. These effects do not depend on the regulation of intracellular accumulation of cisplatin or regulation of caspase activation by estrogen and progesterone. Taking into consideration that the classical receptor antagonist ICI 182,780 and mifepristone were not effective in antagonizing the observed effects, it is likely that these effects are mediated via membrane-bound steroid receptors (53). Further work will be crucial to determine the potential role of progesterone and estrogen in the development of therapy resistance to standard chemotherapy compounds.

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