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Cytotoxicity and accumulation of ergot alkaloids in human primary cells

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ABSTRACT

Ergot alkaloids are secondary metabolites produced by fungi of the species *Claviceps*. Toxic effects after consumption of contaminated grains are described since mediaeval times. Of the more than 40 known ergot alkaloids six are found predominantly. These are ergotamine, ergocornine, ergocryptine, ergocristine, ergosine and ergometrine, along with their corresponding isomeric forms (-inine-forms). Toxic effects are known to be induced by an interaction of the ergot alkaloids as neurotransmitters, like dopamine or serotonin. Nevertheless data concerning cytotoxic effects are missing and therefore a screening of the six main ergot alkaloids was performed in human primary cells in order to evaluate the toxic potential.

As it is well known that ergot alkaloids isomerize easily the stability was tested in the cell medium. Based on these results factors were calculated to correct the used concentration values to the biologically active lysergic (-ine) form. These factors range from 1.4 for the most stable compound ergometrine to 5.0 for the most unstable ergot alkaloid ergocristine. With these factors, reflecting the instability, several controverse literature data concerning the toxicity could be explained.

To evaluate the cytotoxic effects of ergot alkaloids, human cells in primary culture were used. These cells remain unchanged in contrast to cell lines and the data allow a better comparison to the *in vivo* situation than using immortalized cell lines. To characterize the effects on primary cells, renal proximal tubule epithelial cells (RPTEC) and normal human astrocytes (NHA) were used. The parameters necrosis (LDH-release) and apoptosis (caspase-3-activation, DNA condensation and fragmentation) were distinguished. The results show that depending on the individual structure of the peptide ergot alkaloids the toxic properties change. While ergometrine as a lysergic acid amide did not show any effect, the peptide ergot alkaloids revealed a different toxic potential. Of all tested ergot alkaloids ergocristine was the most cytotoxic compound inducing apoptosis in human kidney cells starting at a concentration of 1 μM in RPTEC. Uptake studies underline the cytotoxic properties, with an accumulation of peptide ergot alkaloids and no uptake of ergometrine.

The results represent a new description of effects of ergot alkaloids regarding cytotoxicity and accumulation in human primary cells. For the first time apoptosis has been identified besides well described receptor effects. This gives a hint for a more complex mode of action of ergot alkaloids than described in literature so far.

1. Introduction

Since the Middle Ages ergot had a great impact in terms of mass poisoning (Schiff, 2006) or pharmaceutical approaches (Tfelt-Hansen et al., 2000). The toxic compounds, the so called ergot alkaloids, are secondary metabolites produced by the fungal family of *Clavicipitaceae* with most notably *Claviceps purpurea* parasitizing wheat, barley, and most dominantly rye. The fungus infests the living plant at the time of flowering, forming a persistent dormant body, the sclerotia. Sclerotia are resistant to temperature and remain alive during winter, or during storage of crops. In spring the sclerotia germinates and the produced spores can infect other plants again, continuing this cycle (Guggisberg, 1954).

The consumption of contaminated cereals leads to several illnesses, which result in the discovery of the relevant compounds for this effect. The sclerotia also contain the toxic metabolites of the fungus, with more than 40 different alkaloids, known so far. The intoxication was described in mediaeval literature as St. Anthony's fire, where vasoconstriction and gangrene, as well as neurotoxic symptoms were reported. Today the disease is referred to as ergotism, which can occur in two different forms, the ergotism gangrenous and the ergotism convulsive (Barger, 1931). The con-

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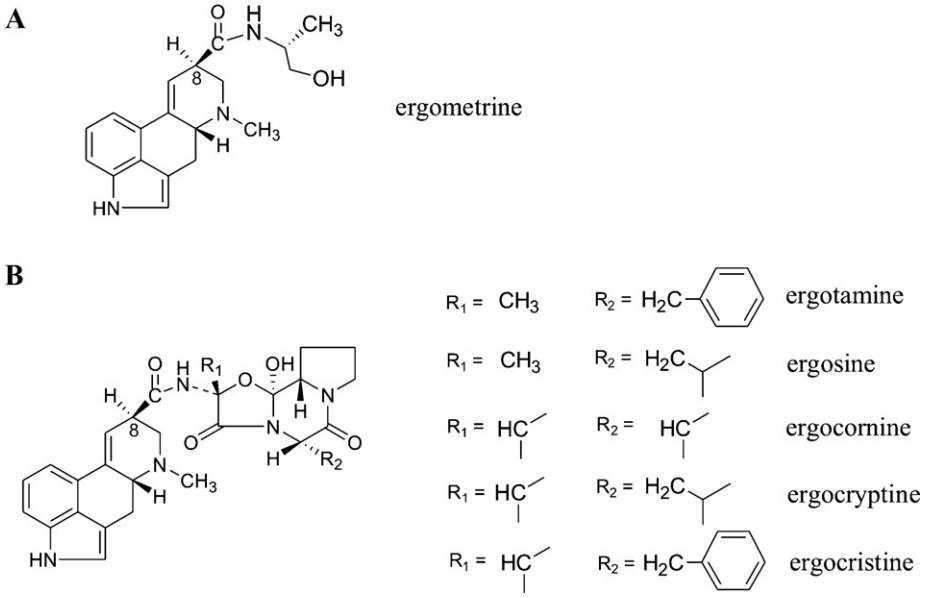

Fig. 1. Structure of relevant ergot alkaloids: lysergic acid amide, ergometrine (A) and peptide ergot alkaloids (B).

vulsive form causes several effects on the central nervous system resulting in spasm, hallucinations or epileptic fits. The gangrenous form (St. Anthony's fire symptoms) affects the poor blood supplied parts of the body, like fingers or toes as the main target. This results in several circulation disorders and ultimately the occurrence of necrotic, black coloured tissue and the loss of the affected body parts (Berde and Stürmer, 1978).

The relevant substances are classified by referring to their structure as clavines, lysergic acid, lysergic acid amines and ergopeptides. The clavines are also called the 8-ergolenes due to the double bond position and the other derivatives belong to the group of the 9-ergolenes. In 2005 the European Food Safety Authority (EFSA) assigned six ergot alkaloids as the most important ones (EFSA, 2005), namely ergotamine, ergocornine, α-ergocryptine, ergosine, ergocristine as peptide ergot alkaloids and ergometrine a lysergic acid amide. The structures for the individual compounds are summarized in Fig. 1.

It is well reported that the toxic effects are caused by the structure similarity of the ergot alkaloids to several neurotransmitters (Eich and Pertz, 1994; Schiff, 2006). Ergot alkaloids can act as partial agonists and antagonists of α-adrenergic, dopaminergic and serotonergic receptors and therefore have a manifold mode of action on the human body, ultimately leading to the toxic effects of the ergotism. Due to effects on the neurotransmitter receptors, the substances have also caused a strong influence on the pharmaceutical industry (Tfelt-Hansen et al., 2000). For example ergotamine with its α-adrenoreceptor-blocking mode of action, first described by Dale (1906), verified by Sachs and Yonkman (1942), is used for the acute therapy of migraine for over 50 years (Tfelt-Hansen et al., 2000).

Various animal experiments lead to several LD_{50} values and many epidemiological descriptions of ergotism are also available (Barger, 1931; Chaumartin, 1946; Guilhon, 1955). Different *in vivo* studies regarding acute toxicity have shown different sensitivities depending on the used animal species, application form and ergot alkaloid. Griffith et al. (1978) have reported rabbits to be the most sensitive species with LD_{50} values between 0.9 and 3.2 mg/kg b.w. after intravenous injection with ergometrine as the least toxic compound and ergocryptine as the most toxic one. These results clearly differ from other species like mice or rats with intravenous LD_{50} values between 30 and 275 mg/kg b.w. whereas the LD_{50} value for an oral dose could often not be calculated due to a very low bioavailability of the peptide ergot alkaloids (Little et al., 1982; Tfelt-Hansen et al., 2000). For humans the toxic effects are referred to the receptor interaction resulting in the acute toxic symptoms of the ergotism, such as vasoconstriction, uterus contraction, lethargy or depression or acute intoxication including diarrhea, collapse and vomiting (Guggisberg, 1954). For *in vitro* experiments only limited data are available for the single substances and their toxic effects on human cells. Most data consist of receptor interaction analysis for single substances in dopamine over expressing cells or tumour cells (Larson et al., 1995, 1999). Experiments using a neurite outgrown model by Oda et al. (2008) indicated a different toxic potential for peptide ergot alkaloids and lysergic acid amide alkaloids.

The aim of this work was to determine the toxic effect of the six mentioned ergot alkaloids using human cells in primary culture. These cells are directly isolated from the corresponding tissue and it is possible to cultivate them over a few passages. The cells remain unchanged and are therefore not immortalized or modified giving a closer look to the *in vivo* situation than normal cell lines. As presented in Königs et al. (2009) there was a major influence regarding trichothecene mycotoxins in comparison between cell lines and primary cells.

To study the influence of the ergot alkaloids on primary cells several parameters like cell viability, necrotic effects (LDH-release) uptake of the ergot alkaloids and triggering apoptosis (caspase-3-activation, sub G1 formation, DNA condensation) were measured. Two cell types, the renal proximal tubule epithelial cells (RPTEC) and normal human astrocytes (NHA) were chosen for a screening of the six main ergot alkaloids. The toxic relevant compounds should be identified and effects besides the well described receptor interaction were investigated. This screening gives the possibility to evaluate and compare the toxic compounds with each other. As the data for toxic effects on humans for the individual compounds are missing and the data from animal studies are difficult to compare with the human situation, the analysis of the effects on primary cells will complete the data to understand the toxicity of ergot alkaloids and their range of effects.

2. Materials and methods

2.1. Chemicals

Ergot alkaloids ergocornine, α-ergocryptine, ergotamine-D-tartrate and ergometrine-maleate were purchased from Sigma–Aldrich (Steinheim, Germany).

Ergosine and T-2 toxin were biosynthetically prepared and isolated in our lab (Beyer et al., 2009; Franzmann, 2010). Ergocristine, as well as ergocristinine, ergosinine, ergotaminine, ergometrinine, ergocorninine and ergocryptinine were purchased from Alfarma (Černošice, Czech Republic). Methysergide maleate was ordered from Biotrend (Wangen, Switzerland).

Cell culture media DMEM/Ham's F-12 and Quantum 333 and all used supplements were obtained from PAA Laboratories (Pasching, Austria). Fetal calf serum (FCS) was purchased from Biochrom AG (Berlin, Germany) and epidermal growth factor (EGF) from BD Bioscience (San Jose, USA). All other chemicals were obtained from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma–Aldrich (Steinheim, Germany). Purified water was generated by a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany).

2.2. Quantification of ergot alkaloids

Determination of the epimerization of the single ergot alkaloids was done using high performance liquid chromatography with fluorescence detection (HPLC-FLD). Therefore the substances were incubated in DMEM/Ham's F-12 cell medium without any cells at a concentration of 1 μM. After several time points (0, 1, 2, 3, 4, 5, 8, 24, 30, 48, 72 h) samples were collected and quantified, using methysergid-maleate as an internal standard (1.6 μg/mL). The separation of the ergot alkaloids was performed on a 150 mm × 4.6 mm i.d., 5 μm, Agilent Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, USA). The HPLC system consists of a binary pump (Merck-Hitachi L-7100, Tokio, Japan), an autosampler (Merck-Hitachi AS-2000A) and a fluorescence detector (Merck-Hitachi FLD F-1050). The injection volume was 20 μL and the mobile phase consisted of acetonitrile (A) and ammonium carbamate buffer, 0.2 g/L (B). Peptide ergot alkaloids were separated using a modified gradient described by Franzmann et al. (2010). Starting with 2 min of 35% A increasing to 60% A in 1 min and again increasing to 80% A in following 2 min, held for 4 min and equilibrating the system to the starting conditions for 4 min, with a total run time of 13 min. Ergometrine and ergometrinine were separated, using a gradient starting with 27% A, which was increased to 35% in 3 min, held for 1.5 min and finally equilibrated to the starting conditions for 5.5 min, with a total run time of 10 min. The flow rate was 1 mL/min and all substances were detected with a fluorescence detector at an exciting wavelength of 330 nm and an emission wavelength of 415 nm. Data analysis was performed using Merck-Hitachi D-7000 HSM HPLC System Manager. The quantification was done via peak area and the internal standard methysergid-maleate was added to the system directly before the measurement.

2.3. Cell culture

Renal proximal tubule epithelial cells (RPTEC) and normal human astrocytes (NHA), both cells in primary culture were obtained from Lonza Group AG (Basel, Switzerland).

RPTEC were cultivated using DMEM/Ham's F-12 medium with the supplements of 15 mM HEPES buffer solution, 10 μg/L epidermal growth factor (EGF), 5 mg/L apotransferrin, 5 mg/L bovine insulin, 0.5 mg/L hydrocortisone, 5 μg/L sodium selenite, 6.5 μg/L thyroxin, 0.5 mg/L epinephrine and 1% FCS.

NHA were cultivated using Quantum 333 medium with the supplements of 10 μg/L EGF, 25 mg/L bovine insulin, 1% non essential amino acids 100 mM (v/v), 1% sodium pyruvate 100 mM (v/v), 1% MEM vitamins 100 mM (v/v) and 10% FCS.

The media was changed twice a week and cells were sub cultivated with a ratio of 1:3 every 14–17 days. Caspase-3-activation, protein content, cellular uptake and LDH-release were performed in 24-well plates with a mean volume of 300 μL and 1×10^5 cells.

For CCK-8 assay 1×10^4 cells were seeded in 96 well plates with at least 100 μL medium and for cell cycle analysis cells were cultivated in 100 mm cell culture dishes with 1×10^6 cells and at least 7 mL medium. For fluorescence microscopy 4×10^5 cells were seeded on alcian blue coated cover slips in 6 well plates in 2 mL medium.

When reaching microscopic confluence of 80–90% at about 2 days after seeding the DMEM/Ham's F-12 medium with 15 mM HEPES and no other supplements was applied for 16 h to exclude any substance binding to serum protein. Ergot alkaloids were applied from a stock solution in serum free media (stock solution: ethanol/0.25 g/L tartaric acid (40:60; v/v)) in concentration ranges from 1 nM to 20 μM for 24 and 48 h. Twenty μM appear to be the upper limit concentration in cell media, because crystallization of the compounds in the cell medium was detectable using higher concentrations. Control cells were incubated with an equal amount of the used solvent. All studies were performed by using passages 3–10 for both used cell types, and under red light or light exclusion due to the instability of the ergot alkaloids.

T-2 toxin as a positive control for apoptosis was added from a 10 mM stock solution (acetonitril/water (50/50; v/v)). All the cultivating and incubating steps were performed without antibiotics at 37 °C with 5.0% CO_2.

2.4. Determination of cellular uptake

For cellular uptake studies cells were seeded in 24 well plates. 1 μM of ergotamine, ergometrine or ergocristine was added after 24 h. After several time points (5, 10, 20, 40 min, 1, 2, 4, 6, 8, 24, 48, 72 h) the toxin containing cell medium was removed and 200 μL of 1% triton-x solution were added. At least 100 μL of cell lysate was used for quantification of ergot alkaloids (see Section 2.2). Additionally cells were counted on each measuring point to determine μmolar toxin concentration in cell lysate.

2.5. Determination of cytotoxicity (CCK-8)

General cytotoxic effects were evaluated using the Cell counting Kit-8 (CCK-8) from Dojindo Laboratories (Tokyo, Japan). Cells were seeded in 96 well plates and were incubated with toxin concentrations from 1 nM to 20 μM after synchronization. After toxin exposure for 48 h the WST-8 solution [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] was added and incubated for 2–4 h according to the manual. The absorbance of each well was measured with a FLUOstar Optima microplate reader (BMG Labtechnologies, Jena, Germany) at 450 nm. The water soluble tetrazolium salt can be reduced in the presence of an electron carrier, e.g. dehydrogenases. Therefore the amount of reduced, coloured formazan dye is directly proportional to the amount of living cells per well. The absorbance of the alkaloid treated wells was compared to the solvent treated control.

2.6. Determination of protein content

The protein content was quantified using the bicinchoninic acid (BCA) assay-kit from Sigma–Aldrich (Steinheim, Germany). The cells were seeded in 24 well plates and were used after 24 and 48 h toxin exposure. The method was done by Königs et al. (2007). 15 μL of cell lysates were incubated with 200 μL of a mixture from BCA and 4% copper sulfate (50:1) in a 96 well plate. The plate was incubated 30 min at 37 °C and for 1 h at room temperature in the dark. The absorbance of the formed violet complex was measured at 560 nm with a FLUOstar Optima microplate reader (BMG Labtechnologies, Jena, Germany). The protein content was quantified with a calibration curve using bovine serum albumin (BSA) in concentration ranges from 50 to 500 μg/mL.

2.7. Determination of caspase-3-activation

For caspase-3-activation studies the cell lysates from a 24 well plate was used after 24 and 48 h of alkaloid exposure. Caspase-3-activation was measured as described by Königs et al. (2007). Thirty μL of cell lysate were used and 33 μL of a reaction buffer mixture was added (50 mM PIPES, 10 mM EDTA, 0.5% CHAPS, 10 mM DTT, 80 μM DEVD-AFC) in a black 96 well plate. After an incubation time of 6 h the fluorescence activity of the released product, due to caspase-3-activity (7-amino-4-trifluoromethylcumarin) was measured at 400 nm excitation and 505 nm emission wavelength, using a FLUOstar Optima microplate reader (BMG Labtechnologies, Jena, Germany). Quantification was done using an AFC calibration curve (0.3–12.8 μM) and was related to the protein content per well.

2.8. Determination of LDH-release

As a marker for LDH activity in cell media, as well as in cell lysate the conversion of NADH to NAD^+ was measured. This reaction is catalyzed from lactate dehydrogenase referring to the conversion of pyruvat to lactate according to the protocol from Bergmeyer (1970). After 24 and 48 h incubation time 40 μL of the cell media and 15 μL of the cell lysates were removed and mixed with a reaction buffer (100 mM HEPES, 0.14 g/L NADH, 1.1 g/L natrium pyruvate, pH 7) to a total volume of 200 μL in a 96 well plate. The absorbence at 355 nm was measured in a FLUOstar OPTIMA microplate reader (BMG Labtechnologies, Jena, Germany) at 37 °C every 2 min. The LDH activity was determined as mU/mL and the LDH-release was expressed as the LDH-activity in the medium in % of total LDH release in comparison to control cells.

2.9. Cell cycle analysis

For DNA analysis cells were seeded on 100 mm cell culture dishes and incubated with alkaloids for 48 h after reaching at least 1×10^6 cells. The cell media was collected and the cells were detached, using Accutase (PAA Laboratories, Pasching, Austria) and transferred to the collected cell media. After centrifugation (5 min, 200 × *g*) the former medium was removed obtaining the pellet, which was washed using phosphate buffered saline (PBS) with 5% FCS (v/v). After additional centrifugation (5 min, 200 x *g*) the cells were fixed in 0.5 mL 70% ethanol (v/v) over night at −20 °C.

After fixation and centrifugation (5 min, 200 × *g*, 4 °C) the pellet was washed twice with PBS. RNase (1 mg/mL) was added to a concentration of 10 μg/mL and the mixture was incubated for 30 min at 37 °C. Propidium iodide was added with 25 μg/mL and after 15 min of incubation time on ice the samples were measured with a FC 500 flow cytometer (Beckman Coulter, Krefeld, Germany). Fluorescence was obtained using FL-3 amplification (620 nm). At least 20,000 cells were recorded for each sample. Cell cycle analysis was performed by using the Software MultiCycle for Windows version 284 (Phoenix Flow System, USA).

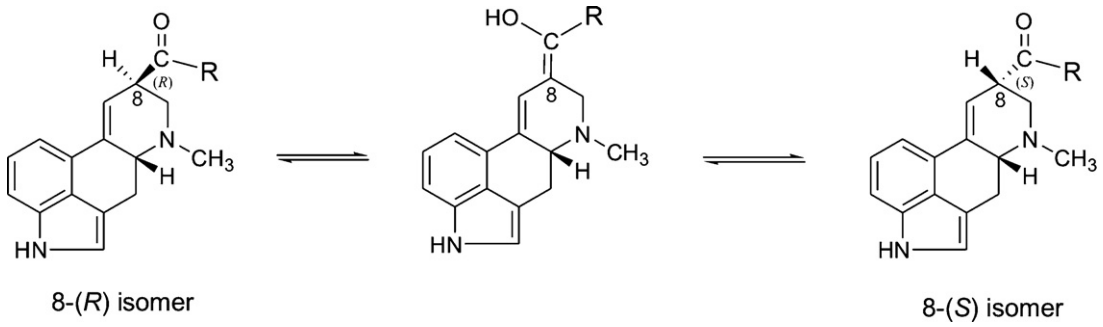

Fig. 2. Epimerization scheme of ergot alkaloids.

2.10. Fluorescence microscopy

Cells were seeded on alcian blue coated cover slips in a 6 well plate. After 48 h incubation time with the alkaloids the media was removed and an equal amount of fixing buffer was added (1.662 g/L $NaH_2PO_4{}^*H_2O$, 11.389 g/L Na_2HPO_4 and 10% Formalin (v/v)). After 20 min the buffer was replaced with a staining solution (10 mg Hoechst 33258 (Bisbenzimide) in 10 mL methanol) for another 20 min. After this the slips were transferred to a microscope slide and pictures were taken with fluorescence microscope Axio Imiger M2 (Zeiss, Oberkochen, Germany) and evaluated with Axiovision 4.5 software (Zeiss, Göttingen, Germany).

2.11. Statistical analysis

All measurements are given as mean value ± SED. All experiments were performed at least in three different passages using a minimum of 4 well per group for LDH-release, caspase-3-activation and protein content and a minimum of 3 samples in three individual experiments for stability studies and cell cycle analysis and a minimum of 2 samples for uptake studies. Thus 6–12 samples were collected for each mentioned parameter. Significant differences were determined using unpaired Student's *t*-test with $p \leq 0.05$ as statistically significant.

3. Results

3.1. Stability of ergot alkaloids

Several ergot alkaloids are commercially available as pure compounds. But the characteristic chemical structure shows two different optically active carbon atoms at position C-5 and C-8 (Hafner et al., 2008). Especially the stereochemistry at position C-8 can change from the lysergic 8-(*R*) isomer to the so called isolysergic 8-(*S*) form (Komarova and Tolkachev, 2001). Chosen suffixes for these forms are -ine and -inine. Both forms can be converted into each other depending on parameters like temperature, light, solvent and pH value. The mechanism is displayed in Fig. 2. To determine epimerization effects the ergot alkaloids were incubated in cell medium as described in Section 2.2. Fig. 3 displays typical chromatograms showing the formation of the -inine epimer after several time points. Depending on the used ergot alkaloid the increase of the -inine peak stops after a certain time point, reaching an equilibrium for both forms. In the example shown in Fig. 3 the equilibrium was reached after 8 h. In our experiments no *aci*-derivatives (Kreilgard and Kisbye, 1974a) were detectable during the incubation period. Similar results were obtained for the other alkaloids (data not shown). The formation of *lumi*-derivatives (Kreilgard and Kisbye, 1974b) could only be detected in larger concentrations following the incubation of ergocristine. In order to reflect the aspect of epimerization during our experiments correction factors were calculated. These factors as well as the equilibrium time are summarized in Table 1 showing a clear difference between each compound. The most stable compounds appear to be ergometrine as a lysergic acid amide with a factor of 1.4 and ergosine as a peptide ergot alkaloid with a calculated factor of 1.6. These factors were used in all experiments to correct the concentration value to the biologically active -ine form (Stadler and Sturmer, 1970). To ensure that the equilibrium was reached, all substances were pre-incubated in the cell culture medium for 24 h at 37 °C before application to the cells.

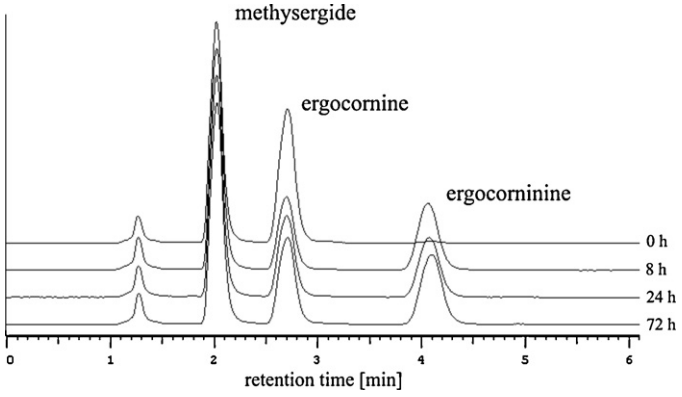

Fig. 3. HPLC-fluorescence detection chromatogram of ergocornine in cell culture media; time dependant formation of ergocorninine after 8, 24 and 72 h with methysergide as an internal standard for quantification.

3.2. Cytotoxic effects of ergot alkaloids

To evaluate the cytotoxic effects on human cells two different primary cells were tested. The renal proximal tubule epithelial cells (RPTEC) as cells derived from the kidney as key organ to eliminate toxic compounds from the body, and human astrocytes as cells from the central nervous system, both in primary culture. Renal cells were reported to have dopamine receptors (Jose et al., 1992), and also effects of ergotamine in the renal ATPase system in rats are described (Aperia et al., 1987; Moubarak et al., 1993). Besides these cells, cultured astrocytes have also been reported to have dopamine and serotonine receptors (Hösli et al., 1987). Therefore both cells seem to be adequate targets to evaluate the toxic effect of the different ergot alkaloids and to conclude weather there are any cytotoxic effects. For a first screening of the cytotoxic potential of the six main compounds we used the CCK-8 assay. Concentration ranges from

Table 1
Calculated correction factors for individual ergot alkaloids after time depending incubation in cell medium; factors were calculated from concentrations of the corresponding ine- and inine-forms adjusting in an equilibrium; number of samples $n = 9$ for each ergot alkaloid; with mean ± SEM.

Ergot alkaloid	Factor in equilibrium	Time adjusting an equilibrium
Ergometrine/-inine	1.4 ± 0.1	24 h
Ergocornine/-inine	2.0 ± 0.2	8 h
Ergotamine/ergotaminine	1.8 ± 0.1	2 h
α-Ergocryptine/-inine	2.4 ± 0.3	5 h
Ergocristine/-inine	5.0 ± 0.4	24 h
Ergosine/-inine	1.6 ± 0.1	8 h

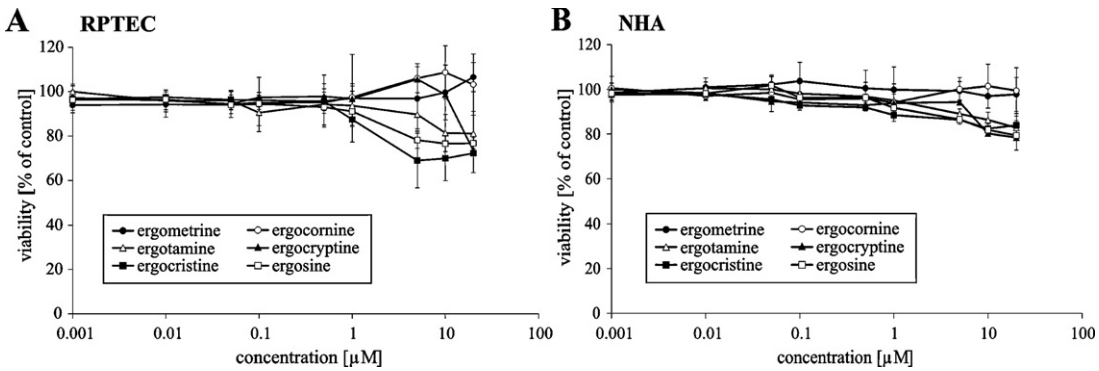

Fig. 4. Concentration-dependent viability of RPTEC (A) and NHA (B) after 48 h incubation time with different ergot alkaloids, determined with CCK-8 assay; number of analysed wells $n = 18$. Significance ($p \leq 0.05$) differences for individual substances: RPTEC and NHA: ergotamine, ergosine (5–20 μM); ergocristine (1–20 μM); ergocryptine (20 μM in RPTEC); ergocryptine (10–20 μM in NHA).

1 nM to 20 μM were applied for a time period of 48 h. All used concentrations were corrected as mentioned in Section 3.1 *stability of ergot alkaloids*. As shown in Fig. 4A and B the impact of the different ergot alkaloids only appeared to be very low. For RPTEC (Fig. 4A) the cell viability only declines to 70% using the peptide ergot alkaloids ergotamine, ergocristine and ergosine. No cytotoxic effect could be detected using ergometrine and ergocornine and only a weak toxic effect for α-ergocryptine. Similar results were obtained using NHA displayed in Fig. 4B. Only ergocristine, ergotamine, ergosine and α-ergocryptine show a weak cytotoxic effect with a decline in viability to about 80%. Ergometrine and ergocornine showed no effect neither in the test system nor visibly. Although the effects are rather weak, a clear significant cytotoxic effect could be observed for the mentioned compounds (ergotamine, ergosine, ergocristine, α-ergocryptine) in comparison to control cells, starting at a concentration of 5 μM, and 1 μM for ergocristine ($p \leq 0.05$). Both cell types showed the same significance with exception of α-ergocryptine showing significant results at 10 μM for NHA. Due to the low effects and the limitation of 20 μM concentration range, no IC_{50} values were calculated.

3.3. LDH release

To underline the results of the general cytotoxicity the necrotic effect of the ergot alkaloids was investigated. The used concentrations were based on the previous cytotoxicity assay with a range from 10 nM to a maximum of 20 μM and the ergot alkaloids were incubated for 24 and 48 h. Fig. 5A–D summarizes the data for RPTEC and NHA (data for control, 10 and 100 nM are not shown as no effect was observed) reflecting similar effects as in the CCK-8 assay. After 24 and 48 h there was no LDH-release induced by the lysergic acid amide ergometrine in RPTEC. The peptide alkaloids showed different results depending on their structure. After 24 h there was no effect for ergotamine and ergocornine in all tested concentrations (Fig. 5A). Ergosine, α-ergocryptine and ergocristine showed a very slight significant LDH-release ($p \leq 0.05$) in RPTEC starting at a concentration of 10 μM with 3–7% in comparison to the control. The LDH-release effect of ergocristine has been statistically significant using already 1 μM with 4.5% (Fig. 5A). An enhanced incubation period revealed similar results. Ergometrine and ergocornine remain with no impact. Ergosine and ergotamine induced a LDH-release about 3–7.5%. While the effect in RPTEC was only little enhanced for ergosine, the effect for the ergotamine was now detectable. The strongest impact on the LDH-release was measured using α-ergocryptine and ergocristine with up to 15% in comparison to the control (Fig. 5B). Very similar results were obtained using NHA (Fig. 5C and D). Ergometrine showed no effect for the selected incubation times. The effects of ergotamine, α-ergocryptine and ergocristine were measurable with a slight LDH-release between 2 and 5% starting at 10 μM (Fig. 5C). Again the effects increased after 48 h of incubation time. Significant results were obtained for ergotamine, α-ergocryptine and ergocristine at a concentration of 1 μM. The effect enhanced for these peptide ergot alkaloids for higher concentrations and also ergocornine showed an LDH-release at a concentration of 10 μM with about 4%. The strongest impact was again measured using α-ergocryptine and ergocristine with up to 15% LDH-release, using 20 μM (Fig. 5D).

3.4. Caspase-3-activation

To further characterize the cytotoxic effects of the ergot alkaloids on human cells, experiments were carried out to distinguish between necrotic and apoptotic cell death. For this, activation of the caspase-3 was measured as a typical parameter for apoptosis. The effective concentrations were again similar to the previous LDH-release assay. The results are summarized in Fig. 6A–D representing the data for 24 and 48 h of used cell types (data for control, 10 and 100 nM are not shown as no effect was observed). For RPTEC again α-ergocryptine and ergocristine showed the strongest effect. To classify these results T-2 toxin was used as a positive control from data by Königs et al. (2009). Ergocristine showed a significant caspase-3-activation after 24 h starting at 1 μM with 243% compared to the control and an enhancing effect at 10 and 20 μM with 450–550% activation (comparison to positive control: 10 μM T-2 toxin; 650% activation). Ergosine and α-ergocryptine also showed a strong effect with 250–350% caspase-3-activation at 10 μM and 20 μM ($p \leq 0.05$) (Fig. 6A). After 48 h incubation time the caspase-3-activation was further enhanced. For ergotamine caspase-3-activation was detectable after 48 h, starting with 160% at 10 μM. For ergosine and α-ergocryptine the activation increased up to 400%. Ergocristine showed the strongest effect with up to 860% caspase-3-activation in comparison to the control (positive control: 10 μM T-2 toxin 1500%) (Fig. 6B). For human astrocytes (NHA) caspase-3-activation was detectable with concentrations above 10 μM after 24 h for ergotamine, α-ergocryptine and ergocristine. Ergometrine, ergocornine and ergosine showed no effect. The effects for α-ergocryptine and ergotamine were similar at 10 and 20 μM with about 230% activation in comparison to the control. Ergocristine showed again the highest effect with 550% and clearly distinguished from the other compounds (Fig. 6C). After 48 h of incubation time the caspase-3-activation in NHA increased for the peptide ergot alkaloids with exception of ergocornine and ergocristine. At a concentration of 10 μM and 20 μM the data were comparable to each other, ranging between 200 and 350%. Ergocristine appeared to have the strongest impact, but declined in its potential after 48 h compared to 24 h (Fig. 6D).

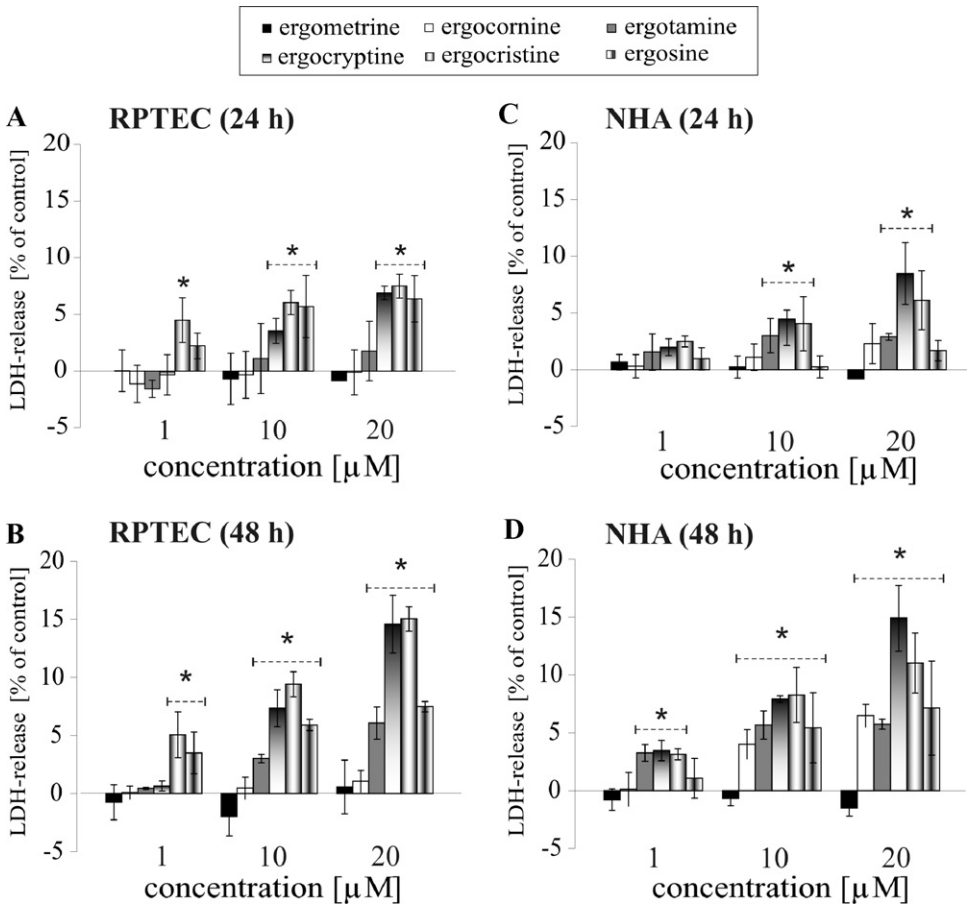

Fig. 5. Concentration and time-dependent LDH-release for RPTEC (A, B) and NHA (C, D) after ergot alkaloid exposure for 24 and 48 h; LDH release in % of control cells was defined as 0%; number of analysed wells $n = 12$; *significant differences from the control ($p \leq 0.05$).

3.5. Cell cycle analysis

For a verification of the apoptotic effect RPTEC were incubated with the six ergot alkaloids for 48 h at a concentration of 10 µM. The distribution is displayed in Fig. 7. The control cells show a characteristic pattern with two main peaks. In contrast to this T-2 toxin as positive control revealed an intensive sub G1 peak while simultaneously the G1 and G2/M peak declined. From the six ergot alkaloids tested ergometrine, ergocornine and ergosine did not show any sub G1 formation after 48 h incubation period. However α-ergocryptine, ergotamine and ergocristine showed a sub G1 signal with different intensities confirming the apoptotic potential of these compounds in agreement with the caspase-3-activation. The data for quantification of the sub G1 area are summarized in Table 2. The formation of the DNA fragments was most notably after incubation with ergocristine with 150% in comparison to the control. Also a significant peak formation was detected for ergotamine with 118% and α-ergocryptine with 127%. The effects of the caspase-3-activation were confirmed with ergosine being an exception without sub G1 formation.

Table 2
Substance-depending formation of sub G1 peak (10 µM) in RPTEC after 48 h incubation time; formation in % of control with control cells as 100% value; number of samples $n = 9$; with mean ± SEM.

Substance	Quantification of subG1 in comparison to control [%]
Control	100.0
T-2 toxin	260.5 ± 3.2
Ergometrine	91.5 ± 0.3
Ergocornine	92.2 ± 5.0
Ergotamine	118.1 ± 2.1
α-Ergocryptine	127.8 ± 0.8
Ergocristine	152.9 ± 2.8
Ergosine	88.8 ± 1.7

3.6. Fluorescence microscopy

To investigate a third parameter and clearly identify an apoptotic effect we stained the DNA using Hoechst 33258 dye. The chromatin condensation and fragmentation was identified by bright spots which are very dominant in the T-2 toxin sample in comparison to the control cells (Fig. 8). But also clear bright spots indicating chromatin condensation could be detected in the ergocristine incubated sample.

3.7. Uptake of ergot alkaloids

Cellular uptake (see Section 2.4) was used as a hint for a accumulation of ergot alkaloids in human primary cells. As shown in Fig. 9A–D concentrations of ergot alkaloids exceed the initial concentration in cell medium (1 µM). Ergotamine/ergotaminine (Fig. 9A) was enriched in the cell lysate up to 160% of initial concentration. Time depending studies revealed a fast transport of the substances from cell medium to the cell interior ultimately leading to an accumulation of the substances with a plateau from 1 to 6 h (180 µM). Fig. 9B shows the time depending accumulation of ergocristine/ergocristinine. While ergocristine was enriched similar to ergotamine up to 180 µM in cell lysate (after 2 h), ergocristinine showed a complete different picture. Cellular con-

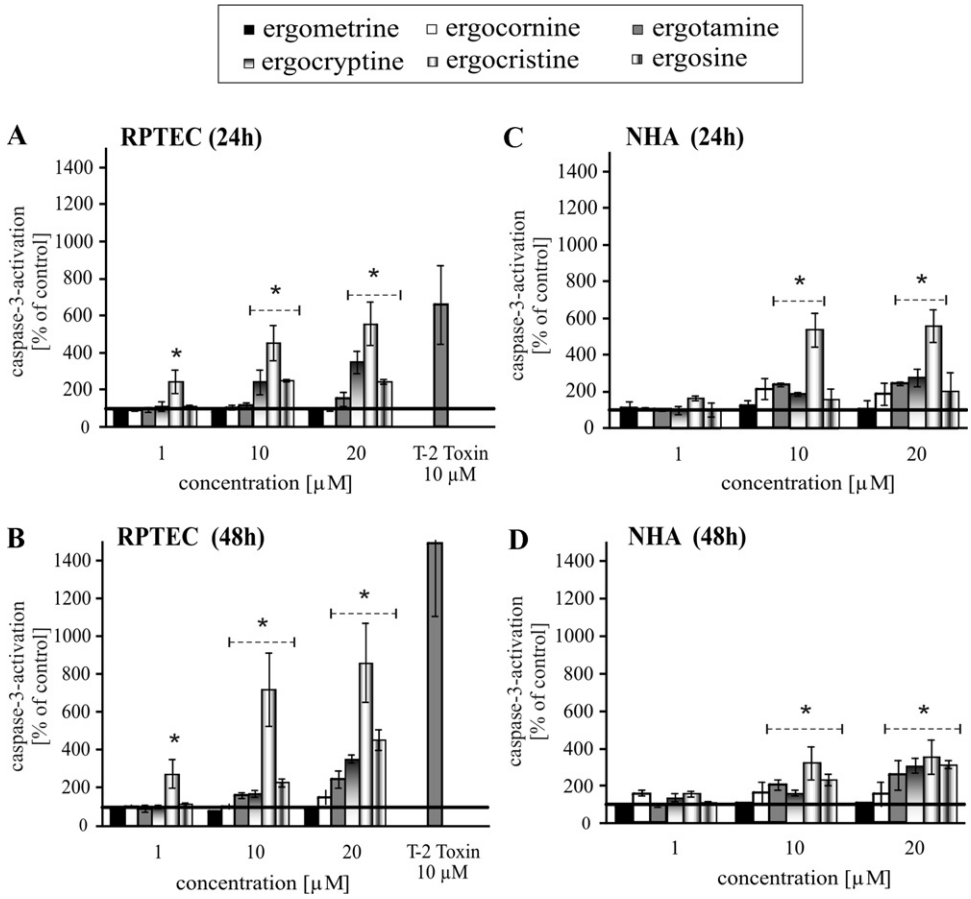

Fig. 6. Concentration and time-depending caspase-3-activation for RPTEC (A,B) and NHA (C,D) after ergot alkaloid exposure for 24 and 48 h; caspase-3-activation in % of control cells was defined as 100%; number of analysed wells $n = 12$; *indicates significant differences from the control ($p \leq 0.05$).

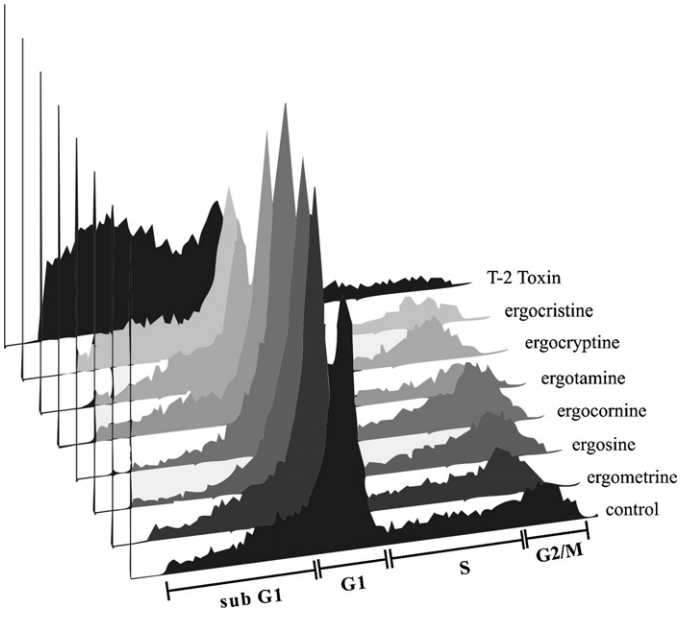

Fig. 7. Histogram plot of cell cycle distribution in RPTEC after 48 h of incubation; control cells displayed in front and positive control T-2 toxin (10 µM) in the back; analysed ergot alkaloids (10 µM) are placed in between; substance depending formation of sub G1 peak.

centration reached a maximum after 4 h incubation time with 600 µM, consequently 600-fold of initial concentration. Due to the fast uptake during the first hour epimerization effects seems to be unlikely. After 8 h of incubation the concentration of ergotamine and ergocristine as well as the corresponding -inine forms decrease again leading to a final concentration of 100–300 µM after 72 h. Similar results were obtained for NHA (Fig. 9C and D). Accumulations of ergocristine/ergocristinine were in a similar range with a maximum concentration of 650 µM ergocristinine in cell lysate after 2 h. Ergotamine/ergotaminine concentration in human astrocytes exceeds up to 400 µM for ergotamine and 200 µM for ergotaminine compared to cell medium concentration. The accumulation of ergometrine/ergometrinine was also studied but both compounds were not detectable in any cell lysate.

4. Discussion

4.1. Stability

The experiments in cell cultivation medium clearly demonstrate that it is essential to determine the stability of the substances before performing any experiments. As presented in literature there is a known epimerization of ergot alkaloids and the formation of an equilibrium between both isomers (-ine and -inine-form) was also described (Kreilgard and Kisbye, 1974a,b; Smith and Shappell, 2002; Hafner et al., 2008). The results of our studies have shown that all the substances are instable in aqueous cell media. As it is presented in Section 3.1 *stability of ergot alkaloids* the peptide ergot alkaloids form rapidly their corresponding -inine form. All of the experiments were performed using only one specific medium for

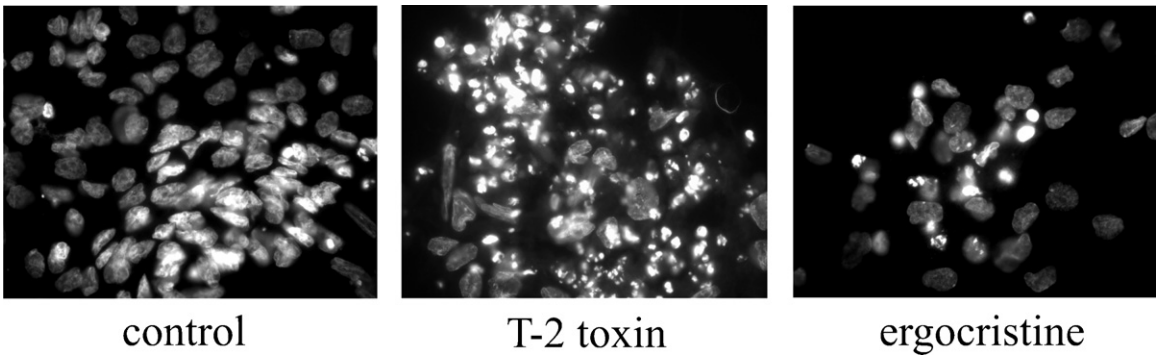

Fig. 8. Fluorescence microscopy of Hoechst stained cell nuclei in RPTEC after 48 h of incuvation; control cells in contrast to positive control cells (T-2 toxin, 10 μM) in comparison with ergocristine (10 μM).

incubation. This is important since the substances show different behaviour using different solvents. After up to 24 h the equilibrium was reached and remained unchanged until 72 h. For this reason the substances were preincubated for 24 h and than applied for 48 h, so it was guaranteed that the calculated factors were constant during the incubation time. Ergometrine showed the highest stability reaching the equilibrium after 24 h with 70% of the initial concentration left. Since the -inine form is described as only weak biologically active the consideration of the epimerization is crucial (Stadler and Sturmer, 1970). The corrected concentrations, as used in this paper, are a suitable way to draw conclusions on an effective concentration range of the ergot alkaloids. According to these data some literature facts concerning toxic effects could be explained. As an example α-ergocryptine has shown the strongest effect in rabbits so far known in literature (LD_{50} (i.v.) 0.34 mg/kg b.w.) and was considered as the most toxic compound in animal studies in contrast to ergocristine (LD_{50} (i.v.) 1.6 mg/kg b.w.) (Griffith et al., 1978). In fact α-ergocryptine did have a high cytotoxic potential in our assays (Sections 3.2–3.6) but did not reach the toxic potential of ergocristine and showed nearly only half of the effect. This controversy could be explained by our stability studies. Since ergocristine is much more unstable in our experiments than α-ergocryptine (factor 5.0 in contrast to 2.4) this could also appear under physiological conditions. Because animal studies have been performed with pure substances or extracts from sclerotia, the concentrations of the more toxic ergocristine could be much more influenced by epimerization than α-ergocryptine. This could result in a higher toxic potential. All these data and suggestions underline the importance to consider the stability of the ergot alkaloids. Therefore we highly recommend investigations of the stability of the substances in the used solvent before performing any experiments.

4.2. Cytotoxicity

In the used cells a low necrotic effect was detected using concentrations of 10 μM or higher for several peptide ergot alkaloids which was increasing with the incubation time. Also lower concentrations seem to have an effect after 48 h. This effect was more distinctive in RPTEC than in NHA but slight differences could be detected. However the necrotic effects appeared to be not very distinctive with only 15% LDH-release in comparison to the control as a maximum effect although the cells were visibly strong influenced after incubation with the substances. Additional experiments which analysed the apoptotic potential allowed a further differentiation between the cytotoxic effects of the substances. The activation of the caspase-3-enzyme was detected in RPTEC and NHA in different concentration ranges for the single substances. In both used cells an activation of the caspase-3-enzyme was detectable

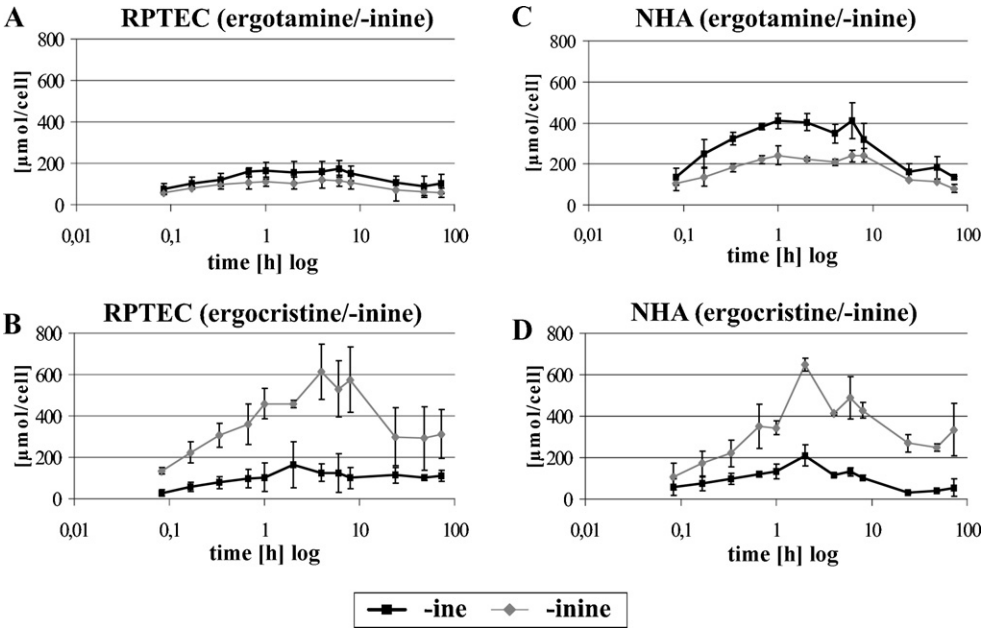

Fig. 9. Time-depending uptake of ergotamine/ergotaminine (1 μM) and ergocristine/ergocristinine (1 μM) in RPTEC and NHA; concentrations were related to cell number and cell volume in [μM/cell]; number of analysed samples $n = 6$.

comparable to T-2 toxin after 24 h. The lowest effective concentracion was 1 μM (609 ng/mL) ergocristine in RPTEC. The most toxic compound ergocristine showed a high apoptotic potential and the results were verified using flow cytometry (increase in sub G1 fraction) and DNA condensation. Overall the apoptotic effect of the peptide ergot alkaloids could be clearly identified using three different assays. Depending on only small changes in the structure the effect was clearly influenced. For both cells it was shown that ergometrine has no cytotoxic effect and the peptide ergot alkaloids differ in their potential but the tendency was the same for both cells, especially after an incubation time of 24 h. Since ergotamine and ergocristine are referred to account 60% of alkaloid content in sclerotia it is an important fact that one of these compounds appear to have the strongest toxic impact (Scott and Lawrence, 1980; Young, 1981; Appelt and Ellner, 2009; Franzmann et al., 2010).

In the literature only a few *in vitro* data are available which can be compared to our results. Effects of ergovaline, another peptide alkaloid have been described in caco-2 cells using the MTT assay. But the effects were notably different in a millimolar range (Shappell, 2003). In our cell culture studies it was not possible to dissolve such high concentrations so we were not able to verify these data. Bromocriptine as a synthetic ergot alkaloid is used in the treatment of Parkinson disease and of pituitary tumours. In GH_3 cells bromocriptine at 25 μM affected p53 content and induced apoptotic and necrotic effects after 24 h, more pronounced after 48 h (Wasko et al., 2004). Additionally Oda et al. (2008) have shown that bromocriptine induced neurite outgrowth in PC12 cells using 20 μM. Besides bromocriptine also ergotamine and ergocornine were tested with the same effective concentration range. While the peptide ergot alkaloids seemed to have an impact in this model system ergometrine has shown no effect. These results underline our findings in the same effective concentration ranges. It appears that the effects of ergot alkaloids exceeded the known receptor interaction, having toxic effects in human primary cells and are somehow not defined very well in literature so far.

Both cell types are good targets since there are several receptors described for them which are known to be responsible for toxic effects (Dale, 1906; Barger and Dale, 1907; Sibley and Creese, 1983; Rowell and Larson, 1999). The different cytotoxic potential of the individual ergot alkaloids observed in our studies are in a higher concentration range, than data of receptor activation. Larson et al. (1995) described EC_{50} values for binding to the dopamine receptor D_2 for ergotamine (2.2 nM) ergometrine (47 nM) and α-ergocryptine (28 nM). The EC_{50} values are considerably lower than cytotoxic concentrations. A possible explanation could be the choice of cells. While Larson et al. (1995) used receptor overexpressing cells our experiments were performed using primary cells from different organs. Further experiments should also regard the aspect of receptor activation of ergot alkaloids in primary cells to evaluate any correlation between apoptotic potential and receptor interaction or evaluate cytotoxic data in receptor overexpressing cells to elucidate weather there is a correlation or not. The receptor binding activity might result in the mentioned cytotoxic secondary effect, but our results also give the first hint that there is a complete different mechanism triggering toxic effects. The described effects in this paper have to be distinguished from receptor effects since substances like ergometrine had shown no cytotoxic effect, but a receptor binding activity in other studies. So both effects have to be considered for risk assessment.

4.3. Accumulation

It was shown that the most lipophilic compound, ergocristine is the most toxic compound in all cytotoxic assays. The cytotoxic effects correlate with the lipophilic properties of the different peptide ergot alkaloids and the structure of each single compound seems to have a high impact on the activity. Since the basic structure appears the same, the characteristic side chains seem to have a strong influence (Fig. 1). More lipophilic compounds like α-ergocryptine and ergocristine have shown the highest apoptotic potential with ergocristine starting at 1 μM. More polar peptide alkaloids like ergotamine and ergosine have shown much lower effects. The comparison of ergotamine and ergocristine is crucial, since their occurrence in sclerotia accounts for 60% (Scott and Lawrence, 1980; Young, 1981; Appelt and Ellner, 2009; Franzmann et al., 2010). Another fact is that the two alkaloids only differ slightly from each other in their structure, but this has a strong influence on their toxic properties (factor 3), although being rather similar in all structural terms. The uptake data underline these theoretical suggestions. In fact the tested substances ergotamine/ergotaminine and ergocristine/ergocristinine show a very high accumulation in the cell lysate. The substances are transported very fast within a few hours. While the concentrations decline over the period of time the concentration in the cells is up to 100-fold higher than in the exterior cell medium after 24 h and stays nearly constant up to 72 h. This fast uptake and accumulation could result in the mentioned cytotoxic effects. Due to the lipophilic character and the strong accumulation effect of the substances a disruption of the cell membrane cannot be excluded resulting ultimately in necrotic effects. An important fact which has to be pointed out is the very high accumulation of ergocristinine in both used cells. Although ergocristinine is described as less active in the receptor interaction it might be converted into the active -ine form under physiological conditions (Stadler and Sturmer, 1970; Komarova and Tolkachev, 2001). The rapid uptake and accumulation of an -inine form gives a possible hint for probably toxic aspects which have to be confirmed by future research. Also these results underline the non-toxic effect of ergometrine/ergometrinine, since the substances were not detectable in the cell lysate at any time point of the experiments. Consequently this substance is not able to enter cells, triggering toxic effects but of course can interact with receptors on the cell surface as shown by Larson et al. (1995).

Regarding the accumulation of the peptide ergot alkaloids the possibility of the formation of a "toxin-depot" in cells has to be highlighted. Referring to a case report of ergotism in 1998 (Stange et al., 1998) an 42 year old farmer suffered from ergotism after several month of inhalation of corn dust, although peptide ergot alkaloids are known to have a low oral bioavailability (Little et al., 1982). The plasma ergotamine level was 9.1 ng/mL (0.015 μM) and even after elimination of any ergot alkaloid source, the level of ergotamine only declined to 4.1 ng/mL after 3 month. The authors described this effect as some kind of "toxin-depot" in the body due to the constant ergotamine plasma level although a toxin exposure was missing. Linking these data to the accumulation results in our study, the low plasma level concentrations could be a result of a slow toxin release from cells. Since the concentration of ergot alkaloids rapidly increases after a single dose in our cell culture model it is crucial to evaluate whether a constantly low level of ergot alkaloids exposure could accumulate in human cells causing toxic effects. In literature uptake curves were measured for plasma level of [^{3}H]-dihydroergotamin in volunteers by Maurer and Frick (1984), which are similar to our results. The substance level rapidly increases to a maximum and is declining after 4–6 h but the concentration did not reach zero even after 60 h. Besides this it is important to mention that ergot alkaloids always occur in a mixture of different compounds (Franzmann et al., 2010) leading to a high accumulation even if the alkaloids are present only in low concentrations.

4.4. Conclusion

For the first time an apoptotic effect of ergot alkaloids was described for human cells in primary culture. Ergocristine had

shown the strongest toxic potential with half to one-third of the effect compared to the mycotoxin T-2. Additionally other peptide ergot alkaloids show weak cytotoxic effects with α-ergocryptine and ergotamine also inducing apoptosis or necrosis. Besides the receptor activation the ergot alkaloids have several secondary effects which are not fully investigated yet. Also it was essential to evaluate the effect of epimerization since the stability of ergot alkaloids differs substantially. Corrected concentration values taking into account the epimerization can explain different results in literature in terms of toxicity and are a crucial point of concern. Uptake studies revealed an accumulation of peptide ergot alkaloids in human cells in association with the cytotoxic effects.

Since the results for all tested compounds obtained in this study differ from each other it is necessary to collect more data in terms of receptor binding, toxicity and bioavailability of the ergot alkaloids in human cells. With such data it might be possible to evaluate the toxic relevant compounds for a risk assessment in food and feed.

Conflict of interest

None.

Acknowledgement

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