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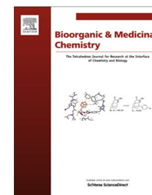

The influence of saponins on cell membrane cholesterol

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ABSTRACT

We studied the influence of structurally different saponins on the cholesterol content of cellular membranes. Therefore a cell culture model using ECV-304 urinary bladder carcinoma cells was developed. To measure the cholesterol content we used radiolabeled ^{3}H-cholesterol which is chemically and physiologically identical to natural cholesterol. The cells were pre-incubated with ^{3}H-cholesterol and after a medium change, they were treated with saponins to assess a saponin-induced cholesterol liberation from the cell membrane. In another experiment the cells were pre-incubated with saponins and after a medium change, they were treated with ^{3}H-cholesterol to assess a saponin-induced inhibition of cholesterol uptake into the cell membrane. Furthermore, the membrane toxicity of all applied saponins was analyzed using extracellular LDH quantification and the general cytotoxicity was analyzed using a colorimetric MTT-assay and DNA quantification. Our results revealed a correlation between membrane toxicity and general cytotoxicity. We also compared the results from the experiments on the saponin-induced cholesterol liberation as well as the saponin-induced inhibition of cholesterol uptake with the membrane toxicity. A significant reduction in the cell membrane cholesterol content was noted for those saponins who showed membrane toxicity (IC_{50} <60 μM). These potent membrane toxic saponins either liberated ^{3}H-cholesterol from intact cell membranes or blocked the integration of supplemented ^{3}H-cholesterol into the cell membrane. Saponins with little influence on the cell membrane (IC_{50} >100 μM) insignificantly altered the cell membrane cholesterol content. The results suggested that the general cytotoxicity of saponins is mainly dependent on their membrane toxicity and that the membrane toxicity might be caused by the loss of cholesterol from the cell membrane.

We also analyzed the influence of a significantly membrane toxic saponin on the cholesterol content of intracellular membranes such as those of endosomes and lysosomes. In these experiments ECV-304 cells were either incubated with ^{3}H-cholesterol or with ^{3}H-cholesterol and 5 μM saponin. After isolation of the endosomes/lysosomes their ^{3}H-cholesterol content was measured. A significant influence of the saponins on the cholesterol content of endosomal/lysosomal membranes was not detected.

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1. Introduction

Saponins are secondary plant compounds with amphiphilic properties and surface activity. These characteristics are derived from the molecular structure consisting of a lipophilic triterpenoid or steroidal scaffold and one or more attached sugar moieties of various lengths and branching. Thus saponins are capable of interacting with physical surfaces such as those of aqueous solutions and lower the surface tension. They can also interfere with biological surfaces such as cell membranes.[1] The latter case may result in haemolysis or cytotoxicity as a consequence of perturbation or loss of the cell membrane integrity. The mechanism behind this typical saponin characteristic is not yet completely understood, but there are different indications that the cell membrane-cholesterol is decisively involved in this process. Saponins have been described to complex cholesterol.[2,3,12] A replacement of the cell membrane-cholesterol by saponins is also conceivable. As cholesterol is known for its cell membrane stabilizing function, a loss of the cholesterol itself or the loss in its ability to carry out this function would surely lead to the observed cell membrane instabilities and membranolysis.[4]

To analyze the influence of saponins on the cell membrane-cholesterol we developed a cell culture model using ECV-304 urinary bladder carcinoma cells and radiolabeled ^{3}H-cholesterol and scrutinized its uptake and its liberation into and from the cell membrane induced by the combination with saponins. ^{3}H-cholesterol is chemically and physiologically identical to natural cholesterol.[5] Hence it was also possible to estimate the behavior of the unmarked cholesterol by measuring the ^{3}H-cholesterol. On the basis of preliminary investigations we tested a selection of different saponins either showing significant membrane toxicity as well as significant general cytotoxicity (EC_{50} and IC_{50} ⩽60 μM) or showing little membrane toxicity as well as little general cytotoxicity (EC_{50} and IC_{50} ⩾100 μM). We analyzed if saponin-induced perturbation

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of the cell membrane and saponin-induced cytotoxicity coincide with influences on the cell membrane-cholesterol content. As saponins can complex cholesterol, in our opinion an interaction of saponins with the cell membrane cholesterol may lead to its displacement from the cell membrane and thereby destabilize it. Thus, saponins that show a stronger membrane perturbation and a higher cytotoxicity in general, might change (lower) the cholesterol content of the cell membrane more than those who show only little effect to the cells and their membranes at same concentrations.

Furthermore, we wanted to answer the question if the cholesterol content of intracellular membranes such as endosomal/lysosomal membranes is also influenced by certain saponins when the cholesterol content of the cytoplasm membrane is influenced by these saponins. Since some saponins are known to synergistically enhance the cytotoxicity of ribosome-inactivating proteins type I (RIP-I), the knowledge about a saponin's influence on the endosomal/lysosomal cholesterol content might contribute to the understanding of mechanism of toxification of the only little cytotoxic activity showing RIP-I. According to Weng et al. after endocytosis of the proteins certain saponins play a crucial role to facilitate the endosomal escape into the cytosol where they can exhibit an N-glycosidase activity at the ribosomes and thus lead to cell death.[6] To be able to analyze the endosomal/lysosomal cholesterol content we incubated the cells with ^{3}H-cholesterol to get an endosome/lysosome fraction from the cells a lysosome isolation kit was used.

2. Materials and methods

2.1. Substances

The listed substances were tested for their general cytotoxicity and membrane toxicity in cell culture. They were also tested for the liberation of cholesterol from intact cell membranes and the influence on the uptake of cholesterol into intact cell membranes. SA1641 was also tested for its influence on the total cholesterol content in endosomal membranes. All structures are given in Figures 1 and 2.

Alpha-hederin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)
Beta-escin (Merck KGaA, Darmstadt, Germany)
Beta-sitosterol (ARCO-Chemikalien, Berlin, Germany)
Glycyrrhizin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)
Hederacoside C (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)
NP-000712 (AnalytiCon discovery GmbH, Potsdam, Germany)
NP-013544 (AnalytiCon discovery GmbH, Potsdam, Germany)
NP-017810 (AnalytiCon discovery GmbH, Potsdam, Germany)
Primulic acid 1 (isolation product from *Primula vulgaris* Huds.; purity > 95%)
SA1641 (isolated from Saponinum album (Merck KGaA, Darmstadt, Germany); purity > 95%).

2.2. Cell culture

Human urinary bladder epithelial carcinoma cells (ECV-304, ACC 310) were obtained from Deutsche Sammlung von Microorganism und Zellkulturen GmbH (Leipniz-Institut DSMZ, Braunschweig, Germany). Epithelial cells ECV-304 cells are adapted to function on surfaces and boundary layers. Therefore this cell line seems suitable for experiments with substances that are known to affect the cell membrane integrity. The cells were cultured in Dulbecco's MEM (DMEM) without phenol red (Biochrom AG, Berlin, Germany) with 10% FCS (fetal calf serum), 5% glutamine, 10% HEPES (Biochrom AG, Berlin, Germany) at 37 °C in saturated water vapor atmosphere containing 5% CO_2. No passage higher than 35 was used for the experiments. The cultures were split 1:5 twice a week using trypsin/EDTA (Biochrom AG, Berlin, Germany).

2.3. General cytotoxicity assays

The general cytotoxicity reflects the tolerance of the cell to proliferate in the presence of different concentrations of a certain saponin. Therefore the cells were disseminated at a density of 5000 cells/well and were grown for 24 h in 96-well plates, transparent and black. The culture medium was then replaced by medium containing different concentrations of saponin. The cells were incubated for another 72 h. The cytotoxicity was evaluated using a MTT assay and via DNA quantification.

The colorimetric MTT assay was performed in transparent 96-well plates. The supernatant was removed and was replaced by DMEM, containing no other additives. The MTT dye (Carl Roth GmbH+Co. KG, Karlsruhe, Germany) was dissolved in PBS and added to the cells to reach a final concentration of 0.5 mg/mL. After 30 min of incubation at 37 °C, living cells transformed the yellowish MTT into a water-insoluble purple formazan. The medium was discarded and the formazan was dissolved in 100 μL/well DMSO (Carl Roth GmbH+Co. KG, Karlsruhe, Germany). After 5 min of careful shaking the absorbance was measured in a Tecan Spectrafluor microplate reader (Tecan Group Ltd, Mainz, Germany) at 580 nm. The absorbance is proportional to the number of viable cells. The cytotoxicity was calculated by comparing saponin-treated wells with untreated control-wells.

The DNA quantification was performed in black 96-well plates. Therefore the supernatant was removed and the cells were washed with isotonic NaCl solution. 100 μL/well ultra-pure water was added into the wells followed by three freeze-thaw-cycles in order to break the cell membranes. After that 100 μL/well double-concentrated DNA buffer, pH 7.4 containing 82 mM $Na_2HPO_4 \cdot 2H_2O$, 18 mM $NaH_2PO_4 \cdot H_2O$, 4 mM EDTA and 4 M NaCl was added. The DNA was stained with 10 μL/well of 10 μg/mL Hoechst 33258 dye (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). The fluorescence was measured in a Tecan infinite F200 microplate reader (Tecan Group Ldt, Mainz, Germany) at 360 nm excitation wavelength and 465 nm emission wavelength. The fluorescence is proportional to the amount of DNA. The cytotoxicity was calculated by comparing saponin-treated wells with untreated control-wells.

IC_{50}-values represent the calculated median cytotoxic concentration.

2.4. Membrane toxicity assay

The membrane toxicity can be rated by quantifying the liberation of the intracellular enzyme lactate dehydrogenase (LDH) into the supernatant. The enzyme may leak through pores or ruptures in the cell membrane caused by the tested substances. Due to the short incubation time enzyme liberations by other processes like apoptosis would not be involved. To assess the LDH liberation the Cytotoxicity Detection Kit (plus)(LDH) (Roche Applied Science, Mannheim, Germany) was used.

The cells were disseminated at a density of 2000 cells/well and were grown for 24 h in transparent 96-well plates. The culture medium was replaced by DMEM without phenol red with 1% FCS, 5% glutamine, 10% HEPES and dissolutions of saponins in different concentrations. The cells were then incubated for 2 h. After 1:45 h half of the control wells were treated with 2% Triton-X100 (Carl Roth GmbH+Co. KG, Karlsruhe, Germany). After 2 h 100 μL/well of the assay-dye in assay-buffer was added and incubated for 5 min at 37 °C. The dye's reaction was then stopped with the stop-solution from the kit. The staining was measured in a Tecan Spectrafluor microplate reader at 450 nm. The membrane toxicity

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