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Membrane organization determines barrier properties of endothelial cells and short-chain sphingolipid-facilitated doxorubicin influx

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ABSTRACT

The endothelial lining and its outer lipid membrane are the first major barriers drug molecules encounter upon intravenous administration. Our previous work identified lipid analogs that counteract plasma membrane barrier function for a series of amphiphilic drugs. For example, short-chain sphingolipids (SCS), like N-octanoyl-glucosylceramide, effectively elevated doxorubicin accumulation in tumor cells, both in vitro and in vivo, and in endothelial cells, whereas other (normal) cells remained unaffected. We hypothesize here that local membrane lipid composition and the degree of lipid ordering define SCS efficacy in individual cells. To this end, we study the differential effect of SCS on bovine aortic endothelial cells (BAEC) in its confluent versus proliferative state, as a model system. While their (plasma membrane) lipidome stays remarkably unaltered when BAECs reach confluency, their lipids segregate to form apical and basolateral domains. Using probe NR12S, we reveal that lipids in the apical membrane are more condensed/liquid-ordered. SCS preferentially attenuate the barrier posed by these condensed membranes and facilitate doxorubicin influx in these particular membrane regions. We confirm these findings in MDCK cells and artificial membranes. In conclusion, SCS-facilitated drug traversal acts on condensed membrane domains, elicited by confluency in resting endothelium.

1. Introduction

In anti-cancer therapy, slow drug diffusion over lipid membranes and poor intracellular drug accumulation areimportant causes of treatment failure [1,2]. The plasma membrane is the main barrier for a drug to enter a cell and reach its intracellular targets. On its turn, the endothelial lining is the first cellular barrier that is faced when a drug is administered intravenously [3–5].

We identified a series of truncated lipid analogs (short-chain sphingolipids; SCS) that, when pre-inserted into the plasma membrane, facilitate transmembrane traversal of amphiphilic drugs. For example, N-octanoyl-glucosylceramide (GC) elevates intracellular levels of doxorubicin, in tumor cells and endothelium, both in vitro and in vivo [6–9]. In contrast, various normal tissues (heart, liver, spleen) or cultured cells (including cardiomyocytes) remain insensitive to the effect of GC. We recently reported that in genetically engineered tumor models co-administration of GC with doxorubicin widens the therapeutic window, yields more effective therapy response and counteracts drug resistance [6].

In previous attempts to elucidate the mechanism of this drug uptake enhancing the effect of SCS, we excluded the involvement of membrane proteins or drug transporters [8] (see also Supplementary data, Fig. S5). They neither induce SCS membrane leakage, nor stimulate endocytic uptake of doxorubicin, nor form a physical complex with the drug. Also, SCS do not act by a general increase in membrane fluidity, while acting specifically in combination with a defined class of drugs [6,8,9]. Instead, SCS readily insert in the plasma membrane, where also doxorubicin is binding and trapped. Such doxorubicin molecule poses significant stress on lipid order in the membrane [10]. SCSs then assemble a lipid channel (or funnel) tightly surrounding a drug molecule. Through this particular channel, the hydrophilic moiety of the drug can readily traverse the membrane [6].

Beside this fundamental mechanism, the differential effect of SCS, including GC, on distinct cell types is not well-understood. Remarkably, bovine aortic endothelial cells (BAEC), when confluent, are sensitive to the effect of GC, however, when sub-confluent, SCS elevate intracellular accumulation in BAECs poorly [6]. Here we used these BAECs as a physiologically relevant cell model to gain insight in what additional factors might govern the differential degrees of SCS-mediated doxorubicin uptake in the cellular context.

Our studies on model membranes have revealed that, in addition to the drug molecule, the surrounding lipids influence channel formation and kinetics [6]. We therefore characterized the lipid composition of BAEC cell and plasma membrane, in both confluent and sub-confluent

Abbreviations: BAEC, bovine aortic endothelial cells; bFGF, basic fibroblast growth factor; CH, cholesterol; Dox, doxorubicin3; DPPC, di-palmitoyl-phosphatidylcholine; DSM, dehydrosphingomyelin; ePC, ether-linked phosphatidylethanolamine; GC, N-octanoyl-glucosylceramide; LUV, large unilamellar vesicle; MDCK, Madin–Darby canine kidney cells; PA, phosphatic acid; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE-Cer, ceramide phosphoethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; POPC, palmitoyl-oleoyl-phosphatidylcholine; SCS, short-chain sphingolipid; SM, sphingomyelin

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states. In addition, we studied lipid domain organization of the membrane when confluent and the relation of SCS-facilitated doxorubicin influx to structural order of the lipids.

2. Materials and methods

2.1. Materials

N-octanoyl-glucosylceramide was from Avanti Polar Lipids (Alabaster, Alabama, USA). Cholesterol, chicken egg SM and salmon sperm DNA were from Sigma (Zwijndrecht, The Netherlands). Hydrogenated Soy PC (HSPC), POPC, DPPC and PEG2000-DSPE were provided by Lipoid GmbH (Ludwigshafen, Germany). Doxorubicin was from Pharmacia (Woerden, The Netherlands). Mouse anti-ZO-1 monoclonal antibody and AlexaFluor 405 goat-anti-mouse antibody were from Invitrogen (Carlsbad, USA). The fluorescent probe NR12S was received from A.S. Klymchenko (Laboratoire de Biophotonique et Pharmacologie, Université de Strasbourg, France).

2.2. Cell culture

Primary bovine aortic endothelial cells (BAECs; passage 14–19) were kindly provided by Dr A Haimovitz-Friedman (Memorial Sloan-Kettering Cancer Center, New York City, USA) and were maintained in 1.0 g/l glucose DMEM containing 10% fetal calf serum, 1.0 ng/ml basic fibroblast growth factor, and 100 IU/ml penicillin and 100 μg/ml streptomycin, up to passage 25. MDCK cells (parental) and the engineered MDCK cell line stably expressing an estrogen-inducible and constitutively active Raf1 mutant protein (MDCK-DDRafER) were kindly obtained from Sabine Macho-Maschler (University of Veterinary Medicine, Vienna, Austria). MDCK-DDRafER cells were cultured in the presence of puromycin (5.0 μg/ml). To induce epithelial to mesenchymal transition, estradiol (1.0 μM) was applied for 5 days, to which TGF-β (5 ng/ml) was added and incubated for at least 3 additional days. For all experiments, cells were deprived of mitogens by serum starvation for at least 4 h (unless indicated otherwise). Cells were subcultured by trypsinization and maintained in a water-saturated atmosphere containing 10% CO2 (BAEC) or 5% CO2 (MDCK) at 37 °C.

2.3. Cell fractionation and lipid analysis

BAECs were cultured in 15 cm dishes, and when confluent for 2 days, or sub-confluent (<80%), washed twice in PBS. After the serum starvation, the cells were homogenized by snap freezing immediate after detachment with a cell scraper under hypotonic conditions (Graham, 1993). An enriched plasmalemma fraction was isolated by two step differential centrifugation. The supernatant from 15 min., 13,000 × *g*, 4 °C centrifugation was subjected to 145,000 × *g*, 4 °C for 75 min. The pellet was harvested and lipids were extracted with chloroform/methanol (Bligh and Dyer, 1959) and dried under a stream of N_2. Lipidomics analysis was performed by the Kansas Lipidomics Research Center (Kansas State University, USA). Plasma membrane enrichment was verified by the enhanced sphingomyelin (marker) content.

2.4. Intracellular doxorubicin accumulation

Cells were cultured in flat-bottom 96-well plates. Short-chain lipids were applied from ethanol for 15 min after which free doxorubicin (50 μM) was added for 1 h. After three washing steps with PBS, cells were lysed in 1.0% (w/v) Triton X-100. Doxorubicin fluorescence was determined in a PerkinElmer Victor Wallac II microplate reader using 485 and 535 nm filters for excitation and emission, respectively. All values were corrected for background fluorescence and for differences in protein content, as determined with the use of bicinchoninic acid assay. Cellular doxorubicin content was quantified using dilution series of known amounts of doxorubicin.

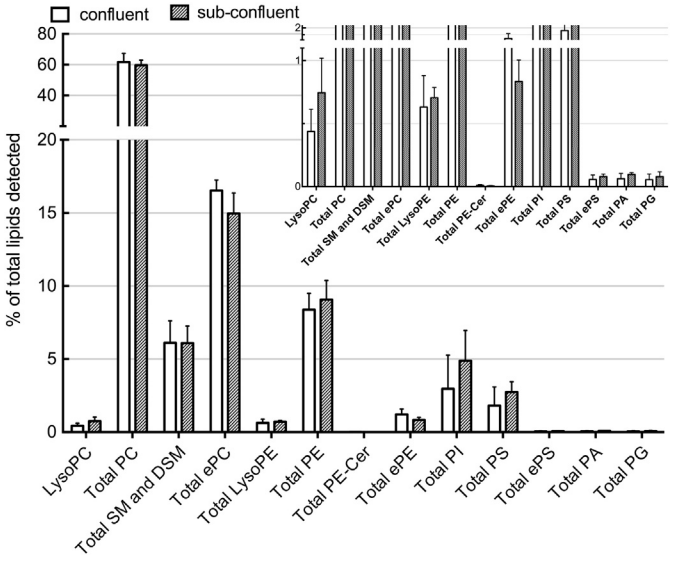

Fig. 1. BAEC lipidome is comparable in confluent and sub-confluent states. None of the major lipid classes significantly alters in total cell lipid extracts. The insert displays a y-axis zoom in. Mean, SD, n = 4.

2.5. Confocal microscopy

Cells were cultured onto glass coverslips, serum-starved for 4 h and then stained with 50 nM NR12S from DMSO (final volume < 0.5%). 40× or 100× oil immersion objectives were used and an argon laser at 488 nm for excitation. Labda emission spectra were recorded on a Leica Sp5-II system from 520 to 680 nm with a 10 nm bandwidth and 5.0 nm step-size. The cells were maintained in a temperature- and CO2-controlled chamber during imaging.

2.6. Immunofluorescence

Cells, cultured onto glass coverslips, were fixed in 2.0% formaldehyde and permeabilized in 0.2% Triton X-100, PBS. 5% Bovine Serum

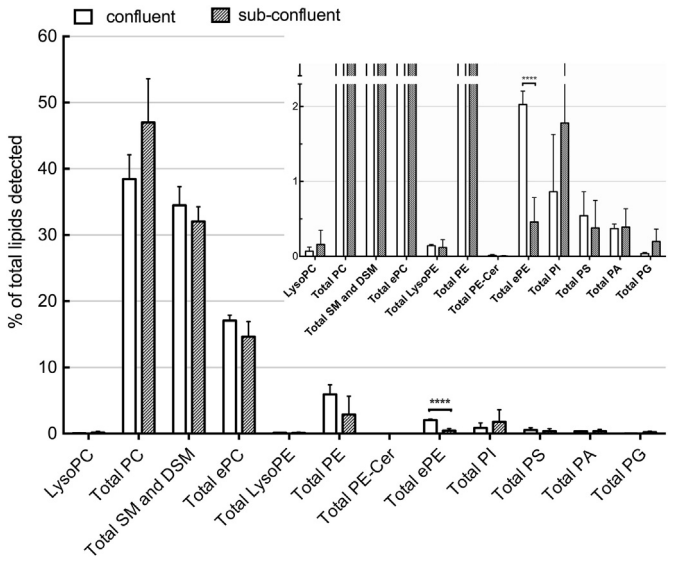

Fig. 2. Plasmalemma lipid composition of confluent and sub-confluent BAEC cultures. ePE (ether-linked PE) is the only lipid species significantly elevated in the confluent endothelium plasmalemma. Cell homogenates were differentially centrifuged to concentrate for the plasma membrane fraction. Enrichment for SM is characteristic for plasma membranes. Mean, SD, n = 3; ****p-value < 0.001.