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Lipidomics reveals membrane lipid remodelling and release of potential lipid mediators during early stress responses in a murine melanoma cell line

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ABSTRACT

Membranes are known to respond rapidly to various environmental perturbations by changing their composition and microdomain organization. In previous work we showed that a membrane fluidizer benzyl alcohol (BA) could mimic the effects of heat stress and enhance heat shock protein synthesis in different mammalian cells. Here we explore heat- and BA-induced stress further by characterizing stress-induced membrane lipid changes in mouse melanoma B16 cells. Lipidomic fingerprints revealed that membrane stress achieved either by heat or BA resulted in pronounced and highly specific alterations in lipid metabolism. The loss in polyenes with the concomitant increase in saturated lipid species was shown to be a consequence of the activation of phopholipases (mainly phopholipase A_2 and C). A phospholipase C–diacylglycerol lipase–monoacylglycerol lipase pathway was identified in B16 cells and contributed significantly to the production of several lipid mediators upon stress including the potent heat shock modulator, arachidonic acid. The accumulation of cholesterol, ceramide and saturated phosphoglyceride species with raft-forming properties observed upon both heat and BA treatments of B16 cells may explain the condensation of ordered plasma membrane domains previously detected by fluorescence microscopy and may serve as a signalling platform in stress responses or as a primary defence mechanism against the noxious effects of stresses.

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1. Introduction

Recent findings indicate that most anticancer agents induce apoptosis, directly or indirectly, through alterations of tumor cell membrane fluidity [reviewed in 1]. Several potential therapeutic applications, including hyperthermia, directed at such fluidity have been proposed [1–3]. Heat shock proteins (Hsps) were first identified as stress proteins that confer resistance to physical stresses such as elevated temperatures in all cellular organisms. Elevated Hsp expression promotes cancer by inhibiting the major known programmed cell death pathways (apoptosis [4], autophagy [5]) and leads to resistance to heat- (thermotolerance) or chemotherapy. It would therefore be undesirable to elevate the intracellular level of Hsps in tumor cells when attempting various cancer treatments. On the other hand, the upregulation of the surface expression of Hsps has been shown to increase immunogenicity of tumor cells [6]. Furthermore, mild heat stress may confer radiosensitization of cancer cells probably through effects on membrane structure [2]. In this investigation we used B16-F10 cells which are a highly metastatic cancer cell line, in order to clarify the role of membrane lipids in this dichotomic feature of thermal stress.

It has been proposed that, during abrupt temperature fluctuations, membranes represent the most thermally-sensitive macromolecular structures [7,8]. Thus, changes in the physical state of membranes of *Synechocystis, Escherichia coli* or yeast [9–11] have been shown to affect profoundly the temperature-induced expression of heat shock

Abbreviations: AA, arachidonic acid; BA, benzyl alcohol; CE, cholesteryl ester; Cer, ceramide; Chol, cholesterol; DAG, diacylglycerol; ESI-MS/MS, electrospray ionization tandem mass spectrometry; FA, fatty acid; FAAH, fatty acid amide hydrolase; FAME, fatty acid methyl ester; FFA, free (non-esterified) fatty acid; GC-MS, gas chromatography-mass spectrometry; HSF1, heat shock factor1; Hsp, heat shock protein; IP_3, inositol-1,4,5-trisphosphate; LPC, lysophosphatidylcholine; MAFP, methyl arachidonyl fluorophosphonate; MAG, monoacylglycerol; MDR, multidrug resistant; PCA, principal component analysis; PC and PC-O, diacyl and 1-alkyl-2-acyl species of phosphatidylcholine, respectively; PC1, PC2 and PC3, first, second and third principal components, respectively; PE, diacyl phosphatidylethanolamine; PE-P, 1-(1Z-alkenyl)-2-acyl species of PE; PG, phosphatidylglycerol; PI, phosphatidylinositol; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PS, phosphatidylserine; SFA, MUFA and PUFA, saturated, monounsaturated and polyunsaturated FAs, respectively; SM, sphingomyelin; THL, tetrahydrolipstatin; TPL, total polar lipid fraction

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protein (*hsp*) genes. Opposite changes in membrane fluidity mimic cold or heat stress activation of distinct plant mitogen-activated protein kinase pathways, respectively [12]. When *Saccharomyces cerevisiae* is exposed to elevated temperatures in the presence of alcohols, the temperature required for the maximal activation of the *hsp* promoter and the concentration of alcohol required decreases as its hydrophobicity increases [13]. In the moss *Physcomitrella patens* it was shown that early sensing of mild temperature increments occurs at the plasma membrane independent of cytosolic protein unfolding. The heat signal is translated into an effective heat shock response by way of a specific membrane-regulated Ca^{2+} influx, leading to thermotolerance [14]. In tobacco BY2 cells, a rapid H_2O_2 burst occurs in response to increases in membrane fluidity, which may be triggered by temperature elevation or alternatively by treatment of cells with benzyl alcohol (BA). This H_2O_2 stimulation is required for the production of small heat shock proteins [15].

Previously we have demonstrated that, irrespective of the origin of membrane perturbations, the formation of isofluid membrane states is accompanied by an essentially identical heat shock response in K562 cells. Heat shock at 42 °C or the administration of 30 mM BA, which is a well-established membrane fluidizing agent [16,17] proved equally effective in the up-regulation of Hsp70 formation. The increase in fluidity of isolated membranes treated by 30 mM BA was identical to that seen during a thermal upshift to 42 °C. Similar to thermal stress, exposing cells to membrane fluidizers elicited a nearly identical rapid rise of cytosolic Ca^{2+}, and caused mitochondrial hyperpolarization to a similar extent as well [18].

In B16 cells, BA treatment elevated the levels of *hsp70* and *hsp25* mRNAs, which peaked at 40 mM levels of the fluidizer [19]. Furthermore, using fluorescent polyethylene glycol-derivatized cholesterol, which specifically recognizes sterol-rich membrane domains, a characteristic rearrangement of the membrane microdomains was observed when B16 cells were exposed to heat or BA treatment [19]. It was suggested that, in B16 cells, a distinct reorganization of cholesterol-rich microdomains is required for the generation and transmission of sufficient stress signals to activate *hsp* genes. Mild, fever-type heat ($\leq$41 °C)-induced raft enlargements were shown also in natural killer cells and have been related to their antitumor function [2].

A commonly-accepted paradigm is that stress-induced protein denaturation serves as the primary stress-sensing machinery that triggers *hsp* gene expression. However, the exposure of mammalian cells to various membrane fluidizers or compounds with the ability to interact with certain membrane lipids substantially modulates Hsp expression without inducing protein unfolding. The above mentioned findings fully supported the paradigm shift toward our "membrane sensor" hypothesis that membranes can act as temperature sensors [20–23].

It is well established that a large number of lipid mediators liberated after various stresses can serve as stress-response modulating factors. Heat shock produced increases in inositol phosphate concentrations comparable in magnitude to those achieved after stimulation with growth factors, indicating that heat shock might initiate transmembrane signalling cascades of potential importance in cellular regulation [24]. Common cellular responses to heat and growth factors also included feedback modulation of phospholipase C (PLC) by its products and the parallel stimulation of phospholipase A_2 (PLA_2) activity [25].

Extracellular exposure of HeLa cells to arachidonic acid (AA) induced heat shock gene transcription in a dose-dependent manner via acquisition of DNA-binding activity and phosphorylation of heat shock factor 1 (HSF1). In addition, exposure of cells to low concentrations of AA reduced the temperature threshold for HSF1 activation indicating that elevated heat shock gene expression can be a direct consequence of an AA-mediated cellular response [26].

This study was aimed at systematically mapping the lipid changes following membrane perturbation caused by heat or fluidizer treatment in order to identify novel lipid alterations during the initial phase of the stress response. Our results highlight the possible links between distinct membrane lipid changes and reorganization of membrane microdomains leading to the generation of stress signals.

2. Materials and methods

2.1. Materials

Methyl arachidonyl fluorophosphonate (MAFP), tetrahydrolipstatin (THL, also referred to as orlistat), U-73122, RPMI-1640 medium and Hanks' balanced salt solution were purchased from Sigma (Steinheim, Germany). 1-Palmitoyl-2-[1-^{14}C]oleoyl-phosphatidylcholine was from DuPont NEN Research Products (Boston, USA), calcein AM was from Molecular Probes (Eugene, OR, USA) and the Flo Scint II scintillation cocktail was from PerkinElmer (Waltham, Massachusetts, USA). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The solvents used for extraction and for mass spectrometric analyses were of gas or liquid chromatographic grade and BA of analytical grade was from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma and were of the best available grade.

2.2. Inhibitors

The inhibitors were dissolved in ethanol and added to the serum-free medium. Their final concentrations were 10 μM for MAFP, 30 μM for THL and 5 μM for U-73122. The final concentration of ethanol was 0.1% or less and this concentration had no detectable effect on growth or metabolism.

2.3. Cell culturing and treatments

B16-F10 (ATCC CRL-6475) mouse melanoma cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 4 mM L-glutamine at 37 °C. Cells were plated in 3×10^6 cell number/10 cm plate and grown additionally for 1 day. For ESI-MS studies, the cells were treated in the plates. Thus, for BA treatments, the growth medium was changed to fresh growth medium containing 40 mM BA. For heat shock experiments, the plates were immersed in a water bath set to the indicated temperature ($\pm$0.1 °C). After 1 h, the cells were scraped into 2 mL ice-cold water and extracted. In the case of arachidonate release, the cells were first scraped, resuspended in 12 mL serum-free medium containing 0.5% fatty acid-free BSA and treated by BA or heat as above. At the indicated time points, 2 mL aliquots (about 3×10^6 cells) were taken out and immediately extracted. For inhibitor studies, the cells were resuspended in a small amount of serum-free medium, divided into equal parts (6×10^6 cells/experiment), diluted to 2 mL with serum-free medium containing the inhibitor at the appropriate concentration and incubated at 37 °C. After 20 min, 2 mL serum-free medium containing the inhibitor (at the appropriate concentration with 1% fatty acid-free BSA) was added to the reaction flasks to control and heat-shocked samples and, for BA treatments, the medium contained additional BA to a final concentration of 40 mM. The treatments were applied for 60 min. The reactions were stopped by placing the flasks on ice and the reaction mixture was immediately extracted for lipids.

Because we have found that culture conditions (e.g. cell number, serum quantity) affect lipid composition and metabolism in B16 cells, we paid very careful attention to the above conditions and only cultured for a few passages. Independently grown samples were used as replicates.

2.4. PLA_2 activity in vitro

PLA_2 in vitro activity was assayed according to [27] with slight modifications.

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