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Squalene is lipotoxic to yeast cells defective in lipid droplet biogenesis

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ABSTRACT

The toxic effect of overloaded lipids on cell physiology and viability was described in various organisms. In this study we focused on the potential lipotoxicity of squalene, a linear triterpene synthesized in eukaryotic cells as an intermediate in sterol biosynthesis. Squalene toxicity was studied in the yeast *Saccharomyces cerevisiae*, a model unicellular eukaryote established in lipotoxicity studies. Squalene levels in yeast are typically low but its accumulation can be induced under specific conditions, e.g. by inhibition of squalene monooxygenase with the antimycotic terbinafine. At higher levels squalene is stored in lipid droplets. We demonstrated that low doses of terbinafine caused severe impairment of growth and loss of viability of the yeast mutant *dga1Δ lro1Δ are1Δ are2Δ* unable to form lipid droplets and that these defects were linked to squalene accumulation. The hypersensitivity of the lipid droplet-less mutant to terbinafine was alleviated by decreasing squalene accumulation with low doses of squalene synthase inhibitor zaragozic acid. Our results proved that accumulated squalene is lipotoxic to yeast cells if it cannot be efficiently sequestered in lipid droplets. This supports the hypothesis about the role of squalene in the fungicidal activity of terbinafine. Squalene toxicity may represent also a limiting factor for production of this high-value lipid in yeast.

1. Introduction

Lipotoxicity is generally defined as the deleterious effect of lipid overload on cellular functions and viability. Such effects have been described for free fatty acids (FA) [1], cholesterol [2], sphingolipids and their derivatives [3] or diacylglycerols [4]. The interest in lipotoxicity was stimulated by the involvement of this phenomenon in the pathogenesis of several human morbidities, e.g. type 2 diabetes, cardiovascular diseases, non-alcoholic hepatosteatosis, cardiomyopathy and metabolic syndrome [5]. The mechanisms of lipotoxicity include mitochondrial damage and production of reactive oxygen species [6,7], impaired ER functions [8,9] or disturbed intracellular signaling [10]. The final outcome of severe lipotoxicity in humans is usually cellular death resulting in organ dysfunction [11].

Toxic effects of accumulated lipids are manifested also in the unicellular eukaryote *Saccharomyces cerevisiae*. Mechanisms of lipid homeostasis are very similar in *S. cerevisiae* and humans and therefore it is not surprising that *S. cerevisiae* was utilized as a model to elucidate lipotoxicity on molecular and cellular levels [12]. Lipotoxicity studies in yeast were focused mostly on the toxic effect of free FA. Excessive FA are efficiently sequestered in lipid droplets (LD) in the form of triacylglycerols (TAG) and steryl esters (SE) in *S. cerevisiae*. The *dga1Δ lro1Δ are1Δ are2Δ* quadruple mutant deficient in four major acyltransferases involved in the synthesis of TAG (Dga1p, Lro1p) and SE (Are1p, Are2p) is viable but unable to form lipid droplets (LDs) [13]. Several studies showed that this LD-less mutant is sensitive to exogenous unsaturated FA [14–17]. Similarly to human cells, the effects induced by exogenous FA in LD-less yeast include production of reactive oxygen species [14], induction of unfolded protein response in the ER [14,15], and induction apoptotic cell death [14].

In this report we investigated possible toxicity of squalene in the yeast *S. cerevisiae*. Squalene is an important lipidic metabolite with many medical and industrial applications. In eukaryotic organisms it is synthesized from acyl-CoA in the mevalonate pathway as the first precursor dedicated to sterol synthesis [18]. Intracellular levels of squalene are usually low due to its rapid conversion to oxidosqualene in an oxygen-dependent reaction. In multicellular organisms, high levels of squalene were observed in some tissues where it fulfills specific functions, e.g. human skin [19] or liver of

Abbreviations: CFU, colony forming units; ER, endoplasmic reticulum; FA, fatty acids; IC_{50}, concentration causing 50% inhibition of growth; LD, lipid droplet; SE, steryl esters; TAG, triacylglycerols; YPD, yeast extract/peptone/dextrose.

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deep-sea sharks [20]. In yeast, increased squalene was found in hypoxic or hem-deficient cells [21–24]. In addition, high amounts of squalene are accumulating in fungi treated with the antimycotic terbinafine, a specific inhibitor of squalene monooxygenase. Ryder [25] suggested that accumulation of squalene may contribute to the fungicidal effect of terbinafine, however, this hypothesis was not experimentally verified. Since squalene is widely used as food supplement, in cosmetics and in pharmacology (reviewed in Ref. [18]), its toxicity may have significant implications also for human subjects. In addition, yeast is a perspective source for industrial production of squalene which makes squalene toxicity a biotechnologically relevant issue.

2. Materials and methods

2.1. Reagents

Terbinafine, zaragozic acid, ergosterol and squalene were obtained from Sigma–Aldrich (Germany). Yeast extract, peptone and agar were from Becton Dickinson (USA). Organic solvents (HPLC grade) were from Merck (Germany). Other chemicals of the highest purity available were purchased from various suppliers.

2.2. Strains and growth conditions

S. cerevisiae strain W303-1B (*α ade2-1 ura3-52 trp1-1 leu2-3,112 his3-11,15 can1-100*) and its LD-less derivative mutant (*α ade2-1 ura3-52 trp1-1 leu2-3,112 his3-11,15 can1-100 dga1Δ lro1Δ are1Δ are2Δ*) [13] were obtained from Scan Bi Ltd. (Alnarp, Sweden). Strains were grown aerobically in liquid YPD media (1% yeast extract, 2% peptone, 2% glucose) on rotatory shaker at 28 °C. Where indicated, media were supplemented with inhibitors terbinafine (stock solution 2 mg/mL in DMSO) and zaragozic acid (stock solution 5 mg/mL in water). Culture growth was determined by counting in Bürker chamber and expressed as the number of generations (population doublings) according to the formula:

$$\text{number of generations} = [\log(N_{24}/N_0]/\log(2)$$

where N_0 is the initial cell count (time = 0 h) and N_{24} is the final cell count (time = 24 h).

2.3. Inhibitor susceptibility assays

Sensitivity to inhibitors (terbinafine and/or zaragozic acid) on agar plates was determined by serial dilution spot assay. Cells grown overnight at 28 °C were counted in Bürker chamber, washed and the cell density was adjusted to 2×10^6 cells/mL. Drops of 10-fold serial dilutions (corresponding to 10^4-10^3-10^2-10^1 cells/drop) were spotted on YPD plates containing indicated concentrations of inhibitors. Growth was evaluated after 3–5 days of incubation at 28 °C.

Growth sensitivity in liquid media was estimated as the decrease of the number of generations after 24 h of cultivation in YPD media with indicated concentrations of inhibitors. IC_{50} was determined as the concentration of terbinafine reducing the number of generations to 50% of the untreated control.

Viability was estimated as colony forming units (CFU) in cultures cultivated for 24 h in YPD media with corresponding inhibitors. Cells were washed in sterile water and counted in Bürker chamber. Washed cells were diluted in sterile water to the density of 1000 cells/mL and 100 and 200 cell aliquots were plated on YPD agar plates. Number of colonies was determined after 3 days of incubation at 28 °C.

2.4. Quantitative analysis of sterols and squalene as non-saponifiable lipids

Cells cultivated in liquid media for 24 h were washed and disrupted in FastPrep 24 instrument (MP Biomedicals) (glass beads diameter 0.4 mm, disruption 2 × 45 s at 6 m/s speed with 5 min cooling on ice between the runs). Cell homogenate was incubated in 3 mL of 60% KOH (w/v) – 50% methanol (v/v) for 2 h at 70 °C. Non-saponifiable lipids were extracted twice with 3 mL of n-hexane, combined extracts were dried under nitrogen. Lipid residue was dissolved in acetone and sterols were analyzed by HPLC on Agilent 1100 instrument equipped with Eclipse XDB-C8 column, diode array detector (Agilent Technologies, USA), and Corona Charged Aerosol Detector (ESA Inc., USA). Sterols were eluted with 95% methanol (flow rate of 1 mL/min, temperature 30 °C). Individual peaks were identified by retention times of ergosterol and squalene standards and verified by their UV spectra. The quantity of sterols was calculated from the output of the Corona CAD detector using calibration curves constructed for ergosterol and squalene standards.

3. Results

3.1. Yeast mutant lacking lipid droplets is hypersensitive to terbinafine

S. cerevisiae quadruple mutant strain carrying deletions of genes encoding four acyltransferases (*dga1Δ lro1Δ are1Δ are2Δ*) is unable to form lipid droplets (LDs) [13]. This LD-less strain cannot sequester excess FA in TAG or SE and is thus sensitive to exogenous unsaturated FA [14–17]. LDs are also the site of squalene storage in human adipose cells [26] or yeast *hem1* mutants [22,23]. The relation between squalene and LDs is indicated also by increased cellular content of LDs in terbinafine-treated yeast cells accumulating high levels of squalene [22,27,28]. Interestingly, *dga1Δ lro1Δ are1Δ are2Δ* strain lacking LDs showed increased sensitivity to terbinafine, an inhibitor of squalene monooxygenase [29]. This terbinafine hypersensitivity might reflect lipotoxicity of accumulating squalene in cells with compromised lipid storage. We therefore analyzed the effect of terbinafine on the LD-less strain in more detail.

The serial dilution spot assay demostrated terbinafine hypersensitivity of the LD-less strain (Fig. 1A) which was confirmed also in liquid media (Fig. 1B, Supplementary Fig. S1). Wild type cells responded to terbinafine treatment by a gradual decline in the number of generations in 24 h with 4 generations at 15 μg/mL terbinafine compared to 8 generations in control culture. On the other hand LD-less strain showed an abrupt loss of the growth ability at terbinafine concentrations over 1 μg/mL with only one generation in 24 h at 3 μg/mL of terbinafine. There was about tenfold difference between both strains in the IC_{50} of terbinafine calculated from the number of generations (15 μg/mL and 1.3 μg/mL for wild-type and LD-less strain, respectively). The growth curves (Fig. S1) revealed no significant lag phase in control and terbinafine-treated cultures. Interestingly, terbinafine treatment did not affect the growth rate of wild-type or LD-less cells during the initial 3–4 h and the concentration-dependent inhibitory effect of terbinafine was evident in both strains only after 4 h of growth (Supplementary Table I). This indicates that terbinafine-induced defects accumulated gradually in cells treated with subinhibitory concentrations of terbinafine.

The viability estimated as CFU (Fig. 1C) corresponded well with the growth ability in both strains. Wild type strain started to lose viability at 10 μg/mL of terbinafine and significant viability (about 34%) was retained even at the highest terbinafine concentration

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