FEMS Microbiology Letters 238 (2004) 241–248

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Low concentrations of the non-ionic detergent Nonidet P-40 interfere with sterol biogenesis and viability of the yeast *Saccharomyces cerevisiae*

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Received 30 April 2004; received in revised form 15 July 2004; accepted 19 July 2004

First published online 29 July 2004

Abstract

Mild non-ionic detergents are used for solubilization of hydrophobic substrates in yeast growth media at concentrations 0.1–1%. Our data show that low concentrations of Nonidet P-40 may significantly affect lipid biogenesis in the yeast *Saccharomyces cerevisiae*. The uptake and esterification of external [4-^{14}C]-cholesterol is strongly reduced in *hem1* mutants treated with low concentrations of Nonidet P-40. Significant inhibitory effect of NP-40 on sterol uptake and esterification was evident both in non-growing and growing cells supplemented with external cholesterol. Increased levels of sterol precursors (squalene, lanosterol) in *hem1* cells grown in complex medium with cholesterol indicated general interference of NP-40 with sterol biosynthesis. NP-40 in the growth medium affected also cell viability estimated as the colony forming ability. More attention should be therefore paid to possible effects of mild detergents at low concentrations generally considered to be harmless, especially in cells with disturbed lipid biogenesis.

Keywords: Detergents; Nonidet P-40/Tergitol; Yeast *Saccharomyces cerevisiae*; Sterol uptake; Sterol esterification; Tergitol heme deficiency

1. Introduction

Membranes are important structural and functional components of all cell types both as boundaries of cells or intracellular compartments and as the environment for enzymes involved in vital cellular processes. Specific lipid composition is important for optimal physical properties and function of individual membranes. Maintenance of membrane lipid composition is severely compromised in yeast *Saccharomyces cerevisiae* with lipid biogenesis disturbed either by genetic changes or external conditions. Anaerobic growth imposes specific constraints on lipid biogenesis in this yeast. Molecular oxygen is involved in the biosynthesis of ergosterol and in fatty acid unsaturation both directly and via heme synthesized itself in an oxygen-dependent manner. Heme plays a key role in the adaptation of yeast cells to anaerobiosis. In addition to its role as the cofactor in several enzymes in the ergosterol biosynthesis pathway and in fatty acid desaturase [1] heme is involved in sensing of oxygen and in the regulation of gene transcription in response to changes in oxygen concentration [2]. Heme deficiency and anaerobiosis induce auxotrophy for ergosterol and unsaturated fatty acids and simultaneously activate the systems involved in the uptake and utilization of sterols present in the medium [3]. This stimulation of sterol uptake is vital for the survival of

Abbreviations: NP-40; Nonidet P-40; δ-ALA; δ-aminolevulinic acid; PI; propidium iodide.

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0378-1097/$22.00
doi:10.1016/j.femsle.2004.07.041

anaerobic cells since the entry of external sterols is efficiently blocked in aerobic cells with normal heme synthesis by a mechanism termed aerobic sterol exclusion [4,5].

Utilization of lipids as growth supplements is complicated by their hydrophobicity. To increase the solubility of lipid supplements, mild non-ionic detergents are usually added to the growth media. The possible detrimental effects of these detergents (e.g., Tween, Nonidet P-40/Tergitol, Tyloxapol) in the concentration range commonly used for solubilization (0.1–1%) were not sufficiently discussed in relevant reports [3,6–12]). During the optimization of a quantitative assay of the uptake of external sterols in anaerobic or heme-deficient *S. cerevisiae* we noticed that these detergents interfere with the sterol uptake process. This prompted us to examine the effect of low concentrations of common non-ionic detergents on yeast sterol biogenesis. The results presented here demonstrate that yeasts with disturbed lipid biogenesis might be particularly sensitive to detergents and that even low concentrations of mild detergents used for the solubilization of sterols as growth supplements may have significant effect on the uptake of external sterols and generally on sterol biogenesis.

2. Material and methods

2.1. Yeast strains and growth conditions

Two yeast strains were used in this study: W303 *hem1* (*MATα*, *ade2 leu2 his1 ura3 trp1 hem1::LEU2*) prepared in our laboratory from W303-1B by disruption of *HEM1* gene with the *LEU2* disruption cassette, and WS 17-5D *h1* (*MATα*, *arg1 leu2 ura3 trp1 hem1::LEU2*) provided generously by Dr. J. Rytka, Poland. Yeast strains were routinely grown in YPD containing 1% yeast extract (Imuna Slovakia), 1% peptone (Imuna Slovakia), 2% glucose, buffered to pH 5.0 with 50 mmol l^{-1} citrate–phosphate buffer. The cultivation was performed on rotary shaker at 28 °C and the growth was estimated by counting the cells in haemocyte chamber under microscope or by measurement of optical density at 600 nm in Shimazu spectrophotometer UV-2401.

2.2. Sterol uptake in non-growing cells

[4-^{14}C]-Cholesterol (Amersham) was used as external probe in all sterol uptake assays. Cholesterol uptake in the absence of cell growth was estimated in *hem1* yeast cells cultivated for 24 h under conditions of heme- and sterol deficiency (YPD medium supplemented only with 0.06% Tween 80 (Sigma) as unsaturated fatty acid source). Sterol-depleted cells were washed in H_2O and suspended in the uptake medium (50 mM citrate–phosphate buffer pH 5.0, 0.5% glucose, [4-^{14}C]-cholesterol (specific activity 2000 dpm μg^{-1}) and Nonidet P-40 (NP-40) (US Biochemical) in the concentrations indicated in Figure legends) to the optical density of 3 or 5 absorbance units at 600 nm (OD_{600}). Cell suspension was incubated at 28 °C with shaking for time periods indicated in Figure legends, cells were collected by centrifugation, washed three times with 0.5% NP-40 and once with H_2O, and resuspended to OD_{600} of three. All washing steps were performed at room temperature. Five ml aliquot was centrifuged and used for the estimation of total sterol uptake and the sediment from 10 ml aliquot of the suspension was used for lipid extraction and analysis.

2.3. Sterol uptake in growing cells

Cells cultivated in YPD with 50 μg ml^{-1} δ-aminolevulinic acid (δALA) for 24 h were inoculated to YPD medium supplemented with 0.06% Tween 80, 20 μg ml^{-1}, [4-^{14}C]-cholesterol (specific activity 500 dpm μg^{-1}) and 0.2% NP-40 (added at time points indicated in figure legend) and grown at 28 °C with shaking. At indicated time points, 45 OD_{600} units were collected from each variant by centrifugation, washed three times with 0.5% NP-40 and once with H_2O. About 15 OD_{600} units were processed for estimation of total sterol uptake and 30 OD_{600} units were collected for lipid extraction and analysis.

2.4. Quantification of total sterol uptake

Total cholesterol uptake was estimated as the radioactivity associated with washed cell pellet. 10 μl of 10% Triton X-100 were added to cell pellet which was frozen quickly in liquid nitrogen. After thawing, cell pellet was solubilized in 10% sodium deoxycholate at 37 °C overnight and the total [4-^{14}C]-cholesterol uptake was estimated by liquid scintillation counting in Beckman LS6000SE.

2.5. Lipid extraction and analysis

Lipid extraction was performed according to Bligh and Dyer [13] with minor modifications. Shortly, 0.5–1×10^9 cells processed as described above were disrupted by vortexing with glass beads (diameter 0.4 mm) 6×1 min, with cooling on ice. Lipids were extracted from cell homogenate by hot methanol (30 min at 65 °C) followed by twofold extraction in chloroform–methanol–H_2O (1:2:0.8) at room temperature. Organic phase containing lipids was withdrawn and evaporated. Dry lipid residue was dissolved in chloroform–methanol (2:1) and aliquots corresponding to $2–3 \times 10^8$ cells were applied to silica gel plates (Merck, Germany). Neutral lipids were separated by ascending two-step thin layer chromatography (first step: petroleum ether – diethyl ether – acetic

acid 70:30:2; second step: petroleum ether – diethyl ether 49:1). Individual lipid spots were visualized by charring with sulfuric acid, transferred to scintillation vials and the radioactivity was estimated by liquid scintillation counting.

2.6. Estimation of cell viability as colony-forming ability

The effect of NP-40 on colony-forming ability was determined in cultures grown in YPD and collected at timepoints as indicated in the Results section. Aliquots of cell suspensions were washed in sterile water, counted under microscope, diluted and plated in duplicates on YPD agar plates with δALA at densities 100–400 cells per plate. The number of formed colonies was estimated after incubation of the plates for three days at 30 °C and the plating efficiency calculated as percentage of plated cells.

2.7. Estimation of cell integrity by vital staining

Cell integrity was estimated at indicated timepoints by staining with the vital dye propidium iodide (PI; Serva, Germany). Aliquots of cell suspensions were washed in distilled water, suspended in water with 5 $\mu g\,ml^{-1}$ of PI (cell density about 1 OD_{600} unit) and incubated 10 min at room temperature. Cells were washed and resuspended in water and the number of total and PI stained cells was counted under fluorescent microscope.

3. Results

3.1. Non-ionic detergents interfere with the uptake of external sterols in non-growing yeast cells

To eliminate the interference of different growth rates with the estimation of uptake of external sterols in various yeast strains we developed an assay for quantification of the uptake of [4-^{14}C]-cholesterol as sterol probe in non-growing heme-deficient yeast cells that have been depleted of internal sterols. During the optimization of this uptake assay we were confronted with the problem of non-specific association of cholesterol with cell surface at concentrations of external cholesterol exceeding 5 $\mu g\,ml^{-1}$. These non-specific aggregates were only poorly solubilized by subsequent wash in 0.5% NP-40 and caused high and variable background interfering with the estimation of the uptake rate. In an attempt to reduce the aggregation of cholesterol by inclusion of NP-40 to uptake medium we observed that the uptake was significantly reduced by low concentrations of this mild non-ionic detergent (Fig. 1(a)). Inhibitory effect of NP-40 on cholesterol uptake was evident at much lower concentrations than those commonly used for solubilization of sterol supplements (typically 0.2–0.5%). This effect was apparently not related to formation of detergent micelles since it was observed also at concentrations below the critical micellar concentration (estimated to be close to 0.005% based on sharp increase in fluorescence intensity of diphenylhexatriene, results not shown). Interference of NP-40 with cholesterol uptake in non-growing cells was maintained over extended time periods (Fig. 1(b)). The inhibitory effect was neither strain-specific nor limited to the detergent NP-40 since similar results were observed in two strains bearing *hem1* disruption in

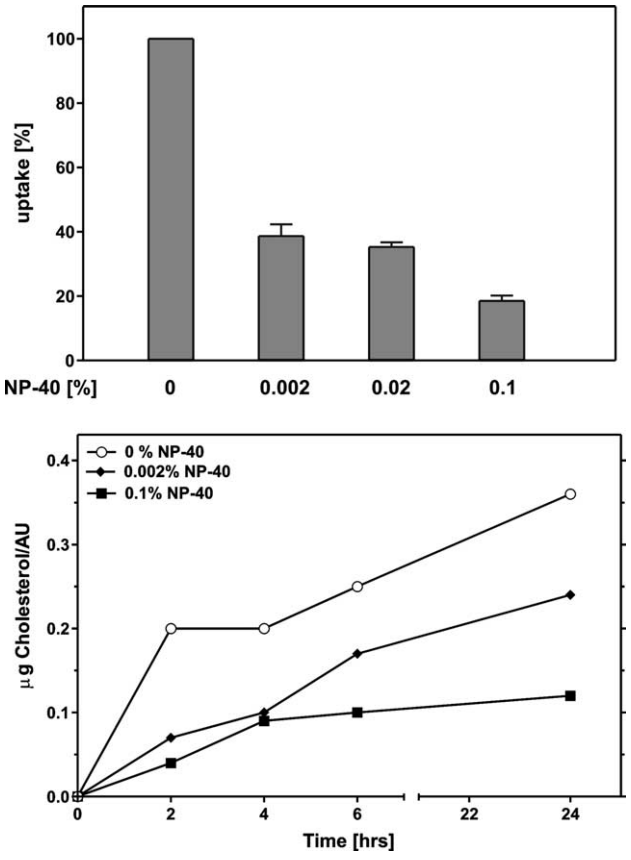

Fig. 1. Cholesterol uptake in non-growing cells is inhibited by NP-40. Sterol uptake was estimated in WS17-D *hem1* cells depleted in sterols by growth in sterol-free medium (YPD with 0.06% Tween80 as the source of unsaturated fatty acids) for 24 h. Washed stationary-phase cells were assayed for the uptake of [4-^{14}C]-cholesterol in the absence of growth as described in Section 2. *Panel a*: Cells were incubated in uptake buffer containing 5 $\mu g\,ml^{-1}$ of cholesterol and different concentrations of NP-40 for 2 h, washed and solubilized in Na-deoxycholate as described in Section 2. Values of cholesterol uptake (in µg per 1 OD_{600} unit) of untreated control were taken as 100%. Average of two independent experiments with the variation between the experiments is shown. *Panel b*: Sterol depleted cells were assayed for uptake of [4-^{14}C]-cholesterol as in (a), except of reduced cholesterol concentration (2 $\mu g\,ml^{-1}$) and extended incubation time (2–24 h). Cholesterol uptake is expressed in µg of cholesterol per OD_{600} unit. Similar results were obtained with 5 $\mu g\,ml^{-1}$ cholesterol in uptake buffer.

different genetic backgrounds (WS17-5D *h1* and W303-1B *hem1*) and in cells treated by another mild non-ionic detergent Tween 80 (results not shown). Since NP-40 (sometimes used under different brand name Tergitol) is most commonly used for solubilization of lipid supplements in yeast, we limited our subsequent studies to this detergent.

Aggregation of cholesterol on the surface of non-growing cells can be overcome by reducing the concentration of cholesterol to 2–5 μg ml^{-1}. As demonstrated in Fig. 2(a), *hem1* mutant cells made competent for heme and ergosterol synthesis by the addition of δ-aminolevulinic acid to growth medium showed extremely low labeling with [4-^{14}C]-cholesterol at this cholesterol concentration even in the absence of detergent in the uptake medium. On the other hand, heme-deficient cells that were depleted of internal sterol stores by cultivation in sterol-free medium are able to take up significant amounts of external cholesterol in a detergent-sensitive manner. The analysis of lipids extracted from these cells showed that reduced levels of cholesterol in detergent-treated cells reflect virtual absence of cholesterol esters in these cells (Fig. 2(b)). These results suggest that the effect of NP-40 on cholesterol uptake is not a simple solubilization effect but a more specific interference of the detergent with the sterol uptake process. Since sterol esterification takes place in endoplasmic reticulum [14] the effect of NP-40 in non-growing sterol-depleted cells may be related either to internalization of cholesterol or its esterification.

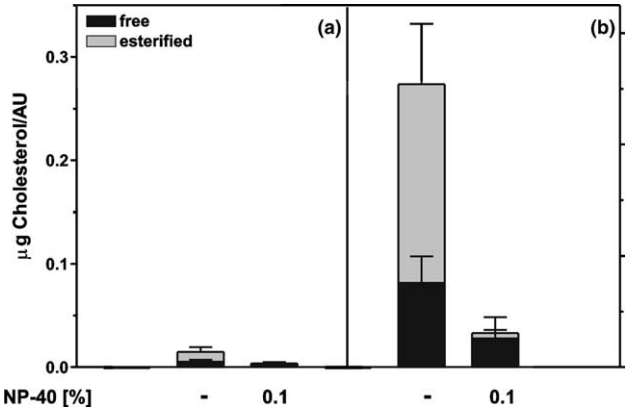

Fig. 2. Reduced uptake of external cholesterol reflects inhibition of sterol esterification by NP-40. [4-^{14}C]-cholesterol uptake was assayed in non-growing WS 17-D *hem1* cells as described in Fig. 1 and in Section 2. The uptake buffer contained 2 μg ml^{-1} cholesterol and 0.1% NP-40 (where indicated). After four hours incubation, lipids were extracted from washed cells and analysed by TLC as described in Section 2. Results shown are average values ± standard deviation of μg cholesterol per OD_{600} unit in sterol esters (grey bars) and free sterols (black bars) from three independent experiments. *Panel a*: Uptake in heme- and sterol-competent cells after growth in YPD medium containing 50 μg ml^{-1} δ-aminolevulinic acid for 24 h. *Panel b*: Uptake in heme-deficient sterol-depleted cells after growth in YPD medium with 0.06% Tween 80.

3.2. NP-40 affects external sterol uptake and esterification in growing cells

Reduced levels of sterol esters could be caused by direct inhibition of sterol esterification by NP-40. We therefore examined the effect of NP-40 on the levels of sterol esters derived from internally synthesized sterols in *hem1* cells made competent for heme and sterol synthesis by δ-aminolevulinic acid supplementation. Analysis of the lipids labeled with ^{14}C-acetate in these cells revealed no changes in sterol ester levels in detergent-treated cells (results not shown). Direct interference of NP-40 with the sterol:acyl-CoA acyltransferases is therefore unlikely and the effect may be related to other processes during the uptake and internalization of external cholesterol.

The effect of NP-40 on the levels of cholesterol esters might reflect some unusual characteristics of sterol-depleted cells assayed in the absence of growth. We therefore examined the effect of NP-40 on cholesterol uptake and esterification during the growth of *hem1* mutant in YPD media. In the absence of detergent, sterol esters showed a typical growth phase-dependent pattern with moderate levels in the exponential phase and extensive accumulation in the stationary phase. Cells treated with 0.2% NP-40 from the beginning of cultivation (Fig. 3(a)) had low levels of cholesterol esters in the exponential phase and reduced efficiency of cholesterol esterification was maintained up to the late stationary phase. This was accompanied by elevated levels of unesterified sterols relative to the control untreated cells suggesting that low sterol ester levels were not caused by general limitation in intracellular sterols due to compromised uptake. When NP-40 was added to cells in the exponential phase (Fig. 3(b)), cholesterol esterification was arrested on the exponential phase level and this low level was maintained up to the late stationary phase. Addition of NP-40 to stationary phase cells did not affect sterol ester levels (Fig. 3(c)) suggesting that detergent-dependent changes were not caused by possible stimulation of sterol ester hydrolysis. The inspection of TLC chromatograms (Fig. 3(d)) of neutral lipids revealed another interesting aspect. Yeast cells exposed to NP-40 from the beginning of growth (lanes 5–8) or from the exponential phase (lane 9) maintained increased levels of sterol precursors squalene and lanosterol (characteristic for exponential phase cells) up to the late stationary phase. These changes were not observed in lipid extracts from cells treated by detergent in the stationary phase cells (lane 10) which indicates that the addition of NP-40 may have a more general effect on sterol metabolism in growing yeast.

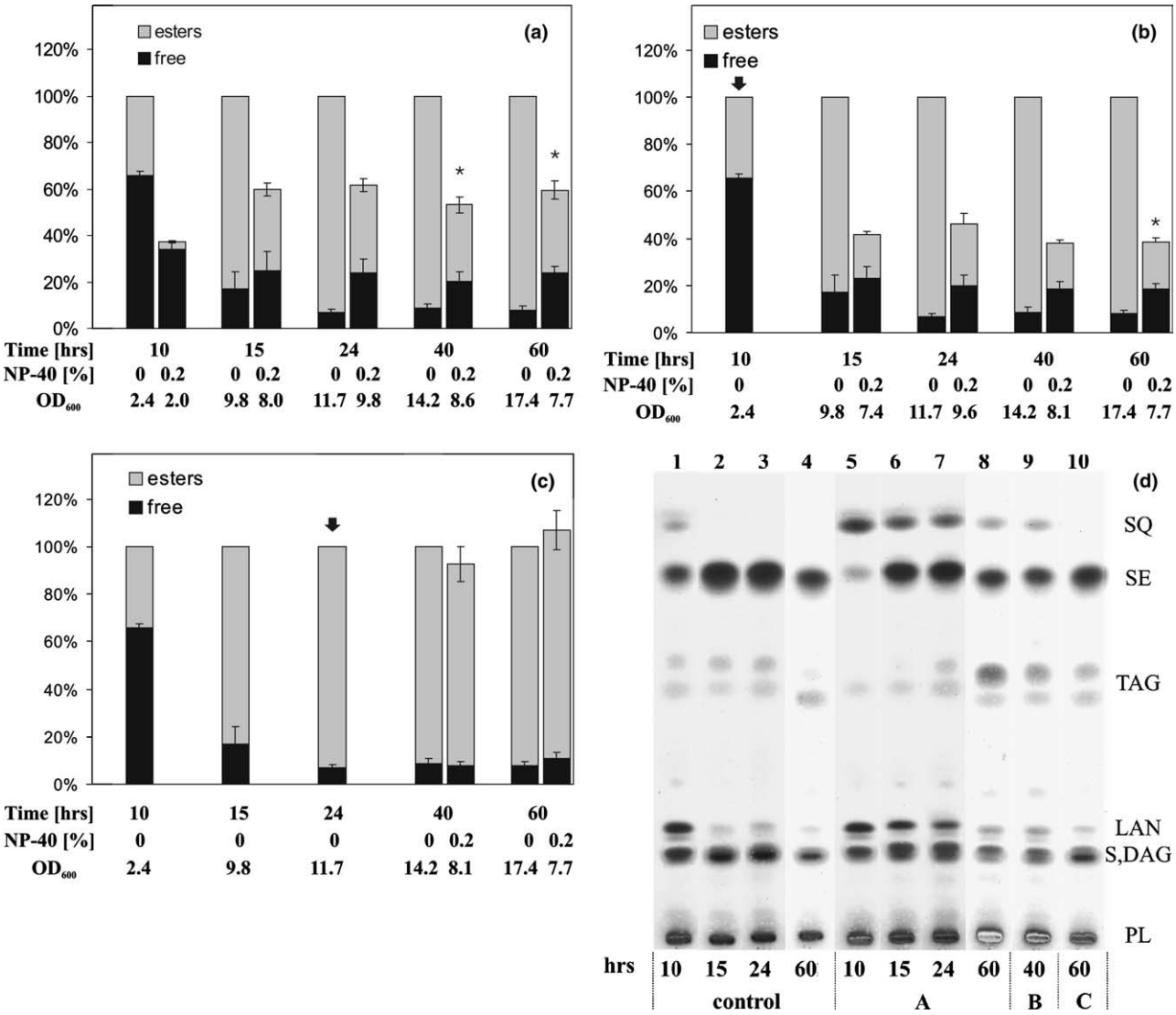

Fig. 3. NP-40 interferes with sterol biogenesis in growing yeast cells. WS17-D *hem1* strain cultivated for 24 h in YPD medium with 50 μg ml^{-1} δ-aminolevulinic acid was inoculated to fresh YPD medium with 0.06% Tween 80 and 20 μg ml^{-1} [4-^{14}C]-cholesterol. Where indicated, NP-40 was added to the final concentration of 0.2% either from the start of cultivation (*Panel a*) or at specific time points (*Panels b and c*). All variants were cultivated at 28 °C with shaking, aliquots of cell suspensions were withdrawn at indicated time points and the total cholesterol uptake and incorporation of cholesterol into free (black bars) and esterified (grey bars) sterol fractions were estimated as described in Section 2. *Panel a*: NP-40 present in the medium from time 0. *Panel b*: NP-40 added during exponential phase at time 10 h. *Panel c*: NP-40 added during stationary phase at time 24 h. Average growth yield determined as optical density at 600 nm is indicated for corresponding time points. Average values ± standard deviation from three independent experiments are expressed as fractions of free and esterified cholesterol from total cholesterol content. Values for NP-40 treated variants were expressed relative to total cholesterol levels in corresponding untreated controls. Arrow – addition of NP-40; * – average values from two experiments. *Panel d:* TLC chromatogram of neutral lipids extracted from cell suspensions sampled during one of the experiments described in *Panels 3(a)–(c)*. Legend to individual samples: *Lanes 1–4:* control samples (no NP-40), sampling time 10–40 from *Panel (a)*. *Lanes 5–8:* NP-40 treated variants (sampling time 10–60 h) from *Panel (a)*. *Lane 9:* NP-40 treated sample (sampling time 40 h) from *Panel (b)*; *lane 10:* NP40-treated sample (sampling time 60 h) from *Panel (c)*. The mobility of lipid standards is shown on the right: SQ, squalene; SE, sterol esters; TAG, triacylglycerols; FFA, free fatty acids; LAN, lanosterol; S, 4-desmethylsterols (ergosterol and cholesterol); DAG, diacylglycerols; PL, phospholipids.

3.3. Effect of NP-40 on cell growth and viability

Another effect of detergents that has not been addressed in most reports using Nonidet or Tergitol as lipid solubilizers is the detrimental effect of these detergents on cell integrity and/or viability. In our experiments, cells supplemented with δALA (i.e., heme-competent) reached typical high cell densities in the stationary phase after 24 h of growth and this growth yield was only slightly reduced by the presence of 0.2% NP-40 in the medium. On the other hand, the growth yield of heme deficient *hem1* cells relying on the uptake of external sterol reached the stationary phase at lower cell densities compared to heme-competent cells ($0.5–1 \times 10^8$ cells ml^{-1} at 24 h)

and they did not grow further until 48 h. Growth yield of these heme-deficient cells was significantly reduced in YPD medium containing 0.2% Nonidet, especially in the 2nd subcultivation in this medium (Fig. 4(a)). Plating tests of the cells cultivated in media containing 0.2% NP-40 for extended time periods revealed that both heme-supplemented and heme-deficient cultures show strongly reduced ability to form colonies (Fig. 4(b)). Interestingly, this plating efficiency was reduced also in heme-competent cells showing no major decrease in growth yields after NP-40 treatment. Apparently, the reduced fraction of living cells is sufficient for relatively normal growth yield after prolonged growth in these long-term experiments.

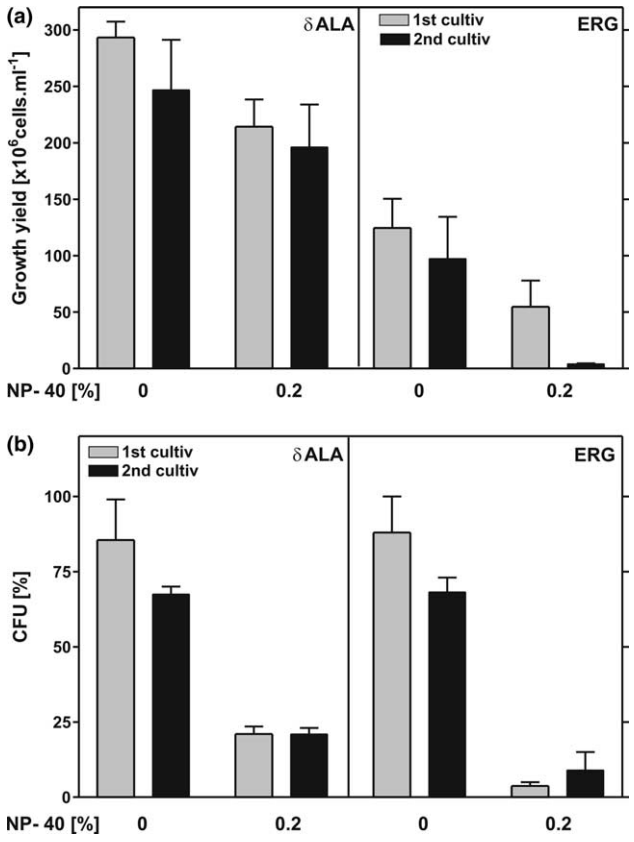

Fig. 4. NP-40 reduces cell growth and viability in yeast cells. Strain WS17-D *hem1* was cultivated for 24 h in YPD medium with 50 μg ml^{-1} δ-aminolevulinic acid and inoculated to fresh medium containing either 50 μg ml^{-1} δ-aminolevulinic acid (δALA) or 20 μg ml^{-1} ergosterol and 0.06% Tween 80 (ERG). NP-40 was added to final concentration of 0.2% where indicated and cells were cultivated at 28 °C for 24 h. After 24 h (1st subcultivation), growth yield was estimated by counting the cells in haemocyte chamber. Viability of the cells was determined as the ability to form colonies after plating as described in Section 2. An aliquot of the cells from the 1st subcultivation was inoculated into fresh medium with the same composition to cell density of 2×10^6 cells per ml and grown for another 24 h (2nd subcultivation). Growth yield and cell viability were determined as described above. Values are averages ± standard deviations from three independent experiments. *Panel a*: Growth yields in stationary phase cultures (24 h). *Panel b*: Colony-forming ability in stationary phase cultures (24 h). Grey bars: 1st subcultivation; black bars: 2nd subcultivation.

Since NP-40 seems to affect cell integrity and/or viability, observed effects on sterol metabolism might be related to the accumulation of dead cells in NP-40 treated cultures. We therefore estimated cell integrity in one of the experiments described in Fig. 3. In the period where the most pronounced changes in sterol pattern were observed (10–15 h), less than 2% of cells were stained by vital dye propidium iodide (PI) in both control and NP-40 treated cultures supplemented with external sterols. During extended cultivation (24–60 h) control cells retained this impermeability to PI while 25–38% of NP-40 treated cells were stained by PI. Colony-forming ability in these NP-40 treated cells was reduced to 13% (24 h) and 1–2% (40 and 60 h), however, high plating efficiency was preserved (75–100%) during the first 15 h of growth even in cells treated by NP-40. This indicates that the changes in sterol metabolism induced by NP-40 precede the loss of cell viability and integrity.

4. Discussion

NP-40 and other mild non-ionic detergents are commonly applied as a means to increase the solubility of lipid supplements in growth media. Typical concentrations used for growth experiments with *S. cerevisiae* strains defective in ergosterol biosynthesis are in the range 0.1–1% [3,6–12]). Since no apparent growth defects were noted, possible detrimental effects of mild non-ionic detergents on yeast physiology were not considered in these reports. High NP-40 concentrations (up to 4%) were used in short-term experiments involving the uptake of external cholesterol [15,16]. Highly reduced viability has been reported in one of these reports, however, ergosterol or heme depletion has been suggested as the cause here [15]. On the other hand, our results clearly show that even low concentrations of NP-40 have significant effect on the physiology of yeast cells. The most striking effect was reduced ability to form colonies after prolonged growth in the presence of NP-40 indicating a general loss of viability. In addition, strong inhibition of uptake and/or esterification of external cholesterol by low concentrations of NP-40 in non-growing cells and significant changes in sterol metabolism in growing heme-deficient cells treated with this detergent indicate a specific effect of low concentrations of detergents on sterol biogenesis in these cells. Since NP-40 treated cells remained impermeable to the vital stain PI under conditions where significant changes in sterol pattern occurred, effects of NP-40 cannot be explained by a disintegration of plasma membrane and cell death.

There are several processes potentially affected by the presence of mild detergents like NP-40 that might

underlie changes in sterol biogenesis observed in this study. Moskvina et al. [17] have found that 0.01% NP-40 causes re-localization of the transcription factor Msn2p to nucleus – a process important for the stress response element-mediated gene expression. This means that changes in the plasma membrane structure caused by the presence of detergents may trigger stress response in yeast. The link between mild non-ionic detergents and stress response is further supported by the report of Nakagawa et al. [18] showing that disruption of the *CCT* gene (encoding catalase) in *Candida albicans* causes increased sensitivity to several detergents including NP-40. Effects of low concentrations of NP-40 observed in our experiments in yeast *S. cerevisiae* might thus reflect a general defect in intracellular communication and signaling caused either by general changes in membrane environment or by more specific interactions with signal transduction machinery.

Another possible explanation for observed effects could be a more direct interference of detergents with the mechanisms of external sterol uptake or internalization in yeast. Proteins of the ABC transporter family were reported to be involved in the uptake of external sterols by anaerobic yeast [3]. Although no data exist on the effects of NP-40 on the activity of pleiotropic drug resistance system in yeast, several compounds with detergent properties were found to affect the activity of PDR pumps in a large-scale screen of several hundred of substances in yeast *S. cerevisiae* [19] . Moreover, NP-40 at low concentrations was reported to reverse the multidrug resistance phenotype in mammalian cells [20]. Reduced uptake and internalization/esterification of cholesterol in the presence of NP-40 in our experiments may thus reflect the direct interference of this detergent with the sterol uptake system.

In conclusion, our data show that mild non-ionic detergents such as NP-40 in concentrations used commonly to solubilize hydrophobic growth supplements may significantly affect cell physiology of the yeast *S. cerevisiae*. Significant decrease of total uptake and esterification of external cholesterol, changes in sterol precursor levels as well as loss of viability indicated that processes both at the level of plasma membrane as well as intracellular processes are disturbed by low concentrations of NP-40. More attention should be therefore paid to the design of experiments and interpretation of their results, especially for strains defective in lipid biogenesis that are dependent on the delivery of lipid supplements.

Acknowledgements

Authors thank M. Kostolanská for technical help with some experiments and Dr. Joanna Rytka (Warszaw, Poland) for providing the strain and *hem1:LEU2* disruption casette. This work was supported by projects VEGA-2/4130/24 and APVT-20-016502 (to I.H.).

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