

Fatty acids trigger mitochondrion-dependent necrosis

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Abbreviations: AIF, apoptosis inducing factor; DAG, diacylglycerol; DHE, dihydroethidium; Eth, ethidium; FFA, free fatty acid; LA, linoleic acid; L3A, linolenic acid; mtDNA, mitochondrial DNA; MPTP, mitochondrial permeability transition pore; MAG, monoacylglycerol; MUFA, monounsaturated fatty acid; OA, oleic acid; PUFA, polyunsaturated fatty acid; PCD, programmed cell death; PI, propidium iodide; QKO, quadruple knock-out; ROS, reactive oxygen species; SFA, saturated fatty acid; TAG, triacylglycerol

Obesity is characterised by lipid accumulation in non-adipose tissues, leading to organ degeneration and a wide range of diseases, including diabetes, heart attack and liver cirrhosis. Free fatty acids (FFA) are believed to be the principal toxic triggers mediating the adverse cellular effects of lipids. Here, we show that various cooking oils used in human nutrition cause cell death in yeast in the presence of a triacylglycerol lipase, mimicking the physiological microenvironment of the small intestine. Combining genetic and cell death assays, we demonstrate that elevated FFA concentrations lead to necrotic cell death, as evidenced by loss of membrane integrity and release of nuclear HMGB1. FFA-mediated necrosis depends on functional mitochondria and leads to the accumulation of reactive oxygen species. We conclude that lipotoxicity is executed via a mitochondrial necrotic pathway, challenging the dogma that the adverse effects of lipid stress are exclusively apoptotic.

Introduction

Over one billion people are currently overweight in the world, 20–30% of which are considered as overtly obese.¹ Obesity, which is generally associated with elevated plasma FFA levels,² has been causally linked to an array of human diseases, including metabolic syndrome, type II diabetes, diverse cardiovascular disorders, hepatosteatosis and an increased propensity to develop cancer.^{1,2} Various lipid species are becoming increasingly recognized as agents that are endowed with an intrinsic potential of cytotoxicity (or ‘lipotoxicity’). While it has been traditionally considered that elevated glucose levels are responsible for the degeneration and death of insulin-producing β -cells, recent advances underscore the pathogenic impact of fatty acids in the development of diabetes.³ Unfortunately, the molecular mechanisms underlying lipotoxicity and their contribution to human pathophysiology remain poorly explored.⁴ Some studies have demonstrated that elevated extracellular concentrations of free fatty acids (FFAs) can stimulate the synthesis of toxic ceramides,^{5,6} which in turn are potent inducers of apoptotic cell death. Nonetheless, not all

FFAs are incorporated into ceramides and ceramide-independent routes of FFA-induced cell death must exist.^{6–8} Such ceramide-independent pathways may involve the release of cytochrome *c* from mitochondria^{9–12} and/or the unfolded protein response (UPR) in the endoplasmic reticulum.^{13,14} In this context, it may appear intriguing that diacyl glycerol (DAG) might be (one of) the second messenger(s) that mediate(s) FFA-induced cell death in the fission yeast *Schizosaccharomyces pombe*.¹⁵

There exist several cell death types by which cells regulate their demise. While apoptosis is a well-accepted subroutine of programmed cell death (PCD), early reports described necrosis as an ‘accidental’ form of cell death based on its presumed unregulated nature.¹⁶ However, extensive evidence has emerged over the past decade that strongly argues for the role of specific genes in controlling the onset and progression of necrosis.^{17,18} Among the factors considered to play a crucial role during necrosis are calpains, cathepsins and cyclophilin D, as well as the serine/threonine kinases RIP1 and RIP3.^{18,19} Necrotic cells characteristically display increased cell volume (oncosis), organelle swelling and rupture of the plasma membrane

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leading to leakage of intracellular material.¹⁶ Similar to apoptosis, mitochondrial-derived production of reactive oxygen species (ROS) also occurs during necrosis.²⁰⁻²² Furthermore, during the necrotic disintegration of cells, DNA is randomly degraded, ATP-levels are depleted, and frequently inflammatory responses by surrounding cells are activated.²³

Necrosis has been reported to occur in several pathological states including tissue ischemia, microbial infection and neurodegenerative disease.^{18,24-26} Furthermore, chronological ageing of budding yeast (*Saccharomyces cerevisiae*) is coupled to the physiological induction of cell death, which can manifest either as apoptosis or as necrosis.²⁷⁻³⁰ Age-associated necrotic yeast cell death is prevented by autophagy, which can be induced by external supply of the naturally occurring polyamine spermidine.^{27,31}

S. cerevisiae represents a well-established model organism to study PCD.³² Both necrosis and apoptosis are likely to share certain mediators, such as ROS or components of the permeability transition pore.^{33,34} Caspases, on the other hand, the proteases involved in many but not all apoptotic scenarios,³⁵ are not directly involved in the execution of necrotic cell death. Since abrogation of one form of PCD often results in cell death through a different pathway, there is presumably a molecular 'switch', whereby cells are directed to die through either apoptosis or necrosis.^{36,37}

S. cerevisiae has a high potential of utilising externally supplied FFAs because its genome encodes as many as six acyl-CoA synthetases with broad substrate specificities. Interestingly, even at high doses, FFAs are not toxic to wild type yeast cells.³⁸ However, a quadruple knockout (QKO) mutant that is defective in triacylglycerol (TAG) synthesis exhibits a marked proliferation of intracellular membranes upon treatment with exogenous oleic acid (OA).³⁹ Thus, cells respond to impaired FFA deposition into triacylglycerols by increasing phospholipid synthesis. Moreover, QKO yeast cells challenged with high doses of FFA can die while they produce elevated ROS levels.¹³ Here, we investigated the mechanisms through which FFA can kill yeast cells and show that OA, linoleic acid (LA) and linolenic acid (L3A) can activate a necrotic cell death program.

Results

Cooking oils induce yeast cell death upon administration of a triacylglycerol lipase. We initially examined the effects of various alimentary oils on yeast cell viability, which was quantified by means of clonogenic assays. Olive, pumpkin seed, linseed (also termed flaxseed), salmon, rapeseed and walnut oils mostly consist of triacylglycerols (TAG) with varying proportions of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (Fig. 1A). Treatment with any of these oils had no effect on the viability of wild-type (BY4741) yeast cultures, except for treatment with linseed or rapeseed oils, which reduced viability slightly (Fig. 1B). We reasoned that the addition of triacylglycerol lipase would mimic the metabolic environment in the mammalian small intestine, in which pancreatic lipases catalyze the conversion of TAG into FFAs, DAG, monoacyl glycerol (MAG) and glycerol.⁴⁰ Importantly, when a triacylglycerol

lipase from *Candida rugosa* was added to the culture medium, all alimentary oils became toxic to yeast, albeit, there were clear differences among the distinct classes of oils (Fig. 1C). Although linseed oil is considered to be among the healthiest oils used for cooking, it was most effective in killing yeast cells in a dose-dependent manner (Fig. 1C and S1).

We next sought to determine the mode of cell death underlying oil-toxicity by using specific markers for apoptosis and necrosis. When combined with triacylglycerol lipase, all tested oils elicited an increase in the proportion of cells stained with propidium iodide (PI), which is indicative of plasma membrane rupture, one of the hallmarks of necrotic death (Fig. 1D). Cells treated with linseed oil displayed a more than 5-fold increase in the number of necrotic cells (Fig. 1D). In contrast, apoptosis was not significantly induced, since the number of cells stained with Annexin V, which is a marker for apoptotic phosphatidylserine externalization onto the outer leaflet of the plasma membrane, remained largely unchanged (Fig. 1D). To test whether ROS levels were elevated upon treatment, we employed an automated assay based on the superoxide-driven conversion of non-fluorescent dihydroethidium (DHE) into fluorescent ethidium (Eth) (Fig. 1E). The accumulation of ROS in cells treated with oils largely reflects the pattern of PI positive cells, suggesting that FFA-mediated necrosis is preceded and accompanied by excessive ROS generation.

FFA-induced necrosis is exacerbated by higher degrees of unsaturation. Cooking oils contain different proportions of fatty acid species. As a means to identify the specific nature of fatty acid-induced cell death, we supplemented growing yeast cultures with different FFA and assessed cell survival. However, the use of wild type cells for such studies is problematic, because FFAs are rapidly activated in yeast by several acyl-CoA synthetases³⁸ and subsequently incorporated into cellular lipids, thus losing their cytotoxic potential even when they are applied at high concentrations (Fig. 2A). To avoid the detoxification of FFAs, we employed a strain lacking *LROI*, *DGAI*, *ARE1* and *ARE2* (hereafter termed quadruple knockout, QKO), which is unable to esterify FFAs.^{13,39} Unlike wild type yeast cells, QKO cells lose their clonogenicity upon treatment with various unsaturated FFAs (Fig. 2A). OA, LA and L3A (Fig. 2A) reduced cell survival, and this effect increased progressively with the degree of their unsaturation (Fig. 2A). Confirming this tendency, saturated FFAs such as palmitic acid and stearic acid were unable to induce cell death at doses at which unsaturated FFAs efficiently killed QKO cells (Fig. 2A). Thus, the severity of FFA-induced cell death correlates with the degree of unsaturation.

We next determined the type of cell death elicited by FFA supplementation in the QKO. Consistent with our findings with alimentary oils, the lipotoxic effect of OA LA and L3A was predominantly necrotic in nature as demonstrated by AnnexinV/PI costaining (Fig. 2B). Only a small fraction of the cells became apoptotic, since only around 10% of the cells showed AnnexinV staining. Again, these treatments strongly induced ROS (Fig. 2C and D). The release of the nuclear HMGB1 (a chromatin-bound non-histone protein) into the cytosol has been established as a specific marker for necrotic cell death.⁴¹ As an additional

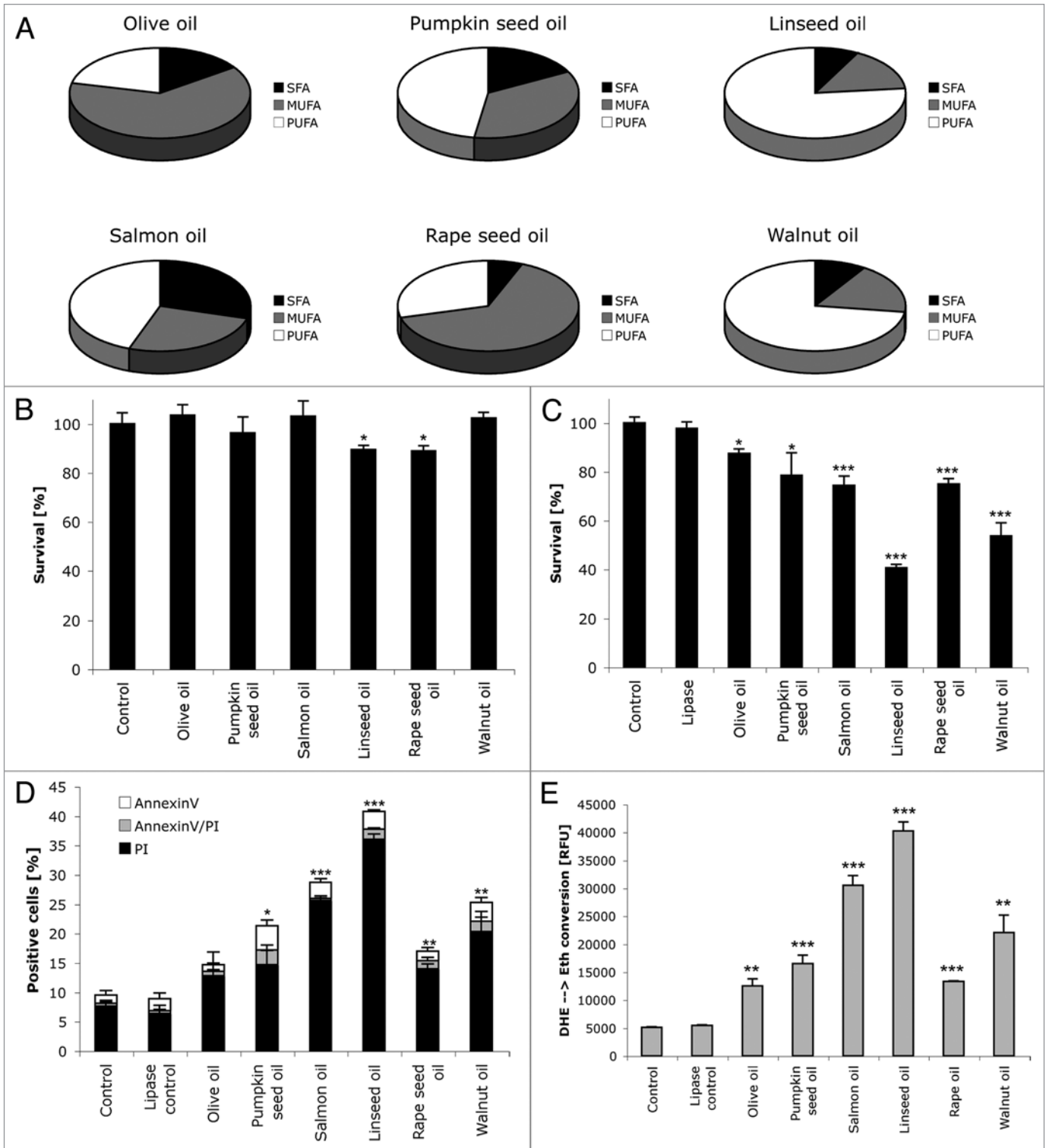


Figure 1. Continuous cooking oil treatment (0.5%) induces yeast necrotic cell death. Error bars show standard errors and asterisks indicate significant difference (Student's t-test) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (A) Fatty acid composition of cooking oils. For pumpkin seed-, salmon- and rape seed oils as depicted on label; for olive-, linseed- and walnut oils as stated by "Deutsches Lebensmittelbuch" and "Deutsche Gesellschaft für Fettwissenschaft" (<http://www.dgfett.de/material/fszus.htm>) in average. (B) Survival based on clonogenicity of wt cells after 20 hr of treatment with indicated cooking oils without lipase treatment. Cells were washed with ddH₂O prior to measurement of cell density with the Casy cell counter. (C) Survival analogous to part B with additional lipase treatment (50 U/ml). (D and E) AnnexinV/PI costaining (asterisks refer to PI) (D) and DHE to ethidium conversion for ROS quantification of lipase treated cells depicted in (E).

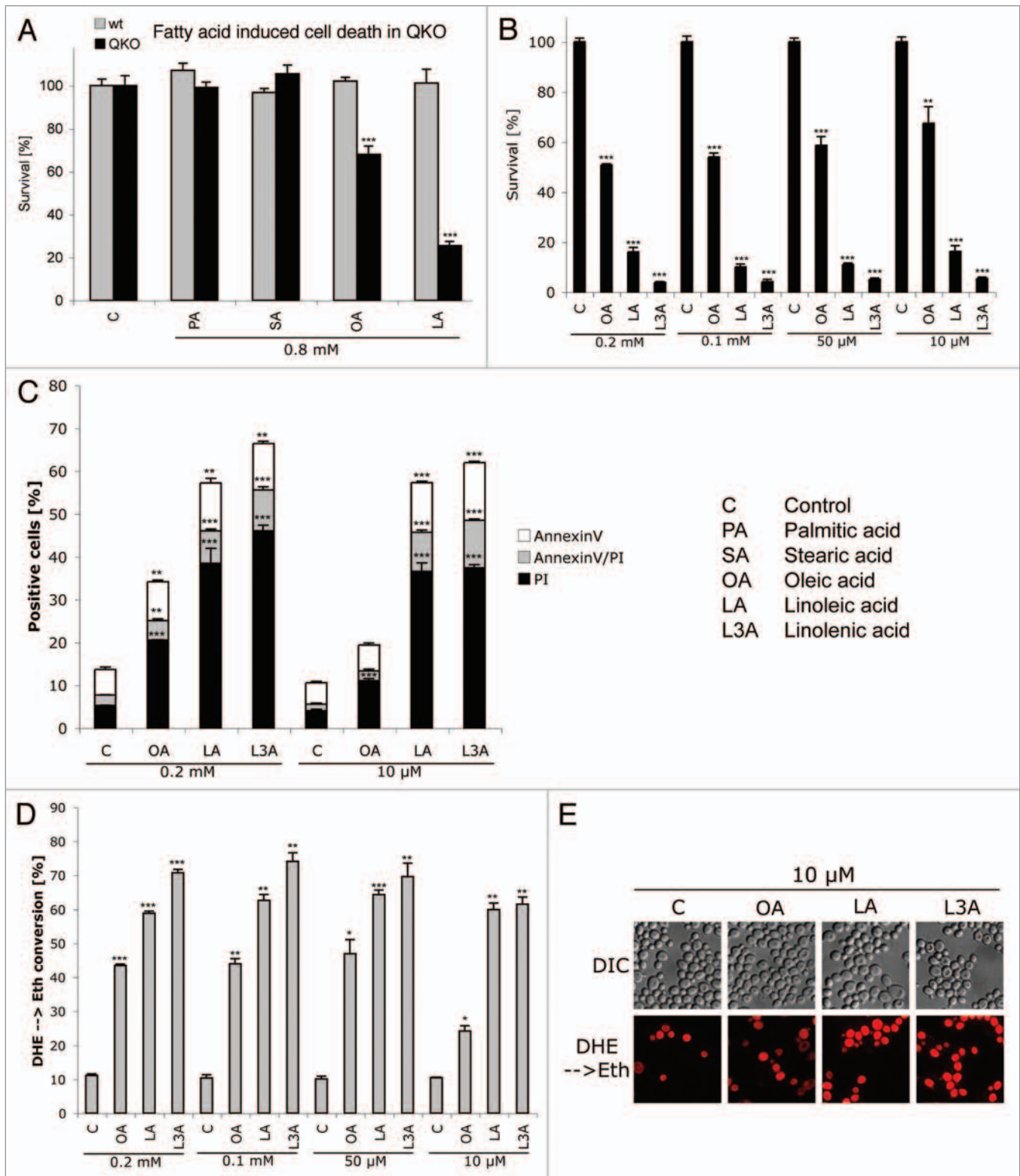


Figure 2. Free fatty acids induce yeast necrosis in dependency on the degree of unsaturation. Error bars show standard errors and asterisks indicate significant difference (Student's t-test) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (A) Survival based on clonogenicity of wt or QKO cells, respectively, after 20 hr of treatment with indicated fatty acids. (B) Survival based on clonogenicity of QKO cells after 4 hr of treatment with indicated fatty acids. (C and D) AnnexinV/PI costaining (C) and DHE to ethidium conversion for ROS quantification depicted in (D). (E) Microscopy pictures of DHE to ethidium conversion.

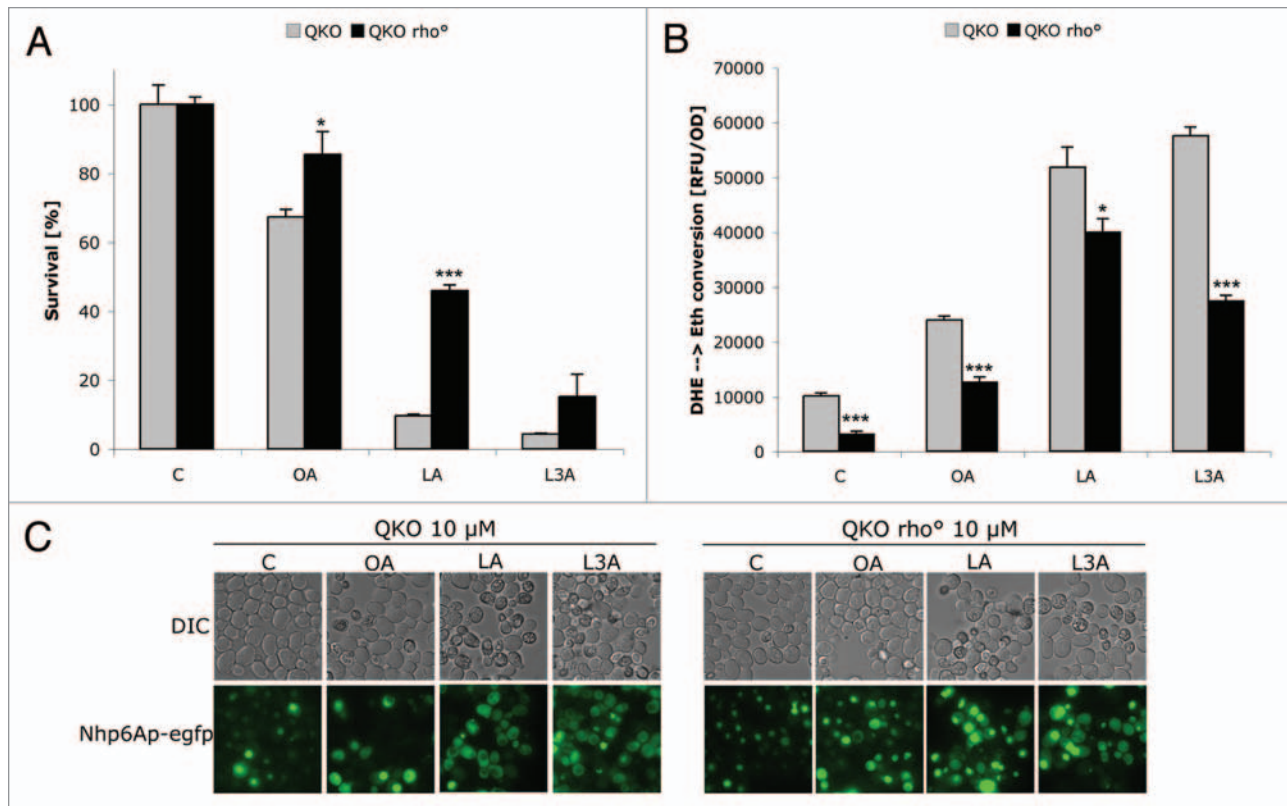


Figure 3. Fatty acid induced cell death is dependent on mitochondrial DNA. Error bars show standard errors and asterisks indicate significant difference (Student's t-test) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (A) Survival based on clonogenicity of QKO and QKO rho⁰ cells after 4 hr of treatment with indicated fatty acids. (B) DHE to ethidium conversion for ROS quantification (normalised to OD₆₀₀). (C) Microscopy for Nhp6Ap-EGFP localisation after 6 hr of treatment with indicated fatty acids.

assay for the mode of death triggered by FFA treatment in yeast, we determined the localization of the yeast HMGB1 ortholog (Nhp6Ap), which was tagged with EGFP. OA, LA and L3A supplementation resulted in nuclear release of Nhp6Ap-EGFP (Fig. 3C). Collectively, our data demonstrate that FFAs stimulate the necrotic demise of yeast cells.

FFA induced cell death depends on mitochondrial DNA. Because budding yeast is a facultative anaerobic organism, respiration-deficient strains are often used to explore the biological impact of oxidative phosphorylation or other mitochondrial processes. To gain further insight into the mechanism of lipotoxic cell death we generated QKO rho⁰ mutants, which lack mitochondrial DNA (mtDNA) and hence are respiration deficient. Intriguingly, rho⁰ mutants were markedly less susceptible to FFA-induced ROS production, plasma membrane permeabilisation (Fig. 3A and B) and nuclear release of Nhp6Ap-EGFP (Fig. 3C). This implies that the cytotoxic effects of FFAs are mediated, at least in part, by mitochondria. Of note, we detected LA (C18:2)-containing lipids in purified mitochondria of LA-stressed cells, indicating that exogenously applied FFAs can be incorporated into organellar membranes (Suppl. Fig. 2). Altogether, these results underscore the contribution of mitochondria to FFA-induced cell death of QKO cells.

Discussion

Using *S. cerevisiae*, an accepted model organism to study PCD, we have investigated FFA-mediated cell lethality. We conclude that cooking oil- or FFA-induced lipotoxicity in wildtype or QKO mutants is primarily necrotic. These findings challenge the hypothesis that lipotoxicity is exclusively apoptotic, as this has previously been suggested.⁹ Rather, our data suggest that FFA can induce necrosis, a finding that might be of pathophysiological relevance because local FFA might trigger pro-inflammatory necrosis in adipose tissue or in atherosclerotic plaques.^{42,43}

We postulate that necrosis is executed in a programmed fashion, which coincides with accumulation of ROS and is dependent on functional mitochondria. In mammalian cells it has already been demonstrated that programmed necrosis depends on mitochondria^{44,45} and frequently involves the mitochondrial permeability transition pore (MPTP).^{36,46-48} Also in yeast, earlier studies have revealed a role for mitochondria during necrosis concomitant with elevations in ROS levels.^{41,49,50} Interestingly, dysfunction of peroxisomes (which play a key role in fatty acid degradation) results in necrotic cell death.^{49,51} It is tempting to speculate a mitochondrial-peroxisomal axis that regulates cell fate as FFAs approach potential cytotoxic concentrations.

We observed that necrosis is enhanced with increasing degrees of FFA unsaturation. Saturated FFAs are not toxic to yeast cells

due to their efficient elongation and desaturation to match the endogenous fatty acid pattern in yeast lipids.³⁹ In contrast, in mammals, saturated FFAs such as palmitate exert the strongest lipotoxic effects.⁶ However, using *Dgat1*^{-/-} mouse embryonic fibroblasts, which represent an analogous system to the yeast QKO system, Listenberger et al. could show that the unsaturated FFA OA is also toxic for mammalian cells.⁵² Interestingly, linseed oil, which exerted the strongest cell death effects upon lipase administration (Fig. 1), harbours the highest amount of PUFAs among the cooking oils tested. This is consistent with our studies ascribing enhanced cytotoxicity to increased unsaturation of FFAs.

The molecular mechanisms by which PUFAs exert their lipotoxic effects are currently unknown. Double bonds in FFA are susceptible to peroxidation, which may involve mitochondrial respiration. Indeed, treatment of yeast cells with fatty acid hydroperoxides,⁵³ or endogenous expression of a heterologous fatty acid desaturase render cells more susceptible to oxidative challenge.⁵⁴ Exogenous FFAs are readily incorporated into cellular lipids and are also prominently present in mitochondrial phospholipids, providing a prime target for respiration-induced peroxidation and ROS production.

Materials and Methods

Yeast strains and growth conditions. All experiments were carried out in strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). The quadruple knock out mutant (QKO), which was generated previously³⁹, was genetically manipulated as follows: *ycr048wΔ::KanMX4 ynr019wΔ::KanMX4 yor245cΔ::KanMX4 ynr008wΔ::KanMX4*.

All strains were grown on SCD medium containing 0.17% yeast nitrogen base (Difco), 0.5% (NH₄)₂SO₄ and 30 mg/l of all amino acids (except 80 mg/l histidine and 200 mg/l leucine), 30 mg/l adenine and 320 mg/l uracil with 2% glucose as carbon source.

For abrogation of the mtDNA (*rho*⁰), QKO cells were grown in complete medium containing 10 μg/ml ethidium bromide for 3 days. The resulting respiratory deficiency was confirmed by a complete lack of growth on obligatory respiratory medium (SC medium with 2% glycerol).

For wild type stress experiments, cultures were inoculated from a stationary overnight culture to an OD₆₀₀ = 0.1, grown on SCD for 4 hr to an OD₆₀₀ = 0.35 and then stressed with 0.5% unmodified cooking oil and 50 U/ml lipase from *Candida rugosa* (Sigma). Survival plating and tests for apoptotic and necrotic markers were performed 20 hr after stress.

For QKO stress experiments, cultures were inoculated from a stationary overnight culture to an OD₆₀₀ = 0.1, grown on SCD to an OD₆₀₀ = 0.35, and then stressed with indicated amounts of

emulsified fatty acids (Sigma). Stock solutions of fatty acids were at a concentration of 50 mg/ml in 1:5 tyloxapol/ethanol except for Figure 2A, where stock solutions were at 50 mg/ml in 1:1 tyloxapol/ethanol. Survival plating and tests for apoptotic and necrotic markers were performed 4 hr after stress.

Survival plating and test for apoptotic/necrotic markers. For survival plating, the cell concentrations of culture dilutions were determined with a CASY cell counter (Schaerfe Systems), and aliquots containing 500 cells were plated on YPD plates. The number of colonies formed was determined after 2 days at 28°C for *rho*⁺ or after 3 days for *rho*⁰ cells. AnnexinV/PI co-staining was performed as previously described.⁵⁵ 30,000 cells were evaluated using flow cytometry and analysed using BD FACSDiva software.

For dihydroethidium staining, 5 x 10⁶ cells were harvested by centrifugation, resuspended in 250 μl of 2.5 μg/ml DHE in PBS, and incubated in the dark for 5 min. Relative fluorescence units (RFU) were determined using a fluorescence reader (Tecan, GeniusPRO) or positive cells were counted using flow cytometry. The same samples were analysed by fluorescence microscopy on a Zeiss Axioskop microscope equipped with a rhodamine filter set.

The translocation of the yeast HMGB1 homologue (Nhp6Ap) from the nucleus to the cytosol was monitored by epifluorescence microscopy of the ectopically expressed chimeric fusion protein, Nhp6Ap-EGFP.²⁷ Therefore, yeast QKO and QKO *rho*⁰ cells transformed with pUG35/NHP6A were grown on SCD lacking uracil. Cultures were inoculated from an overnight culture to an OD₆₀₀ of 0.1 and grown to an OD₆₀₀ of 0.35, then stressed with FA and subjected to epifluorescence microscopy with the use of small-band EGFP filter (Zeiss) on a Zeiss Axioskop microscope to monitor intracellular localization of Nhp6Ap-EGFP. It should be noted that the expression of Nhp6Ap-EGFP appears to be enhanced through stress inflicted by FFAs, since the total green fluorescence signal was stronger in LA and L3A treated cells compared to controls. We, therefore, reduced the tonal value adjustment of the images of LA and L3A treated cells in order to be comparable to the wt-control and OA-treated cells.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/RockenfellerCC9-14-sup.pdf

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