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Original Contribution

Small amounts of isotope-reinforced polyunsaturated fatty acids suppress lipid autoxidation

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ABSTRACT

Polyunsaturated fatty acids (PUFAs) undergo autoxidation and generate reactive carbonyl compounds that are toxic to cells and associated with apoptotic cell death, age-related neurodegenerative diseases, and atherosclerosis. PUFA autoxidation is initiated by the abstraction of bis-allylic hydrogen atoms. Replacement of the bis-allylic hydrogen atoms with deuterium atoms (termed site-specific isotope-reinforcement) arrests PUFA autoxidation due to the isotope effect. Kinetic competition experiments show that the kinetic isotope effect for the propagation rate constant of Lin autoxidation compared to that of 11,11-D_2-Lin is 12.8 ± 0.6. We investigate the effects of different isotope-reinforced PUFAs and natural PUFAs on the viability of coenzyme Q-deficient *Saccharomyces cerevisiae coq* mutants and wild-type yeast subjected to copper stress. Cells treated with a C11-BODIPY fluorescent probe to monitor lipid oxidation products show that lipid peroxidation precedes the loss of viability due to H-PUFA toxicity. We show that replacement of just one bis-allylic hydrogen atom with deuterium is sufficient to arrest lipid autoxidation. In contrast, PUFAs reinforced with two deuterium atoms at mono-allylic sites remain susceptible to autoxidation. Surprisingly, yeast treated with a mixture of approximately 20%:80% isotope-reinforced D-PUFA:natural H-PUFA are protected from lipid autoxidation-mediated cell killing. The findings reported here show that inclusion of only a small fraction of PUFAs deuterated at the bis-allylic sites is sufficient to profoundly inhibit the chain reaction of nondeuterated PUFAs in yeast.

Introduction

PUFAs are essential nutrients and are avidly taken up by cells [1]. As components of phospholipids, PUFAs are key building blocks of membrane bilayers; they facilitate the assembly and stability of protein complexes, and play major roles in cellular metabolism [2]. PUFAs can be metabolized into several major classes of hormones by oxidative enzymes, including cyclooxygenases, lipoxygenases, and epoxygenases, generating prostaglandins, hydroxyl-fatty acids, leukotrienes, and other mediators collectively known as eicosanoids [3,4].

PUFAs comprise the most vulnerable components of cells and are highly susceptible to nonenzymatic oxidation by reactive oxygen species (ROS) [5,6]. ROS initiate the free radical chain reaction of PUFA autoxidation, resulting in changes in membrane permeability and fluidity due to accumulation of lipid peroxides

Abbreviations: αLnn, α-linolenic acid (C18:3, *n*–3); ARA, arachidonic acid (C20:4, *n*–6); BHT, butylated hydroxytoluene; D, deuterium; D_2-Lin, 11,11-D_2-linoleic acid or ethyl ester; D_4-αLnn, 11,11,14,14-D_4-α-linolenic acid; EPA, eicosapentaenoic acid (C22:5, *n*–3); EtOAc, ethylacetate; GC–MS, gas chromatography–mass spectrometry; HODE, hydroxyoctadecadienoic acid or hydroxyoctadecadienoate; IE, isotope effect; KIE, kinetic isotope effect; KODE, keto-octadecadienoic acid or ketooctadecadienoate; Lin, linoleic acid or ethyl ester (C18:2, *n*–6); MeOAMVN, 2,2′-azobis(4-methoxy-2-dimethylvaleronitrile); Ole, oleic acid (C18:1, *n*–9); PPh_3, triphenylphosphine; PUFA, polyunsaturated fatty acid; Q, coenzyme Q or ubiquinone; QH_2, coenzyme QH_2 or ubiquinol; ROS, reactive oxygen species; YPD, rich growth medium with dextrose.

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[7] and *cis* to *trans* isomerization [8]. The lipid peroxides resulting from PUFAs autoxidation may play a role in DNA damage [9] and carcinogenesis [10]. Because of their ability to generate oxyradicals, lipid peroxides may initiate degenerative processes and promote disorders, including inflammation [11] and cancer [12]. A separate class of nonenzymatic lipid peroxidation products comprises arachidonic acid-derived isoprostanes, which play a role in cellular signaling [13], and PUFA-derived resolvins and protectins, which act as lipid mediators to resolve inflammation [14]. Oxidative damage to PUFAs also leads to a smorgasbord of reactive carbonyl electrophiles including products such as *trans*-4-hydroxy-2-nonenal, *trans,trans*-2,4-decadienal, malondialdehyde, crotonaldehyde, 4-hydroxyhexenal, acrolein, and others [15–17]. These carbonyl electrophiles cause harm by reacting with cellular components such as proteins and nucleic acids [16,18,19]. The majority of cellular electrophiles are generated from PUFAs by a peroxidation chain reaction that is readily triggered by ROS, but propagates without their further input. Thus, the formation of lipid-derived electrophiles such as *trans*-4-hydroxy-2-nonenal is relatively insensitive to the level of initiating ROS, but depends mainly on the availability of PUFAs and O_2. This is consistent with observations that life span is inversely correlated to membrane peroxidizability [20].

We have demonstrated that PUFAs harboring deuterium atoms at the bis-allylic sites are much more resistant to autoxidation reactions [21]. This is due to the isotope effect, whereby abstraction of the bis-allylic H atom, the rate-limiting step of PUFA autoxidation, is substantially slowed down by the presence of the D atoms at the bis-allylic site. Isotope-reinforced PUFAs were shown to protect coenzyme Q-deficient (*coq*) mutants of *Saccharomyces cerevisiae* and heat-stressed wild-type yeast against the toxic effects of lipid autoxidation products [21]. Isotope-reinforced PUFAs are not diluted by endogenous PUFAs in yeast, because yeast synthesize only saturated and monounsaturated fatty acids and do not require PUFAs as essential nutrients [22]. Thus, PUFAs content can be readily manipulated, and isotope-reinforced PUFAs can provide the sole source of PUFAs in the yeast cell.

However, PUFAs are essential components of animal cells, and the total replacement of essential PUFAs in animals with isotope-reinforced PUFAs is a daunting prospect. We report herein the kinetic isotope effect of autoxidation of 11,11-D_2-Lin in solution. Inclusion of only a small fraction of PUFAs deuterated at the bis-allylic sites is sufficient to profoundly inhibit the chain reaction in nondeuterated PUFAs in yeast. The exogenously added D-PUFAs slow detrimental lipid autoxidation within live yeast cells, and are effective even when present at low ratios in cell lipids. The results suggest that it may be practical to ameliorate ROS-initiated PUFA damage with the isotope-reinforcement approach.

Experimental procedures

Fatty acids

The fatty acids used in this study are shown in Fig. 1. Ole, Lin, and αLnn (99% pure) were from Sigma-Aldrich. ARA and EPA were from Matreya (Pleasant Gap, PA). The synthesis of 11,11-D_2-Lin and 11,11,14,14-D_4-αLnn was described previously [21]. The synthesis of 8,8-D_2-Lin, 11,11-D,H-Lin, and 11-^{13}C-Lin is described in Supplementary Material.

Radical clock and co-oxidation experiments

Determination of rate constants for peroxidation of Lin and D_2-Lin were performed as previously described [23,24]. PUFAs

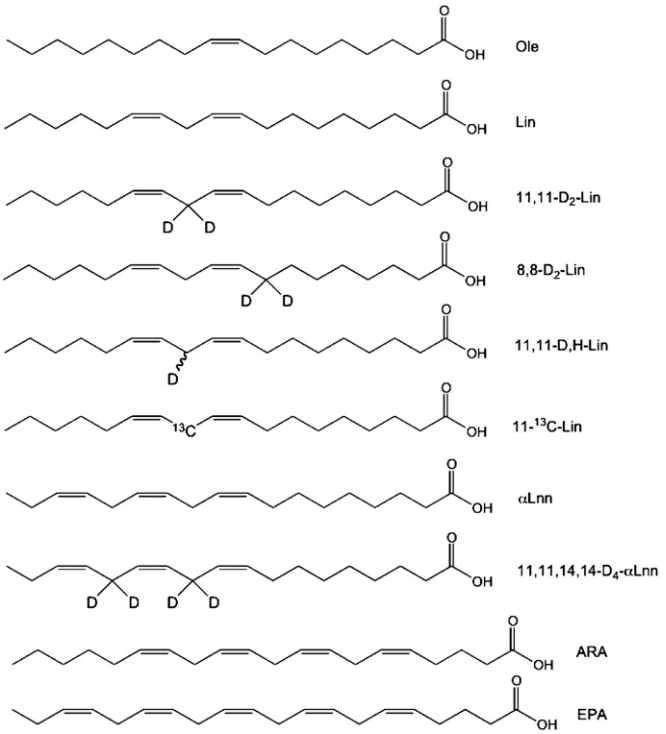

Fig. 1. Structures of fatty acids used in this study. Ole, oleic acid (18:1, *cis*-9-octadecenoic acid); Lin, linoleic acid (18:2, *cis,cis*-9,12-octadecenoic acid); 11,11-D_2-Lin (11,11-D_2-18:2; 11,11-D_2-*cis,cis*-9,12-octadecenoic acid); 8,8-D_2-Lin (8,8-D_2-18:2; 8,8-D_2-*cis,cis*-9,12-octadecenoic acid); 11,11-D,H-Lin (11,11-D,H-18:2; 11,11-D,H-*cis,cis*-9,12-octadecenoic acid); 11-^{13}C-Lin (11-^{13}C-18:2; 11-^{13}C-*cis,cis*-9,12-octadecenoic acid); αLnn, linolenic acid (18:3, *cis,cis,cis*-9,12,15-octadecenoic acid); 11,11,14,14-D_4-αLnn (D_4-18:3; 11,11,14,14-D_4-*cis,cis,cis*-9,12,15-octadecenoic acid); ARA, arachidonic acid; EPA, eicosapentaenoic acid.

were purified by flash column chromatography (10% EtOAc in hexanes to 20% EtOAc in hexanes) and dried overnight in vacuum. A stock solution of 0.1 M 2,2′-azobis(4-methoxy-2,4-dimethyl)-valeronitrile (MeOAMVN) in benzene was used to initiate all reactions. Standards used in analysis were 4-methoxybenzyl alcohol (HPLC-UV) and D_4-13-*trans,cis*-HODE (HPLC-MS). In all clocking or competition experiments, reagents were added in the order of: (1) benzene, (2) Lin/11,11-D_2-Lin (ethyl esters or free acids), (3) MeOAMVN. Reaction vials were vortexed for 5 s, and then heated at 37 °C for 1 h. Each reaction was quenched by the addition of 25 μL of both 0.5 M BHT (to quench radicals) and 0.5 M PPh_3 (to reduce hydroperoxide to alcohol). All experiments were carried out in triplicates.

In clocking experiments, Lin or 11,11-D_2-Lin ethyl esters were used and the oxidation products, HODEs, were analyzed by normal phase HPLC-UV (250 × 4.6 mm silica column; 5 μm; elution solvent, 0.5% 2-propanol in hexanes; monitoring wavelength, 234 nm). The residual amount of 11-D_1-Lin and D_0-Lin in the 11,11-D_2-Lin starting material was determined to be 2.9 and 0.8 mol%, respectively, from ^{1}H NMR analysis and these values were used to correct the data from 11,11-D_2-Lin assuming that 11-D_1-Lin is half as reactive as D_0-Lin.

In competition experiments, the total amount of Lin free acids (Lin + 11,11-D2-Lin) in each experiment was held constant at 0.64 M. After the reaction was quenched by addition of BHT and PPh_3 (vide supra), 4-methoxybenzyl alcohol was added as an internal standard for HPLC-UV analysis and each reaction was divided into two parts. One part was analyzed by HPLC-UV for quantification of total HODEs formed in each reaction (250 × 4.6 mm silica column; 5 μm; elution solvent, 1.4% 2-propanol and 0.1% acetic acid in hexanes; monitoring wavelength, 234 nm). The other part