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Spin-labelled lutein as a new antioxidant in protection against lipid peroxidation

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Abstract
A new potentially antioxidant compound, spin-labelled lutein (SL-lut), was synthesized and incorporated into egg yolk phosphatidylcholine (EYPC) liposome membrane. The approximate location of nitroxide free radical groups of SL-lut was determined based on electron paramagnetic resonance (EPR) spectra. Then the ability of SL-lut to protect EYPC liposomes against lipid peroxidation (LPO) was compared to the antioxidant effects of lutein and a nitroxide spin label 3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxy (3-CP). Two free radical generation systems were used—a thermal decomposition of 2,2'-azobis (2,4 dimethyl-valeronitrile) (AMVN) and a modified Fenton reaction using ferric-8-hydroxyquinoline ($Fe(HQ)_3$). Determination of the amount of thiobarbituric acid reactive species (TBARS) was used as a measure of LPO. SL-lut was the most powerful antioxidant, reducing LPO by about 6-times in AMVN-treated liposomes and 7-times in $Fe(HQ)_3$-treated liposomes. Lutein alone gave only a moderate protection in both systems, while 3-CP was as efficient as SL-lut in the presence of AMVN, but not efficient whatsoever in the presence of $Fe(HQ)_3$. The results suggest that a nitroxide part of SL-lut plays an important role in enhancing the antioxidant activity of lutein and makes SL-lut a powerful antioxidant efficient under different conditions.

Keywords: *Carotenoids, lutein, nitroxide, antioxidant activity, alkyl radicals, peroxyl radicals*

Introduction

There is a growing amount of evidence that carotenoids, which are natural pigments present in many fruits and vegetables, may serve the antioxidant function *in vivo*. Although their antioxidant action results mainly from their ability to quench singlet oxygen [1–4], they are also able to scavenge a wide range of free radicals [5,6]. In model studies, carotenoids have been shown to react with free radicals in organic solvents [7], as well as in liposomes, where they protected lipids against peroxidation [4,8–10]. It has been suggested that the chemical structure, especially the number of conjugated double bonds and the presence of polar hydroxyl groups at the ends of a carotenoid molecule, affects the reactivity of carotenoids with radicals [7,8]. In membranes, however, the chemical reactivity of a carotenoid does not seem to be the only factor determining its ability to protect lipids against peroxidation. There are reports showing that the position and orientation of a carotenoid molecule in the bilayer are important as well [8,10].

Lutein is one of the most widely distributed carotenoids found in frequently consumed fruits and vegetables. Interestingly, both lutein and zeaxanthin are found selectively at high concentrations in the centre of the human retina, at the macula lutea, where they are believed to protect photoreceptors

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against oxidative damage [11,12]. Studies on models of photoreceptor outer segment membranes showed that both carotenoids were preferentially located in membrane domains enriched in unsaturated lipids [13,14]. Such a selective carotenoid accumulation in the environment susceptible to peroxidation may be helpful in their antioxidant action. Other model studies reported that the orientation of zeaxanthin and lutein in the membrane was different. Zeaxanthin was shown to span a lipid bilayer with its two polar groups (β-ionone rings) interacting with opposite hydrophilic surfaces of the membrane [15], while for lutein two orientations in the membrane were suggested. Sujak et al. [10] reported the presence of two fractions of lutein in the membrane, one oriented like zeaxanthin, i.e. perpendicular to the membrane surface, and the other oriented parallel with β-ionone rings placed at the same side of the membrane.

Since lipid peroxidation (LPO) has been proven to be a main process that damages the integrity of lipid membranes leading to many disorders, a lot of effort has been invested in the search for effective synthetic antioxidants. Among others, non-toxic nitroxides, which are stable free radicals widely used as spin labels in electron paramagnetic resonance (EPR) spectroscopy, have been identified as novel antioxidants protecting isolated macromolecules, cells, organs and whole animals from diverse insults [16–19]. These low molecular weight compounds can be present in both lipophilic and hydrophilic compartments, they can also react with a wide range of reactive species and protect against the damage caused by the oxidative chain reaction [19].

In our study, we have compared the protective effects against lipid peroxidation of lutein, a naturally occurring carotenoid, and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxy (3-CP), a synthetic nitroxide spin label, with the effect of spin-labelled lutein (SL-lut), a compound synthesized by us. We were mostly interested in determining whether SL-lut would be a more effective antioxidant than lutein and 3-CP and, if so, what would be the main mechanism for its increased efficiency. On the one hand, we expected that antioxidant abilities of nitroxides and carotenoids would add in a combined molecule of SL-lut. On the other hand, SL-lut, which is longer compared to lutein and has polar nitroxide moieties on both ends of its molecule, should adopt only one perpendicular orientation with respect to the membrane surface and therefore should be able to react with free radicals at all depths in the membrane.

Materials and methods

Materials

Egg-yolk phosphatidylcholine (EYPC, type XI-E, in chloroform solution), $FeCl_3$ and 8-hydroxyquinoline were purchased from Sigma Chemical Co. (St. Louis, MO), thiobarbituric acid (TBA), trichloracetic acid (TCA) and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxy (3-CP) from Sigma Aldrich (Germany) and 2,2'-azobis (2,4 dimethyl-valeronitrile) (AMVN) from Polysciences, Inc. (Warrington, PA). 16-DOXYL-stearic acid spin label (16-SASL) and cholestane spin label (CSL) were purchased from Molecular Probes (Eugene, OR). Lutein was a generous gift from Kemin Industries, Inc. (IA) and 4-(*N*-2-hydroxyethyl-*N*,*N*-dimethyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl-phosphatidic acid ester spin label (Tempo-PC) was a gift from Prof. San-Ichy Ohnishi from Kyoto University (Japan).

Spin labelling of lutein

One gram of lutein was added to 40 ml of dry benzene and stirred under argon for 30 min. The solution was filtered on a glass filter and benzene was removed under reduced pressure to leave 620 mg of semi-solid brown material. TLC analysis (Silufol, UV-254, eluent chloroform–methanol 100:1) showed that the material consisted mainly of one dark brown compound. The material was dissolved in benzene (4 ml) under argon and a solution of 3-isocyanato-2,2,5,5-tetramethyl-2,5-dihydropyrrol-1-yloxy [20] (500 mg, 2.7 mmol) in benzene (3 ml) was added upon stirring. The resulting solution was allowed to stand under argon at 25°C for 48 h. The TLC control showed the consequent formation of two dark brown products with lower R_f. The compound with the lowest R_f was the final and main product after 48 h. The reaction mixture was poured on a chromatography column filled with silica gel (Kieselgel 60, Merck) and eluted with chloroform. The compound left a strong green trace on silica gel upon elution, however ~150 mg of solid dark brown material was collected. The TLC analysis (Silufol, UV-254, eluent: chloroform–methanol 100:1) showed that the material consisted of a single compound. The EPR spectrum of the sample in methanol showed a broadened triplet, with no trace of biradical features (i.e. the nitroxide moieties are far away from each other). The spectrum was isotropic, with the hyperfine splitting constant $A_{iso} = 15.4$ G. The IR spectrum, obtained with the Bruker Vector 22 FT-IR spectrometer in KBr pellets (the concentration 0.25%; the pellet thickness 1 mm), ν_{max} (KBr)/cm^{-1}: 3312, 3076, 2976, 2929, 2855, 1736, 1660, 1547, 1515, 1465, 1433, 1371, 1361, 1327, 1241, 1218, 1162, 1050, 1024, 971, 819. The element analysis data: Found: C, 66.96; H, 8.55; N, 6.23. Calc. for $C_{58}H_{82}N_4O_6 \times CHCl_3$: C, 67.45; H, 7.96; N, 5.33.

The idea of synthesizing a spin-labelled lutein came from Dr Witold K. Subczynski and thanks to his effort that compound was obtained. To the best of our knowledge, this was the first spin-labelled carotenoid.

The molecular structures of lutein and SL-lut are shown in Figure 1.

Preparation of liposomes

Multilamellar liposomes made of EYPC, with or without 1 mol% lutein or SL-lut, were prepared by the following method [13,14,21]. Chloroform solutions of lipids, lutein and SL-lut were mixed to attain the desired compound concentrations, chloroform was then evaporated with a stream of nitrogen and the lipid film on the bottom of the test tube was thoroughly dried under reduced pressure (~ 0.1 mmHg) for 12 h. A buffer solution (0.1 M phosphate buffer, pH 7.0) was added to the dried film and vortexed vigorously. Then the liposome suspension was centrifuged (7000 rpm, 4°C). In case of preparation of SL-lut-containing unilamellar liposomes, discarding of the supernatant and resuspending of the pellet in the fresh buffer helped to remove the spin label not bound to the membrane. When desired, 160 μM 3-CP (in a buffer solution) was added to liposome samples. Unilamellar liposomes were prepared by extrusion of multilamellar liposomes according to MacDonald et al. [22]. The multilamellar liposome suspension was freeze–thawed several times and then extruded 15 or 17 times through LiposoFast extruder equipped with polycarbon filters of 100 nm diameter (both from Avestin, Inc., Ottawa, ON, Canada).

EPR measurements of spin labelled lutein

EPR measurements were performed using a Bruker ESP 300E spectrometer operating at the X-band and equipped with a temperature control unit. Suspension of multilamellar EYPC liposomes containing 1 mol% SL-lut or other spin labels was placed in a gas permeable capillary (i.d. 0.9 mm) made of the methylpentene polymer TPX [23] and located inside the EPR dewar insert in a resonant cavity of the spectrometer. The sample was thoroughly deoxygenated with nitrogen gas, which was also used for temperature control. EPR spectra were obtained at room temperature and at −130°C.

Free radical generation

Lipid peroxidation (LPO) in EYPC liposomes was initiated either by 5 μM ferric-8-hydroxyquinoline [$Fe(HQ)_3$] in the presence of 50 μM ascorbate at room temperature or with 15 mM AMVN at 50°C. $Fe(HQ)_3$ complex was used because of its lipophilic character and good penetration properties through the liposomal membrane. The complex was prepared according to the modified method described by Korytowski et al. [24]. Stock solution of 1 mM $Fe(HQ)_3$ was prepared by mixing 1 mM $FeCl_3$ in 4 mM HCl with 3 mM 8-hydroxyquinoline in 50% ethanol. LPO was initiated by $Fe(HQ)_3$ and ascorbate, which was used as a reducer of Fe^{3+} to Fe^{2+}. AMVN is a hydrophobic compound that undergoes thermal decomposition and can be used as a source of free radicals that are produced at the controlled rate [25]. Both ethanol solution of AMVN (final ethanol concentration in the sample 3%) and $Fe(HQ)_3$ complex were freshly prepared before each experiment. Liposomes without and with lutein, SL-lut or 3-CP were incubated for up to 75 min. EYPC concentration was 3 mg/ml (in case of $Fe(HQ)_3$) and 6 mg/ml (in case of AMVN). AMVN appeared to be less effective in free radical generation, so we increased the lipid concentration in order to improve the sensitivity of the method used for LPO determination. Samples were collected every 15 min and the reaction was stopped by freezing in liquid nitrogen.

Measurement of lipid peroxidation—TBARS

During thawing of liposome samples, equal volumes of 20% TCA with addition of 0.5 mM BHT (to prevent further oxidation) were added and samples were centrifuged. Thereafter, 200 μl of the supernatant was transferred to a microcentrifuge tube,

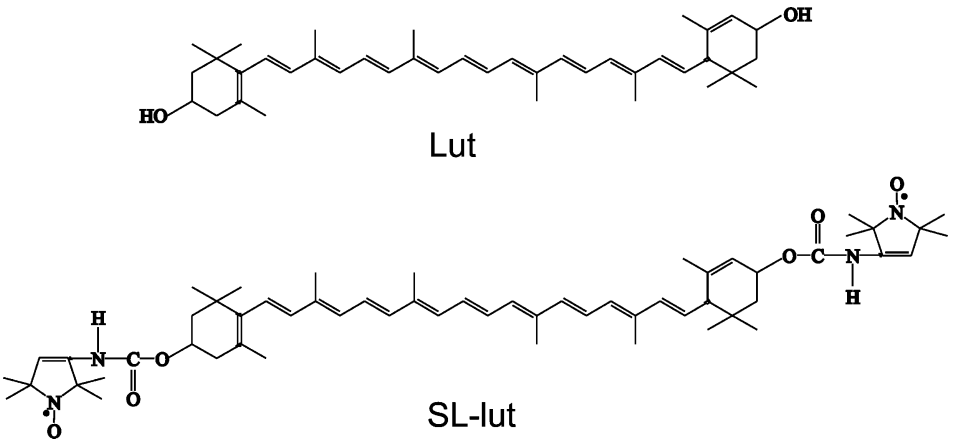

Figure 1. Molecular structures of lutein (lut) and spin-labelled lutein (SL-lut).

mixed with 200 µl of 0.5% TBA and boiled for 20 min. After cooling down the samples on ice, the absorbance at 532 nm was measured using a quartz cuvette (Hellma, 108-002B-QS, light path 10 mm, volume 500 µl) and a Hewlett Packard 8452A UV/VIS diode array spectrophotometer. The concentration of the TBA adduct with malondialdehyde (MDA) was determined based on a calibration curve for MDA standard prepared according to the method described by Suttnar et al. [26] and expressed as MDA equivalents in mmol per mol EYPC.

Results and discussion

Membrane localization of spin-labelled lutein

Different EPR spectral parameters of spin labels give information about the local environment of the nitroxide free radical moiety. To determine the localization of the free radical moiety of SL-lut in the membrane, we have chosen the A_z parameter (z component of the hyperfine interaction tensor), which in frozen systems depends on the local polarity of the environment [21,27,28]. $2A_z$ can be measured directly from EPR spectra, as illustrated in Figure 2A,

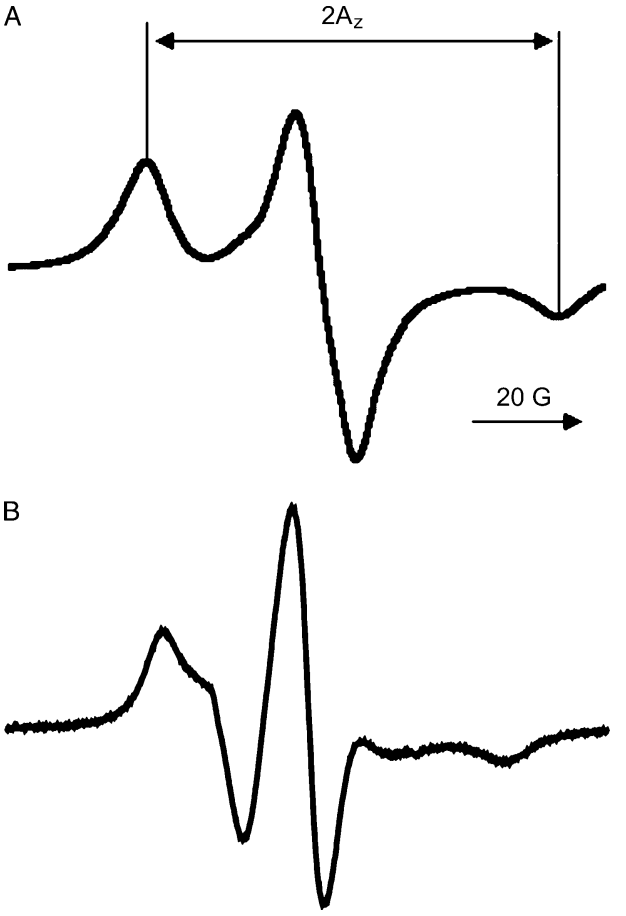

Figure 2. EPR spectra of SL-lut in EYPC liposomes, in a frozen suspension (at −130°C) (A) and at room temperature (B). The measured parameter $2A_z$ is shown.

which shows the EPR spectrum of SL-lut incorporated into the EYPC membrane recorded at −130°C. A_z increases with increasing polarity. The EPR spectrum of SL-lut in EYPC liposomes recorded at room temperature is also shown (Figure 2B) and its shape reflects a restricted motion characteristic for a membrane-bound spin label. Table I shows the $2A_z$ value for SL-lut in the EYPC membrane compared with the $2A_z$ values for the following spin labels: Tempo-PC (with the nitroxide moiety located in the polar headgroup region of the EYPC membrane), CSL (with the nitroxide moiety located very close to the polar headgroup region) and 16-SASL (with the nitroxide group attached to the 16^{th} carbon in the stearic acid chain, i.e. in the membrane centre). Comparison of these $2A_z$ values indicates that the nitroxide moieties of SL-lut are located in the polar headgroup region of the membrane. It has to be pointed out however that this is an approximate location only, because the A_z values of nitroxides are also slightly dependent on the overall structure of a spin label [29] and the spin labels which we used have different ring structures.

AMVN–induced MDA production

Figure 3 shows the effect of antioxidants (lutein, SL-lut and the nitroxide spin label 3-CP) on lipid peroxidation in EYPC liposomes incubated with 15 mM AMVN at 50°C. The amount of MDA produced in liposomes without antioxidants clearly increases with time of incubation (Figure 3A). In the presence of SL-lut and 3-CP the rate of MDA production is significantly slowed down. Lutein alone has only a minor effect on lipid peroxidation. Figure 3B summarizes the effect of all three compounds on MDA production after 30 min of liposomes incubation with AMVN. Lutein decreases the MDA amount by about 2.2-times compared to the control, while SL-lut and 3-CP are much more effective and reduce the MDA amount by about 6-times.

Our data on MDA production show that carotenoids, lutein and SL-lut protect EYPC liposomes against LPO induced by AMVN. Simultaneously, both lutein and SL-lut were clearly bleaching during the incubation of liposomes with AMVN (data not shown). According to Woodall et al. [8], in the case of AMVN–induced LPO, carotenoids react more rapidly with peroxyl radicals than do the unsaturated

Table I. $2A_z$ values (in gauss) of lipid spin labels incorporated into EYPC membranes, measured at −130°C.

Spin label	$2A_z$ [G]
SL-lutein	69.3
Tempo-PC	68.9
CSL	68.1
16-SASL	66.8

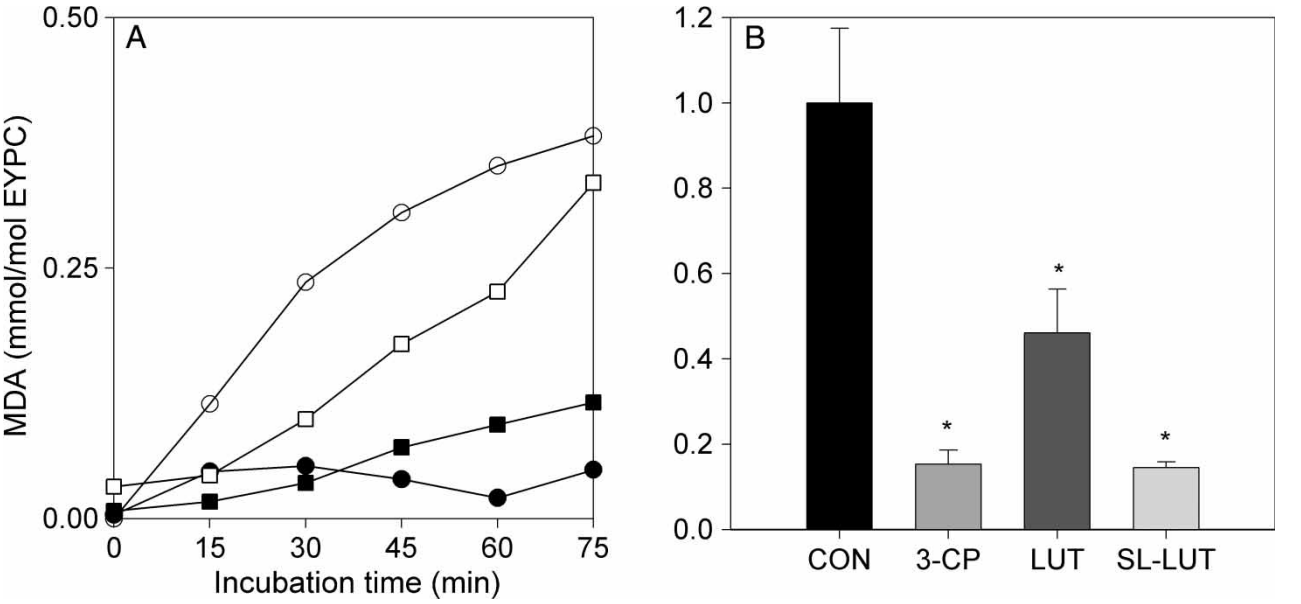

Figure 3. Kinetics of MDA production in EYPC liposomes incubated with 15 mM AMVN at 50°C (A) expressed as mmol MDA per mol EYPC, and histograms of normalized mean value ± SD of MDA accumulation after 30 min of incubation with AMVN of at least 4 repeats (B). Symbols: ○ control, ● 3-CP, □ lutein and ■ SL-lut–containing liposomes. *indicates statistically significant difference (the Student's t-test, $p \leq 0.05$) relative to control EYPC liposomes.

acyl chains of lipids. However, the effect of SL-lut was stronger than that of lutein, which can only be explained by the contribution of the nitroxide parts of the SL-lut molecule to the antioxidant process. It has previously been shown that nitroxides can undergo radical–radical reactions with free radicals deriving from azo-initiators. This takes place before azo-compounds initiate lipid peroxidation, therefore nitroxides contribute to the overall inhibition of LPO [19]. Our data show the strong protective effect of 3-CP alone against LPO in liposomes incubated with AMVN (Figure 3) and confirm that the nitroxide part of the molecule is partially responsible for the antioxidant effect of SL-lut.

It has to be pointed out that the two parts of the SL-lut molecule are located in two different environments: within the membrane (carotenoid polyene chain) and at the membrane surface, exposed to water (nitroxide moieties) and in our opinion react independently with free radicals. AMVN is lipophilic, therefore should be located within the EYPC membrane and produce free radicals in the lipid phase. At higher temperatures it decomposes homolytically giving carbon-centred radicals, which in turn produce peroxyl radicals when oxygen is present [25,30]. In our experiments, AMVN was added to the unilamellar liposome suspension as a concentrated ethanol solution and probably not all was incorporated into the membranes, some might have remained in solution or adsorbed at the membrane surface. We suspect that, after the thermal decomposition, alkyl radicals were generated from AMVN also in solution and at the membrane surface, where they might have been scavenged by free nitroxides (3-CP) or nitroxide moieties of SL-lut. This may be a valid mechanism since nitroxides were proven to react with alkyl radicals competitively to oxygen, giving a stable diamagnetic adduct (alkylated hydroxylamine) [31,32]. The rate constant for the reaction of most carbon-centred radicals with oxygen is almost diffusion-controlled (10^9 M^{-1} s^{-1}) and the reaction of most alkyl radicals with nitroxides is in the same order of magnitude [31,33]. Therefore, since the competition between nitroxide and oxygen is possible, we suggest that in our system nitroxides react effectively with alkyl radicals before peroxyl radicals are formed. In fact, nitroxides were found to efficiently trap carbon-centred radicals [31], whereas there is a disagreement about their ability to protect from peroxyl radicals. Damiani et al. [33] have shown a strong inhibition of oxygen consumption by nitroxides during peroxidation of linolenic acid micelles induced by 2,2′azobis(2-amidopropane)dihydrochloride (AAPH)—the azo-compound producing radicals in a way similar to AMVN but in polar solvents [30]. They attributed this effect to the reaction between the carbon-centred radicals generated from AAPH and nitroxides. Also, Damiani et al. [34] and Offer and Samuni [35] suggested that nitroxides protected DNA by scavenging carbon-centred radicals. Conversely, several reports concluded that nitroxides did not remove peroxyl radicals [32,34,36,37].

The strong protective effect of 3-CP can be attributed to the distribution of this spin label in three different environments: solution, membrane surface and membrane interior. In contrast to the

nitroxide moieties of SL-lut, which are anchored at the membrane surface, 3-CP can freely diffuse and is able to react with alkyl radicals produced in solution and in the membrane.

$Fe(HQ)_3$-induced MDA production

Figure 4 shows the results of the EYPC liposomes treatment with $Fe(HQ)_3$. Like in the case of AMVN-treated liposomes, the amount of MDA produced in this system in control liposomes increases clearly with time of incubation (Figure 4A). The final MDA amount measured after 75 min of liposomes incubation with $Fe(HQ)_3$ was higher than in the case of AMVN, even though the lipid concentration was twice lower. This suggests that in our model system $Fe(HQ)_3$ is a more effective free radical generator. The presence of both lutein and SL-lut slows down the rate of MDA production, however the effect of SL-lut is much stronger. Interestingly, practically no effect of 3-CP on MDA production was observed. Figure 4B summarizes the antioxidant effect of all three compounds after 30 min of liposomes incubation with $Fe(HQ)_3$. SL-lut is clearly the most effective antioxidant which decreases the amount of MDA by about 7-times compared to the control after 30 min of incubation. Lutein alone gives only moderate protection and reduces the amount of MDA by about 2.2-times. 3-CP does not exert any antioxidant effects in the presence of $Fe(HQ)_3$, whereas it is a very efficient inhibitor of lipid peroxidation in the system with AMVN.

$Fe(HQ)_3$ generates free radicals differently than AMVN. Unsaturated lipids are often contaminated with traces of lipid peroxides. After adding $Fe(HQ)_3$ into such a sample, lipid peroxides decompose giving alkoxyl and peroxyl radicals [25]. In our system, we used liposomes made of EYPC, which were slightly contaminated by lipid peroxides. The best antioxidant in this system was SL-lut, while, in contrast to the AMVN–induced LPO, the effect of 3-CP was very weak. This indicates that 3-CP does not react with peroxyl radicals, which would be in agreement with our previous suggestion (see the first part of Results and discussion section and [32,34, 36,37]). Therefore, the nitroxide parts of the SL-lut molecule are probably not involved here in direct free radical scavenging, but may play a structural role. We think that SL-lut adopts one orientation in the membrane, perpendicular to the surface, which incresases the efficiency of peroxyl radicals scavenging by the carotenoid polyene chain at different depths in the membrane.

Final conclusions

Our results show that spin-labelled lutein is the most effective antioxidant in both systems studied. The nitroxide parts of SL-lut play an important role in enhancing the antioxidant activity of a carotenoid molecule, either by adding the antioxidant properties of nitroxides, which effectively react with alkyl radicals (like in the case of AMVN-induced LPO) or by a better positioning of a carotenoid polyene chain, which effectively reacts with peroxyl radicals in the membrane (as observed in case of $Fe(HQ)_3$-induced LPO). Spin-labelled lutein may therefore act

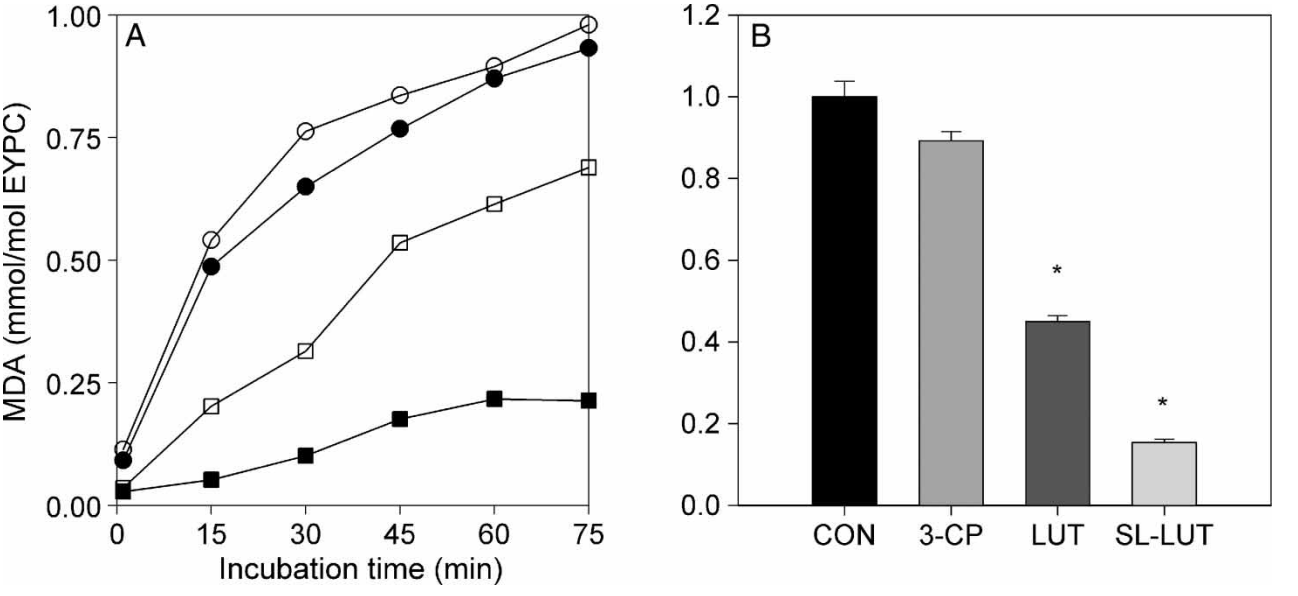

Figure 4. Kinetics of MDA production in EYPC liposomes incubated with 5 μM $Fe(HQ)_3$ complex at room temperature (A) expressed as mmol MDA per mol EYPC, and histograms of normalized mean value ± SD of MDA accumulation after 30 min of incubation with $Fe(HQ)_3$ of at least 4 repeats (B). Symbols: ○ control, ● 3-CP, □ lutein and ■ SL-lut–containing liposomes. *indicates statistically significant difference (the Student's *t*-test, $p \leq 0.05$) relative to control EYPC liposomes.

as a carotenoid, additionally better oriented in the membrane, reacting with peroxyl radicals formed within the membrane, and also as a nitroxide, scavenging alkyl radicals at the membrane surface. This makes it a powerful antioxidant efficient under different conditions.

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References

[1] Krinsky NI. Antioxidant functions of carotenoids. Free Radic Biol Med 1989;7:617–635.
[2] Conn PF, Schalch W, Truscott TG. The singlet oxygen and carotenoid interaction. J Photochem Photobiol B: Biol 1991;11:41–47.
[3] Cantrell A, McGarvey DJ, Truscott TG, Rancan F, Böhm F. Singlet oxygen quenching by dietary carotenoids in a model membrane environment. Arch Biochem Biophys 2003;412:47–54.
[4] Wrona M, Korytowski W, Rozanowska M, Sarna T, Truscott TG. Cooperation of antioxidants in protection against photosensitized oxidation. Free Radic Biol Med 2003;35:1319–1329.
[5] Liebler DC, McClure TD. Antioxidant reactions of β-carotene: identification of carotenoid-radical adducts. Chem Res Toxicol 1996;9:8–11.
[6] Mortensen A, Skibsted LH, Truscott TG. The interaction of dietary carotenoids with radical species. Arch Biochem Biophys 2001;385:13–19.
[7] Woodall AA, Lee SW-M, Weesie RJ, Jackson MJ, Britton G. Oxidation of carotenoids by free radicals: relationship between structure and reactivity. Biochim Biophys Acta 1997;1336:33–42.
[8] Woodall AA, Britton G, Jackson MJ. Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: relationship between carotenoid structure and protective ability. Biochim Biophys Acta 1997;1336:575–586.
[9] Stahl W, Junghans A, de Boer B, Driomina ES, Briviba K, Sies H. Carotenoid mixtures protect multilamellar liposomes against oxidative damage: synergistic effects of lycopene and lutein. FEBS Lett 1998;427:305–308.
[10] Sujak A, Gabrielska J, Grudzinski W, Borc R, Mazurek P, Gruszecki WI. Lutein and zeaxanthin as protectors of lipid membranes against oxidative damage: the structural aspects. Arch Biochem Biophys 1999;371:301–307.
[11] Kirschfeld K. Carotenoid pigments: their possible role in protecting against photooxidation in eyes and photoreceptor cells. Proc R Soc Lond B 1982;216:71–85.
[12] Rapp LM, Maple SS, Choi JH. Lutein and zeaxanthin concentrations in rod outer segment membranes from perifoveal and peripheral human retina. Invest Ophthalmol Vis Sci 2000;41:1200–1209.
[13] Wisniewska A, Subczynski WK. Accumulation of macular xanthophylls in unsaturated membrane domains. Free Radic Biol Med 2006;40:1820–1826.
[14] Wisniewska A, Subczynski WK. Distribution of macular xanthophylls between domains in a model of photoreceptor outer segment membranes. Free Radic Biol Med 2006;41:1257–1265.
[15] Gruszecki WI, Sielewiesiuk J. Orientation of xanthophylls in phosphatidylcholine multibilayer. Biochim Biophys Acta 1990;1023:405–412.
[16] Nilsson UA, Olsson LI, Carlin G, Bylund FA. Inhibition of lipid peroxidation by spin labels. Relationship between structure and function. J Biol Chem 1989;264:11131–11135.
[17] Miura Y, Utsumi H, Hamada A. Antioxidant activity of nitroxide radicals in lipid peroxidation of rat liver microsomes. Arch Biochem Biophys 1993;300:148–156.
[18] Mitchell JB, Xavier S, DeLuca AM, Sowers AL, Cook JA, Krishna MC, Hahn SM, Russo A. A low molecular weight antioxidant decreases weight and lowers tumor incidence. Free Radic Biol Med 2003;34:93–102.
[19] Samuni AM, Barenholz Y. Site-activity relationship of nitroxide radical's antioxidative effect. Free Radic Biol Med 2003;34:177–185.
[20] Hideg K, Hankovszky OH. Chemistry of spin-labeled amino acids and peptides: Some new mono- and bifunctionalized nitroxide free radicals. In: Berliner LJ, Reuben J, editors. Biological magnetic resonance. Vol. 8. Spin labelling. Theory and applications. New York; London: Plenum Press; 1989. p 427–488.
[21] Wisniewska A, Subczynski WK. Effect of polar carotenoids on the shape of the hydrophobic barrier of phospholipid bilayers. Biochim Biophys Acta 1998;1368:235–246.
[22] MacDonald RC, MacDonald RI, Menco BP, Takeshita K, Subbarao NK, Hu LR. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. Biochim Biophys Acta 1991;1061:297–303.
[23] Hyde JS, Subczynski WK. Spin-label oximetry. In: Berliner LJ, Reuben J, editors. Biological magnetic resonance. Vol. 8. Spin labelling. Theory and applications. New York; London: Plenum Press; 1989. p 399–425.
[24] Korytowski W, Wrona M, Girotti AW. Radiolabeled cholesterol as a reporter for assessing one-electron turnover of lipid hydroperoxides. Anal Biochem 1999;270:123–132.
[25] Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. New York: Oxford University Press; 1999.
[26] Suttnar J, Cermak J, Dyr JE. Solid-phase extraction in malondialdehyde analysis. Anal Biochem 1997;249:20–23.
[27] Griffith OH, Dehlinger PJ, Van SP. Shape of the hydrophobic barrier of phospholipids bilayers (evidence for water penetration into biological membranes). J Membr Biol 1974;15:159–192.
[28] Subczynski WK, Wisniewska A, Yin J-J, Hyde JS, Kusumi A. Hydrophobic barriers of lipid bilayer membranes formed by reduction of water penetration by alkyl chain unsaturation and cholesterol. Biochemistry 1994;33:7670–7681.
[29] Marsh D. Electron spin resonance: spin labels. In: Grell E, editor. Membrane spectroscopy. Molecular biology, biochemistry and biophysics. Vol. 31. Berlin: Springer-Verlag; 1981. p 51–142.
[30] Niki E. Free radical initiators as source of water- or lipid-soluble peroxyl radicals. Methods Enzymol 1990;186:100–108.
[31] Beckwith ALJ, Bowry VW, Ingold KU. Kinetics of nitroxide radical trapping. 1. Solvent effects. J Am Chem Soc 1992;114:4983–4992.
[32] Kocherginsky N, Swartz HM. Chemical reactivity of nitroxides. In: Kocherginsky N, Swartz HM, editors. Nitroxide spin labels. Reactions in biology and chemistry. Boca Raton; New York; London; Tokyo: CRC Press; 1995. p 25–65.
[33] Damiani E, Belaid C, Carloni P, Greci L. Comparison of antioxidant activity between aromatic indolinoic nitroxides and natural and synthetic antioxidants. Free Radic Res 2003;37:731–741.

[34] Damiani E, Kalinska B, Canapa A, Canestrari S, Wozniak M, Olmo E, Greci L. The effects of nitroxide radicals on oxidative DNA damage. Free Radic Biol Med 2000;28: 1257–1265.

[35] Offer T, Samuni A. Nitroxides inhibit peroxyl radical-mediated DNA scission and enzyme inactivation. Free Radic Biol Med 2002;32:872–881.

[36] Blough NV. Electron paramagnetic resonance measurements of photochemical radical production in humic substances. 1. Effects of O2 and charge on radical scavenging by nitroxides. Environ Sci Technol 1988;22:77–82.

[37] Damiani E, Castagna R, Astolfi P, Greci L. Aromatic and aliphatic mono- and bis-nitroxides: a study on their radical scavenging abilities. Free Radic Res 2005;39:325–336.