Biophysical Chemistry 137 (2008) 56–62

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH• techniques

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ARTICLE INFO

Article history:
Received 3 June 2008
Received in revised form 1 July 2008
Accepted 2 July 2008
Available online 6 July 2008

Keywords:
Conjugated linoleic acids
Linoleic acid
Lipid peroxidation
Chemiluminescence
DPPH assay

ABSTRACT

The chemiluminescent response of conjugated linoleic acid isomers (CLAs), linoleic acid (LA) and methyl linoleate (LAME) against the prooxidant *t*-butyl hydroperoxide (tBHP) was analyzed. The c9, t11-CLA and t10, c12-CLA isomers showed significant photoemission at the highest concentration used, while photoemission was not detected at any concentration of LA and LAME analyzed. These results show that CLAs are more susceptible to peroxidation than LA and LAME. Likewise, the effect of CLA, LA and LAME on lipid peroxidation of triglycerides rich in C20:5 ω3 and C22:6 ω3 (Tg ω3-PUFAs) was investigated. For that, chemiluminescence produced by triglycerides in the presence of tBHP, previously incubated with different concentrations of CLAs, LA and LAME (from 1 to 200 mM) was registered for 60 min. Triglycerides in the presence of *t*-BHP produced a peak of light emission (3151 ± 134 RLUs) 5 min after addition. CLAs produced significant inhibition on photoemission, t10, c12-CLA being more effective than the c9, t11-CLA isomer. LA and LAME did not have an effect on lipid peroxidation of Tg ω3-PUFAs.

CLA isomers, LA and LAME were also investigated for free radical scavenging properties against the stable radical (DPPH•). Both CLA isomers reacted and quenched DPPH• at all tested levels (from 5 to 25 mM), while LA and LAME did not show radical quenching activity even at the highest concentration tested. These data indicate that CLAs would provide protection against free radicals, but LA and LAME cannot.

1. Introduction

Conjugated linoleic acid (CLA) is a generic term used to describe a group of positional and geometric isomers of octadecadienoic acid [linoleic acid (LA), 18:2 ω-6]. In CLA, two double bonds are conjugated, whereas they are methylene-interrupted in LA [1]. The most abundant isomer of CLA in nature is the *cis*-9, *trans*-11 (c9, t11) CLA [2], intermediary product of the biohydrogenation of linoleic acid by the ruminal bacterium *Butyrivibrio fibrisolvens* [3]. It is found naturally in foods derived from ruminant animals. Diverse beneficial effects are attributed to it such as: metabolic regulator, hypocholesterolemic, antiatherogenic, anticarcinogenic, antioxidant, and also presents favorable effects in the prevention and treatment of certain nourishing allergies [4]. This isomer, found in the milky fat, has been recognized by the National Academy of Sciences, of the U.S.A., like the only fatty acid that has presented the property unequivocally to inhibit the carcinogenesis in experimental animals [5].

The role of CLAs in local oxidative status and stress has been investigated in a few models and biological systems because their influences on oxidative stress may explain some of the physiological responses [6]. Some authors showed that CLA had antioxidative activity and proposed this as a possible explanation for the anticarcinogenic and antiatherogenic effects. However, other investigations have found that CLA was a prooxidant. At the present the mechanism (s) involved in the biological actions of CLAs are still not well understood [7,8].

As far as we know, there is no information about the capacity of conjugated linoleic acid isomers and related compounds to act as chain-breaking during peroxidation of pure lipids enriched in ω3-PUFAs. Therefore, this study was undertaken to gain more insight into the mechanism of potential CLA antioxidant activity. It was tested whether CLAs could protect triglycerides rich in C20:5 ω3 and C22:6 ω3 from oxidative damage. The progress of oxidation was determined by direct photoemission capacity that is an indication of lipid peroxyl radical formation [9]. As a complement, the free radical scavenging

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doi:10.1016/j.bpc.2008.07.001

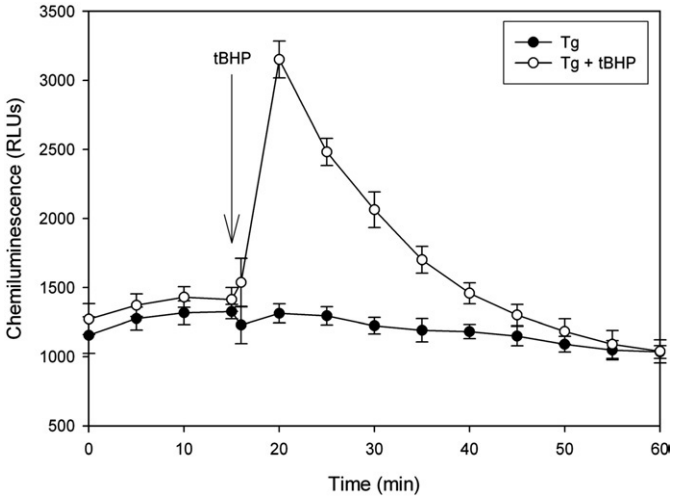

Fig. 1. Chemiluminescence as a function of time during *tert*-butyl hydroperoxide induced lipid peroxidation of triglycerides ω3-PUFAs (100 μl) in chloroform solution, final volume 1 ml, 37 °C. Without *t*-BHP ●–●, with *t*-BHP ○–○. Vertical bars represent the standard deviation of each data point.

activities of CLA isomers, LA and LAME were tested by their ability to bleach the stable radical 2, 2, diphenyl-1-picrylhydrazyl (DPPH•). In contrast to the complexity of much of the earlier work, the present *in vitro* assay eliminates the contributions of enzyme induction and activation, measuring only the direct antioxidant properties of the compounds tested.

2. Materials and methods

2.1. Materials

Commercial fish oil (triglycerides enriched in ω3-polyunsaturated fatty acids = Tg ω3-PUFAs) that was unstabilized, deodorized, refined, and bleached (eicosapentaenoic acid or EPA 20%; docosahexaenoic acid or DHA 40%) was donated by Winterization Europe Fécamp Cedex, France. Conjugated linoleic acid isomers: CLA c9, t11 and CLA t10, c12 90% — FFA were from Natural ASA, Lysaker, Norway. 2,2-Diphenyl-2-picrylhydrazyl (DPPH•) as free radical form (90% purity), linoleic acid free acid ≥99% (GC) and linoleic acid methyl ester ≥99% (GC) were from Sigma (St. Louis, MO, USA). All other chemicals were reagent grade or better and were obtained from Sigma-Aldrich Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

2.2. Measurements of lipid peroxidation of c9, t11 CLA, t10, c12 CLA, linoleic acid and its methyl ester by detection of photoemission

CLA isomers, LA and LAME (final concentrations: 1, 4, 10, 20, 100 and 200 mM of each) were incubated for 60 min in chloroform solution. Lipid peroxidation was started by the addition of 10 μl of methanol solution of *tert*-butyl hydroperoxide (final concentration: 36.4 mM). Reagents were introduced in eppendorf tubes placed into thermostatic (37 °C) camera. All the solutions were freshly prepared daily. In all cases, controls without *tert*-butyl hydroperoxide were assayed and these values were subtracted from the samples peroxidized. Lipid peroxidation was measured by monitoring low-level chemiluminescence that was recorded with a photon counter equipment-20/20n Luminometer (Turner Byosystems, Sunnyvale CA 94085 USA), spectral response range 350–650 nm. The intensity of the light emitted in a reaction was monitored as a function of time, $I=f(t)$. Relative luminic units (RLUs) were recorded every 5 min to establish the time course of lipid peroxidation. The areas under the chemiluminescence curves, taken between 15 and 45 min, were used to calculate the total chemiluminescence (see Figs. 2–5, A and B). All the procedures for determining chemiluminescence were performed in darkness.

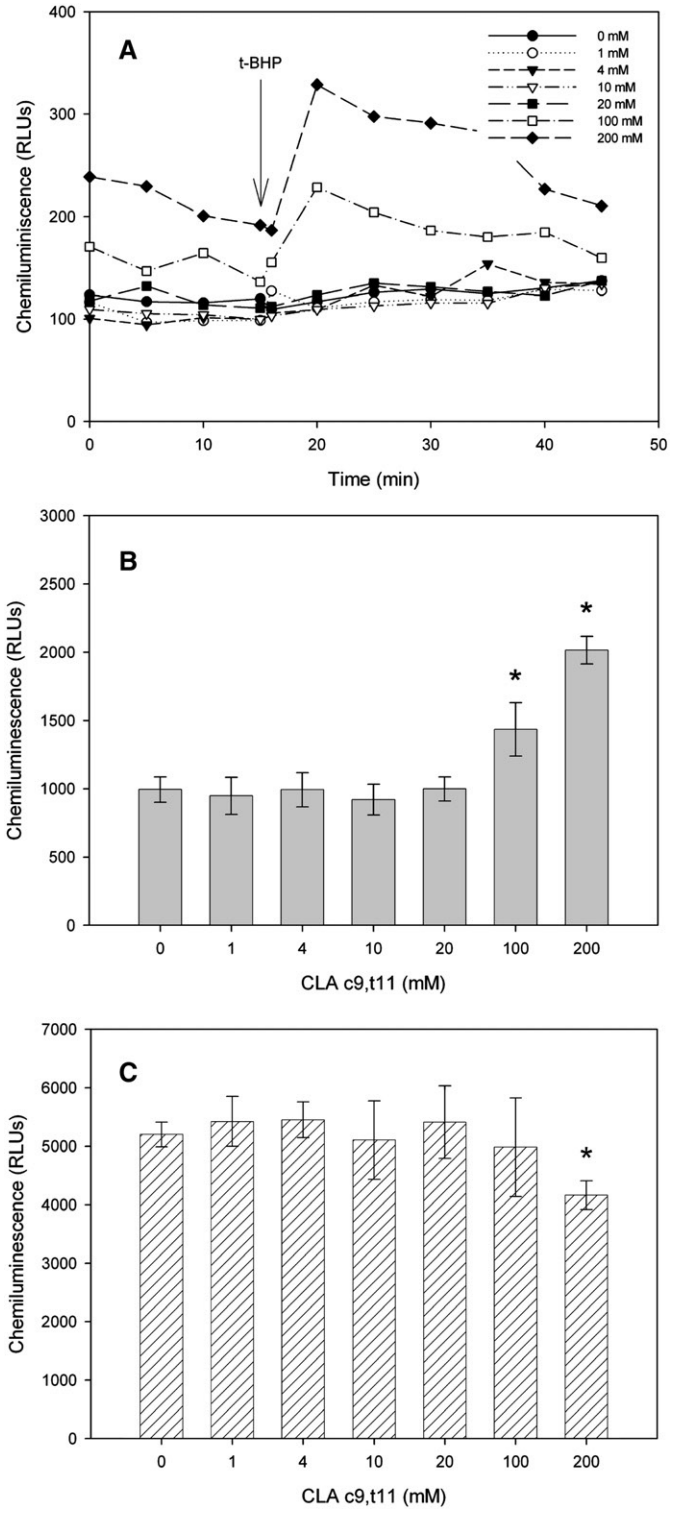

Fig. 2. A) Chemiluminescence as a function of time during *tert*-butyl hydroperoxide induced lipid peroxidation of c9, t11 CLA in chloroform solution, final volume 1 ml, measured at 37 °C. B) The areas under the chemiluminescence curves, taken between 15 and 45 min, were used to plot the total chemiluminescence as a function of c9, t11-CLA concentration. C) Effect of c9, t11-CLA concentration on lipid peroxidation of Tg ω3-PUFAs. An asterisk (*) at the top of a column indicates that a significant difference ($p<0.05$) was detected between control (0 mM) and that concentration. Vertical bars represent the standard deviation of at least four assays.

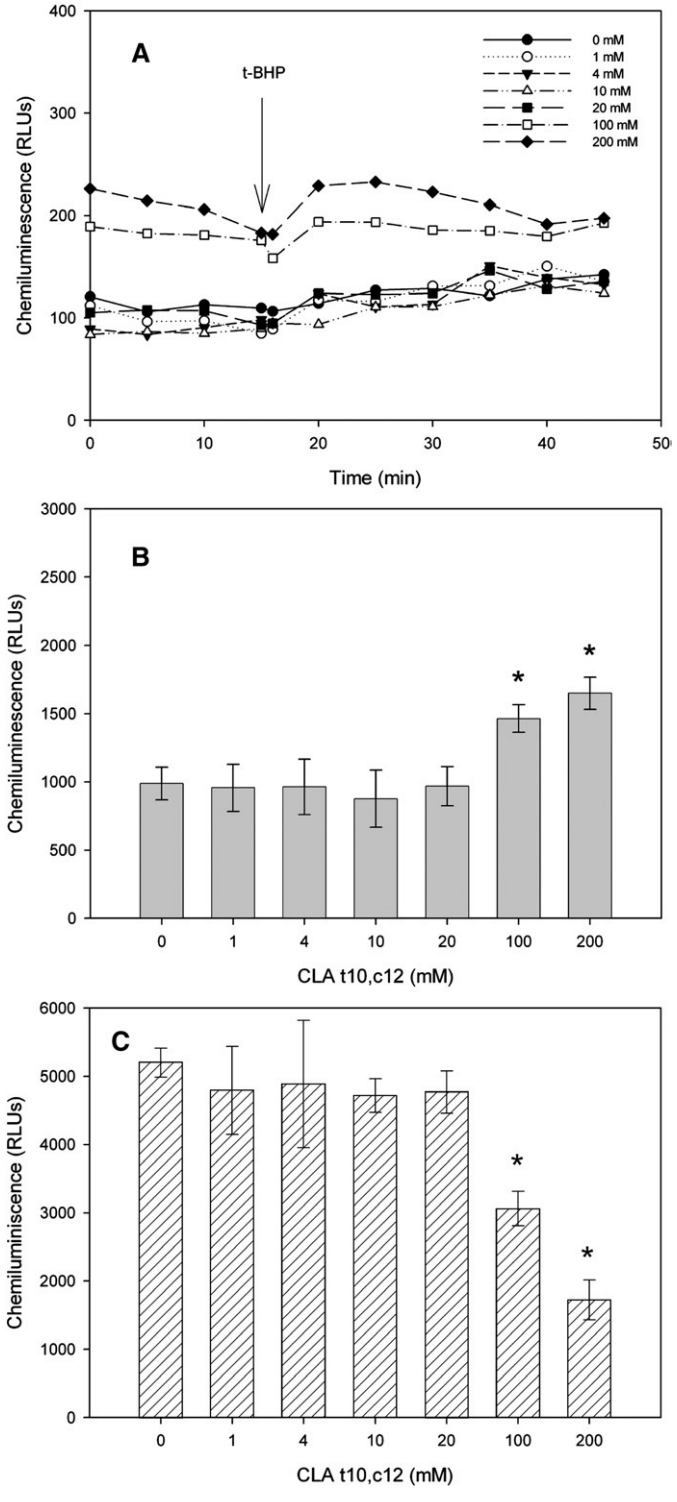

Fig. 3. A) Chemiluminescence as a function of time during *tert*-butyl hydroperoxide induced lipid peroxidation of t10, c12 CLA in chloroform solution, final volume 1 ml, measured at 37 °C. B) Total chemiluminescence (∑15–45 min) as a function of t10, c12-CLA concentration. C) Effect of t10, c12-CLA concentration on lipid peroxidation of Tg ω3-PUFAs. An asterisk (*) at the top of a column indicates that a significant difference ($p<0.05$) was detected between control (0 mM) and that concentration. Vertical bars represent the standard deviation of at least four assays.

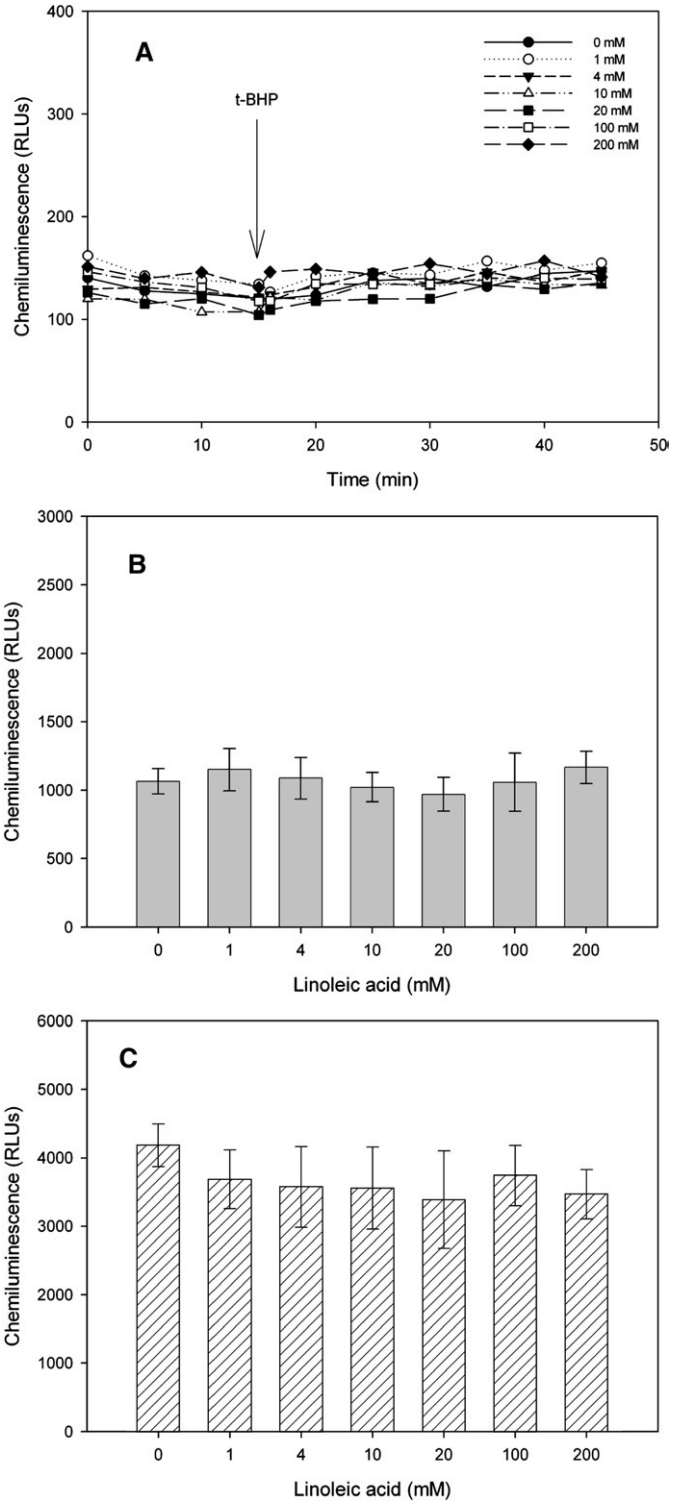

Fig. 4. A) Chemiluminescence as a function of time during *tert*-butyl hydroperoxide induced lipid peroxidation of linoleic acid (LA) in chloroform solution, final volume 1 ml, measured at 37 °C. B) Total chemiluminescence (∑15–45 min) as a function of LA concentration. C) Effect of LA concentration on lipid peroxidation of Tg ω3-PUFAs. Vertical bars represent the standard deviation of at least four assays.

2.3. Measurements of lipid peroxidation of triglycerides enriched in ω3-polyunsaturated fatty acids by detection of photoemission: effect of addition of CLAs, LA or LAME

100 μl of winterized fish oil (Tg ω3-PUFAs) were incubated in chloroform solution during 60 min. Lipid peroxidation was started by the addition of 10 μl of methanol solution of *tert*-butyl hydroperoxide (final concentration: 36.4 mM) (see Fig. 1). The same procedure was done with triglycerides previously incubated with increasing concentration (from 1 to 200 mM) of CLAs, linoleic acid or its methyl ester (dissolved in chloroform), final volume 1 ml. Reagents were introduced in eppendorf tubes placed into thermostatic (37 °C) camera. All the solutions were freshly prepared daily. In all the cases, controls without *tert*-butyl hydroperoxide were assayed, and these values were subtracted from the samples peroxidized. Lipid peroxidation was measured by monitoring low-level chemiluminescence as indicated above. The calculated total chemiluminescence was used to compare the inhibitory effect produced by different antioxidants (see Figs. 2C–5C).

2.4. Radical scavenging capacity assay of conjugated linoleic acid isomers, linoleic acid and its methyl ester

The free radical scavenging activities of CLA isomers, LA and LAME were tested by their ability to bleach the stable radical 2, 2, diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) [10]. The spectrophotometric technique employs DPPH, which shows a characteristic UV–VIS spectrum with a maximum of absorbance close to 518 nm in ethanol. The addition of an antioxidant, which can donate hydrogen, results in a decrease of absorbance proportional to the concentration and antioxidant activity of the compound itself [11]. This method presents the advantage of using a stable and commercially available free radical. The DPPH$^{\bullet}$ assay was run by the following procedure: 1 ml of ethanol solutions to six concentrations (final concentration from 5 to 25 mM) of the CLA isomers, LA or LAME was added to 1 ml of DPPH ethanol solution (final concentration: 31.5 μM). Absorbance of each reaction mixture at 518 nm was measured from 0 to 120 min, against an ethanol blank, using an Agilent Chem Station with diode-array UV–VIS spectrophotometer. The initial absorbance was close to 0.400 always.

The EC_{50} is the concentration of CLA required to quench 50% of the initial concentration of DPPH$^{\bullet}$ radical under experimental conditions [12]. The EC_{50} of CLAs was obtained plotting the percent DPPH$^{\bullet}$ remaining at steady state against the corresponding CLA concentration. The percentage of DPPH$^{\bullet}$ remaining at the steady state was determined as

$$\%\mathrm{DPPHrem} = (A_f/A_o) \times 100.$$

A_o and A_f correspond to the absorbance at 518 nm of the mixture at the beginning and at steady state, respectively. The percentage of the DPPH$^{\bullet}$ remaining is inversely proportional to the antioxidant activity.

2.5. Expression of results and statistical analysis

Data were reported as mean±SD, for at least three replications, except in Figs. 6A, B and 7, where there is only one measure to each point. Statistical analyses were conducted using a multiple range test based in the method of Tukey HSD ($p<0.05$) to identify statistically significant differences among means.

3. Results

3.1. Measurements of lipid peroxidation of c9, t11 CLA, t10, c12 CLA, linoleic acid and its methyl ester by detection of photoemission

The addition of *tert*-butyl hydroperoxide to different concentrations of c9, t11 CLA or t10, c12 CLA produced significant photoemissions at 100 and 200 mM for both CLA isomers (Figs. 2A, B, 3A and B). Photoemission observed at lower concentrations was not significant.

Linoleic acid and its methyl ester did not undergo measurable lipid peroxidation when incubated with *tert*-butyl hydroperoxide in chloroform solution at 37 °C (Figs. 4A, B, 5A and B).

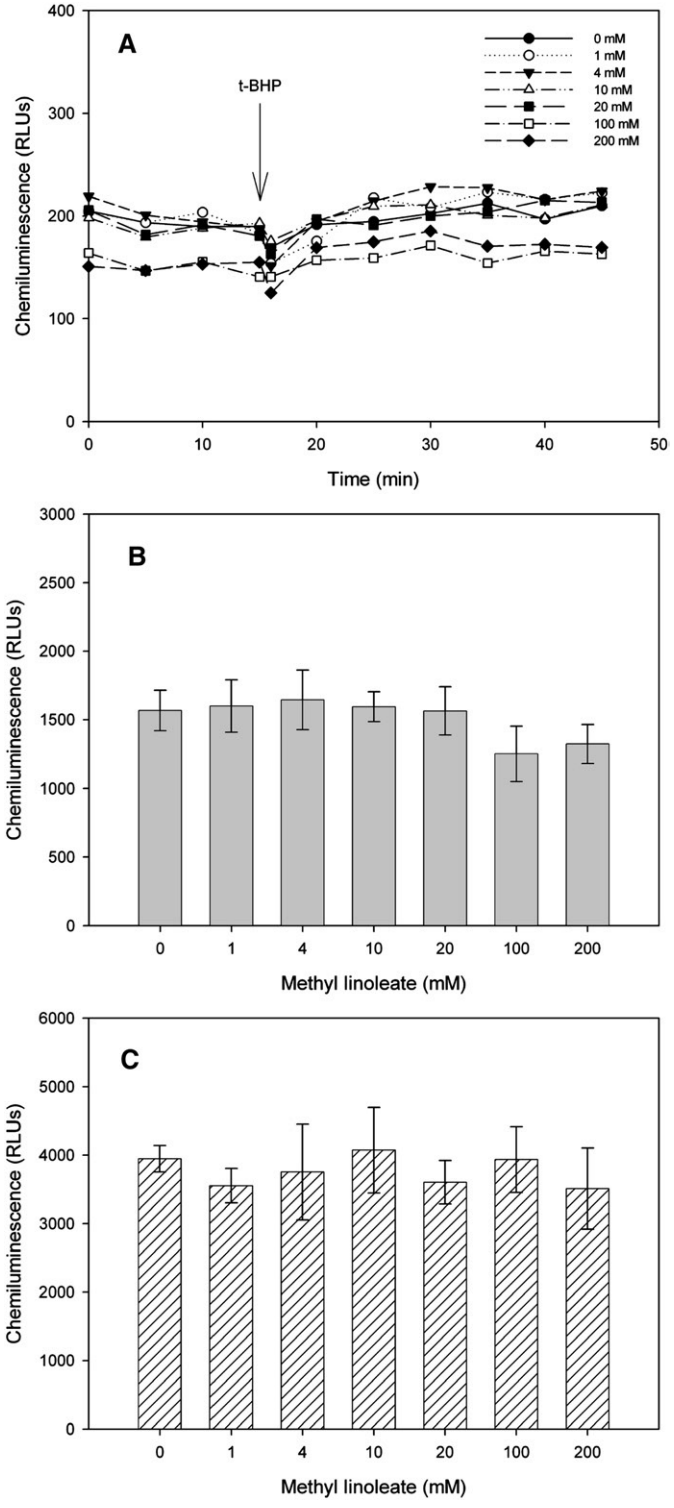

Fig. 5. A) Chemiluminescence as a function of time during *tert*-butyl hydroperoxide induced lipid peroxidation of linoleic acid methyl ester (LAME) in chloroform solution, final volume 1 ml, measured at 37 °C. B) Total chemiluminescence (∑15–45 min) as a function of LAME concentration. C) Effect of LAME concentration on lipid peroxidation of Tg ω3-PUFAs. Vertical bars represent the standard deviation of at least four assays.

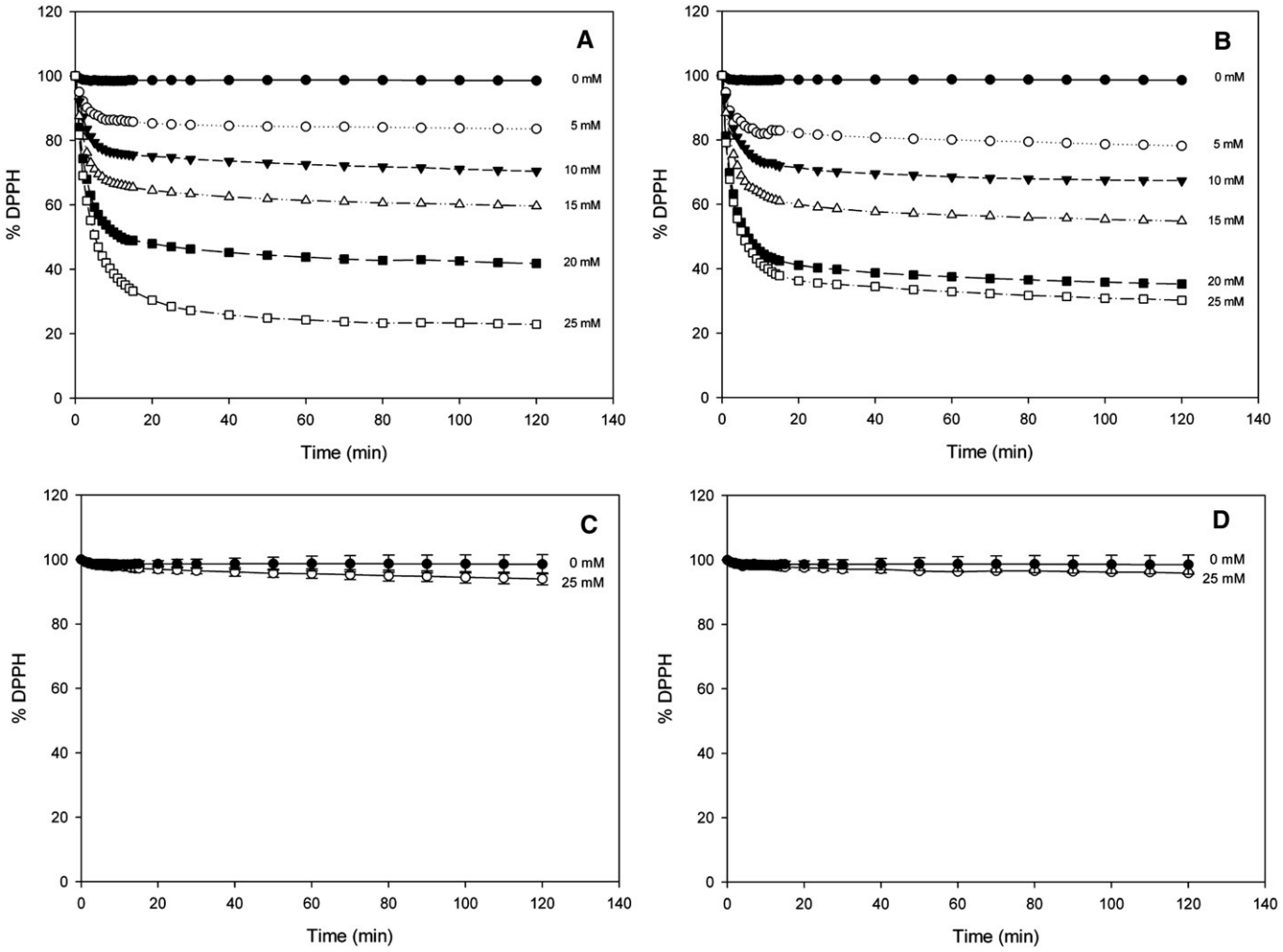

Fig. 6. A) Kinetic curves of CLA c9, t11-DPPH radical reaction. B) Kinetic curves of CLA t10, c12-DPPH radical reaction. C) Kinetic curve of LA-DPPH radical reaction. D) Kinetic curve of MLA-DPPH radical reaction. The final DPPH radical concentration was 31.5 μM in all reaction mixtures.

In graphs A (Figs. 2A–5A), the standard deviation of each point has not been represented for best visualization of results.

3.2. Measurements of lipid peroxidation of triglycerides enriched in ω3-polyunsaturated fatty acids by detection of photoemission: effect of addition of CLAs, LA or LAME

Triglycerides enriched in polyunsaturated fatty acids (Tg ω3-PUFAs) undergo lipid peroxidation when they are incubated with tBHP in chloroform solution at 37 °C. 5 min after the addition of tBHP, a maximum light emission (3151 ± 134 RLUs) was observed (Fig. 1).

Tg ω3-PUFAs were incubated in chloroform solution at 37 °C with different concentrations (ranging from 1 to 200 mM) of c9, t11 CLA, t10, c12 CLA, linoleic acid or its methyl ester. After 15 min, tBHP was added to start the reaction. Both CLAs isomers were effective in reducing chemiluminescence, t10, c12-CLA being slightly more efficient (effective at 100 and 200 mM) than the c9, t11-CLA isomer (effective at 200 mM) (Figs. 2C and 3C). Linoleic acid and its methyl ester did not have an effect on lipid peroxidation of triglycerides ω3-PUFAs (Figs. 4C and 5C).

3.3. Radical scavenging capacity assay of conjugated linoleic acid isomers, linoleic acid and its methyl ester

Both CLA isomers presented free radical scavenging activity in a concentration dependent manner (Fig. 6A and B). The concentrations assayed ranged from 5 mM to 25 mM. The percent of DPPH remaining at steady state was plotted against the corresponding CLA concentration to obtain the EC_{50}. The EC_{50} of CLA c9, t11 was 16.86 mM and for CLA t10, c12 was 16.38 mM.

There was no significant radical scavenging capacity detected for LA or LAME (Fig. 6C and D), even at the highest concentration used in this study (25 mM). For this reason, EC_{50} was not calculated.

4. Discussion

Pariza et al. [13] reported that grilled ground beef contained both bacterial mutagens and a substance that inhibited mutagenesis. Pariza et al. went on to identify the new anticarcinogen as conjugated linoleic acid, (CLA) [14,15]. Ha et al. reported that CLA is an effective antioxidant, more potent than α-tocopherol and almost as effective as butylated hydroxytoluene (BHT) [16]. Several researchers proposed that the antioxidant effect of CLA might be partly responsible for its anticarcinogenic property [16–18]. However, other investigators have shown that CLA functions more as a prooxidant. In this regard, Chen et al. [19] and Van den Berg et al. [20] showed that CLA does not act as an efficient radical scavenger in any way comparable to vitamin E or BHT.

CLA has been claimed to have many biological effects. The reported effects of CLA on lipid metabolism and body composition, and at least some of the effects of CLA on the immune system, are due to t10, c12 CLA. Ip et al. [18] have demonstrated that the c9, t11 and t10, c12 CLA

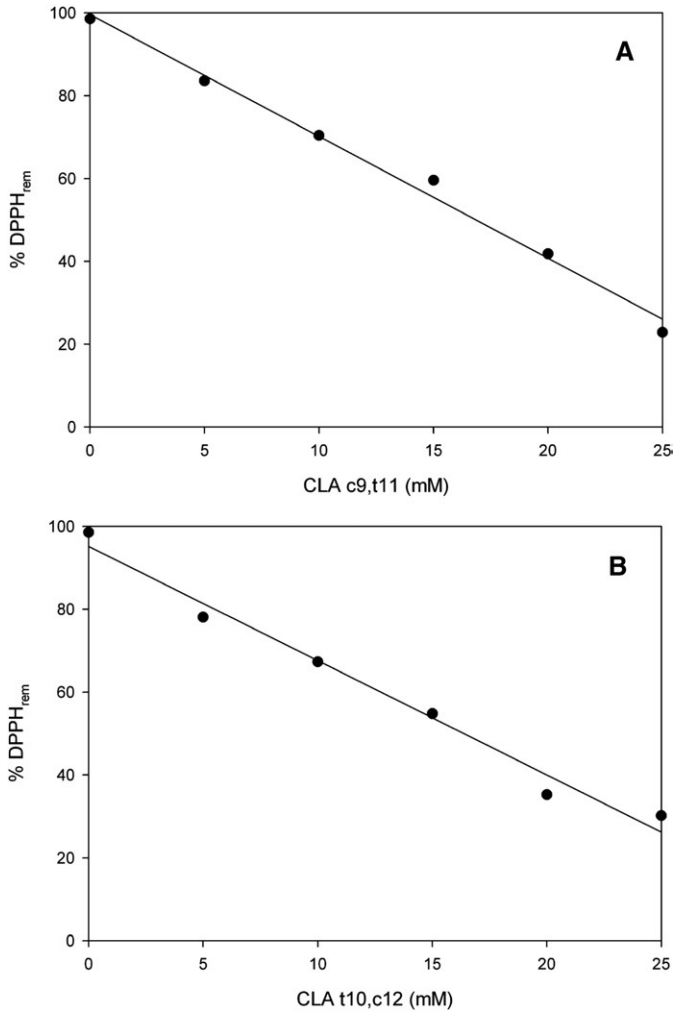

Fig. 7. A) % $DPPH_{rem}$ as a function of c9, t11-CLA concentration. The calculated EC_{50} value was 16.86 mM. B) % $DPPH_{rem}$ as a function of t10, c12-CLA concentration. The calculated EC_{50} value was 16.38 mM.

isomers may be equally effective in inhibiting carcinogenesis. Pariza et al. [21] obtained some degree of evidence indicating that c9, t11 CLA may be important in effecting the CLA induced growth enhancement in young rodents and further that t10, c12 CLA may interfere with the growth enhancement induced by the c9, t11 isomer. Different isomers appear to produce different effects although the biochemical mechanism/s still needs to be studied.

To better understand the beneficial effects of individual CLA isomers, it is important to investigate whether these isomers and related compounds differ in their reactions with free radicals.

Taken into account these considerations, this study was conducted to (i) address how c9, t11 CLA, t10, c12 CLA, linoleic acid or its methyl ester affect lipid peroxidation of triglycerides rich in C20:5 ω3 and C22:6 ω3 and (ii) to study the radical scavenging properties of these compounds against the stable DPPH free radical.

Non-enzymatic lipid peroxidation is a free radical-driven chain reaction in which one radical can induce the oxidation of a large number of lipid molecules (LH) containing polyunsaturated fatty acids (PUFA) [9,22]. Lipid hydroperoxide (LOOH) is the first, relatively stable product of the lipid peroxidation reaction [23]. Under conditions where lipid peroxidation is continuously initiated, a termination reaction limits the extent of this process, yielding non-radical products (NRP), and destroying two radicals at once:

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow NRP + \varphi h\nu(\text{chemiluminescence}).$$

This reaction is particularly interesting because it is accompanied by chemiluminescence whose intensity (I) may serve as a measure of lipoperoxide free radical ($LOO^{\bullet}$) concentration in accord to the following equation:

$$I = K\varphi k[LOO^{\bullet}]^2.$$

where φ represents the chemiluminescence quantum yield, K is the coefficient depending on the net sensitivity of the instrument and k the rate constant. $LOO^{\bullet}$ is a free radical produced from lipid molecules [24].

Chain-breaking antioxidants are able to react with lipid peroxyl radicals, eventually terminating the chain reaction:

$$LOO^{\bullet} + AntOH \rightarrow LOOH + AntO^{\bullet}.$$

This reaction between a lipidic peroxyradical ($LOO^{\bullet}$) and a molecule of antioxidant (AntOH) prevents the emission of light [25]. Thus, we have used the photoemission technique in order to measure the peroxidation process of specific lipids and the antioxidant activity of conjugated linoleic acid isomers and related compounds.

In our assay system the use of $DPPH^{\bullet}$ provides a precise way to evaluate the antiradical activities of antioxidants. The addition of an antioxidant, in our case both CLA isomers, which can donate hydrogen, produce a decrease of absorbance proportional to the concentration and antioxidant activity of the compound itself

$$DPPH^{\bullet} + CLA - H \rightarrow DPPH - H + CLA^{\bullet}$$

However linoleic acid or its methyl ester cannot donate hydrogen and a decrease of absorbance is not observed even at the highest concentration used (25 mM).

It has been previously demonstrated that CLA, with two conjugated double bonds, might be more vulnerable to autoxidation than linolenic acid with three methylene-interrupted double bonds [26]. This is because CLA can readily donate an electron or hydrogen to form a CLA free radical intermediate due to resonance delocalization.

In fact, these CLA free radicals have been shown to be rapidly decomposed to furan fatty acids. However, the oxidation systems used have to be taken into consideration when the oxidative stability of CLA is assessed [27]. Our results agree with those observations since both CLA isomers were more susceptible to oxidation than linoleic acid or its methyl ester in our *in vitro* system. The comparison between structural analogues revealed that both CLA isomers were more effective than linoleic acid or its methyl ester on preventing lipid peroxidation of triglycerides ω3-PUFAs. Furthermore with both CLA isomers, free radical scavenging activity against $DPPH^{\bullet}$ was detected in a concentration dependent manner, however, there was no significant radical scavenging capacity for linoleic acid or its methyl ester at the highest concentration tested, (25 mM). It will be necessary to identify the intermediaries and reaction products in order to elucidate the mechanism for CLA-radical reaction. Knowledge of this mechanism securely will confirm that the conjugated double bonds made important contributions to the radical scavenging capacity of CLA [28,29].

In conclusion, CLA isomers showed capacity to directly react and quench free radicals as measured by photoemission and spectrophotometric methods. CLA can provide immediate prevention against free radicals but LA or LAME cannot.

Additional studies are necessary to show free radical scavenging activity of CLAs, linoleic acid and its methyl ester in different radical systems and under physiological conditions, and to determine whether there is any link between their radical scavenging properties and their biological effects.

Acknowledgement

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), PICT 13399 granted to AC.

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