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Effect of conjugated linoleic acid on non enzymatic lipid peroxidation of mitochondria obtained from different rat tissues

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Abstract

Palacios, A.; Piergiacomi, V.A.: ***Effect of conjugated linoleic acid on non enzymatic lipid peroxidation of mitochondria obtained from different rat tissues.*** *Rev. vet. 16: 1, 3–8, 2005*.
The polyunsaturated fatty acid composition, chemiluminescence and peroxidizability index of mitochondria obtained from rat liver, kidney, lung and heart, were studied after oral administration of conjugated linoleic acid (CLA). After incubation of mitochondria in an ascorbate Fe^{++} system (120 min at 37°C), it was observed that the total counts per min/mg protein originated from light emission: chemiluminescence, was lower in liver and kidney mitochondria in the CLA group than in the control group. When peroxidized mitochondrias were compared to natives, in CLA and control groups it was observed that 18:2 n–6 decreased in all tissues used in this work. In the control group, it also decrease C20:4 n–6 and C22:6 n–6 in kidney and liver, while in lung it was affected C22:6 n–3. In CLA group, C20:4 n–6 decreased in liver and kidney, whereas in liver it also decreased C22:6 n–3. As a consequence, the peroxidizability index –a parameter based on the maximal rate of oxidation of fatty acids– showed significant changes in liver and kidney mitochondria. These changes were less pronounced in membranes derived from rats receiving CLA *per os*. Our results confirm and extend previous observations that indicated that CLA may act as an antioxidant, protecting membranes from deleterious effects.

Key words: conjugated linoleic acid, mitochondria, peroxidation, chemiluminescence.

Resumen

Palacios, A.; Piergiacomi, V.A.: ***Efecto del ácido linoleico conjugado en un sistema de peroxidación lipídica no enzimática de mitocondrias obtenidas de diferentes tejidos de ratas.*** *Rev. vet. 16: 1, 3–8, 2005*. La composición de ácidos grasos poliinsaturados, quimioluminiscencia y el índice de peroxidabilidad de las mitocondrias obtenidas de hígado, riñón, pulmón y corazón de ratas, fueron estudiados luego de la administración oral de ácido linoleico conjugado (ALC). Luego de la incubación de las mitocondrias en un sistema ascorbato–Fe^{++} (120 min a 37°C) se observó que el total de cuentas por minuto/mg de proteína originados por emisión lumínica: quimioluminiscencia fue menor en las mitocondrias de hígado y riñón del grupo ALC con respecto al grupo control. En las mitocondrias obtenidas del grupo control, los ácidos grasos más sensibles a la peroxidación fueron el ácido linoleico C18:2 n–6, el ácido araquidónico C20:4 n–6 y el ácido docosahexaenoico C22:6 n–3 en hígado y riñón. Cuando se compararon las mitocondrias peroxidadas con las nativas, se observó que tanto para el grupo control como para ALC el C18:2 n–6 fue afectado en todos los tejidos utilizados en este estudio. En el grupo control disminuyeron el C20:4 n–6 y C22:6 n–3 en hígado y riñón, mientras que en pulmón lo hizo el C22:6 n–3. En el grupo ALC disminuyó el C20:4 n–6 en hígado y riñón, además en hígado disminuyó el C22:6 n–3. Como una consecuencia del índice de peroxidabilidad –parámetro utilizado para medir la máxima capacidad de oxidación de los ácidos grasos– se hallaron cambios significativos en las mitocondrias obtenidas de hígado y riñón. Estos cambios fueron menos pronunciados en los animales que recibieron ALC por vía oral. Nuestros resultados concuerdan con observaciones previas en las que se menciona al ALC como un posible antioxidante que protege a las membranas.

Palabras clave: ácido linoleico conjugado, mitocondria, peroxidación, quimioluminiscencia.

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INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term generally referred to a mixture of positional and geometric conjugated dienoic isomers of linoleic acid [14]. Numerous CLA isomers are found in milk–fat, cheese and beef [26,,32]. The cis–9, trans–11 isomer of CLA (the principal dietary form), is produced in the rumen of cattle and other ruminants during the microbial biohydrogenation of linoleic and linolenic acids [13, 31]. CLA is an *in vitro* antioxidant, and in the cells it protects membranes from oxidative attack [33]. It is evident that CLA reduces tumor incidence as well as tumor multiplicity [8, 30].

The mechanism through which CLA inhibits tumor genesis is proposed [1]. In relation to other important dietary antioxidants, it quenches singlet oxygen more effectively than α–tocopherol. It appears to act as a chain–breaking antioxidant, by trapping chain–propagating free radicals. Pariza and Ha (1990) have demonstrated that CLA is a potent antioxidant and that cis–9, trans–11 isomer is selectively incorporated into cellular phospholipids, which may explain, at least in part, the anticarcinogenic activity of CLA as antioxidant [23] . Other reports indicated that CLA exhibited antioxidant properties *in vivo* [9] and *in vitro* [8].

The relationship between antioxidant–enzyme defense responses and cellular growth suppression in human cancer cells, exposed to CLA in cultures, has also been studied. The activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (Gpx) were induced in cell lines exposed to CLA. The data indicate that CLA–induced cytotoxicity against cancer lines is related to the extent of lipid peroxidation of CLA treated cells, and affirm that the CLA–induced antioxidant enzymes failed to protect these cells from cytotoxic lipid peroxidation products [20, 29].

One might speculate that the inhibition of carcinogenesis by CLA could result from the combined effects of a number of CLA activities, possibly including direct effects of one or more CLA isomers/metabolites on cell differentiation and the effects of one or more CLA isomers on prostaglandin metabolism, which may also influence cancer development at some sites [17, 19]. Animal fat, which has been maligned for so long, may actually contribute with a potent therapeutic component to our diet [15].

In previous works, we have demonstrated that rat liver microsomal and mitochondrial membranes are protected by vitamin A and CLA, when subjected to non enzymatic lipoperoxidation [21]. The present study aims to compare the polyunsaturated fatty acid composition and non enzymatic lipid peroxidation of rat liver, kidney, lung and heart mitochondria obtained from animals supplemented with conjugated linoleic acid [22, 36] .

MATERIAL AND METHODS

Materials. Female Wistar AH/HOK rats were supplied by Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata. Conjugated octadecadienoic acid (CLA) and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO). BSA (fraction V) was obtained from Wako Pure Chemicals Industries Ltd, Japan. Standards of fatty acids methyl esters were kindly supplied by NU Check Prep. Inc, Elysian, MN, USA. L[+] ascorbic acid and boron trifluoride–methanol complex were from Merck Laboratories.

Animals. Female Wistar AH/HOK rats (7 weeks–old, 120–137 g) were used. Two groups of three rats were considered, group A: received conjugated linoleic acid (CLA), group B: controls. All rats were fed with commercial rat chow and water was administered *ad libitum*. Group A received daily 12.5 mg oral administration of CLA for 10 days. On day 11, all the rats were sacrificed by cervical dislocation and the liver, kidneys, lungs and heart were rapidly removed.

Preparation of mitochondria. Liver, kidney, lung and heart were cut into small pieces and washed extensively with 0.15 M NaCl. An homogenate 30% (w/v) was prepared in a 0.25 M sucrose solution, 10 mM Tris–HCl pH 7.4 using a Potter–Elvejhem homogenizer. The homogenate was centrifuged at 3,000 G for 5 min; pellets were discarded, and the supernatant was centrifuged at 10,000 G for 10 min to obtain mitochondrial pellets. All operations were performed at 4°C and under dim light.

Lipid peroxidation of mitochondria. Chemiluminescence and lipid peroxidation were initiated by adding ascorbate to mitochondria [37]. Organelle (0.5 mg mitochondrial protein) were incubated at 37°C with 0.01 M phosphate buffer pH 7.4, 0.4 mM ascorbate, final volume 2 ml. Phosphate buffer is contaminated with sufficient iron to provide the necessary ferrous or ferric iron (final concentration in the incubation mixture was 2.15 μM) for lipid peroxidation [35]. Organelle preparations, without ascorbate, were analyzed simultaneously. Membrane light emission was determined over a 120 min period, chemiluminescence was recorded as counts per minute (cpm) every 10 min and the sum of the total was used to calculated cpm/mg protein. Chemiluminescence was measured as counts per min in a Packard 1900 TR liquid scintillation analyzer equipment with a special software for this parameter.

Fatty acid analysis. Mitochondrial lipids were extracted with chloroform/methanol (2:1 v/v) [6] from both native or peroxidized membranes. Fatty acids were transmethylated with F_3B in methanol at 60°C for 3 h. Fatty acids methyl esters were analyzed with a GC–14 A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a packed column (1.80 m x 4 mm i.d.) GP 10 % DEGS–PS on 80/100 Supelcoport. Nitrogen was used as the carrier gas. The injector and detector temperatures were maintained at 250°C, column temperature was held at 200°C. Fatty acid methyl ester peaks were identified by comparison of the retention times with those of standards.

Table 1. Polyunsaturated fatty acid composition (area %) of mitochondria native and peroxidized from control group ($\bar{x}$ ± SD of three experiments).

fatty acid	liver		kidney		lung		heart	
	native	perox.	native	perox.	native	perox.	native	perox.
C18:2 (n–6)	15.621± 1.328[a]	7.524± 2.021[a]	24.742± 2.084[b]	6.932± 2.123[b]	12.386± 2.921[c]	6.975± 1.945[c]	10.911± 4.167[d]	3.424± 0.982[d]
C20:4 (n–6)	21.733± 1.431[e,1]	2.831± 0.408[e,3]	17.921± 1.934[f]	8.432± 2.831[f]	10.883± 1.984	7.432± 1.234	9.983± 1.937	8.324± 2.021
C22:6 (n–3)	8.931± 0.924[g,2]	0.841± 0.197[g,2]	3.715± 0.298[h]	0.814± 0.137[h,4]	2.514± 0.765[i]	0.924± 0.295[i]	6.324± 0.895	5.998± 1.537

Perox.: peroxidized; native: without incubation in ascorbate Fe^{++} system; peroxidized: with incubation in the presence of 0.4 mM ascorbate. Statistically differences between native vs. peroxidized mitochondria of different tissues are indicated by $p^a < 0.005$, $p^b < 0.001$, $p^c < 0.05$, $p^d < 0.04$, $p^{e-g-h} < 0.0001$, $p^f < 0.009$, $p^i < 0.03$. Statistically differences between fatty acids of Table 1 and 2 are indicated by $p^1 < 0.009$, $p^2 < 0.03$, $p^3 < 0.002$ and $p^4 < 0.01$.

Table 2. Polyunsaturated fatty acid composition (area %) of mitochondria native and peroxidized from CLA group ($\bar{x}$ ± SD of three experiments).

fatty acid	liver		kidney		lung		heart	
	native	perox.	native	perox.	native	perox.	native	perox.
C18:2 (n–6)	14.432± 2.843[a]	6.921± 1.056[a]	22.932± 2.856[b]	7.521± 1.962[b]	10.338± 0.986[c]	5.959± 1.786[c]	12.911± 1.253[d]	4.836± 0.693[d]
C20:4 (n–6)	16.467± 1.234[e,1]	11.431± 1.857[e,3]	18.526± 1.254[f]	8.634± 1.576[f]	9.818± 1.610	6.924± 1.267	10.263± 0.786	9.312± 1.582
C22:6 (n–3)	6.842± 0.621[g,2]	0.924± 0.263[g]	3.986± 0.379[4]	3.355± 0.934	1.914± 0.956	0.542± 0.298	7.355± 1.426	6.832± 1.321

Perox.: peroxidized; native: without incubation in ascorbate Fe^{++} system; peroxidized: with incubation in the presence of 0.4 mM ascorbate. Statistically differences between native vs. peroxidized mitochondria of different tissues are indicated by $p^a < 0.01$, $p^b < 0.002$, $p^{c-e} < 0.02$, $p^d < 0.001$, $p^{f-g} < 0.001$. Statistically differences between fatty acids of Table 1 and 2 are indicated by $p^1 < 0.009$, $p^2 < 0.03$, $p^3 < 0.002$ and $p^4 < 0.01$.

Peroxidizability Index. Peroxidizability index (PI) was calculated according to the formula PI = (percent of monoenoic acids x 0.025) + (percent of dienoic acids x 1) + (percent of trienoic acids x 2) + (percent of tetraenoic acids x 4) + (percent of pentaenoic acids x 6) + (percent of hexaenoic acids x 8) [27].

Other methods. Proteins were determined by the method of Lowry [18].

Statistical analysis. Results are expressed as mean ($\bar{x}$) ± standard deviation (SD) of three independent determinations. Data were statistically evaluated by one–way analysis of variance (ANOVA) and Tukey test. The statistical criteria for significance was selected at different p values, which were indicated in each case.

RESULTS

Polyunsaturated fatty acid composition of total lipids from different tissues of mitochondria (native vs. peroxidized). The polyunsaturated fatty acid composition of total lipids isolated from rat liver, kidney, lung and heart mitochondria obtained from CLA and control groups is showed in Tables 1 and 2. The changes in fatty acids composition of organelles from control group were more pronounced than those from CLA group. When peroxidized mitochondria were compared to natives, in CLA and control group it was observed that C18:2 n–6 decreased in all tissues used in this work. In control group, C20:4 n–6 and C22:6 n–6 also decreased in kidney and liver, while in lung it was affected C22:6 n–3. In CLA group, C20:4 n–6 decreased in liver and kidney, whereas in liver C22:6 n–3 decreased as well.

Effect of conjugated linoleic acid treatment on fatty acid composition of native mitochondria. When Tables 1 and 2 were compared, it was observed that the effect of conjugated linoleic acid treatment on the total fatty acid composition of native liver mitochondria was evidenced by a statistically significant decrease of arachidonic acid C20:4 n–6 and docosahexanoic acid C22:6 n–3 when compared with control group, whereas changes were not detected in kidney, lung and heart.

Effect of conjugated linoleic acid treatment on fatty acid composition of peroxidized mitochondria. From the analysis of Tables 1 and 2, it was observed that the effect of conjugated linoleic acid treatment on the total fatty acid composition of peroxidized liver mitochondria was evidenced by a statistically significant decrease of arachidonic acid C20:4 n–6, when compared with control group. In kidney, statistically significant differences were found in the content of C22:6 n–3, while changes were not detected in lung and heart.

Table 3. Total chemiluminescence of rat liver and kidney mitochondria induced by ascorbate–Fe^{++} ($\bar{x}$ ± SD of three experiments).

	liver		kidney	
	control	CLA	control	CLA
light emission	1538 ± 147	1126 ± 102	1264 ± 127	952 ± 63
inhibition (%)	0	26,7[a]	0	24,7 [b]

CLA: conjugated dienoic derivatives of linoleic acid. Statistically differences between CLA vs. control group are indicated by $p^a < 0.006$, $p^b < 0.008$.

Table 4. Peroxidizability index ($\bar{x}$ ± SD of three experiments).

		liver	kidney	lung	heart
control group	native	156.139± 14.425[a]	118.712± 8.634	70.996± 10.537	88.841± 9.624
	perox.	18.848± 2.452[b]	45.536± 5.064[c]	42.239± 5.757	72.714± 16.784
CLA group	native	127.352± 6.215[a]	120.952± 7.975	61.094± 3.457	98.093± 8.648
	perox.	58.189± 5.864[b]	62.187± 3.529[c]	36.907± 4.885	83.076± 11.625

CLA: conjugated dienoic derivatives of linoleic acid, Perox.: peroxidized. Statistically differences are indicated by $p^a < 0.03$, $p^b < 0.001$ and $p^c < 0.01$.

Light emission of mitochondria obtained from different tissues during lipid peroxidation. The incubation of mitochondria in the presence of ascorbate–Fe^{++} system resulted in the peroxidation of membranes, as evidenced by emission of light (chemiluminescence). It was observed that the total cpm/mg of protein originated from light emission, was significantly lower in liver and kidney mitochondria in CLA treated animals than in the control group (Table 3). These changes were not detected in lung and heart.

Peroxidizability Index. There were marked differences when peroxidizability index of peroxidized liver and kidney mitochondria from control and CLA groups were compared; in this case, the PI of CLA group was higher, meaning this more protection against lipid peroxidation (Table 4). Significant changes in the peroxidizability index were observed in mitochondria from liver and kidney. These changes were less pronounced in those membranes derived from rat receiving CLA. The PI of native liver mitochondria obtained from CLA group was lower than the PI of those obtained from liver mitochondria of control group, but higher in peroxidized organelles of CLA group. This suggests that CLA acts by decreasing polyunsaturated fatty acids content in natives membranes, thus protecting them against lipid peroxidation. The changes in the fatty acid composition of membranes subjected to lipid peroxidation in the presence of ascorbate –Fe^{++}, produced an important decrease in the relative content of the more polyunsaturated fatty acids. As a result, the peroxidizability index of the peroxidized membranes obtained from liver, kidney and lung in the CLA group was significantly higher than in the control group.

DISCUSSION

Although considerable research has already been performed to characterize the changes in structure, composition and physical properties of membranes subjected to oxidation [11, 28], it is important to know how biological compounds with antioxidant properties contribute to the protection of specialized membranes against deleterious effects produced by reactive oxygen species and other free radicals. Evidence leading to the recognition of the anticarcinogenic activity of the conjugated dienoic derivatives of linoleic acid (CLA) has been reviewed. New data indicated that CLA has potent antioxidant activity [12]. Because the cis–9, trans–11 CLA isomer is esterified with phospholipids, it may represent a heretofore unrecognized *in situ* defense mechanism against membrane attack by oxygen radicals [24].

In relation to other important dietary antioxidants, it quenches less singlet oxygen, being therefore more effective than α–tocopherol. It appears to act as a chain–breaking antioxidant, by trapping chain propagating free radicals [34]. One might speculate that the inhibition of carcinogenesis by CLA could result from the combined effects of a number of CLA activities, possibly including direct effects of one or more CLA isomers/metabolites on cell differentiation [10], effects of CLA on vitamin A metabolism that would also influence cell differentiation, and effects of one or more CLA isomers on prostaglandin metabolism [9], which may also influence cancer development at some sites [17].

In vitro lipid peroxidation studies are useful for the elucidation of a possible mechanism of peroxide formation *in vivo* [4], since the high concentration of polyunsaturated fatty acids in membranes causes susceptibility to lipid peroxidative degradation. Previous investigations from our laboratory have shown that the fatty acid composition of rat liver microsomes is modified after non–enzymatic peroxidation in the presence of ascorbate–Fe^{++} [5]. In the study reported here, the effect of conjugated linoleic acid (CLA) on the polyunsaturated fatty acid composition, chemiluminescence and peroxidizability index of mitochondria isolated from rat liver, kidney, lung and heart, was analyzed.

The effect of CLA on the polyunsaturated fatty acid composition of native liver mitochondria was evidenced by a statistically significant decrease of arachidonic acid C 20:4 n6 and docosahexaenoic acid C 22:6 n3, when compared with control group. Rat liver and kidney or mitochondria obtained from CLA group were protected against lipid peroxidation when compared to similar organelles obtained from control rats, as shown by the results obtained by chemiluminescence, polyunsaturated fatty acid composition and peroxidizability index. As a

result, from the analysis of PI of peroxidized mitochondrial and microsomal membranes, it was shown that CLA group was more effective against lipid peroxidation, compared to the other group. In our experimental conditions, before incubation in an ascorbate Fe^{++} system, we found changes in the polyunsaturated fatty acid composition of mitochondria from liver of rats treated with CLA and vitamin A when compared to control group, being the latter in agreement with previous results of other investigators [3, 7].

Our results show that CLA decrease polyunsaturated fatty acids; another author states that CLA does not appear to act as an antioxidant, its ability to decrease polyenoic fatty acid concentration could decrease the formation of highly cytotoxic lipid peroxidation products [3]. In contrast to this, other investigators suggested an antioxidant mechanism of CLA [24, 25]. Nevertheless, the same authors postulate that CLA may induce both non–enzymatic and enzymatic *in vivo* lipid peroxidation [2, 16].

In conclusion, our results are consistent with the hypothesis that formation and action of CLA represents a previously unrecognized *in situ* defense mechanism against membrane attack by oxygen radicals. Further studies are needed for a more adequate evaluation of these observations.

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