

Conjugated linoleic acid induces lipid peroxidation in humans

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Abstract Conjugated linoleic acid (CLA) is shown to have chemoprotective properties in various experimental cancer models. CLA is easily oxidised and it has been suggested that an increased lipid oxidation may contribute to the antitumorigenic effects. This report investigates the urinary levels of 8-iso-PGF_{2α}, a major isoprostane and 15-keto-dihydro-PGF_{2α}, a major metabolite of PGF_{2α}, as indicators of non-enzymatic and enzymatic lipid peroxidation after dietary supplementation of CLA in healthy human subjects for 3 months. A significant increase of both 8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} in urine was observed after 3 months of daily CLA intake (4.2 g/day) as compared to the control group ($P < 0.0001$). Conjugated linoleic acid had no effect on the serum α -tocopherol levels. However, γ -tocopherol levels in the serum increased significantly ($P = 0.015$) in the CLA-treated group. Thus, CLA may induce both non-enzymatic and enzymatic lipid peroxidation in vivo. Further studies of the mechanism behind, and the possible consequences of, the increased lipid peroxidation after CLA supplementation are urgently needed.

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Key words: Conjugated linoleic acid; Prostaglandin; Isoprostane; Lipid peroxidation; Human

1. Introduction

Conjugated linoleic acid (CLA) is the common denomination of a group of fatty acids with 18 carbon atoms consisting of a mixture of positional and geometrical isomers with two conjugated double bonds, unlike linoleic acid which is a non-conjugated diene. The two double bonds in CLA are usually in the C-9 and C-11 or C-10 and C-12 positions and can be in either the *cis* or *trans* configuration. CLA is a naturally occurring minor constituent in certain human foods such as ruminant meat and dairy products [1].

Since the early work by Pariza and colleagues on antimutagenic and anticarcinogenic properties of CLA several studies on the beneficial effects of CLA have been reported [2,3]. Most of these studies were performed in experimental animals showing unique inhibitory effects of CLA on mammary gland cancer, skin cancer and forestomach neoplasia and development of body fat content [1–6]. We have recently shown that

the proportion of body fat in humans was reduced after daily supplementation of CLA for 3 months [7].

Structurally CLA belongs to the family of linoleic acids, the parent compound of arachidonic acid. Recent studies have shown that dietary CLA reduces arachidonic acid content and prostaglandin (PG) E₂ biosynthesis in murine keratinocytes [8]. Dietary CLA also inhibits phorbol ester-induced skin tumour promotion and PGE₂ production in mouse epidermis [9] and the CLA-induced cytotoxicity in cancer cell lines is associated with increased lipid oxidation [10,11]. Thus, the availability of CLA in the tissue or circulation seems to affect the endogenous linoleic acid-related compounds and their metabolites. It is well known that arachidonic acid metabolism through non-enzymatic and enzymatic pathways leads to the formation of isoprostanes and prostaglandins that control many physiological and pathophysiological consequences of the body [12–19].

We have recently developed highly specific and sensitive radioimmunoassays (RIA) for 8-iso-PGF_{2α}, a major F₂-isoprostane, and 15-keto-dihydro-PGF_{2α}, a major metabolite of PGF_{2α}, as indicators of lipid peroxidation in vivo, through raising unique antibodies in rabbits [20,21]. The antibodies discriminate these two very closely related substances which increase during two different important biochemical manifestations of lipid peroxidation, namely non-enzymatically and enzymatically catalysed oxidation of arachidonic acid [22].

The effect of CLA on the lipid peroxidation in humans is unknown. In this work we have studied the biochemical effect of CLA on the non-enzymatic and enzymatic lipid peroxidation by measuring both 8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} in urine, and antioxidant status in serum or plasma samples, collected from healthy human subjects following 3 months of daily supplement of a mixture of CLA isomers in a double-blind manner.

2. Materials and methods

2.1. Materials

Unlabelled 8-iso-PGF_{2α}, 15-K-DH-PGF_{2α} and other related isoprostanes and prostaglandins were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Tris-HCl, tris-base, EDTA disodium salt and bovine γ -globulin were purchased from Sigma Chemicals (St. Louis, MO, USA). Hi-Safe scintillation cocktail was obtained from Wallac Inc. (Turku, Finland). Polyethylene glycol (MW 4000) was purchased from Merck (Germany). Tris-HCl buffer 0.05 M, pH 7.8 was used in the RIA. Unlabelled 8-iso-PGF_{2α}, 15-K-DH-PGF_{2α} standards, tritium-labelled tracer and working antibody solution were prepared in the RIA buffer. The tritium-labelled 8-iso-PGF_{2α} (specific activity: 608 GBq/mmol) was synthesised and purified as described previously [20]. The tritium-labelled 15-K-DH-PGF_{2α} (specific activity: 6.77 TBq/mmol) was obtained from Amersham (Buckinghamshire, UK). Antibodies against both 8-iso-PGF_{2α} and 15-K-DH-PGF_{2α} were raised at our laboratory and have been well characterised [20,21]. Carbon tetrachloride was purchased from Merck, Germany.

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Abbreviations: COX, cyclooxygenase; PG, prostaglandin; 8-iso-PGF_{2α}, 8-iso-prostaglandin F_{2α}; 15-keto-dihydro-PGF_{2α}/15-K-DH-PGF_{2α}, 15-keto-13,14-dihydro-prostaglandin F_{2α}; PGF_{2α}, prostaglandin F_{2α}; RIA, radioimmunoassay; CLA, conjugated linoleic acid; MDA, malondialdehyde; HPLC, high pressure liquid chromatography

2.2. Experimental protocol

2.2.1. Subjects. Fifty-three healthy men and women aged 23–63 years were included in the study. The participants were randomly assigned to either a CLA-treated group or a control group before entering the study. The treatment followed a double-blind fashion. Some of the baseline data of the two groups are shown in Table 1. There was no significant difference between the groups at the beginning of the study. The study plan was approved by the University Ethics Committee, Medical Faculty, Uppsala University, Sweden.

2.2.2. Study design. All subjects were given control capsules containing olive oil during two initial weeks. The following 12 weeks the subjects in the CLA group were given capsules containing 4.2 g of mixed isomers of CLA, mainly consisting of equal amounts of the *cis* 9 *trans* 11 and *trans* 10 *cis* 12 CLA isomers. The CLA preparation used in this study contained no isoprostanes or prostaglandins. The subjects in the control group continued taking olive oil during the same period of time. The control and CLA capsules were identical in appearance. The capsules were kindly provided by Natural Ltd. ASA, Oslo, Norway. The participants were requested not to change their habits regarding food and physical activity and not to use any dietary supplements such as vitamins, minerals or fatty acids. Urinary samples were collected both in the morning and once daily (24 h) during the initial 2 weeks and during the twelfth week according to the earlier described method of urinary sample collection [23]. All samples were stored frozen at -70°C until analysis.

2.3. Methods

2.3.1. Radioimmunoassay of 8-iso-PGF_{2α} (non-enzymatic lipid peroxidation indicator). The plasma and urinary samples from this study were analysed for free 8-iso-PGF_{2α} without any extraction by a newly developed radioimmunoassay [20]. In brief, an antibody was raised in rabbits by immunisation with 8-iso-PGF_{2α} coupled to bovine serum albumin (BSA) at the carboxylic acid by the 1,1'-carbonyldiimidazole method. The cross-reactivity of the antibody with 8-iso-15-keto-13,14-dihydro-PGF_{2α}, 8-iso-PGF_{2β}, PGF_{2α}, 15-keto-PGF_{2α}, 15-keto-13,14-dihydro-PGF_{2α}, thromboxane (TX) B₂, 11β-PGF_{2α}, 9β-PGF_{2α} and 8-iso-PGF_{3α} was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6%, respectively. The detection limit of the assay was about 23 pmol/l. The urinary levels of 8-iso-PGF_{2α} were adjusted for creatinine values, which were measured by a commercial kit (IL[®] Test by Monarch Instrument, Amherst, NH, USA).

2.3.2. Radioimmunoassay of 15-keto-dihydro-PGF_{2α} (enzymatic lipid peroxidation indicator). The urinary samples (24 h) from this study were also analysed for 15-keto-dihydro-PGF_{2α} without any extraction by a newly developed radioimmunoassay [21]. In brief, an antibody was raised in rabbits by immunisation with 15-keto-dihydro-PGF_{2α} coupled to BSA at the carboxylic acid by the 1,1'-carbonyldiimidazole method. The cross-reactivity of the antibody with PGF_{2α}, 15-keto-PGF_{2α}, PGE₂, 15-keto-13,14-dihydro-PGE₂, 8-iso-15-keto-13,14-dihydro-PGF_{2α}, 11β-PGF_{2α}, 9β-PGF_{2α}, TXB₂ and 8-iso-PGF_{3α} was 0.02, 0.43, <0.001, 0.5, 1.7, <0.001, <0.001, <0.001, 0.01%, respectively. The detection limit of the assay was about 45 pmol/l. The urinary levels of 15-keto-dihydro-PGF_{2α} were adjusted for creatinine values.

2.3.3. Measurement of tocopherol. Plasma α- and γ-tocopherol levels were assayed by using HPLC with fluorescence detection [24]. In brief, 500 μl plasma was extracted with 500 μl ethanol containing 0.005% butylated hydroxytoluene and 2 ml hexane. A volume of 20 μl of the supernatant was injected to a HPLC column (LiChrospher 100 NH₂ 250×4 mm). The fluorescence detector had an excitation wavelength of 295 nm and an emission wavelength of 327 nm. Plasma tocopherol levels were adjusted for serum lipid concentrations.

2.3.4. Malondialdehyde measurement. Malondialdehyde (MDA), a product of lipid peroxidation, was measured in plasma as an additional parameter for comparison with 8-iso-PGF_{2α} measurement by using a HPLC and fluorescence detector after minor modification as described previously [25]. In brief, a thiobarbituric acid reaction was carried out by mixing 750 μl of 0.15 M phosphoric acid with 300 μl of water and 250 μl of thiobarbituric acid with 200 μl plasma sample. The reaction mixture was incubated in a boiling water bath for 60 min and then cooled in ice. The MDA-thiobarbituric acid complex was extracted with methanol and quantified. A volume of 20 μl of sample was injected into the HPLC column. The fluorescence detector had an excitation wavelength of 532 nm and an emission wavelength of 553 nm. The mobile phase contained methanol:50 mM phosphate buffer (2:3).

2.3.5. Statistical analyses. The results of this investigation were analysed using the software systems Statistical Analysis System and STATA (Stata Corporation, TX, USA). For analyses of differences between the changes in the two treatment groups unpaired *t*-tests were used. Variables with a skewed distribution were logarithmically transformed prior to the *t*-test.

3. Results

This was a double-blind controlled trial in 53 healthy subjects who were treated with CLA (4.2 g/day) or a corresponding amount of a placebo preparation containing olive oil for 3 months. No participant experienced any side effects during the study period.

3.1. Non-enzymatic lipid peroxidation as assessed by urinary and plasma F₂-isoprostanes

Non-enzymatic lipid peroxidation was assessed by the measurement of one of the major F₂-isoprostanes, 8-iso-PGF_{2α}, in urine samples collected in the morning as well as during 24 h at the beginning and at the end of this study. Ingestion of CLA for 3 months resulted in an increase in the basal levels of both morning and 24 h urinary levels of 8-iso-PGF_{2α} (Table 2). The morning urinary levels of 8-iso-PGF_{2α} increased significantly (difference between the changes in the control and CLA groups, $P=0.0001$) to about four times the basal level (0.47 nmol/mmol creatinine) in these healthy subjects who were supplemented with CLA for 3 months (Table 2). Similarly, the 24 h urinary 8-iso-PGF_{2α} levels increased ($P<0.0001$) more than three times from the basal level (0.51 nmol/mmol creatinine) (Table 2). No such increment in the morning or 24 h urinary 8-iso-PGF_{2α} levels was seen in the control group (morning urine: before 0.33 and after 0.32 nmol/mmol creatinine; 24 h urine: before 0.34 and after 0.36 nmol/mmol creatinine).

Supplementation of CLA increased plasma 8-iso-PGF_{2α} levels (CLA group: before 74 ± 10.1 pmol/l (mean $\pm$ S.E.M.) and after 107 ± 15.6 pmol/l) whereas in the control group there was no change (control group: before 55 ± 6.7 pmol/l and after 58 ± 10.0 pmol/l) ($P=0.0181$ for difference between changes in the groups). However, the results from only 34 of the total of 53 subjects could be used in the results because of technical difficulties (many of the heparinised plasma samples were haemolysed and haemolysed samples cannot be assayed by this RIA).

3.2. Enzymatic lipid peroxidation as assessed by urinary 15-keto-dihydro-PGF_{2α}

Enzymatic lipid peroxidation was assessed by the measurement of 15-keto-dihydro-PGF_{2α} in 24 h urine samples. The urinary levels of 15-keto-dihydro-PGF_{2α} increased significantly ($P<0.0001$) from the basal level (0.70 nmol/mmol cre-

Table 1
The baseline values of the healthy human subjects included in this study given as mean (S.D.)

Age (years)	45.4 (11.7)
Sex (men/women)	27/26
Weight (kg)	75.7 (15.3)
BMI (kg/m ²)	25.1 (4.1)
Serum TAG (mmol/l)	1.39 (0.77)
Serum cholesterol (mmol/l)	5.60 (1.07)

BMI = body mass index, TAG = triacylglycerides.

Table 2

The levels and change (%) of 8-iso-PGF_{2α}, 15-keto-dihydro-PGF_{2α}, MDA and tocopherols in the body fluids in control and CLA-treated groups

	Control (<i>n</i> = 25)		Change (%) ^a	CLA (<i>n</i> = 28)		Change (%) ^a	<i>P</i> value for difference between groups
	Before (mean ± S.E.M.)	After (mean ± S.E.M.)		Before (mean ± S.E.M.)	After (mean ± S.E.M.)		
u-8-iso-PGF _{2α} (nmol/mmol creatinine, morning)	0.33 ± 0.04	0.32 ± 0.04	8.3	0.47 ± 0.07	1.85 ± 0.35	407	0.0001
u-8-iso-PGF _{2α} (nmol/mmol creatinine, 24 h)	0.34 ± 0.03	0.36 ± 0.04	17.0	0.50 ± 0.07	1.65 ± 0.29	333	< 0.0001
u-15-keto-dihydro-PGF _{2α} (nmol/mmol creatinine, 24 h)	0.51 ± 0.07	0.54 ± 0.07	13.0	0.70 ± 0.09	1.30 ± 0.13	129	< 0.0001
p-MDA (μmol/l)	0.63 ± 0.03	0.60 ± 0.03	−0.7	0.60 ± 0.03	0.56 ± 0.03	1.8	n.s.
s-α-tocopherol ^b (mg/mmol)	1.60 ± 0.02	1.60 ± 0.04	0.5	1.59 ± 0.05	1.59 ± 0.04	−0.1	n.s.
s-α-tocopherol ^c (μmol/l)	26.2 ± 1.08	26.0 ± 0.96	−0.1	25.0 ± 0.95	25.3 ± 1.1	1.0	n.s.
s-γ-tocopherol ^b (mg/mmol)	0.08 ± 0.01	0.09 ± 0.01	6.4	0.10 ± 0.01	0.14 ± 0.01	40.2	0.015
s-γ-tocopherol ^c (μmol/l)	1.44 ± 0.11	1.49 ± 0.12	5.9	1.70 ± 0.07	2.33 ± 0.26	43.7	0.027

p = plasma, u = urine, s = serum, n.s. = non-significant, *n* = number of subjects in each group.

^aPercent change is calculated as ((value after − value before)/value before) × 100.

^bLipid-corrected.

^cWithout lipid correction.

atinine) and were approximately doubled in the subjects who were supplemented with CLA for 3 months (Table 2). No such increment in the urinary 15-keto-dihydro-PGF_{2α} levels was seen in the control group (before 0.51 and after 0.54 nmol/mmol creatinine). The individual changes in the concentrations of 15-keto-dihydro-PGF_{2α} in the urine were significantly correlated with those of urinary 8-iso-PGF_{2α} (*r* = 0.46, *P* = 0.0006).

3.3. MDA levels in plasma

No significant difference in changes of the MDA levels in plasma was seen in the CLA (before 0.60 and after 0.56 μmol/l) or in the control group (before 0.63 and after 0.60 μmol/l) (Table 2).

3.4. Antioxidant levels in plasma

The serum α-tocopherol levels in the CLA and in the control group subjects were unchanged, whereas the γ-tocopherol levels in the CLA group increased significantly from the basal level (0.10 mg/mmol) by 40% (*P* = 0.015) in the CLA-supplemented group (Table 2). No such increase was seen in the control group (before 0.08 and after 0.09 mg/mmol).

4. Discussion

The main aim of this study was to investigate non-enzymatic and enzymatic lipid peroxidation in the body in CLA-treated humans. Our study clearly shows that both 8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} levels in the urine and also the plasma levels of 8-iso-PGF_{2α} were significantly elevated indicating a direct effect of CLA on *in vivo* lipid peroxidation. The 8-iso-PGF_{2α} is formed *in vivo* in humans and animals by free radical-catalysed oxidation of arachidonic acid and is claimed to be a unique biomarker of non-enzymatic lipid peroxidation [14,15,20,22]. Increased amounts of this compound are found both in the plasma and in the urine

in various human and animal models of oxidant injury or dietary supplementation studies [15,17,19,26–29]. Measurement of 15-keto-dihydro-PGF_{2α} in peripheral plasma has been applied in various species for many years as an indicator of endogenous PGF_{2α} secretion as a result of cyclooxygenase-catalysed lipid peroxidation [13,16,21,30]. Recent studies on 15-keto-dihydro-PGF_{2α} in urinary samples showed that measurement of this metabolite in urinary samples could also serve as a parameter of endogenous PGF_{2α} release [21,29].

This study reports for the first time that CLA can upregulate both isoprostane and prostaglandin formation in humans. This shows that CLA can modulate both free radical-induced and cyclooxygenase-catalysed arachidonic acid oxidation. The mechanism of induction of these two important biochemical pathways cannot be determined from this study. Conjugated linoleic acid has been proposed to affect prostaglandin biosynthesis besides its anticarcinogenic and other beneficial properties [8,9]. Prostaglandins have earlier been shown to possess a cytotoxic effect in cell growth [31]. In concert, CLA-induced cytotoxicity in cancer cell lines has been shown to be associated with increased lipid peroxidation [10,11]. Whether the increase of lipid peroxidation products *in vivo* as demonstrated in this study is of importance for the anti-tumorigenic effect of CLA cannot be evaluated from this study.

The total fat consumption was, also after addition of 4–5 g extra fat, well within the range of a normal diet. Oleic acid was used as a placebo control. Alternatively, linoleic acid could have been used. The choice of placebo preparation (oleic vs. linoleic acid) should be of no major importance for the results regarding the increased lipid peroxidation after CLA supplementation. We have earlier studied the effect of adding 3–4 times more linoleic acid in the context of a linoleic acid-rich diet, and compared the effects with those of a diet enriched in oleic acid. There was no difference in the urinary concentration of F₂-isoprostane between these diets [28].

Although the levels of both 8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} increased significantly, the relative increase was much higher for 8-iso-PGF_{2α} than for 15-keto-dihydro-PGF_{2α}. This indicates that CLA probably has a more direct effect on the non-enzymatic than on the enzymatic conversion of arachidonic acid. The individual changes in the concentrations of 15-keto-dihydro-PGF_{2α} in the urine were significantly correlated with those of 8-iso-PGF_{2α}. It was recently shown that intravenous administration of 8-iso-PGF_{2α} may lead to cyclooxygenase activation and subsequent prostaglandin F_{2α} formation in the rabbit (Basu, unpublished results). Thus, formation of 8-iso-PGF_{2α} after CLA treatment in this study may possibly directly affect the cyclooxygenases with sequential formation of PGF_{2α}. However, the magnitude of the increase of 8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} in this study is not comparable to the earlier described inflammatory response-related [17,18] or oxidative injury-induced [19] formation of these compounds.

The concentration of MDA in plasma, another lipid oxidation indicator in vivo, did not increase after CLA treatment, in contrast to urinary 8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α}. These results corroborate previously described comparisons of 8-iso-PGF_{2α} and thiobarbituric acid reactive substances or MDA measurements in hydrogen peroxide- or carbon tetrachloride-induced lipid peroxidation studies [19,28,32]. This is possibly because these two biomarkers of lipid peroxidation reflect different stages of lipid peroxidation and/or insufficient sensitivity to detect lipid peroxidation products by measuring MDA in these experimental models. Conjugated linoleic acid had no effect on α -tocopherol. The γ -tocopherol levels in the CLA group increased significantly.

In conclusion, dietary supplementation of CLA induces both non-enzymatically and enzymatically catalysed lipid peroxidation in humans. The simultaneous measurement of 8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} in body fluids opens excellent opportunities to study the role of non-enzymatic and enzymatic lipid peroxidation in various human diseases and dietary or therapeutic conditions. Further studies of the mechanism behind, and the possible consequences of, the increased lipid peroxidation after CLA supplementation are urgently needed.

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