

Conjugated linoleic acid induces lipid peroxidation in men with abdominal obesity

S. BASU*, U. RISÉRUS*, A. TURPEINEN† and B. VESSBY*

*Sections of Geriatrics and Clinical Nutrition Research, Faculty of Medicine, Uppsala University, Box 609, SE-751 25 Uppsala, Sweden, and †Department of Applied Chemistry and Microbiology, Division of Nutrition, University of Helsinki, FIN-00014 Helsinki, Finland

A B S T R A C T

Conjugated linoleic acid (CLA) has been shown in experimental studies to have chemoprotective properties, and may decrease the deposition of body fat. CLA is prone to oxidation, and it has been suggested that increased lipid oxidation may contribute to the anti-tumorigenic effects of this agent. The present study investigates the urinary levels of 8-iso-prostaglandin F_{2x} (8-iso-PGF $_{2x}$), a major isoprostane, and of 15-oxo-dihydro-PGF $_{2x}$, a major metabolite of PGF $_{2x}$, as indicators of non-enzymic and enzymic arachidonic acid oxidation respectively after dietary supplementation with CLA in middle-aged men (mean age 53 years) with abdominal obesity for 1 month in a randomized controlled trial. Significant increases in the levels of both 8-iso-PGF $_{2x}$ and 15-oxo-dihydro-PGF $_{2x}$ in urine ($P < 0.0001$ and $P = 0.0013$ respectively) were observed after 1 month of daily CLA intake (4.2 g/day) as compared with the control group. The lipid peroxidation parameters had returned to their basal levels at 2 weeks after the cessation of CLA intake, and remained at the same levels for a further 2 weeks until the end of the study. CLA had no effect on serum α -tocopherol and γ -tocopherol levels, or on the urinary levels of 2,3-dinor-thromboxane B_2 . Thus CLA may induce both non-enzymic and enzymic lipid peroxidation *in vivo* in middle-aged men with abdominal obesity, without any side effects. The consequences of the increased lipid peroxidation after CLA supplementation are unknown.

INTRODUCTION

Conjugated linoleic acid (CLA) is the name given to a group of unsaturated fatty acids with 18 carbon atoms, consisting of a mixture of positional and geometrical isomers with two conjugated double bonds (unlike linoleic acid, with 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 the *trans* configurations. CLA, which is a naturally occurring minor constituent of dairy products and of meat from ruminants, is usually produced by bacterial hydrogenation in the ruminant gut [1].

Chemoprotective properties of CLA have been reported in several studies [2–5]. Most of these studies were performed in experimental animals, and showed unique inhibitory effects of CLA on mammary gland cancer,

skin cancer and forestomach neoplasia and development of body fat content [1–6]. Our recent studies in humans showed that the proportion of body fat was decreased after daily supplementation with CLA for 3 months in healthy subjects [7].

Structurally, CLA belongs to the same family as linoleic acid, the precursor compound of arachidonic acid. Dietary CLA decreases both arachidonic acid content and prostaglandin E_2 (PGE $_2$) biosynthesis in murine keratinocytes [8]. Dietary CLA also inhibits phorbol ester-induced skin tumour promotion and PGE $_2$ production in the mouse epidermis [9], and 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 endogenous linoleic acid-related compounds and their metabolites, such as arachidonic acid. It is well known

Key words: abdominal obesity, CLA, human, isoprostanes, lipid peroxidation, metabolic syndrome, prostaglandins.

Abbreviations: CLA, conjugated linoleic acid; PGF $_{2x}$ (etc.), prostaglandin F_{2x} (etc.); TXB $_2$, thromboxane B_2 .

Correspondence: Dr S. Basu (e-mail samar.basu@geriatrik.uu.se).

that the metabolism of arachidonic acid, through both non-enzymic and enzymic pathways, leads to the formation of isoprostanes and prostaglandins respectively, which control many physiological and pathophysiological mechanisms in mammals [12–20].

We have recently developed highly specific and sensitive RIAs for 8-iso-PGF_{2α}, a major F₂-isoprostane, and 15-oxo-dihydro-PGF_{2α} (15-keto-dihydro-PGF_{2α}), a major metabolite of PGF_{2α} as indicators of non-enzymically and enzymically catalysed lipid peroxidation *in vivo* respectively [21–23]. With the aid of these assays, we have recently shown that daily dietary intake of CLA for 3 months may induce both non-enzymic and enzymic lipid peroxidation *in vivo* in normal healthy subjects [24], since levels of both 8-iso-PGF_{2α} and 15-oxo-dihydro-PGF_{2α} in urine and/or in plasma were significantly increased. The aim of the present study was to see if these findings could be reproduced during the short-term intake (1 month) of CLA in a population of obese middle-aged men, and to investigate the levels of these lipid oxidation parameters 2 and 4 weeks after the cessation of CLA intake. We also examined the levels of the thromboxane A₂ metabolite 2,3-dinor-thromboxane B₂ (2,3-dinor-TXB₂) in urine, since this compound may arise from arachidonic acid through the cyclo-oxygenase pathway via thromboxane A isomerase and β-oxidation.

MATERIALS AND METHODS

Materials

Unlabelled 8-iso-PGF_{2α}, 15-oxo-dihydro-PGF_{2α} and other related isoprostanes and prostaglandins were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Tris/HCl, Tris base, EDTA (disodium salt) and bovine γ-globulin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hi-Safe scintillation cocktail was obtained from Wallac Inc. (Turku, Finland). Poly(ethylene glycol) 4000 was purchased from Merck. Tris/HCl buffer (0.05 M, pH 7.8) was used in the RIA. ³H-labelled 8-iso-PGF_{2α} (specific radioactivity 608 GBq·mmol⁻¹) was synthesized and purified as described previously [21]. ³H-labelled 15-oxo-dihydro-PGF_{2α} (specific radioactivity 6.77 TBq·mmol⁻¹) was obtained from Amersham (Little Chalfont, Bucks., U.K.). Antibodies against both 8-iso-PGF_{2α} and 15-oxo-dihydro-PGF_{2α} were raised in our laboratory, and have been well characterized [21,22].

Subjects

A total of 24 middle-aged obese men (waist circumference > 94 cm; waist/hip ratio > 0.95; body mass index > 27 and < 39 kg/m²) between 39 and 65 years of age with signs of metabolic syndrome (intra-abdominal obesity, dyslipidaemia, hypertension and impaired fasting glu-

Table 1 Baseline characteristics of the obese men included in the present study

Values are means ± S.D.

Parameter	CLA	Placebo
No. of subjects	14	10
Age (years)	54 ± 5.7	52 ± 7.8
Body mass index (kg/m ²)	32.2 ± 3.4	31.4 ± 1.9
Waist/hip ratio	1.06 ± 0.07	1.04 ± 0.04
Weight (kg)	106.9 ± 12.3	103 ± 12.7
Serum triacylglycerols (mmol/l)	2.78 ± 1.49	2.81 ± 1.20
Serum cholesterol (mmol/l)	6.27 ± 1.25	6.31 ± 1.01
Glucose (mmol/l)	5.44 ± 0.83	5.28 ± 0.73

ucose) were included in the study after having given their informed consent. 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 protocol. Some of the baseline data of the two groups are shown in Table 1. There were no significant differences between the groups at the beginning of the study. The study plan was approved by the University Ethics Committee, Medical Faculty, Uppsala University, Sweden.

Study protocols

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, each day for 1 month. The CLA preparation used in this study contained no isoprostanes or prostaglandins. The subjects in the control group received placebo (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 intake and physical activity, and not to use any dietary supplements of vitamins, minerals or fatty acids. Urinary samples were collected in the morning before and after 1 month of CLA intake, and 2 and 4 weeks after the cessation of CLA intake, using a previously described method of urinary sample collection [25]. Blood samples were withdrawn from an antecubital vein into heparinized or serum tubes. The samples were centrifuged for 10 min at 3000 g and plasma or serum was separated out. All samples were stored frozen at -70 °C until analysis.

RIA of 8-iso-PGF_{2α} (an indicator of non-enzymic lipid peroxidation)

The urinary samples obtained were analysed for free 8-iso-PGF_{2α} without any extraction, using a newly developed RIA [21]. In brief, antibodies were raised in

rabbits by immunization with 8-iso-PGF_{2α} coupled to BSA at the carboxylic acid by the 1,1'-carbonyldi-imidazole method. The cross-reactivities (%) of the antibody with 8-iso-15-oxo-13,14-dihydro-PGF_{2α}, 8-iso-PGF_{2β}, PGF_{2α}, 15-oxo-PGF_{2α}, 15-oxo-13,14-dihydro-PGF_{2α}, TXB₂, 11β-PGF_{2α}, 9β-PGF_{2α} and 8-iso-PGF_{3α} were 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 approx. 23 pmol/l. The intra-assay accuracy in human plasma for the low and high standards was 95.6% and 101% respectively. Similarly, when precision was determined, the intra-assay coefficient of variation of the low and high standards was 14.5% and 12.2% respectively. Urinary levels of 8-iso-PGF_{2α} were adjusted for creatinine values, which were measured using a commercial kit (IL[®] Test; Monarch Instrument, Amherst, NH, U.S.A.).

RIA of 15-oxo-dihydro-PGF_{2α} (an indicator of enzymic lipid peroxidation)

The urinary samples obtained were also analysed for 15-oxo-dihydro-PGF_{2α} without any extraction, by a newly developed RIA [22]. In brief, antibodies were raised in rabbits by immunization with 15-oxo-dihydro-PGF_{2α} coupled to BSA at the carboxylic acid by the 1,1'-carbonyldiimidazole method. The cross-reactivities (%) of the antibody with PGF_{2α}, 15-oxo-PGF_{2α}, PGE₂, 15-oxo-13,14-dihydro-PGE₂, 8-iso-15-oxo-13,14-dihydro-PGF_{2α}, 11β-PGF_{2α}, 9β-PGF_{2α}, TXB₂ and 8-iso-PGF_{3α} were 0.02, 0.43, < 0.001, 0.5, 1.7, < 0.001, < 0.001, < 0.001 and 0.01% respectively. The detection limit of the assay was approx. 45 pmol/l. The intra-assay accuracy in human plasma for the low and high standards was 108.6% and 103.3% respectively. Similarly, when precision was determined, the intra-assay coefficients of variation for the low and high standards were 12.2% and 14.0% respectively. Urinary levels of 15-oxo-dihydro-PGF_{2α} were adjusted for creatinine values.

Measurement of 2,3-dinor-TXB₂

Urinary excretion of 2,3-dinor-TXB₂ was analysed using morning urine samples, which were kept at -70 °C until analysis. After Sep-Pak purification of the urine samples [26], 2,3-dinor-TXB₂ was measured by solid-phase enzyme immunoassay, as described previously [27]. Commercial reagents were purchased from Cayman Chemical Co. All samples from each subject were analysed in duplicate on the same plate. The intra- and inter-assay coefficients of variation for this method were 7% and 14% respectively.

Measurement of tocopherol levels

Plasma levels of α- and γ-tocopherol were assayed by using HPLC with fluorescence detection [28]. In brief, 500 μl of plasma was extracted with 500 μl of ethanol

containing 0.005% butylated hydroxytoluene and 2 ml of hexane. A volume of 20 μl of the supernatant was injected into an HPLC column (LiChrospher 100 NH₂; 250 mm × 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 the sum of cholesterol and triacylglycerol concentrations in serum.

Statistical analyses

The results were analysed using the software systems Statistical Analysis System and STATA (Stata Corp.). For analyses of differences between the two treatment groups, unpaired Student's *t* tests were used. All variables (except 2,3-dinor-TXB₂ levels) had a skewed distribution, and were logarithmically transformed prior to the *t*-test.

RESULTS

This was a double-blind controlled trial in 24 middle-aged men with abdominal obesity who were treated for 1 month with mixed isomers of CLA (4.2 g/day) or a corresponding amount of a placebo preparation containing olive oil. No participant experienced any side effects during the study period. There were no significant effects on blood pressure, blood lipids or glucose levels.

Non-enzymic lipid peroxidation, as assessed by urinary F₂-isoprostanes

Non-enzymic lipid peroxidation was assessed by the measurement of one of the major F₂-isoprostanes, 8-iso-PGF_{2α}, in morning urine samples collected before and at the end of the study period, as well as 2 and 4 weeks after the cessation of CLA treatment. Daily ingestion of CLA for 1 month resulted in an increase in basal urinary levels of 8-iso-PGF_{2α} (difference between changes in control and CLA groups: *P* < 0.0001), to about four times the mean basal level (Table 2). No such increment in the urinary levels of 8-iso-PGF_{2α} was seen in the control group. Urinary 8-iso-PGF_{2α} levels had decreased back to the basal level at 2 weeks after the cessation of CLA intake, and remained at the same level 4 weeks after the cessation of CLA intake (Figure 1)

Enzymic lipid peroxidation, as assessed by urinary 15-oxo-dihydro-PGF_{2α}

Enzymic lipid peroxidation was assessed by measurement of 15-oxo-dihydro-PGF_{2α} in morning urine samples. The urinary levels of 15-oxo-dihydro-PGF_{2α} increased significantly (*P* = 0.0013) from the basal level, being approximately doubled, in the subjects who received CLA supplements for 1 month (Table 2). No such

Table 2 Urinary levels of 8-iso-PGF_{2α}, 15-oxo-dihydro-PGF_{2α} and 2,3-dinor-TXB₂, and serum concentration of tocopherols, in control and CLA-treated groups before and after 4 weeks of treatment

Tocopherol values were corrected for lipid. The percentage change was calculated as: $\{[(\text{value after}) - (\text{value before})]/(\text{value before})\} \times 100$. Values are means \pm S.E.M. The *P*-values are for the differences between the groups; n.s., not significant

Metabolite	Control (<i>n</i> = 10)			CLA (<i>n</i> = 14)			<i>P</i> value
	Before	After	Change (%)	Before	After	Change (%)	
Urine							
8-Iso-PGF _{2α} (nmol/mmol of creatinine)	0.25 \pm 0.03	0.26 \pm 0.03	4.0	0.20 \pm 0.01	1.01 \pm 0.23	405	< 0.0001
15-Oxo-dihydro-PGF _{2α} (nmol/mmol of creatinine)	0.31 \pm 0.07	0.31 \pm 0.05	0	0.21 \pm 0.01	0.42 \pm 0.06	100	< 0.0013
2,3-Dinor-TXB ₂ (pg/mmol of creatinine)	75 \pm 5.4	78 \pm 14.6	4.9	66 \pm 8.9	77 \pm 29.6	17.0	n.s.
Serum							
α-Tocopherol (μmol/mmol)	3.43 \pm 0.12	3.36 \pm 0.12	-2.0	3.31 \pm 0.12	3.22 \pm 0.12	-3.0	n.s.
γ-Tocopherol (μmol/mmol)	0.28 \pm 0.02	0.26 \pm 0.02	-8.4	0.28 \pm 0.02	0.28 \pm 0.02	0	

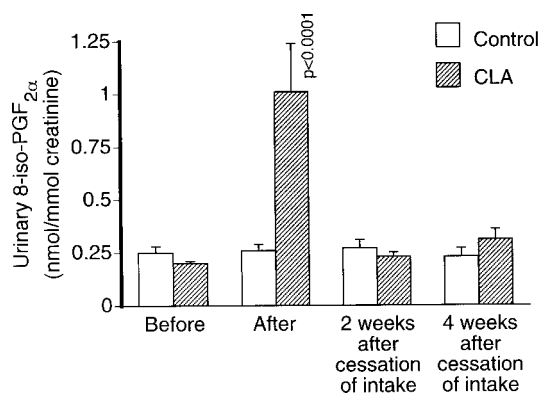


Figure 1 Urinary levels of 8-iso-PGF_{2α} in control subjects and subjects treated with CLA for 1 month

Urine was collected in the morning, before and after 1 month of dietary CLA supplementation, and 2 and 4 weeks after the cessation of treatment. The *P*-value indicates the significance of the difference between the changes in the two groups.

increment in urinary 15-oxo-dihydro-PGF_{2α} levels was seen in the control group. Urinary 15-oxo-dihydro-PGF_{2α} levels had decreased to the basal level at 2 weeks after the cessation of CLA intake, and remained at the same level 4 weeks after the cessation of CLA intake (Figure 2)

2,3-Dinor-TXB₂ levels in urine

There was no significant change in the 2,3-dinor-TXB₂ level in the urine in either group. Levels increased from 75 to 78 pg/mmol in the control group and from 66 to 77 pg/mmol in the CLA-treated subjects (Table 2). The levels of 2,3-dinor-TXB₂ at 2 and 4 weeks after cessation of CLA intake were unchanged (72 and 75 pg/mmol respectively).

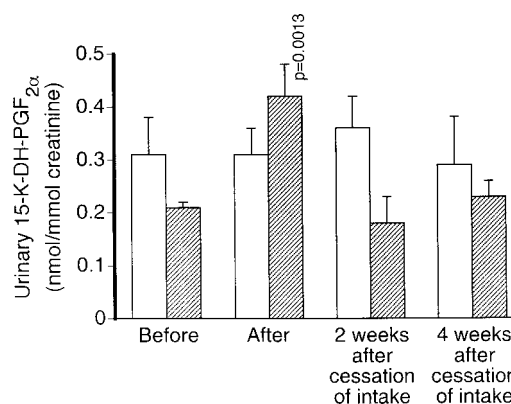


Figure 2 Urinary levels of 15-oxo-dihydro-PGF_{2α} (15-K-DH-PGF_{2α}) in control subjects and subjects treated with CLA for 1 month

Urine was collected in the morning, before and after 1 month of dietary CLA supplementation, and 2 and 4 weeks after the cessation of treatment. The *P*-value indicates the significance of the difference between the changes in the two groups.

Tocopherol levels in plasma

The lipid-adjusted serum α-tocopherol levels in the CLA and control groups were unchanged both during and after CLA supplementation (Table 2).

DISCUSSION

The major goal of the present study was to investigate both non-enzymic and enzymic lipid peroxidation in a cardiovascular high-risk group of middle-aged men with marked visceral obesity and metabolic disorders following 1 month of daily supplementation with CLA. Further aims were to study the effects on lipid peroxidation at 2 and 4 weeks after the cessation of daily CLA intake, and to investigate possible effects on thromboxane production and tocopherol concentration.

This study clearly showed that levels of both 8-iso-PGF_{2x} and 15-oxo-dihydro-PGF_{2x} in urine were significantly increased in the CLA-treated patients, demonstrating a clear effect of CLA on arachidonic acid metabolism. Recently we have shown that levels of both 8-iso-PGF_{2x} and 15-oxo-dihydro-PGF_{2x} in urine and/or plasma had increased after a 3-month supplementation with CLA in healthy subjects [24]. The present study was performed with only a 1-month intake of CLA in subjects with abdominal obesity, in order to acquire knowledge on the effects of CLA on lipid peroxidation in another population group, and on the effects of the duration of CLA intake on the induction of lipid peroxidation. We demonstrate that CLA supplementation for only 1 month is sufficient to induce a significant elevation in both F₂-isoprostane and prostaglandin metabolite levels in the urine in obese patients. The lipid oxidation parameters had returned to baseline levels just 2 weeks after the cessation of CLA intake.

8-Iso-PGF_{2x} is formed in humans and animals *in vivo* by the free-radical-catalysed oxidation of arachidonic acid, and is claimed to be a unique biomarker of non-enzymic lipid peroxidation [14,15,21]. Increased amounts of this compound are found in both the plasma and the urine in various human and animal models of oxidant injury and in dietary supplementation studies [14,15,17–20,24,29–33]. Measurement of 15-oxo-dihydro-PGF_{2x} in peripheral plasma has been applied in various species for many years as an indicator of endogenous PGF_{2x} secretion that occurs as a result of cyclo-oxygenase-catalysed lipid peroxidation [12,13,16,22,23].

The present study reports for the first time that CLA can modulate both free-radical-induced and cyclo-oxygenase-catalysed arachidonic acid oxidation in this group of subjects with a high risk of development of cardiovascular disorders. The mechanism of induction of these two important biochemical pathways cannot be evaluated from the present results. However, dietary supplementation with CLA was well tolerated, with no adverse effects on cardiovascular risk factors. CLA has been proposed to affect prostaglandin biosynthesis, in addition to its anti-carcinogenic and other beneficial properties [8,9]. Prostaglandins have been shown previously to have a cytotoxic effect on cell growth [34]. In addition, CLA-induced cytotoxicity in cancer cell lines has been shown to be associated with increased lipid peroxidation [10,11].

Although the levels of both 8-iso-PGF_{2x} and 15-oxo-dihydro-PGF_{2x} increased significantly, the relative increase was much greater for 8-iso-PGF_{2x} than for 15-oxo-dihydro-PGF_{2x}, which corroborates our earlier study in healthy subjects [24]. This indicates that CLA probably has a more direct effect on the non-enzymic than on the enzymic conversion of arachidonic acid. The concentration of 2,3-dinor-TXB₂ was unchanged after CLA treatment, indicating that the production of

thromboxanes was not affected. However, we have seen a wide variability in the levels of thromboxane metabolites in various stages and groups. CLA had no effect on α -tocopherol and γ -tocopherol levels.

The total fat consumption of our subjects was, even after addition of 4–5 g of extra fat, well within the range of that in a normal diet. We used oleic acid as a placebo control; alternatively, linoleic acid could have been used. The choice of placebo preparation (oleic or linoleic acid) should be of no major importance for the results of increased lipid oxidation after CLA supplementation. We have shown previously 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 subjects on these different diets [32].

In conclusion, dietary supplementation with CLA induces both non-enzymically and enzymically catalysed oxidation of arachidonic acid in obese patients. Further studies of the mechanism behind, and the possible consequences of, the increased lipid peroxidation that occurs following CLA supplementation are urgently required.

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