Conjugated linoleic acid increased C-reactive protein in human subjects

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We previously showed that conjugated linoleic acid (CLA) increases 15-keto-dihydro-prostaglandin $F_{2\alpha}$, a marker for cyclooxygenase-mediated lipid peroxidation and thus an indicator of cyclooxygenase-mediated inflammation. The aim of the present study was to investigate the effects of CLA on other indicators of inflammation in human subjects, including C-reactive protein, TNF-α, TNF-α receptors 1 and 2, and vascular cell adhesion molecule-1. In a double-blind, placebo-controlled study, fifty-three human subjects were supplemented with a mixture (4·2 g/d) of the isomers *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA or control oil for 3 months. CLA supplementation increased levels of C-reactive protein ($P=0·003$) compared with the control group. However, no changes in TNF-α, TNF-α receptors 1 and 2, and vascular cell adhesion molecule-1 were detected.

Conjugated linoleic acid (CLA) is widely sold as an antiobesity product, mainly based on results from animal and *in vitro* studies. However, human studies have shown conflicting results (Blankson *et al.* 2000; Zambell *et al.* 2000; Kelly, 2001; Mougios *et al.* 2001; Risérus *et al.* 2001, 2004*a*; Smedman & Vessby, 2001), and recent investigations have even suggested an aggravated risk profile for people with the metabolic syndrome after *trans*-10,*cis*-12 CLA supplementation (Risérus *et al.* 2001, 2002*b*, 2004*a*).

Population studies show strong associations between indices of inflammation and abnormal lipid and carbohydrate metabolism, obesity and atherosclerosis (Van Lente, 2000; Grimble, 2002; Grundy, 2003; Lind, 2003; Ridker *et al.* 2003). It is, however, unclear whether different biomarkers of inflammation are merely indicators or whether they contribute to the progression and development of atherosclerotic disease (Lind, 2003).

Results from studies of the effects of CLA on parameters of inflammation have been variable. In a number of different animal and *in vitro* models, CLA has been suggested to have anti-inflammatory and/or immune-ameliorating effects (Yu *et al.* 2002). However, only a limited number of studies performed in human subjects have shown effects on inflammation. We previously found increases in C-reactive protein (CRP) levels in obese men after supplementation with CLA *trans*-10,*cis*-12 (Risérus *et al.* 2002*b*). Kelley *et al.* (2000) did not find any alterations in indices of immune status in healthy women of a mixture of CLA isomers. Albers *et al.* (2003) observed beneficial effects on the initiation of a specific response to hepatitis B vaccination in healthy women after CLA supplementation. However, the same group did not observe any effects on TNF-α response in *ex vivo* mononuclear cells (Albers *et al.* 2003). In an *ex vivo* study of rats, TNF production was decreased by CLA (Turek *et al.* 1998). In our group, we have repeatedly observed considerable increases in 15-keto-dihydro-prostaglandin (PG) $F_{2\alpha}$ after CLA supplementation in human subjects (Basu *et al.* 2000*a*,*b*; Risérus *et al.* 2002*b*; Smedman *et al.* 2004). 15-Keto-dihydro-$PGF_{2\alpha}$ is a major metabolite of $PGF_{2\alpha}$ synthesised through cyclooxygenase-catalysed lipid peroxidation, and has been shown to be an indicator of cyclooxygenase-mediated inflammation (Basu, 1998, 2003).

CRP is an acute-phase protein and a circulatory indicator of inflammation that has been suggested to be a component of the metabolic syndrome (Van Lente, 2000; Lind, 2003; Ridker, 2003). Mildly increased concentrations of CRP, within the clinical normal range, may predict future cardiovascular events (Ridker *et al.* 1997). Visceral fat has been found to be a promoter of low-grade inflammation, which can explain part of the association between CRP and features of the metabolic syndrome (Forouhi *et al.* 2001).

TNF-α is a cytokine secreted partly by the adipose tissue that, in addition to its established role in the immune system, affects the metabolism of adipose tissue and glucose homeostasis. Human obesity is positively related to TNF-α expression in adipose and muscle tissue (Hotamisligil *et al.* 1995; Nilsson *et al.* 1998), and plasma levels of TNF-α are positively associated with BMI, fasting glucose and serum triacylglycerides, and inversely associated with HDL cholesterol (Nilsson *et al.* 1998). Thus, increased TNF-α levels are associated with abnormalities characteristic of the metabolic syndrome and have further been suggested to be involved in the pathogenesis of non-insulin-dependent diabetes mellitus and insulin resistance (Nilsson *et al.* 1998).

Two soluble receptors of TNF-α,1 and 2 (sTNFR1 and sTNFR2), have been proposed to be involved in the regulation of TNF-α activity (Hotamisligil *et al.* 1997). Present at low concentrations, the soluble receptors are, under normal conditions, believed to protect the organism from the deleterious effects of TNF-α on distant targets (Hotamisligil *et al.* 1997). Serum levels of sTNFR2 have been shown to be markedly increased in obese subjects compared with lean controls (Hotamisligil *et al.* 1997). There was no corresponding difference for sTNFR1 (Hotamisligil *et al.* 1997), although sTNFR1 has been suggested to be produced by adipose tissue (Mohamed-Ali *et al.* 1999). Both sTNFR1 and sTNFR2 are positively correlated with BMI and percentage body fat (Mohamed-Ali *et al.* 1999) and insulin resistance (Hauner *et al.* 1998).

Vascular cell adhesion molecule-1 (VCAM-1) is believed to play a key role in the initiation of the atherosclerotic process by mediating the interactions between circulation leucocytes and the blood vessel wall (Krieglstein & Granger, 2001; Dichtl *et al.* 2002). VCAM-1 is directly involved in the recruitment of inflammatory cells to the vessel wall and has been shown to be directly up-regulated by several proinflammatory molecules such as CRP (Pasceri *et al.* 2000) and TNF-α (Marui *et al.* 1993).

Because the effects of CLA in human subjects we have previously observed include decreased body fat content, decreased insulin sensitivity and increased cyclooxygenase-mediated inflammation, it is important to study the effects on markers of inflammation closely related to the clinical variables previously studied.

The aim of the present study was to evaluate the effects of supplementation with equal proportions of the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12, a mixture commonly used in commercial CLA preparations, on cytokine-mediated inflammation using the biomarkers CRP, TNF-α, sTNFR1 and sTNFR2, and the effect on vascular inflammatory activation as indicated by sVCAM-1, in human subjects. A secondary aim was to investigate the change in CRP, TNF-α, sTNFR1, sTNFR2 and sVCAM-1 in relation to the increase in 15-keto-dihydro-PGF$_{2\alpha}$ previously seen. In two previous publications, we have reported on other effects of CLA supplementation in human subjects, based on the same intervention study (Basu *et al.* 2000*b*; Smedman & Vessby, 2001).

Subjects and methods

Subjects

Fifty-three women and men aged 23–63 years were included and randomly assigned to either a CLA-treated group or a control group. The study group and the study design have been described in detail elsewhere (Smedman & Vessby, 2001). Table 1 shows the baseline characteristics of the participants. At baseline, there were no statistical differences between the groups with regard to these variables (Smedman & Vessby, 2001). All subjects gave their informed consent, and the study was approved by the Ethical Committee of the Faculty of Medicine at Uppsala University.

Study design

The study was performed in a randomised, double-blind, placebo-controlled manner, as previously described in detail (Smedman & Vessby, 2001). In brief, all subjects were given control capsules containing olive oil during the first 2 weeks. For the following 12 weeks, the subjects in the CLA group were given capsules containing 4·2 g CLA/d, and the control group continued taking control capsules containing the corresponding amount of olive oil. The CLA capsules contained 75·9 % CLA with equal amounts of the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12, respectively, and only minor amounts of other isomers (Smedman & Vessby, 2001). All capsules were provided by Natural Ltd A/S (Oslo, Norway). On three occasions, before the start and during the fifth and ninth weeks of the intervention period, the subjects performed 3 d weighed dietary records, including 2 weekdays and 1 weekend day. The intake did not change during the study (Smedman & Vessby, 2001).

Examinations and samplings

The main investigations, on which the calculations were based, were made on the first day (referred to as the baseline) and the last day of the trial. The examinations and blood samplings were made in the morning after an overnight fast. Morning urinary samples were collected from the first morning urine on each examination day. Blood samples were immediately placed on ice; all the serum and plasma samples were stored at −70 °C within 1 hour after collection, and the urinary samples within 1 hour after the subjects' arrival at the clinic, until analysis. No heavy physical activity was allowed in the evening or in the morning prior to sample collection. The participants were requested not to change their dietary and physical activity habits and to abstain from any dietary supplementation with vitamins, minerals or fatty acids before and during the study. At the end of the study, the participants reported that they had not made any changes in lifestyle or physical activity during the study (data not shown).

Table 1. Baseline characteristics of the participants of the two treatment groups

(Mean values, standard deviations and ranges)

	Control (*n* 25)			CLA (*n* 28)		
	Mean	SD	Range	Mean	SD	Range
Sex (men/women)	10/15			17/11		
Age (years)	47·8	10·1	27·4–59·9	43·3	12·8	23·0–63·4
Body weight (kg)	73·2	15·5	54–109	78·1	15·0	53–105
BMI (kg/m²)	24·4	4·2	19·1–34·5	25·8	3·9	19·5–33·5
Sagittal abdominal diameter (cm)	21·8	3·1	18–28	22·3	3·2	18–31
Body fat (%)	29·4	6·8	15·9–46·2	29·1	6·9	11·4–46·7

CLA, conjugated linoleic acid.

Analysis of CRP, TNF-α, sTNFR1, sTNFR2 and sVCAM-1

The highly sensitive methods for analysing CRP, TNF-α, sTNFR1 and sTNFR2 were developed at the Department of Medicine, University Hospital MAS at Lund University. Plasma CRP was measured with the use of a rabbit anti-human CRP (Dako A/S, Glostrup, Denmark) as capture antibody, rabbit anti-human CRP (peroxidase conjugated, P0227; Dako) for detection, and human CRP high control (X0926; Dako) as standard. For all reactions, unless indicated otherwise, tetramethyl benzidine one substrate (S1600; Dako) was used as substrate. The detection limit was 0·1 μg/l and the interassay CV 8 %.

Plasma TNF-α was measured using mouse anti-human TNF-α (R&D Systems Europe; Abingdon, Oxfordshire, UK) as capture antibody, rabbit anti-human TNF-α (biotin conjugated, BAF210; R&D Systems Europe) and streptavidin conjugated with alkaline phosphatase for detection and AMPAK (K6200; Dako) as substrate. The detection limit was 0·5 pg/ml and the interassay CV 18 %.

sTNFR1 mouse anti-human TNFR1 (MAB625; R&D Systems Europe) was used for coating, and biotinylated goat anti-human TNFR1 (BAF225; R&D Systems Europe) and avidin conjugated with HRP (Dako) was used for detection. The detection limit was 6·25 pg/ml and the interassay CV 12 %.

sTNFR2 was measured in a similar way with rabbit anti-human TNFR2 (HP9003; Hbt, Uden, The Netherlands) used as coating antibody, and biotinylated mouse anti-human TNFR2 (HM2008; Hbt) and avidin conjugated with horseradish peroxidase (Dako) for detection. The detection limit was 15·6 pg/ml and the interassay CV 12 %.

sVCAM-1 was analysed with mouse anti-human VCAM-1 (MCA907, Serotec Ltd, Oxford, UK) as coating antibody and biotinylated mouse anti-human VCAM-1 (MCA1479B; Serotec) and avidin conjugated with horseradish peroxidase (Dako) for detection. The detection limit was 3·75 ng/ml and the interassay CV 7 %.

15-keto-dihydro-PGF$_{2\alpha}$

Samples of urine were analysed for 15-keto-dihydro-PGF$_{2\alpha}$, without extraction, by a radioimmunoassay as described by Basu (1998). The urinary levels of 15-keto-dihydro-PGF$_{2\alpha}$ were adjusted for urinary creatinine values.

Anthropometric measurements

The procedures for anthropometric measurements (Table 1) have previously been described in detail (Smedman & Vessby, 2001).

Statistical analyses

Skewed variables (CRP, sTNFR1 and 15-keto-dihydro-PGF$_{2\alpha}$) were logarithmically transformed until normality (Shapiro-Wilk's test for normality, $W > 0.95$). TNF-α, sTNFR2, sVCAM-1 and sagittal diameter were still not normally distributed after logarithmic transformation. For analyses of differences within a group, Wilcoxon matched-pairs signed-rank tests were used. Wilcoxon rank-sum tests were used for analyses of differences between the groups. For a *post hoc* regression analysis of the association between the changes of two variables, an analysis of the standard residuals of the regression was performed. A significance level of $P < 0.05$ was regarded as statistically significant. The statistical analyses were performed using the software STATA 6.0 (Stata Corporation, College Station, TX, USA).

Results

CRP increased after CLA supplementation compared with the control group (Table 2). In contrast, CLA supplementation did not have any statistically significant effect on the differences between the groups with regard to plasma levels of TNF-α, sTNFR1, sTNFR2 or sVCAM-1 (Table 2). There was no effect of gender or BMI (data not shown). In a *post hoc* regression analysis of the whole population (n 53), the change in CRP was correlated to the change in 15-keto-dihydro-PGF$_{2\alpha}$ (r 0·315, $P = 0.024$; data not shown). There was no correlation between the change in TNF-α and the change in 15-keto-dihydro-PGF$_{2\alpha}$.

Discussion

In this randomised, double-blind, placebo-controlled study of CLA supplementation in volunteers, we found an increase in CRP compared with control treatment (Table 2). In contrast to the effects on CRP, there was no effect on TNF-α, sTNFR1, sTNFR2 or sVCAM-1. Supplementation with *trans*-10,*cis*-12 CLA has previously been observed to increase concentrations of CRP (Risérus *et al.* 2002*b*). There are no previous results published on the effect of CLA on soluble TNF-α in human subjects *in vivo*. However, Albers *et al.* (2003) investigated the TNF-α response in mononuclear cells *ex vivo* and did not find any change after CLA supplementation in human subjects. In an *ex vivo* study of CLA-fed rats, Turek *et al.* (1998) observed decreased TNF-α production. To our knowledge, this is the first time that the effects of

Table 2. Levels of indicators of inflammation before and after the study, given as medians and ranges

| | Control (n 25) | | | | CLA (n 28) | | | | |
| | Before | | After | | Before | | After | | P for differences between the groups |
	Median	Range	Median	Range	Median	Range	Median	Range	
CRP (mg/l)	1·76†	0·07–31·5	1·24	0·08–46·4	3·27	0·02–20·6	4·95**	0·09–27·3	0·003
TNF-α (pg/ml)	4·23†	1·18–14·1	4·00	1·21–7·41	4·81	0·17–20·6	3·53*	0·87–7·42	0·174
sTNFR1 (pg/ml)	631†	291–4590	676	257–1890	528	168–1790	575	302–1620	0·219
sTNFR2 (ng/ml)	9·8†	0·8–750	12·6	0·02–640	9·5	1·6–500	7·8	1·4–590	0·485
sVCAM-1 (ng/ml)	930†	190–13 210	1010	50–25 030	680	180–7500	620	190–9480	0·912

Differences within the groups are based on Wilcoxon matched-pairs signed-rank tests, and differences between the groups are based on Wilcoxon rank-sum tests. * $P < 0.05$, ** $P < 0.01$.
CLA, conjugated linoleic acid; CRP, C-reactive protein; sTNFR, soluble TNF-α receptor; sVCAM-1, soluble vascular cell adhesion molecule-1.
† n 24.

CLA supplementation on plasma concentrations of sTNFR1, sTNFR2 and VCAM-1 have been studied in human subjects.

An increased CRP concentration, even at sub-clinical levels, is associated with the presence of the metabolic syndrome and predicts incident myocardial infarction, stroke, peripheral arterial disease and sudden cardiac death (Ridker, 2003). Like CRP, TNF-α has been associated with the metabolic syndrome (Sethi & Hotamisligil, 1999). In contrast to CRP, however, TNF-α has, in *in vitro* studies, been connected with several mechanistic steps of importance for the development of the metabolic syndrome (Sethi & Hotamisligil, 1999).

There was no effect on sTNFR1, sTNFR2 or sVCAM-1, although these have been related to obesity and insulin resistance (Hauner *et al.* 1998; Mohamed-Ali *et al.* 1999), which have been suggested to be affected by CLA or purified CLA isomers (Smedman & Vessby, 2001; Risérus *et al.* 2002a).

CLA supplementation in human subjects has repeatedly been shown to increase urinary concentrations of 15-keto-dihydro-PGF$_{2\alpha}$, a marker of cyclooxygenase-catalysed lipid peroxidation and an indicator of inflammation (Basu *et al.* 2000a,b, Risérus *et al.* 2002b, 2004b; Smedman *et al.* 2004). Moreover, the increases in CRP and 15-keto-dihydro-PGF$_{2\alpha}$ are correlated. Together, these results suggest a proinflammatory effect of CLA in human subjects, which may possibly be mediated by an increased free radical-induced lipid peroxidation.

The effects of CLA are often isomer specific. In a previous study, the effect on CRP was mainly ascribed to *trans*-10,*cis*-12 CLA (Risérus *et al.* 2002b), whereas no change in CRP was seen in a smaller intervention study with *cis*-9, *trans*-11 CLA (U. Risérus, personal communication). In the present study, we used a mixture of isomers, with equal proportions of the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12. It is thus interesting that, with smaller absolute amounts of *trans*-10,*cis*-12, we still could obtain significant indications of proinflammatory effects. *Trans*-10,*cis*-12 CLA is also known to induce larger increases in 15-keto-dihydro-PGF$_{2\alpha}$ than does *cis*-9,*trans*-11 CLA (Basu *et al.* 2000a; Smedman *et al.* 2004).

In conclusion, we have observed increases in CRP, a biomarker of cytokine-mediated inflammation in human subjects after supplementation with equal proportions of the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12, a mixture commonly used in commercial CLA preparations. It is interesting that CLA supplementation increased the level of CRP but not of the other inflammatory markers investigated in the present study. The clinical implications of the findings of this study are uncertain, but the results are of concern, considering the widespread use of CLA.

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