Supplementation With Conjugated Linoleic Acid Causes Isomer-Dependent Oxidative Stress and Elevated C-Reactive Protein

A Potential Link to Fatty Acid–Induced Insulin Resistance

Ulf Risérus, MMed; Samar Basu, PhD; Stefan Jovinge, MD, PhD; Gunilla Nordin Fredrikson, PhD; Johan Årnlöv, MD; Bengt Vessby, MD, PhD

Background—Conjugated linoleic acids (CLAs), a group of fatty acids shown to have beneficial effects in animals, are also used as weight loss supplements. Recently, we reported that the \( t_{10}c_{12} \) CLA-isomer caused insulin resistance in abdominally obese men via unknown mechanisms. The aim of the present study was to examine whether CLA has isomer-specific effects on oxidative stress or inflammatory biomarkers and to investigate the relationship between these factors and induced insulin resistance.

Methods and Results—In a double-blind placebo-controlled trial, 60 men with metabolic syndrome were randomized to one of 3 groups receiving \( t_{10}c_{12} \) CLA, a CLA mixture, or placebo for 12 weeks. Insulin sensitivity (euglycemic clamp), serum lipids, in vivo lipid peroxidation (determined as urinary 8-iso-PGF\(_2\alpha\), [F2-isoprostanes]), 15-ketodihydro PGF\(_2\alpha\), plasma vitamin E, plasma C-reactive protein, tumor necrosis factor-\( \alpha \), and interleukin-6 were assessed before and after treatment. Supplementation with \( t_{10}c_{12} \) CLA markedly increased 8-iso-PGF\(_2\alpha\) (578%) and C-reactive protein (110%) compared with placebo (\( P<0.0001 \) and \( P<0.01 \), respectively) and independent of changes in hyperglycemia or dyslipidemia. The increases in 8-iso-PGF\(_2\alpha\), but not in C-reactive protein, were significantly and independently related to aggravated insulin resistance. Oxidative stress was related to increased vitamin E levels, suggesting a compensatory mechanism.

Conclusions—\( t_{10}c_{12} \) CLA supplementation increases oxidative stress and inflammatory biomarkers in obese men. The oxidative stress seems closely related to induced insulin resistance, suggesting a link between the fatty acid–induced lipid peroxidation seen in the present study and insulin resistance. These unfavorable effects of \( t_{10}c_{12} \) CLA might be of clinical importance with regard to cardiovascular disease, in consideration of the widespread use of dietary supplements containing this fatty acid. (Circulation. 2002;106:1925-1929.)

Key Words: fatty acids ■ inflammation ■ free radicals ■ insulin ■ syndrome x
and enzymatic lipid peroxidation in vivo, respectively, F2-isoprostanes are probably the most reliable and clinically relevant marker of oxidative stress available. Inasmuch as CLA supplements are widely used among obese subjects, it seemed relevant to study men with abdominal obesity—a high-risk group for cardiovascular disease that might be particularly vulnerable to the possible proinflammatory effects of CLA.

Methods

Subjects
This is the second report from a controlled trial in Uppsala, Sweden, involving men aged 35 to 65 years. Inclusion criteria were set to recruit men with metabolic syndrome: waist circumference >102 cm, waist to hip ratio >0.95, body mass index 27 to 39 kg/m², triglycerides (TG) >1.7 mmol/L and/or high-density lipoprotein (HDL) cholesterol <0.9 mmol/L. Subjects on antidiabetic/lipid-lowering drugs, nonsteroidal antiinflammatory drugs, or dietary supplements, and subjects with CRP >10 mg/L or previously diagnosed heart, liver, or renal disease were excluded. All subjects gave their written consent, and the Ethics Committee of Uppsala University, Medical Faculty, approved the protocol.

Protocol
Urinary 8-iso-PGF₂α and 15-K-DH-PGF₂α, plasma CRP, TNFα, IL-6, and vitamin E concentrations were determined before and after a 3-month intervention. In addition to these variables, insulin sensitivity, fasting glucose, and lipoprotein lipid levels were previously determined, and metabolic data, including protocol, have been described. In brief, 60 men were randomly assigned to receive 6 capsules/d of 3.4 g CLA (isomeric mixture), 3.4 g purified r10c12 CLA, or 3.4 g placebo (olive oil). The major isomer content of the CLA preparation (80% free fatty acids) was 35.9% r10c12 CLA and 35.4% 9/11 CLA. In r10c12 CLA preparation, it was 76.5% r10c12 CLA and 11.4% 18:1n-9. All preparations (identical in appearance) were prepared by Natural Lipids Ltd (Hovebygda, Norway), which also generated the randomization numbers and the double-blind labeling. All men had fasted for 12 hours and had restrained from smoking, alcohol, and exercise in the morning and the day before visits. For each subject, all blood and urine samples were collected on the same morning. Subjects were encouraged to maintain their usual diet and exercise habits throughout the study.

Nonenzymatic Lipid Peroxidation
Urinary samples obtained (first urination in the morning) were analyzed for free 8-iso-PGF₂α, without any extraction, with the use of a highly specific and sensitive radioimmunoassay, as previously described. The radioimmunoassay has a detection limit of 23 pmol/L. Intra- and inter-coefficient of variation (CV) were 4.5% and 7.5%, respectively, 8-iso-PGF₂α levels were adjusted for creatinine values measured with a commercial kit (IL Test, Monarch Instrument).

Enzymatic Lipid Peroxidation
Urinary samples were also analyzed for 15-K-DH-PGF₂α, a major metabolite of PGF₂α, without any extraction by radioimmunoassay, as described previously.

Vitamin E
Plasma tocopherols were assayed by high-pressure liquid chromatography with fluorescence detection as described previously. Tocopherol levels were adjusted for the sum of serum cholesterol and TG levels.

CRP, TNFα, and IL-6
Highly sensitive methods for analyzing CRP, TNFα, and IL-6 have been developed in the Department of Medicine at the University Hospital MAS at Lund University. Plasma CRP was measured with the use of a rabbit antihuman CRP (Dako A/S, Glostrup, Denmark) as capture antibody, rabbit antihuman CRP ( Peroxidase conjugated, Dako P0227), Human CRP high control (Dako x0926) as standard, and TMB one substrate (Dako S1600) as substrate. The detection limit was 0.1 µg/L (Inter-CV=8%). Plasma TNFα was measured with the use of mouse antihuman TNFα (R&D Systems Europe, Abingdon, Oxon, United Kingdom) as capture antibody, rabbit antihuman TNFα (Biotin conjugated, R&D BAF210) as detection antibody, and standard Streptavidin conjugated ALP (AMPAK Dako K6200) as substrate. Detection limit was 0.5 pg/mL (Inter-CV=18%). IL-6 was measured in EDTA-plasma with the use of mouse antihuman IL-6 (R&D systems MAB206) as capture, goat antihuman IL-6 (Biotin conjugated, R&D BAF 206-IL) as detection, and substrate as for TNFα. Detection limit was 0.2 pg/mL.

Euglycemic Clamp
A euglycemic hyperinsulinemic clamp was used to determine insulin sensitivity in vivo according to the method described by DeFronzo et al, slightly modified as previously described. Plasma glucose levels were assayed in a Beckman Glucose analyzer II (Beckman Instruments) by use of an enzymatic method. Insulin sensitivity (M) was calculated as the glucose infusion rate adjusted for body weight during the last hour of the clamp (mg · kg body wt · min⁻¹). Biochemical Analyses
Venous blood was drawn into vacuum tubes, coagulated, and centrifuged at room temperature and then frozen at −20°C. Serum samples were stored at −70°C. All samples from each subject were analyzed within the same analytic run. Plasma insulin was measured with the use of ELISA-kit (Merckodia AB) in a Bio-Rad Coda automated ELA analyzer (Bio-Rad Laboratories AB). Lipoproteins were isolated from fresh serum with a combination of preparative ultracentrifugation and precipitation with a sodium phosphotungstate and magnesium chloride solution. Serum lipoproteins were assayed by enzymatic techniques with a Monarch 2000 centrifugal analyzer (Instrumentation Laboratories).

Statistics
Values are mean±SD. Variables with skewed distributions were logarithmically transformed to achieve a normal distribution. Differences between the 3 groups from baseline to 12 weeks were assessed by use of an overall test by use of ANOVA. In case of a significant overall test, ANOVA and ANCOVA were used to test the differences between the 2 groups. Pearson’s or Spearman’s correlation coefficient was determined by pairwise and partial correlations. If not otherwise stated, correlations are calculated on n=56. A two-tailed probability value <0.05 was regarded as significant. For statistics, JMP software package was used (SAS Institute Inc).

Results
At baseline, there were no significant differences between the groups, except that CRP was significantly lower in the CLA group (Table 1). Of 60 randomized patients, 57 completed the study. Reasons for withdrawal included weight gain (r10c12 CLA), gastrointestinal symptoms (CLA), and hypertension (placebo). For CRP and IL-6 analysis, one subject (on placebo) was excluded for having CRP >10 mg/L because of a common cold. Urinary sample collection was incomplete in one subject (r10c12 CLA).

Treatment Effects
The r10c12 CLA isomer markedly increased lipid peroxidation as measured by both 8-iso-PGF₂α and 15-K-DH-PGF₂α (Figure 1, A and B). The significant increase from baseline in 8-iso-PGF₂α, 15-K-DH-PGF₂α and CRP after r10c12 CLA was 1.04±0.7 (578%), 0.30±0.31 (77%), and 2.89±3.66
TABLE 1. Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=19)</th>
<th>CLA (n=19)</th>
<th>t10c12 CLA (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>53±10.1</td>
<td>51±7.1</td>
<td>55±7.1</td>
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<tr>
<td>Body mass index</td>
<td>30.2±1.8</td>
<td>30.1±1.8</td>
<td>31.2±2.5</td>
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<tr>
<td>VLDL triglycerides, mmol/L</td>
<td>1.5±0.9</td>
<td>1.2±0.4</td>
<td>1.9±2.1</td>
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<td>LDL cholesterol, mmol/L</td>
<td>4.0±0.9</td>
<td>3.8±0.7</td>
<td>4.0±1.1</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.0±0.1</td>
<td>1.0±0.2</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/L</td>
<td>5.7±0.6</td>
<td>5.9±0.7</td>
<td>5.6±0.6</td>
</tr>
<tr>
<td>M, mg·kg body weight−1·min−1</td>
<td>3.7±1.6</td>
<td>4.5±1.5</td>
<td>3.9±1.5</td>
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<tr>
<td>Urinary 8-iso-PGF2α, nmol/mmol creatinine</td>
<td>0.14±0.07</td>
<td>0.16±0.05</td>
<td>0.18±0.06</td>
</tr>
<tr>
<td>Urinary 15-K-DH-PGF2α, nmol/mmol creatinine</td>
<td>0.42±0.15</td>
<td>0.39±0.15</td>
<td>0.39±0.14</td>
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<tr>
<td>α-Tocopherol, μmol/100 mL</td>
<td>3.16±0.31</td>
<td>2.96±0.34</td>
<td>3.19±0.39</td>
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<tr>
<td>γ-Tocopherol, μmol/100 mL</td>
<td>0.20±0.07</td>
<td>0.20±0.09</td>
<td>0.24±1.0</td>
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<tr>
<td>C-reactive protein, mg/L</td>
<td>2.14±2.09</td>
<td>1.17±0.82*</td>
<td>2.62±1.77</td>
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<tr>
<td>TNFα, pg/mL</td>
<td>1.8±2.7</td>
<td>0.6±1.0</td>
<td>1.96±3.29</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>0.92±1.11</td>
<td>0.56±1.34</td>
<td>1.18±1.67</td>
</tr>
</tbody>
</table>

Values are mean±SD.
M indicates insulin sensitivity.
*P<0.05 vs t10c12CLA.

(110%), respectively. The absolute change in these parameters after CLA treatment was 0.25±0.07, 0.14±0.18, and 0.48±0.75, respectively; after placebo treatment it was 0.01±0.17, 0.02±0.17, and −0.17±1.76, respectively. The increase of 8-iso-PGF2α after t10c12 CLA was independent of changes in all other variables, including smoking at baseline. The t10c12 CLA induced insulin resistance as previously reported.8 The significant reduction of insulin sensitivity (M) was abolished when the changes (Δ) of Δ8-iso-PGF2α and Δ very low density lipoprotein (VLDL)-TG were corrected for (ANCOVA analysis), but remained statistically unchanged after correction for changes in CRP and all other measured variables.

CRP increased significantly compared with placebo after t10c12 CLA (110%; P=0.007) but not after CLA (P=0.10) (Figure 1C). CRP significantly increased within the CLA group compared with baseline (41%; P=0.009). The t10c12 CLA–induced increase in CRP was independent of changes in cytokines, metabolic variables, and body mass index, but was abolished when adjusted for changes of 8-iso-PGF2α (P>0.78). TNFα, IL-6, and α- and γ-tocopherol were not significantly changed in any group (data not shown).

Baseline Correlations
At baseline, there were no correlations between insulin sensitivity and markers of oxidative stress, inflammation, lipoproteins, and glucose are shown in Table 2. The significant correlation between insulin sensitivity and 8-iso-PGF2α (Figure 2) was independent of changes in all other variables, including smoking. The significant associations between Δ8-iso-PGF2α and ΔVLDL-TG with Δinsulin sensitivity are shown as scatter diagram in Figure 2.

Changes in CRP only correlated to those of Δ8-iso-PGF2α (Figure 2), and this relationship persisted after adjustment for changes in all measured variables. The only significant Δcorrelations within the t10c12 CLA group (n=19) were between Δ8-iso-PGF2α and 15-K-DH-PGF2α (r=0.68, P<0.001), and between Δ8-iso-PGF2α and Δα- and Δγ-tocopherol (r=0.58, P<0.05 and r=0.57, P<0.05, respectively).

Correlations Between Changes Over Time
Correlations between changes over time (Δ) in insulin sensitivity and markers of oxidative stress, inflammation, lipoproteins, and glucose are shown in Table 2. The significant correlation between insulin sensitivity and 8-iso-PGF2α (Figure 2) was independent of changes in all other variables, including smoking. The significant associations between Δ8-iso-PGF2α and ΔVLDL-TG with Δinsulin sensitivity are shown as scatter diagram in Figure 2.

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Discussion
This randomized, double-blind, placebo-controlled trial demonstrates that dietary supplementation with t10c12CLA causes isomer-specific oxidative stress that is related to
induced insulin resistance. These results are strengthened by the methods used because 8-iso-PGF$_{2\alpha}$ is probably the most valid direct measure of oxidative stress in vivo, and the euglycemic clamp is regarded as the gold standard for determining insulin action.

In contrast to the antioxidative effects observed in vitro, CLA, and especially t10c12 CLA, is pro-oxidative in vivo in humans, which is supported by our earlier data on CLA mixtures. Furthermore, elevated CRP and 15-K-DH-PGF$_{2\alpha}$ indicate a marked proinflammatory effect of t10c12 CLA. Whether or not the CRP increase (41%) within the CLA mixture group, which was not significantly different from placebo, is clinically relevant is uncertain, but the strong link between elevated CRP and coronary risk might cause some concern.

We have recently reported that t10c12 CLA treatment increased insulin resistance and that the impairment of insulin sensitivity was related to ΔVLDL-TG. Interestingly, in the present study, the t10c12 CLA–induced insulin resistance was statistically abolished when Δ8-iso-PGF$_{2\alpha}$, but not the changes of CRP or cytokines were adjusted for, suggesting that oxidative stress might independently contribute to the fatty acid–induced insulin resistance.

The increase in 8-iso-PGF$_{2\alpha}$ after t10c12 CLA was independently related to insulin resistance and hyperglycemia. Furthermore, Δ8-iso-PGF$_{2\alpha}$ and ΔVLDL-TG were both related to insulin resistance, but independently from each other, which is in accord with the lack of a correlation between ΔVLDL-TG and Δ8-iso-PGF$_{2\alpha}$. However, oxidative stress might contribute more to the t10c12 CLA–induced insulin resistance than VLDL-TG does, inasmuch as there was a significant correlation between VLDL-TG and M at baseline that remained unchanged after intervention. However, there was no correlation between M and 8-iso-PGF$_{2\alpha}$ at baseline until after the treatment (Table 2). This suggests an intervention-mediated correlation between changes in oxidative stress and impairment of insulin sensitivity.

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Correlation between changes in lipid peroxidation, insulin sensitivity, inflammation, and VLDL-TG. Pearson correlation coefficients are presented.

To our knowledge, this is the first evidence from a controlled trial linking increased F2-isoprostanes to insulin resistance, here induced by t10c12CLA. Caranoni et al suggested that lipid peroxidation is an early sign of insulin resistance, a hypothesis that is supported by our results.

There are several possible explanations for how oxidative stress might contribute to insulin resistance. In vitro studies indicate that oxidative stress impairs GLUT-4 translocation, which is relevant because insulin resistance involves defective postreceptor insulin signaling. Furthermore, oxidized lipids may promote endothelial dysfunction which could mediate oxidative stress–induced insulin resistance. Indeed,
observational data by Gopaul et al. suggest that lipid peroxidation could precede insulin resistance and endothelial dysfunction, and in subjects with type 2 diabetes, plasma 8-iso-PGF2α correlated to both endothelial dysfunction and insulin resistance. Here, endothelial function was not measured. Vasoconstrictive effects of 8-iso-PGF2α might also contribute to insulin resistance via decreased blood flow.

Neither CLA preparation affected serum tocopherol levels, but the correlation between Δ8-iso-PGF2α and tocopherols within the t10c12 CLA group suggests a compensatory response to elevated oxidative stress. The correlation between Δ8-iso-PGF2α and fasting glucose seen in the present study (Table 2) is in accord with previous associations between improved metabolic control and decreased 8-iso-PGF2α levels in non-insulin-dependent diabetes mellitus and findings that 8-iso-PGF2α plays a role in acute hyperglycemia. Notably, 8-iso-PGF2α here correlated to insulin resistance independently of Δglucose.

Similar to t10c12 CLA, smoking also increases lipid peroxidation and impairs insulin action. The relative increase (578%) in urinary F2-isoprostanes after t10c12 CLA supplementation is considerably higher than that observed in heavy smokers. Elevated 8-iso-PGF2α is present in human atherosclerotic lesions and subjects with type 2 diabetes have 2-fold higher urinary levels of 8-iso-PGF2α compared with controls. Thus, the t10c12 CLA–induced lipid peroxidation is a unique example of aggravated oxidative stress, insulin resistance, and inflammation that might be clinically relevant. The dietary content of t10c12 CLA is very small, but CLA supplements usually contain 40% t10c12 CLA, an amount that might be proatherogenic if consumed on a long-term basis by subjects with metabolic syndrome. Clearly, the pro-oxidative effects of CLA are isomer specific and stand in contrast to the cardioprotective fish oil supplements that do not increase F2-isoprostanes.

Acknowledgments

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References

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