Conjugated Linoleic Acid Deteriorates Insulin Resistance in Obese/ Diabetic Mice in Association with Decreased Production of Adiponectin and Leptin

Atsuko OHASHI, Yukiko MATSUSHITA1, Haruki SHIBATA2, Kazuhiro KIMURA1, Kazuo MIYASHITA2 and Masayuki SAITO1,*

Department of Clinical Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Tohbetsu, Hokkaido 061-0293, Japan
1Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
2Morinaga Institute of Biological Science, Tsurumi, Yokohama 230-8504, Japan
3Department of Bioresources Chemistry, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan

(Received June 28, 2004)

Summary Dietary supplementation of conjugated linoleic acids (CLA) is known to have some beneficial effects such as anti-carcinogenic and anti-obesity effects in several animal species, while it also induces insulin resistance and fatty liver, especially in mice. To explore the possible factors responsible for the CLA-induced insulin resistance, we examined the plasma and mRNA expression levels of several adipocytokines, which are likely involved in the regulation of insulin sensitivity, in normal C57BL, mildly obese/diabetic KK and morbidly obese/diabetic KKAy mice. Feeding a diet supplemented with 0.5% CLA oil consisting of 30.5% c9, t11-CLA and 28.9% t10, c12-CLA for 4 wk resulted in a decrease in white adipose tissue (WAT), an increase in liver weight with excess accumulation of triglyceride, and insulin resistance associated with hyperglycemia and hyperinsulinemia. The plasma and WAT mRNA levels of leptin were higher in KK and KKAy mice than C57BL mice, whereas those of adiponectin were higher in C57BL mice. CLA-feeding decreased the levels of leptin, adiponectin and resistin, especially in KK and KKAy mice. In contrast, tumor necrosis factor-α (TNFα) mRNA levels were higher in KK and KKAy mice than C57BL mice, and were increased by CLA feeding. The present results thus indicate that CLA feeding promotes insulin resistance in obese/diabetic mice by at least inverse regulation of leptin and adiponectin, and TNFα, adipocytokines known to either ameliorate or deteriorate insulin sensitivity, respectively.

Key Words adipocytokine, CLA, mouse, insulin resistance

Conjugated linoleic acids (CLA) are a class of geometric and positional isomers of linoleic acid found in some foods such as ruminant meat and dairy products. Previous reports have demonstrated some beneficial effects of dietary CLA in various animal species (1, 2) such as anticarcinogenic properties in rodents (3), and delaying the onset of atherosclerosis in rabbits (4) and hamsters (5). CLA-supplemented diets also cause massive reduction of body fat mass in rodents (6), pigs (7), and probably in humans (8). This CLA-induced body fat loss seems to be due to increased energy expenditure associated with sympathetic nerve activation, rather than decreased food intake (9), and is isomer specific to t10,c12-CLA, but not c9,t11-CLA (10, 11).

Since excessive accumulation of body fat usually leads to impaired glucose and lipid metabolism including insulin resistance and fatty liver, CLA feeding would be expected to improve such metabolic disorders (1, 2). However, the effects of dietary CLA on glucose and lipid metabolism are rather controversial in various animal species. Houseknecht et al. (12) reported that CLA feeding improved insulin sensitivity and glucose tolerance in Zucker diabetic fatty fa/fa (ZDF) rats. In contrast, CLA feeding has been reported to induce hyperglycemia, hyperinsulinemia and insulin resistance in both obese and normal mice (13, 14) and also in human subjects (8, 15). Moreover, dietary CLA supplementation resulted in marked hepatomegaly associated with an excessive accumulation of intracellular triglyceride, probably due to hyperinsulinemia (11, 14).

The objectives of the present study are to confirm the adverse metabolic effects of CLA and to examine the underlying mechanisms, with special references to the...
Adipocytokines in CLA-Induced Insulin Resistance

possible involvement of adipocyte-derived cytokines, adipocytokines (16), in insulin resistance. We used three strains of mice, normal C57BL, mildly obese/diabetic KK, and morbidly obese/diabetic KKAy mice, and examined the blood levels and mRNA expression of leptin, resistin, adiponectin and tumor necrosis factor-α (TNFα) in white adipose tissue (WAT), all of which are proposed as adipocytokines for the modulation of glucose metabolism and insulin sensitivity (16–21).

MATERIALS AND METHODS

Animals and diets. Female C57BL, KK, KKAy mice were obtained from CLEA Japan Inc. (Tokyo) at 6 wk of age and kept at 23°C with a 12-h light-dark cycle. They were fed on laboratory chow (Nosan, Yokohama, Japan) for 1 wk to stabilize the metabolic conditions, and then on a semi-synthetic diet either with or without CLA for 4 wk. The composition of the diet (w/w) was: 40.5% corn starch, 13.0% dextrin, 10.0% sucrose, 20.0% casein, 5% cellulose, 10% mineral mixture, 1% vitamin mixture, and either 7% soybean oil (control diet) or 6.5% soybean oil plus 0.5% CLA oil (CLA diet). CLA oil, made up of 79.8% triacylglycerol, 13.6% diacylglycerol, 5.0% free fatty acid, and 1.6% monoacylglycerol, was kindly provided by Kaneka Co. (Hyogo, Japan). The fatty acid composition of the CLA oil was 30.5% c9, t11-CLA, 28.9% t10, c12-CLA, 25.1% oleic acid, and 8.5% palmitic acid. Thus the total CLA content in the diet was 0.15% c9, t11-CLA and 0.15% t10, c12-CLA. The food intake and body weight were monitored at regular intervals. On week 3, mice were fasted overnight (for 14–16 h), and used for an insulin tolerance test. On week 4, they were fasted for 2, and sacrificed by cervical vertebral dislocation, and blood was collected in heparinized tubes to prepare plasma samples. Liver, spleen, gastrocnemius muscle, parametrial WAT, and interscapular brown adipose tissue (BAT) were isolated, weighed and preserved in RNA Later (Ambion, Austin, USA) until RNA extraction. The experimental procedures and care of animals were approved by the Animal Care and Use Committee of Hokkaido University.

Insulin tolerance test. For insulin tolerance testing, human insulin (Novo, Nordisk, Denmark) was injected intraperitoneally (0.75 U/kg body weight), and blood samples were obtained from the tail vein before and 15–120 min after the insulin injection. Blood glucose was measured immediately upon collection using an automatic glucose oxidase apparatus (Medi-Safe, Terumo, Tokyo) and integrated for the 120 min period.

Northern blot analysis. Total RNA was extracted from WAT using TRIzol (Invitrogen, CA, USA), and subjected to Northern blot analysis of leptin, adiponectin, TNFα and resistin mRNAs. Briefly, 20 μg of total RNA was separated by electrophoresis in 0.17% formaldehyde/1% agarose gels, transferred to nylon membranes (Hybond-N+, Amersham Bioscience, NJ, USA). The following cDNA probes were synthesized by PCR and labeled with [32P]dCTP by Megaprime DNA labeling systems (Amersham Bioscience): mouse leptin (+57–+557bp, Accession No. NM_008493), mouse adiponectin (+383–+721bp, Accession No. NM_009605), mouse TNFa (+258–+695bp, Accession No. NM_013693), and mouse resistin (+134–+429bp, Accession No. AF 323080). The membranes were hybridized overnight at 65°C, washed, and exposed to an imaging plate and quantitated with an image analyzer (BAS 2500, Fuji Film, Tokyo). The membranes were also hybridized with a cDNA probe of mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH, +566–+1017, Accession No. M32599) as a reference.

Biochemical analyses. The plasma glucose was determined by a glucose oxidase kit (Wako Pure Chemicals, Osaka, Japan), while plasma levels of insulin, leptin and resistin were assayed by respective ELISA kits (Morinaga Institute of Biological Science, Yokohama, Japan). Plasma levels of adiponectin and TNFα were assayed by a mouse adiponectin RIA kit (LINCO Research, Inc., Missouri, USA) and mouse TNFα ELISA kit (Bender MedSystems, Vienna, Austria), respectively. Triglyceride content in the liver was measured using a triglyceride assay kit (Wako Pure Chemicals). The homeostasis model assessment of insulin resistance (HOMA-R) values were calculated from plasma glucose (mg/dL) × plasma insulin (μU/mL)/405.

Table 1. Body and organ weights, triglyceride content in the liver, and food intake in C57BL, KK and KKAy mice fed on a control or a CLA diet for 4 wk.

<table>
<thead>
<tr>
<th></th>
<th>C57BL</th>
<th></th>
<th>KK</th>
<th></th>
<th>KKAy</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CLA</td>
<td>Control</td>
<td>CLA</td>
<td>Control</td>
<td>CLA</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>23.0±0.2</td>
<td>22.7±0.4</td>
<td>38.0±0.9</td>
<td>34.9±1.2*</td>
<td>44.5±0.8</td>
<td>37.0±1.0*</td>
</tr>
<tr>
<td>BAT (mg)</td>
<td>63±9</td>
<td>45±5</td>
<td>225±27</td>
<td>177±10</td>
<td>269±32</td>
<td>284±32</td>
</tr>
<tr>
<td>WAT (mg)</td>
<td>431±43</td>
<td>382±51</td>
<td>3.83±1.17</td>
<td>2.66±0.225*</td>
<td>5.36±163</td>
<td>3.28±178*</td>
</tr>
<tr>
<td>Liver (mg)</td>
<td>1,023±42</td>
<td>1,265±70*</td>
<td>1.469±67*</td>
<td>2.048±109*</td>
<td>2.083±91</td>
<td>2.308±89</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>79±7</td>
<td>82±3</td>
<td>116±5</td>
<td>124±6</td>
<td>123±7</td>
<td>129±10</td>
</tr>
<tr>
<td>Muscle (mg)</td>
<td>107±9</td>
<td>105±9</td>
<td>107±8</td>
<td>117±5</td>
<td>125±6</td>
<td>109±6</td>
</tr>
<tr>
<td>TG (mg/g liver)</td>
<td>89±7</td>
<td>121±14</td>
<td>103±10</td>
<td>226±13*</td>
<td>184±15</td>
<td>262±27*</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>3.29±0.60</td>
<td>3.21±0.37</td>
<td>4.21±0.25</td>
<td>4.29±0.65</td>
<td>4.88±0.63</td>
<td>4.71±0.43</td>
</tr>
</tbody>
</table>

Each value represents the mean±SE for 12 mice. *p<0.05 vs. control.

BAT, interscapular brown adipose tissue; WAT, parametrial white adipose tissue; TG, triglyceride content in the liver.
Data analysis. All values were expressed as means±SE. Statistical analysis was performed by ANOVA with a post-hoc testing.

RESULTS

Effects of CLA on food intake, body and organ weights

Table 1 summarizes food intake, body weight, and organ weight of C57BL, KK, KKAy mice fed on diets with or without CLA for 4 wk. On the control diet without CLA, KKAy, KK and C57BL mice, in this order, ate more and had heavier body and WAT weights, confirming KKAy and KK mice as models of morbid and mild obesity, respectively. The diet with CLA oil had no significant effect on body or WAT weights of C57BL mice, while it reduced those of KK and KKAy mice. However, the CLA-diet significantly increased liver weight, in association with excess accumulation of triglyceride, in C57BL and KK. These effects of CLA-diet were not obvious in morbidly obese KKAy mice, which had heavier livers and excess accumulation of triglycerides even on the control diet. The CLA-diet, compared with the control diet, did not produce any noticeable changes in food intake or the weights of BAT, spleen or skeletal muscle of the three strains of mice. All these results are consistent with previous reports (10, 11, 13, 14) and collectively indicate that dietary CLA supplementation reduces body fat and induces fatty liver to varying degrees between obese/diabetic and normal mice.

CLA-induced hyperglycemia, hyperinsulinemia and insulin resistance

The plasma glucose and insulin in KK and KKAy mice were, as expected, higher than those in normal C57BL mice. As shown in Fig. 1, dietary CLA supplementation increased plasma glucose and insulin levels and the HOMA-R value, calculated from the plasma glucose and insulin levels as an index of insulin resistance, was significantly increased in all strains of mice fed on the CLA-diet. Further, insulin resistance induced by CLA feeding was confirmed by the insulin tolerance test (Fig. 2). Although there was no significant difference in the blood glucose curves between the control and CLA-fed mice, hypoglycemic effects of insulin were less marked in KK and KKAy mice fed on the CLA diet. These results indicate that dietary CLA supplementation deteriorates insulin resistance, especially in obese/diabetic mice.

![Fig. 1. Effects of CLA feeding on plasma glucose and insulin levels. C57BL, KK and KKAy mice were fed on a control (open columns) or a CLA diet (shaded columns) for 4 wk. HOMA-R values were calculated by plasma glucose (mg/dL)×plasma insulin (μU/mL)/405. Values are means±SE for 12 mice. *p<0.05, **p<0.01.](image1)

![Fig. 2. Effects of CLA feeding on insulin tolerance. Mice were fed on a control (open symbols, open columns) or a CLA diet (closed symbols, shaded columns) for 3 wk. After overnight fasting, 0.75U/kg insulin was given intraperitoneally, and plasma glucose was monitored for 120 min. The changes in plasma glucose for 120 min are also integrated. Values are means±SE for 6 mice. *p<0.05.](image2)
Adipocytokines in CLA-Induced Insulin Resistance

Plasma and mRNA expression levels of adipocytokines

To explore the possible factors responsible for the CLA-induced insulin resistance, we examined plasma levels and mRNA expression in WAT of several adipocytokines proposed to regulate insulin sensitivity (16-21): that is, leptin and adiponectin improve insulin sensitivity, whereas TNFα and resistin impair it. As shown in Figs. 3 and 4, the plasma levels of leptin and its mRNA expression in WAT were 10-20 times higher in KK and KKAY mice than C57BL mice, and were decreased by CLA feeding. In contrast, the plasma levels and mRNA expression of adiponectin were lower in obese/diabetic mice than C57BL mice, and were decreased markedly by CLA feeding.

The plasma and mRNA expression of TNFα were much higher in KKAY and KK mice than C57BL mice. Interestingly, the plasma TNFα levels were not influenced by CLA feeding (Fig. 3) while the TNFα mRNA expression in WAT was increased by CLA feeding in C57BL and KK, but not KKAY mice (Fig. 4). The plasma and mRNA expression of resistin were lower in obese/diabetic mice than C57BL mice and CLA feeding significantly decreased the plasma levels in all three strains of mice while the mRNA expression in WAT was significantly decreased in only CLA-fed KKAY mice.

DISCUSSION

In most previous studies on the effects of dietary CLA supplementation in various animal species and also in humans, CLA has been used in the form of free fatty acids, and not the triglyceride-bound form occurring in ordinary foods such as dairy products (1). In the present study, we prepared a diet supplemented with CLA oil at 0.15% c9, t11-CLA and 0.15% t10, c12-CLA (w/w), about 80% of which was triglyceride-bound CLA. Four-week feeding of this diet produced essentially the same changes of reduced body fat content, fatty liver, and insulin resistance as reported previously using diets supplemented with free CLA (11, 13, 14). It is, thus, likely that the effects of the present diet are attributed to free CLA released by digestion of the triglyceride form (22).

It is well known that obese animals, including KK and KKAY mice used in this study, show insulin resistance (23), which is usually ameliorated by reduction of body fat. CLA-feeding would thus be expected to improve obesity-associated insulin resistance (1, 2), but this was not the case in the present study. Instead, the CLA-supplemented diet caused significant reduction of adipose tissue mass, especially in obese KK and KKAY mice, but paradoxically deteriorated insulin resistance. Of interest, insulin resistance is also known to be intimately associated with lipodystrophy (24). In fact, prolonged feeding of CLA diet of C57BL mice resulted in apoptosis of adipocytes and finally a state resembling lipoatrophic diabetes with marked reduction of adipose tissue, hepatomegaly, and severe insulin resistance (14). Further, the CLA-induced lipoatrophic insulin resistance, like other models of lipoatrophic diabetes, was reversed by leptin treatment, suggesting that leptin depletion is the critical factor for the development of insulin resistance (17). In the present study, however, the CLA-induced reduction of body fat was rather modest, and the mice still had considerable adipose tissue, particularly KK and KKAY mice, unlike the state of lipodystrophy. Moreover, the plasma leptin remained at rather higher levels even after CLA-feeding, although its mRNA expression in adipose tissue was markedly decreased. Collectively, it is difficult to consider leptin as the sole factor for the CLA-induced insulin resistance observed in the present study.

There is growing evidence that adiponectin, another protein secreted from adipocytes, is an important factor
for the regulation of glucose and lipid metabolism, and also insulin sensitivity (18, 19, 25). Plasma adiponectin and mRNA expression are reduced in obese/diabetic mice and humans (26, 27), and also in lipatrophyic mice, and adiponectin treatment reverses insulin resistance in the lipatrophyic mice (28). Furthermore, Yamauchi et al. (29) demonstrated that adiponectin improves insulin resistance in obese mice by stimulating glucose utilization and fatty-acid oxidation and thereby decreases triglyceride content in muscle and liver. Our present results also confirm decreased expression of adiponectin in obese/diabetic mice. Moreover, CLA feeding further decreased adiponectin mRNA expression and its plasma levels. Given that the lipodystrophic insulin resistance was completely reversed by the combination of physiological doses of adiponectin and leptin, but only partially by either of the adipocytokines (28), it is likely that, here, the decreased expression of adiponectin, in combination with that of leptin, contributes to the CLA-induced insulin resistance.

Instead of leptin and adiponectin, some adipocytokines have been proposed to impair insulin sensitivity. Hotamisligil and his group (20, 30) have demonstrated that TNFα is a critical cytokine responsible for obesity-associated insulin resistance. TNFα production is increased in adipose tissue of obese animals, and impairs insulin actions both in vitro (31) and in vivo (32). In fact, our results also confirm increased TNFα mRNA expression in adipose tissue of obese KK and KKAY mice, and CLA-feeding increased the expression in adipose tissue of C57BL and KK, but not KKAY mice. Moreover, Maeda et al. (33) recently reported that adiponectin-deficient mice had elevated plasma levels and expression of TNFα in adipose tissue, and administration of adiponectin decreased the TNFα levels. Thus, it appears that decreased adiponectin by CLA feeding causes the increased TNFα expression, which contributes to the CLA-induced apoptosis of adipocytes and insulin resistance (14). However, in the present study, although CLA-feeding markedly elevated TNFα mRNA expression in C57BL mice, it did not induce apparent insulin resistance. Moreover, CLA-feeding did not change the plasma TNFα or mRNA expression in KKAY mice although it deteriorated insulin resistance. These results collectively suggest a minor role of TNFα for the CLA-induced insulin resistance, at least under our experimental conditions.

Resistin may be the other adipocytokine that induces insulin resistance (21), although its precise roles seem controversial (34). In the present study, CLA-feeding paradoxically reduced plasma resistin levels in all the three-mouse strains and the mRNA expression in KKAY mice. At present, our findings do not support the notion that resistin may significantly contribute to the CLA-induced insulin resistance, although further studies to resolve the precise role of resistin are necessary.

The present results obtained in mice are quite a contrast with those reported in ZDF rats: that is, in ZDF rats CLA feeding improves glucose tolerance and the insulin sensitivity of skeletal muscle (12). Moreover, a recent report by Nagao et al. (35) demonstrated that CLA enhances mRNA expression and plasma level of adiponectin in ZDF rats and alleviates hyperinsulinemia and hypertension seen in ZDF rats. The reasons for such quite opposite effects of CLA feeding between the mouse and rat are not clear at present.

In summary, CLA-feeding reduces body fat mass in obese/diabetic mice, but further deteriorates insulin resistance. The CLA-induced insulin resistance may be due to decreased adipose production of adiponectin and possibly of leptin, adipocytokines known to be critical in amelioration of insulin resistance in both obese and lipodystrophic mice. This is a contrast with the effects of food restriction on obese animals, which reduces body fat, increases adiponectin production, and ameliorates insulin resistance.

Acknowledgments

This work was supported by a grant of PROBRAIN from Bio-oriented Technology Research Advancement Institution, Japan.

REFERENCES

12) Houseknecht KL, Vanden Heuvel JP, Moya-Camarena
Adipocytokines in CLA-Induced Insulin Resistance 421

25) Beltowski J. 2003. Adiponectin and resistin—new hor-


23) Robinson SW, Dinulescu DM, Cone RD. 2000. Genetic-


