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Dietary Conjugated Linoleic Acid Lowered Tumor Necrosis Factor-α Content and Altered Expression of Genes Related to Lipid Metabolism and Insulin Sensitivity in the Skeletal Muscle of Zucker Rats

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Type-2 diabetes is characterized by obesity-related insulin resistance. Insulin resistance and accompanying hyperinsulinemia have been reported to play an important role in pathogenesis of the metabolic syndrome. Conjugated linoleic acid (CLA), a mixture of positional and geometric isomers of linoleic acid, has attracted considerable attention because of its potentially beneficial biological effects. Previous studies showed that dietary CLA alleviates diabetes through improvement of glucose tolerance and insulin-stimulated glucose transport activity in skeletal muscle of diabetic rats. Skeletal muscle plays an important role both in insulin-mediated glucose metabolism and in lipid metabolism. In the present study, we evaluated comprehensively the effect of dietary CLA on the expression of genes related to lipid metabolism and insulin sensitivity in the skeletal muscle of obese, diabetic Zucker rats. After 8 weeks of feeding, expression of lipogenic genes was decreased in tendency, while expression of lipolytic genes was markedly increased by dietary CLA. Additionally, expression of genes-related insulin sensitivity, such as adiponectin receptor 1, was significantly enhanced, and mRNA level of peroxisome proliferator activated receptor-α, known as a transcriptional factor related lipid metabolism and insulin signaling in skeletal muscle, was markedly increased in CLA-fed rats. We also showed that dietary CLA significantly decreased the level of tumor necrosis factor-α (TNF-α), associated with the development of insulin resistance, in the skeletal muscle of Zucker rats. We suppose that the attenuated TNF-α accumulation in skeletal muscle may contribute to the alteration of expression of several genes and the alleviation of insulin resistance in CLA-fed Zucker rats.

KEYWORDS: Conjugated linoleic acid; metabolic syndrome; type-2 diabetes; peroxisome proliferator activated receptor-α; tumor necrosis factor-α; Zucker (fa/fa) rats

INTRODUCTION

Diabetes is a serious and growing health problem in developing and industrialized countries. Predicted global prevalence of diabetes in adults, on the basis of data collected by the World Health Organization Ad Hoc Diabetes Reporting Group, will rise from 135 million in 1995 to 300 million in the year 2025 (*1*). An increased risk of heart disease is well recognized as a complication of diabetes, and both type-1 and type-2 diabetes are associated with a several-fold increase in the incidence of atherogenesis (*2*−*5*). Type-2 diabetes is characterized by obesity-related insulin resistance. It has also emerged that insulin resistance and compensatory hyperinsulinemia easily evoke the metabolic syndrome, a syndrome in which multiple atherogenic risks cluster in an individual (*6*). Zucker rats develop a syndrome with multiple metabolic and hormonal disorders that share many features with human obesity (*7*−*9*). Zucker rats have hyperphagia, because they have a missense mutation on the leptin receptor gene, and become obese, developing hyperinsulinemia, diabetes, hypertension, and nonalcoholic fatty liver disease (NAFLD). Therefore, the Zucker rat is a good model for the metabolic syndrome.

Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid with conjugated double bonds. It is found in meat and dairy products, such as beef, milk, and processed cheese (*10*). CLA has attracted considerable attention because of its potentially beneficial biological effects in inhibiting carcinogenesis, attenuating atherosclerosis, reducing body fat, and alleviating hypertension in animal models and humans (*11*−*16*). In previous studies, CLA treatment has been also shown to alleviate diabetes in animal models. Houseknecht et al. demonstrated that dietary

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CLA normalized glucose tolerance and prevented the progression to hyperglycemia and diabetes in young, prediabetic Zucker diabetic fatty (ZDF) rat (*17*). Moreover, Ryder et al. showed that the alleviation of diabetes revealed that a mixture of CLA isomers could not be reproduced by the 9-cis,11-trans-isomer alone in the ZDF rat and concluded that the antidiabetic property can be specifically ascribed to the actions of the 10-trans,12-cis-isomer (*18*). Recently, we also reported that, in Zucker rats, dietary CLA improved insulin resistance induced hyperinsulinemia, NAFLD, and hypertension through the enhanced production of adiponectin, which is an adipose-derived insulin-sensitizing cytokine (*19*, *20*).

Skeletal muscle plays a major role in glucose and lipid metabolism. Because more than half of the insulin-mediated glucose metabolism occurs in skeletal muscle (*21*), an insulin-resistant state of that tissue results in a reduced sensitivity to insulin in the whole body. In fact, previous studies suggested that the abilities of CLA treatment to alleviate diabetes are associated with an improvement of glucose tolerance and insulin-stimulated glucose transport activity in insulin-resistant skeletal muscle of Zucker rats (*18*, *22*). In addition, Teachey et al. supported the possible use of combined CLA and lipoic acid as an intervention to improve insulin action on skeletal muscle glucose transport in Zucker rats (*23*). In the present study, we evaluated comprehensively the effect of dietary CLA on the expression of genes related to lipid metabolism and insulin sensitivity in the skeletal muscle of obese, diabetic Zucker rats.

MATERIALS AND METHODS

Animals and Diets. All aspects of the experiment were conducted according to the guidelines provided by the ethical committee of experimental animal care at Saga University. Male Zucker rats 6-weeks old were purchased from Japan SLC, Inc. (Shizuoka, Japan). The rats were housed individually in metal cages in a temperature-controlled room (24 °C) under a 12-h light/dark cycle. After a 1-week adaptation period, the rats were assigned to two groups (six rats each) that were fed one of two diets: a semisynthetic diet supplemented with 5% corn oil plus 1% high-linoleic safflower oil (control group) or a semisynthetic diet supplemented with 5% corn oil and 1% CLA (CLA group). The basal semisynthetic diets were prepared according to recommendations of the AIN-76 (*24*) and contained the following (in weight %): casein, 20; cornstarch, 15; cellulose, 5; vitamin mixture (AIN-76), 1; mineral mixture (AIN-76), 3.5; DL-methionine, 0.3; choline bitartrate, 0.2; fat, 6; and sucrose, 49. The rats consumed the diets for 8 weeks.

Measurement of Triglyceride Level and Fatty Acid Composition in Skeletal Muscle. Lipids were extracted from skeletal muscle and were purified according to the method of Folch et al. (*25*). The concentration of triglyceride (TG) was measured according to the method of Fletcher (*26*). Fatty acid composition of total lipid fraction was analyzed by gas−liquid chromatography.

Measurement of Tumor Necrosis Factor-α Content in Skeletal Muscle. For the assay of tumor necrosis factor-α (TNF-α) content in skeletal muscle, a piece of tissue was homogenized in a 0.1 mol/L tris-HCl buffer (pH 7.6) that contained 1 mol/L NaCl, 2% fat-free bovine serum albumin, 2 mmol/L EDTA, 80 units/L aprotinin, and 0.02% NaN_3 at 15% wet tissue wt/vol. The homogenate was centrifuged at 13 500*g* for 30 min, and the supernatant was subjected to commercial ELISA assay kit (rat TNF-α, UltraSensitive, BioSource International, Inc., Camarillo, CA).

Analysis of mRNA Expression. Total RNA was extracted from 30 mg of skeletal muscle, using a RNeasy Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan). A TaqMan Universal PCR Master Mix (Applied Biosystems, Tokyo, Japan); Assays-on-Demand, Gene Expression Products (Rn00595644_m1 for acetyl-CoA oxidase 2 (ACO2), Rn00566242_m1 for carnitine palmitoyltransferase 1b (CPT1b), Rn00580220_m1 for delta-6 desaturase, Rn00569117_m1 for fatty acid synthase (FAS), Rn00567070_m1 for insulin receptor (Ins-r), Rn00580555_m1 for monocyte chemoattractant protein 1 (MCP1), Rn00566193_m1 for peroxisome proliferator activated receptor-alpha (PPAR-α), Rn00565707_m1 for PPAR-δ, Rn00440945_m1 for PPAR-γ, Rn0000565874_m1 for uncoupling protein 3 (UCP3), Hs99999901_s1 for 18S RNA, Applied Biosystems, Tokyo, Japan), and TaqMan MGB Gene Expression Kits for adiponectin receptor (ADP-r) 1, glucose transporter 4 (GLUT4), insulin receptor substrate 1 (IRS1), and stearoyl-CoA desaturase 1 (SCD1) were used for the quantitative real-time RT-PCR analysis of ACO2, CPT1b, delta-6 desaturase, FAS, Ins-r, MCP1, PPAR-α, PPAR-δ, PPAR-γ, UCP3, 18S RNA, ADP-r1, GLUT4, IRS1, and SCD1 expression in skeletal muscle. The details of the TaqMan MGB Gene Expression Kits were as follows: ADP-r1 (forward primer, 5′-GCCTTTATGCTGCTCGGATT-3′; reverse primer, 5′-GATGAGACTGGAACCATATGTCAAA-3′; and TaqMan MGB probe, 5′-FAM-AGCGCTTCTTTCCTG-MGB-3′), GLUT4 (forward primer, 5′-AGGATGAGAAACGGAAGTTGGA-3; reverse primer, 5′-GCTGCCGGTGGGTGC-3; and TaqMan MGB probe, 5′-FAM-CAGCTCCTGGGCAGC-MGB-3′), IRS1 (forward primer, 5′-AGTTGCTCCAACCCCATCAG-3′; reverse primer, 5′-TGGTTTCCCACCCACCATAC-3′; and TaqMan MGB probe, 5′-FAM-ATCTCGAACTGAGAGCAT-MGB-3′), SCD1 (forward primer, 5′-AGCCTGTTCGTCAGCACCTT-3′; reverse primer, 5′-CACCCAGGGAAACCAGGAT-3′; and TaqMan MGB probe, 5′-FAM-CACTCTGGTGCTCAAC-MGB-3′). The amplification was performed with a real-time PCR system (ABI Prism 7000 Sequence Detection System; Applied Biosystems). Results were quantified with a comparative method and were expressed as a relative value after normalization to the 18S RNA expression.

Statistical Analysis. All values are expressed as means ± SEM, $n = 6$. Statistical analysis was carried out with Stat View J-4.5 (Abacus Concepts, Berkeley, CA). The significance of differences between means for two groups was determined by Student's *t*-test. Differences were considered significant at $P < 0.05$.

Table 1. Fatty Acid Composition of Dietary Oils[a]

	corn oil	high-linoleic safflower oil	CLA oil
16:0	11.1	6.6	5.7
16:1	0.1	0.1	n.d.
18:0	2.0	2.4	2.0
18:1	29.7	15.2	8.1
18:2	55.0	73.2	2.3
18:2-CLA	n.d.	n.d.	80.9[b]
18:3	0.9	0.2	n.d.
20:0	0.4	0.4	n.d.
20:1	0.3	0.2	n.d.
22:0	0.1	0.3	n.d.
others	0.4	1.4	1.0

[a] Wt %; n.d., not detected. [b] Contained different isomers: 46.0% of 9c,11t; 47.3% of 10t,12c; 3.2% of 9c,11c/10c,12c; and 3.5% of 9t,11t/10t,12t.

RESULTS

Although there was no difference in final body weight, body weight gain, food intake, and food efficiency between rats fed both diets, the CLA diet markedly alleviated hyperinsulinemia and nonalcoholic fatty liver disease in Zucker rats. The data from the same rats were indicated in detail in our earlier studies (*19*, *20*). Relevant data on expression of genes related to lipid metabolism and insulin sensitivity in the skeletal muscle are presented in **Table 2**. Expression of lipogenic genes, such as FAS and SCD1, tended to decrease in CLA-fed rats. In contrast, expression of lipolytic genes, such as ACO2, CPT1b, and UCP3, was markedly increased by the CLA diet. In CLA-fed rats, whose severe hyperinsulinemia and hyperglycemia were markedly alleviated to 35% and 86% of control rats, respectively, expression of ADP-r1, which relates to insulin sensitivity, was significantly enhanced in skeletal muscle. The tendency of expression of IRS1, Ins-r, and GLUT4 was also increased by the CLA diet. mRNA level of MCP1, an inflammatory molecule,

Table 2. mRNA Expression Levels in Skeletal Muscle of Rats Fed the Control and CLA Diets[a]

	control	CLA
Genes Related to Lipid Metabolism, Arbitrary Unit		
FAS	100 ± 30	46.9 ± 9.9
SCD1	100 ± 41	40.2 ± 13.6
Δ6-desaturase	100 ± 17	108 ± 8
ACO2	100 ± 14	172 ± 28[b]
CPT1b	100 ± 10	130 ± 8[b]
UCP3	100 ± 12	197 ± 22[b]
Genes Related to Insulin Sensitivity and Inflammatory, Arbitrary Unit		
Ins-r	100 ± 12	177 ± 36
IRS1	100 ± 8	156 ± 37
GLUT4	100 ± 10	139 ± 19
ADP-r1	100 ± 17	175 ± 26[b]
MCP1	100 ± 10	67.3 ± 10.3[b]
Genes of Transcriptional Factors, Arbitrary Unit		
PPAR-α	100 ± 21	167 ± 13[b]
PPAR-δ	100 ± 16	140 ± 22
PPAR-γ	100 ± 15	106 ± 14

[a] Values are means ± SEM, $n = 6$. [b] Different from the control group, $P < 0.05$.

Table 3. Triglyceride Concentration and Fatty Acid Composition of Total Lipid Fraction in Skeletal Muscle of Rats Fed the Control and CLA Diets[a]

	control	CLA
triglyceride concentration, mg/g tissue	20.6 ± 3.6	11.0 ± 1.1[b]
fatty acid composition, wt %		
14:0	1.66 ± 0.08	1.71 ± 0.10
16:0	32.3 ± 0.2	33.2 ± 0.5
16:1	9.24 ± 0.56	7.12 ± 0.47[b]
18:0	7.85 ± 0.52	9.75 ± 0.56[b]
18:1	26.1 ± 1.1	18.2 ± 0.6[b]
18:2	14.1 ± 0.4	16.5 ± 0.4[b]
9-cis,11-trans-CLA	n.d.	0.318 ± 0.041
10-trans,12-cis-CLA	n.d.	0.257 ± 0.029
20:3	0.501 ± 0.053	0.739 ± 0.050[b]
20:4	4.07 ± 0.46	5.98 ± 0.54[b]
22:4	0.225 ± 0.026	0.390 ± 0.029[b]
22:5 n-6	n.d.	0.301 ± 0.028
22:5 n-3	0.660 ± 0.074	0.831 ± 0.066
22:6	3.31 ± 0.44	4.71 ± 0.42[b]
Δ6-desaturation index (20:4/18:2)	0.287 ± 0.030	0.363 ± 0.033
Δ9-desaturation index (16:1/16:0)	0.286 ± 0.016	0.214 ± 0.011[b]
(18:1/18:0)	3.44 ± 0.37	1.91 ± 0.17[b]

[a] Values are means ± SEM, $n = 6$. [b] Different from the control group, $P < 0.05$.; n.d., not detected.

was significantly decreased in CLA-fed rats compared with the control diet. Transcriptional factors, PPAR-α, -δ, and -γ, have been reported to relate lipid metabolism and insulin signaling in skeletal muscle. In the present study, the PPAR-α mRNA level was markedly increased, and the PPAR-δ mRNA level tended to increase in rats fed CLA. These results indicate that CLA alters expression of genes related to lipid metabolism and insulin sensitivity in the skeletal muscle, and it may contribute to the alleviation of hyperinsulinemia in CLA-fed Zucker rats.

As shown in **Table 3**, CLA consumption resulted in an incorporation of CLA isomers into skeletal muscle. Consistent with the data on expression of genes related to lipid metabolism (shown in **Table 2**), TG content was decreased and fatty acid Δ9-desaturation was suppressed by CLA in total lipid fraction of skeletal muscle.

In addition, the CLA diet significantly decreased the level of TNF-α, whose accumulation in the tissue has been supposed to be a risk factor for insulin resistance, in the skeletal muscle

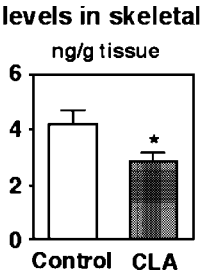

Figure 1. TNF-α content in skeletal muscle of Zucker rats fed the control and CLA diets.

of Zucker rats (**Figure 1**). The data suggested that the attenuated TNF-α accumulation in skeletal muscle also contributes to the alteration of expression of several genes and the alleviation of insulin resistance in CLA-fed Zucker rats.

DISCUSSION

We investigated the effects of dietary CLA on the expression of genes related to lipid metabolism and insulin sensitivity in obese, diabetic Zucker rats. The results indicated that dietary CLA alleviates type-2 diabetes through alterations of gene expressions related to lipid metabolism and insulin sensitivity in the skeletal muscle of Zucker rats.

Type-2 diabetes is characterized by obesity-related insulin resistance. Insulin resistance and compensatory hyperinsulinemia have been reported to play an important role in the pathogenesis of the metabolic syndrome (*2*, *6*). Skeletal muscle plays an important role both in insulin-mediated glucose metabolism and in lipid metabolism. It has been reported that more than 80% of insulin-stimulated glucose uptake is accounted for by skeletal muscle and that accumulation of lipid content in skeletal muscle can disturb insulin action and result in insulin resistance (*27*, *28*). In the present study, expression of lipogenic genes, such as FAS and SCD1, tended to decrease in CLA-fed rats. In contrast, the expression of lipolytic genes, such as ACO2, CPT1b, and UCP3, was markedly increased by the CLA diet. FAS is a key enzyme of fatty acid synthesis, and SCD-1 is a microsomal rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids (MUFAs). ACO2 and CPT1b are muscle abundant isoforms of key enzymes for long-chain fatty acid oxidation, and UCP3 is implicated in the regulation of thermogenesis as well as fatty acid metabolism in the skeletal muscle. Consistent with those alterations of gene expression, TG content was decreased and fatty acid Δ9-desaturation was suppressed by CLA in total lipid fraction of skeletal muscle. Previous studies have suggested that muscle TG content is negatively related to insulin action (*27*, *28*). Moreover, it has been recognized that MUFAs are the major fatty acid form in fat depots, and alterations in the ratio of saturated fatty acid (SFA) to MUFA have been implicated in various disease states including cardiovascular disease, obesity, and diabetes (*28*−*30*). Therefore, the ratio of SFA to MUFA is of physiological importance in normal and disease states. Ntambi et al. demonstrated that the SCD1 knockout mice were resistant to diet-induced obesity and had increased insulin sensitivity relative to the wild-type mice (*31*, *32*). We supposed that downregulated lipogenesis, including Δ9-desaturation, and upregulated lipolysis by CLA feeding contribute to the improvement of insulin sensitivity in Zucker rats.

In the present study, the expression of genes related to insulin sensitivity, such as IRS1 and ADP-r1, was enhanced in skeletal muscle by the CLA diet. Insulin-stimulated tyrosine phosphorylation of IRS1 increases glucose transport through the

enhancement of phosphatidylinositol 3-kinase activity. ADP-r1 is ubiquitously expressed, most abundantly in skeletal muscle, and exhibits high affinity to adiponectin (*33*). Adiponectin is an adipocyte-derived circulatory protein and a mediator of insulin sensitivity (*34*). Adiponectin has been shown to augment insulin-stimulated tyrosine phosphorylation of IRS-1 in the skeletal muscle of mice (*35*). ADP-r1 was also reported to mediate enhancement of fatty acid oxidation and glucose transport activity through activation of AMP kinase and PPAR-α (*33*). In skeletal muscle, insulin-induced glucose transport into cells is stimulated by increasing the translocation of GLUT4 from an intracellular pool to the plasma membrane. The intracellular signaling pathway by which insulin mediates glucose transport involves signal transduction through the Ins-r. In the present study, expression of Ins-r and GLUT4 was increased in tendency in CLA-fed Zucker rats. Evaluation of the effect of CLA on the activity of Ins-r and the translocation of GLUT4 should be addressed in future studies.

PPARs, nuclear receptors related to the modulation of environmental and dietary stimuli, are involved in gene regulation and are important regulators of glucose and lipid metabolism (*36*). In the present study, the PPAR-α mRNA level was markedly increased in rats fed CLA. Because fibrates treatment has been shown to exert lipid-lowering activity and insulin-sensitizing activity via PPAR-α in humans and rodents (*37*, *38*), we suggest that dietary CLA improves lipid metabolism and insulin sensitivity in skeletal muscle partially through the transcriptional regulation of PPAR-α. In addition, the PPAR-δ mRNA level tended to increase in rats fed CLA. Although the physiological function of PPAR-δ was not fully understood, Luquet et al. reported that treatment of obese animals by specific PPAR-δ agonists normalized metabolic parameters and reduced adiposity (*39*). This result suggests that the increased tendency of PPAR-δ mRNA level might be associated with alterations of genes related to lipid metabolism in skeletal muscle of CLA-fed rats. Although thiazolidinediones, known as insulin sensitizers, have been reported to act through the activation of PPAR-γ (*40*), we could not find any alteration in mRNA levels of PPAR-γ by CLA feeding. We need to further evaluate the effect of CLA on the activity of PPAR-γ.

Although TNF-α was identified as a preinflammatory cytokine that kills tumor cells or microorganisms, many studies suggest that TNF-α is associated with the development of insulin resistance in obese patients and in obese animal models (*41*, *42*). It has been suggested that TNF-α lowers insulin sensitivity associated with the induction of inflammatory molecules, such as MCP1, and the downregulation of the PPAR-α expression (*43*−*45*). In the present study, the CLA diet significantly decreased the level of TNF-α in skeletal muscle of Zucker rats. We also previously reported that CLA feeding suppressed mRNA expression of TNF-α in the liver of Zucker rats (*20*). Therefore, we suppose that the attenuated TNF-α accumulation in skeletal muscle may contribute to the alteration of expression of several genes and the alleviation of insulin resistant in CLA-fed Zucker rats.

ABBREVIATIONS USED

ACO, acetyl-CoA oxidase; ADP-r, adiponectin receptor; CLA, conjugated linoleic acid; CPT, carnitine palmitoyltransferase; FAS, fatty acid synthase; GLUT, glucose transporter; Ins-r, insulin receptor; IRS, insulin receptor substrate; MCP, monocyte chemoattractant protein; MUFA, monounsaturated fatty acid; PPAR, peroxisome proliferator activated receptor; SCD, stearoyl-CoA desaturase; SFA, saturated fatty acid; TG, triglyceride; TNF-α, tumor necrosis factor-α; UCP, uncoupling protein.

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