Isomer-specific actions of conjugated linoleic acid on muscle glucose transport in the obese Zucker rat

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The fatty acid-conjugated linoleic acid (CLA) enhances glucose tolerance and insulin action on skeletal muscle glucose transport in rodent models of insulin resistance. However, no study has directly compared the metabolic effects of the two primary CLA isomers, cis-9,trans-11-CLA (c9,t11-CLA) and trans-10,cis-12-CLA (t10,c12-CLA). Therefore, we assessed the effects of a 50:50 mixture of these two CLA isomers (M-CLA) and of preparations enriched in either c9,t11-CLA (76% enriched) or t10,c12-CLA (90% enriched) on glucose tolerance and insulin-stimulated glucose transport in skeletal muscle of the insulin-resistant obese Zucker (fa/fa) rat. Animals were treated daily by gavage with either vehicle (corn oil), M-CLA, c9,t11-CLA, or t10,c12-CLA (all CLA treatments at 1.5 g total CLA/kg body wt) for 21 consecutive days. During an oral glucose tolerance test, glucose responses were reduced (P < 0.05) by 10 and 16%, respectively, in the M-CLA and t10,c12-CLA animals, respectively, whereas insulin responses were diminished by 21 and 19% in these same groups. There were no significant alterations in these responses in the c9,t11-CLA group. Insulin-mediated glucose transport activity was enhanced by M-CLA treatment in both type I soleus (32%) and type IIb epitrochlearis (58%) muscles and by 36 and 48%, respectively, with t10,c12-CLA. In the soleus, these increases were associated with decreases in protein carbonyls (index of oxidative stress, r = −0.616, P = 0.0038) and intramuscular triglycerides (r = −0.631, P = 0.0028). Treatment with c9,t11-CLA was without effect on these variables. These results suggest that the ability of CLA treatment to improve glucose tolerance and insulin-stimulated glucose transport activity in insulin-resistant skeletal muscle of the obese Zucker rat is associated with a reduction in oxidative stress and muscle lipid levels and can be specifically ascribed to the actions of the t10,c12 isomer. In the obese Zucker rat, the c9,t11 isomer of CLA is metabolically neutral.

Conjugated linoleic acid (CLA) is a naturally occurring group fatty acid that is a dienoic derivative of linoleic acid, and CLA has been used as an intervention against cancer and heart disease (21). In addition, CLA has been shown to reduce body fat and induce nutrient partitioning in growing mice (6, 24, 25) and cause fat mass loss in adult humans (1). Moreover, CLA treatment has been shown to enhance glucose tolerance and insulin-stimulated glucose transport activity and glycogen synthase activity in skeletal muscle of the Zucker diabetic fatty (ZDF) rat (16, 33), a rodent model of type 2 diabetes. Although the mechanism of action of CLA for the induction of enhanced insulin action has not yet been fully identified, there is evidence that CLA can activate the peroxisome proliferator-activated receptor-γ in a variety of tissues (16). In addition, CLA possesses significant antioxidant properties (20), and this could underlie at least a part of the beneficial metabolic effect of CLA treatment.

The two major forms of CLA are the cis-9,trans-11 (c9,t11-CLA) isomer and the trans-10,cis-12 (t10,c12-CLA) isomer. Ryder et al. (33) demonstrated that the improvements in glucose homeostasis in the ZDF rat brought about by a 50:50 mixture of the c9,t11- and t10,c12-isomers (M-CLA) could not be reproduced with c9,t11-CLA alone and concluded that the metabolic improvements caused by CLA were therefore the result of the t10,c12-isomer, although this hypothesis was not tested directly. To our knowledge, there has been no study that has directly compared the individual metabolic effects of treatment with enriched preparations of c9,t11-CLA and t10,c12-CLA on whole body and skeletal muscle insulin action in an animal or human model.
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model of insulin resistance. Moreover, the potential antioxidative effects of these CLA isomers have not been studied in conditions of oxidative stress and insulin resistance.

In the context of the foregoing information, the purposes of the present investigation were 1) to determine whether the chronic treatment of insulin-resistant obese Zucker rats with M-CLA leads to an improvement of oral glucose tolerance and insulin-stimulated glucose transport in skeletal muscle; 2) to ascertain whether the chronic effects of CLA on insulin action in obese Zucker rats can be attributed to either the c9,t11 isomer or the t10,c12 isomer; and 3) to establish whether the metabolic actions of CLA isomers in obese Zucker rats are associated with reductions in oxidative stress (i.e., a diminution in the skeletal muscle level of protein carbonyls) and in intramuscular lipid concentrations.

METHODS

Animals and treatments. Female obese Zucker rats (Hsd/Ola;ZUCKER-fa; Harlan, Indianapolis, IN) were received at 7–8 wk of age. Starting at 8–9 wk of age, animals received either vehicle (corn oil), M-CLA (a mixture of CLA isomers consisting primarily of equal parts of c9,t11-CLA and t10,c12-CLA ethyl esters), a preparation 76% enriched in the ethyl ester of c9,t11-CLA (with 24% being t10,c12-CLA), or a preparation 90% enriched in the ethyl ester of t10,c12-CLA (with 10% being c9,t11-CLA) daily by gavage for 21 consecutive days at a dosage of 1.5 g total CLA/kg body wt. Animals had free access to water and chow (Teklad, Madison, WI). All procedures were approved by the University of Arizona Animal Care and Use Committee.

Oral glucose tolerance tests. After 18 days of treatments, animals were restricted to 4 g of chow after 6:00 PM the evening before the test. Starting at 8:00 AM, these animals underwent an oral glucose tolerance test (OGTT). These animals received a 1 g/kg body wt glucose load by gavage. Blood was collected from a small cut at the tip of the tail immediately before and at 30, 60, 90, and 120 min after glucose administration, thoroughly mixed with EDTA (final concentration of 18 mg/ml), and centrifuged at 13,000 g to isolate the plasma. The plasma was stored at −80°C and subsequently assayed for glucose (Sigma Chemical, St. Louis, MO) and insulin by RIA (Linco, St. Charles, MO). Basal plasma samples were also assayed for free fatty acid concentrations (WAKO, Richmond, VA). Immediately after the completion of the OGTT, all animals received 2.5 ml sterile 0.9% saline subcutaneously to compensate for plasma loss. Treatments resumed for three further days.

Glucose transport activity. Between 8:00 and 10:00 AM, ~16 h after the final treatment and again after an overnight food restriction, animals were deeply anesthetized with pentobarbital sodium (50 mg/kg ip). Both epitrochlearis muscles (predominantly type IIb fibers) and one soleus muscle (mainly type I fibers) were surgically removed and prepared for in vitro incubation. Epitrochlearis muscles were incubated intact, whereas soleus muscles were prepared in strips (weighing ~25–30 mg; see Ref. 13). Glucose transport activity, assessed as 2-deoxyglucose (2-DG) uptake, was determined in the absence or presence of a maximally effective concentration of insulin (5 mU/ml) exactly as described previously (15). This method for assessing glucose transport activity in isolated muscle has been validated (11).

Assessment of total abdominal fat. The total fat in the abdominal cavity was assessed as the sum of mesenteric fat, peritoneal and retroperitoneal fat, and perimetrial fat.

Biochemical assays. At the time of muscle dissection, the second soleus muscle was removed and frozen in liquid nitrogen. A piece of this muscle was homogenized in 30 volumes of ice-cold 20 mM HEPES (pH 7.4) containing 1 mM EDTA and 250 mM sucrose. These homogenates were used for determination of total protein content by using the bicinchoninic acid method (Sigma Chemical), GLUT4 protein level (14), and citrate synthase activity (37). A second piece of soleus was used for the measurement of protein carbonyl levels, using the spectrophotometric assay of Reznick and Packer (28). A third piece of the soleus was used for the assessment of muscular triglyceride concentration, using the chloroform-methanol extraction described by Folch et al. (9) followed by the processing method of Frayn and Maycock (10), as modified by Denton and Randle (7). Glycerol was ultimately assayed spectrophotometrically using a commercially available kit (Sigma Chemical).

Statistical analysis. All data are presented as means ± SE. The significance of differences between multiple groups was assessed by a factorial ANOVA with a post hoc Fisher’s protected least significant difference test (StatView version 5.0; SAS Institute, Cary, NC). Differences between two groups were determined by an unpaired Student’s t-test. A P value <0.05 was considered to be statistically significant.

RESULTS

Body weights and total abdominal fat weights. The initial body weights for the obese Zucker rats in the vehicle-treated group, the M-CLA-treated group, the c9,t11-CLA-treated group, and the t10,c12-CLA-treated group were 280 ± 3, 273 ± 4, 280 ± 5, and 282 ± 6 g, respectively. As shown in Fig. 1, the chronic administration of M-CLA to the obese Zucker rat caused a significant decrease (15%, P < 0.05) in body weight gain over the 21-day treatment period com-

Fig. 1. Effect of conjugated linoleic acid (CLA) isomers on body weight gain of obese Zucker rats during the treatment period. Animals were treated with either vehicle, a mixture of the cis (c)9, trans (t)12 and the t10,c12 isomers of CLA (50:50), 76% c9,t11-CLA (c9,t11), or 90% t10,c12-CLA (t10,c12). Values are means ± SE for 5 animals/group. *P < 0.05 vs. vehicle-treated group. **P < 0.05 vs. all other groups.
pared with the vehicle-treated group. Moreover, this relative difference in body weight gain was even more pronounced (44%) in the t10,c12-CLA group. In contrast, there was no significant alteration in body weight gain in the c9,t11-CLA-treated group relative to the control group. The wet weights of the heart and of the soleus and plantaris muscles were also not different among the various groups (data not shown).

The total fat content in the abdominal cavity was also assessed. In vehicle-treated obese Zucker rats, the content of total abdominal fat was 36.0 ± 1.0 g, and as a percentage of body weight was 10.8 ± 0.3%, comparable to previously reported values from female obese Zucker rats of comparable size and age (23). These values for total abdominal fat were not significantly different from control in the M-CLA-treated animals (34.0 ± 0.6 g and 10.7 ± 0.1%) and the c9,t11-CLA-treated animals (36.7 ± 0.6 g and 10.9 ± 0.1%). However, in the t10,c12-CLA-treated animals, total abdominal fat was significantly different (*P < 0.05) from all other groups in both absolute terms (31.5 ± 0.9 g) and relative terms (10.1 ± 0.1%).

**Plasma variables and OGTT.** Whereas fasting plasma glucose was not altered by chronic treatment with M-CLA or c9,t11-CLA (Fig. 2), treatment with t10,c12-CLA was associated with an 11% decrease in this variable compared with the vehicle-treated control group. In addition, both M-CLA (15%) and t10,c12-CLA (12%), but not c9,t11-CLA, caused decreases in the fasting plasma insulin concentration (Fig. 2). The fasting plasma free fatty acid (FFA) concentration was lowered 14% by M-CLA and 27% by t10,c12-CLA, but was not significantly altered by c9,t11-CLA (Fig. 2).

The glucose response during an OGTT (Fig. 3) was lowered significantly compared with the vehicle-treated group by chronic treatment with M-CLA (14% at 30 min) or t10,c12-CLA (23% at 30 min and 13% at 60 min), and the glucose area under the curve (AUC; Fig. 4) was likewise lowered in the M-CLA (10%) and decreased to the greatest extent in the t10,c12-CLA (16%) groups. The insulin response during the OGTT was also decreased by treatment with chronic CLA (16% at 30 min, 22% at 60 min, and 25% at 90 min) or t10,c12-CLA (20% at 30 min, 22% at 60 min, and 23% at 90 min), with corresponding decreases in the insulin AUC in these groups (18 and 19%, respectively) compared with control. Neither the glucose response nor the insulin response was altered by treatment with c9,t11-CLA. The glucose-insulin index, defined as the product of the glucose AUC and the insulin AUC, with a reduction in this value reflecting an increase in whole body insulin sensitivity (26, 34), was significantly less in the chronically M-CLA-treated (27%) and t10,c12-CLA-treated (33%) groups compared with the vehicle-treated obese group but was not changed in the c9,t11-CLA-treated group (Fig. 4).

**Muscle glucose transport activity.** The interventions did not alter the basal rate of 2-DG uptake in skeletal muscle (data not shown). After chronic treatment with either M-CLA or t10,c12-CLA, the insulin-mediated increase in 2-DG uptake was enhanced in epitrochleas (43 and 48%) and in soleus (23 and 46%) muscles (Fig. 5). Treatment with c9,t11-CLA did not improve insulin-mediated muscle 2-DG uptake.

**Muscle biochemistry.** The level of protein carbonyl in the soleus, reflective of oxidative damage (4), was reduced relative to the vehicle-treated control group by M-CLA (48%) and by t10,c12-CLA (56%) but not by c9,t11-CLA (Fig. 6). In addition, the concentration of triglycerides in soleus muscle was reduced by treatment with M-CLA (29%) or t10,c12-CLA (34%; Fig. 7). No alteration in soleus triglyceride concentration was elicited by treatment with c9,t11-CLA. In addition, there was no effect of any CLA treatment on total GLUT4 protein expression or on citrate synthase activity (index of muscle oxidative capacity) in soleus muscle (data not shown).

**Correlational analysis.** The improvements in insulin-mediated 2-DG uptake in soleus muscle after chronic CLA treatment were significantly correlated with the enhancement of whole body insulin sensitivity, as assessed by the reduction in the glucose-insulin index (r = −0.743, P < 0.05; Fig. 8A). Because oxidative stress may play a role in the development of insulin resistance (3, 12), the correlation between protein carbonyl level and insulin-mediated 2-DG uptake

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**Fig. 2.** Effect of CLA isomers on plasma glucose (A), insulin (B), and free fatty acids (C) in obese Zucker rats. Values are means ± SE for 5 animals/group. *P < 0.05 vs. vehicle-treated and c9,t11 groups.
in soleus muscle from the various experimental groups was examined (Fig. 8B). A highly significant inverse correlation was observed \((r = -0.616, P < 0.05)\). In addition, a role of elevated muscle lipid in the etiology of insulin resistance has been recognized (17, 19, 22), and we therefore investigated whether the CLA-mediated improvements in insulin-mediated 2-DG uptake in soleus muscle were correlated with the reductions in muscle triglyceride. As shown in Fig. 8C, a highly significant correlation exists between these two variables \((r = -0.532, P < 0.05)\) between muscle triglycerides and the protein carbonyl level in soleus muscles of the various groups of obese Zucker rats treated with CLA isomers (Fig. 8D).

**DISCUSSION**

In the present investigation, we have demonstrated that the chronic oral treatment of insulin-resistant female obese Zucker \((fa/fa)\) rats with the fatty acid CLA leads to a significant enhancement of oral glucose tolerance that is associated with enhanced insulin-mediated glucose transport activity in both type I and type IIb skeletal muscle. Moreover, we have made the novel observation that these metabolic improvements resulting from CLA treatment can be ascribed specifically to the t10,c12 isomer, with the c9,t11 isomers being nearly metabolically neutral in this animal model of insulin resistance.

There were also several other significant metabolic improvements observed in the obese animals treated with the t10,c12 isomer of CLA. These included a reduction in developmental body weight gain (Fig. 1), small but significant decreases in fasting plasma glucose and insulin (Fig. 2), and a substantial diminution of fasting plasma FFA levels (Fig. 2). Moreover, total abdominal fat content was 13% less in the t10,c12-CLA-treated animals compared with controls, possibly contributing to the improvement in whole body and skeletal muscle insulin action (Figs. 3–5) in these animals and consistent with the hypothesis that a loss of visceral fat can positively influence insulin sensitivity in skeletal muscle (18). Collectively, these adaptive responses in the obese Zucker rat to the t10,c12-CLA treatment would represent a substantial reduction in overall cardiovascular disease risk.

The effects of CLA and its specific isomers on glucose and lipid metabolism in various rodent models have been controversial, and, in some cases, apparently contradictory data have appeared in the literature. The present results derived from the female obese Zucker rat demonstrating an enhancement of oral glucose tolerance (Figs. 3 and 4) and insulin action on skeletal muscle glucose transport (Fig. 5), and favorable adaptations in the plasma insulin and lipid profile (Fig. 2),...
are in agreement with those data obtained from the male ZDF rat (16, 33). In our study and those studies utilizing the ZDF rat (16, 33), a diminution of body weight gain (Fig. 1) was observed. In contrast, CLA feeding induced insulin resistance and increases in plasma lipids in C57BL/6J mice (36). In the male ob/ob C57BL-6 mouse, a murine model of obesity, insulin resistance, and dyslipidemia, a diet containing primarily the t10,c12 isomer of CLA induced increases in postprandial plasma levels of glucose and insulin, reflecting an insulin-resistant state, whereas feeding of the c9,t11-CLA led to a reduction in plasma lipids (31). Moreover, recent clinical studies (29, 30) have demonstrated that treatment of abdominally obese men with the t10,c12 isomer slightly worsens fasting glycemia and insulin resistance. These contradictory effects of CLA may be attributable to the different animal models of obesity-associated insulin resistance employed (obese Zucker rat, ZDF rat, and obese C57BL-6 mouse) and the specific metabolic state of the human subjects. As recently discussed by Roche et al. (31), the decreases in fat mass caused by treatment with t10,c12-CLA in the ZDF rat (33) are associated with improvements in insulin action, whereas this same treatment in the obese C57BL-6 mouse causes the development of a lipodystrophic state, a condition associated with marked insulin resistance (31, 38). It is clear that the specific animal model employed and human population selected to investigate the metabolic actions of CLA are critical considerations in the assessment of the potential beneficial effects of this fatty acid in the context of insulin resistance.

There is accumulating evidence that oxidative stress, hallmarkd by an imbalance of prooxidants relative to antioxidant defenses in plasma and tissues, may play a role in the etiology of insulin resistance (3, 12). We have demonstrated previously that protein carbonyl formation, an index of oxidative stress-associated tissue damage (4, 28), is enhanced in skeletal muscle of the obese Zucker rat and that this increase in protein carbonyl level is associated with insulin resistance of skeletal muscle glucose transport (34). Moreover, interventions such as exercise training or chronic antioxidant (α-lipoic acid) treatment, which reduce tissue protein carbonyl levels, also induce enhancements of insulin-mediated glucose transport activity in skeletal muscle (34). We have shown in the present investigation that the t10,c12 isomer of CLA can act as an antioxidant and decrease the level of protein carbonyls in soleus muscle (Fig. 6) and that this decrease in protein carbonyls is associated with an increase in insulin action on glucose transport (Fig. 8B). These findings are consistent with the in vitro results of Leung and Liu (20), who demonstrated that t10,c12-
CLA acts as a much stronger scavenger of reactive oxygen species than c9,t11-CLA. Therefore, the beneficial metabolic actions of t10,c12-CLA may be mediated, at least in part, by the antioxidant properties of this CLA isomer.

There is generally an inverse relationship between the concentration of intramuscular lipid and the ability of insulin to stimulate muscle glucose transport activity (17, 19, 22, 35). In the soleus muscle of obese Zucker rats, we have observed triglyceride levels that are threefold higher than those in soleus muscle of age-matched lean Zucker rats, and reductions in these lipid levels induced by exercise or antioxidant treatment (α-lipoic acid) are associated with improvements in muscle insulin action (35). Similarly, t10,c12-CLA treatment induced a reduction in the muscle triglyceride concentration of the soleus (Fig. 7) that was significantly correlated with the enhancement of insulin-mediated glucose transport activity (Fig. 8C). These findings are consistent with the hypothesis that the diminution in the muscular lipid concentration could account for a portion of the enhancement in insulin action caused by the t10,c12-CLA treatment. Previous investigations have attributed the reductions in triglyceride concentration elicited by t10,c12-CLA treatment of murine and human adipocytes to inhibition of lipogenesis (2, 8). Whether this same mechanism is functional in skeletal muscle from CLA-treated obese Zucker rats remains to be determined.

We also observed a highly significant correlation between the muscle level of triglycerides and the muscle level of protein carbonyls in the various groups (Fig. 8D). These data indicate a potential link between the degree of elevated muscle lipid and the local level of oxidative stress (as reflected by protein carbonyl level), possibly through an elevation in oxidation of this enhanced lipid substrate. This increase in local oxidative stress may contribute to insulin resistance in these muscles (3, 12). The CLA-mediated decreases in muscle triglycerides could reverse this relationship by reducing oxidative stress and allowing insulin action to increase. Further investigation is required to more definitively test the cellular mechanisms underlying this provocative concept.

The muscle level of the glucose transport isoform GLUT4 is an important determinant of the muscle capacity for glucose transport activation by insulin or contractions (13). However, in the present investigation, there was no enhancement of GLUT4 protein expression or muscle oxidative capacity because of the CLA treatments. These findings contrast with those of Tsuboyama-Kasaoka and colleagues (38), who showed that chronic treatment with CLA induced a 50% increase in GLUT4 mRNA level in the gastrocnemius muscle of C57BL/6J mice. These contradictory findings
again emphasize that the rodent model employed in CLA studies is a critical consideration.

In summary, we have demonstrated that the t10,c12 isomer of the fatty acid CLA can elicit a number of beneficial metabolic responses in the obese Zucker (fa/ fa) rat, a rodent model of insulin resistance, glucose intolerance, hyperinsulinemia, dyslipidemia, and central obesity. These metabolic improvements include enhanced glucose tolerance, decreases in fasting plasma glucose, insulin, and FFAs, improved insulin-mediated glucose transport activity in skeletal muscle, and decreases in total abdominal fat and skeletal muscle protein carbonyl and lipid levels. In contrast, the c9,t11 isomer of CLA was metabolically neutral. These results highlight the potential of the t10,c12 isomer of CLA as a nutriceutical intervention in specific conditions of glucose intolerance and insulin resistance.

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