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Original Research Communication

Metabolic Effects of γ-Linolenic Acid–α-Lipoic Acid Conjugate in Streptozotocin Diabetic Rats

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ABSTRACT

Data suggesting the involvement of increased oxidative stress in the pathophysiology of diabetes has raised interest in the potential therapeutic benefit of antioxidants. Although beneficial metabolic effects of antioxidant supplementation have been suggested, an antioxidant mode of action, particularly in skeletal muscle, has not been documented. In the present study, we evaluate the metabolic effects of a γ-linolenic acid–α-lipoic acid conjugate (GLA-LA) in streptozotocin-induced diabetic rats, and assess its potential mode of action by comparing its effects with equimolar administration of LA and GLA alone. Ten days of oral supplementation of 20 mg/kg body weight GLA-LA, but not LA or GLA alone, caused a mild reduction in fasting blood glucose concentration as compared with vehicle-treated diabetic rats (375 ± 11 vs. 416 ± 16 mg/dl, $p = 0.03$), with no change in fasting plasma insulin levels. A peripheral insulin-sensitizing effect could be observed with GLA-LA, LA, and GLA treatments, as demonstrated by a significant ($p < 0.04$) 23%, 13%, and 10% reduction, respectively, in the area under the glucose curve following an intravenous insulin tolerance test. This effect was associated with a 67% and 50% increase in GLUT4 protein content in the membranes of gastrocnemius muscle of GLA-LA and LA-treated animals, respectively; however, no change was observed with GLA treatment alone. Interestingly, both GLA-LA and LA treatments corrected a diabetes-related decrease in the gastrocnemius muscle low-molecular-weight reduced thiols content. These data demonstrate insulin-sensitizing properties of the GLA-LA conjugate by distinct mechanisms attributable to each of its components, which are associated with antioxidant effects. Antiox. Redox Signal. 1, 523–535.

INTRODUCTION

ACCUMULATING DATA IN RECENT YEARS have suggested that diabetes mellitus is one of an increasing number of human diseases in which oxidative stress plays a pathogenic role (Baynes, 1991). Oxidative stress has been linked to the pathogenesis of several diabetes-associated conditions, including the accelerated development of atherosclerosis (Ceriello and Giugliano, 1997), the increased incidence of abnormal embryonic development in pregnancies of diabetic mothers (Ericksson and Borg, 1993), as well as the development of diabetic peripheral polyneuropathy (Yagihashi, 1995). The documentation of an oxidant-antioxidant imbalance in diabetes includes reports suggestive of increased reactive oxygen species (ROS) production (Baynes, 1991; Wolff *et al.*, 1991), and diminished antioxidant defense mechanisms (Jones *et al.*, 1988; Cunningham *et al.*, 1991). This results in increased lipid and DNA oxidation, as assessed by both elevated plasma levels of lipid and DNA oxidation products (Gallou *et*

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al., 1993; Dandona *et al.*, 1996; Laaksonen *et al.*, 1996), as well as by increased erythrocyte membranes malondialdehyde production and decreased GSH content (Chari *et al.*, 1984; Nagasaka *et al.*, 1989). In addition, plasma of type 2 diabetic patients was significantly more susceptible to lipid peroxidation induced by a free radical initiator, as compared to normal subjects (Haffner *et al.*, 1995), suggesting increased plasma oxidizability. Yet, despite these various markers of "systemic" oxidative stress, relatively little information exists regarding stress markers in tissues which play a role in the pathogenesis of diabetes (Duarte *et al.*, 1993). This limits the ability to correlate oxidative stress to the actual abnormalities in intracellular metabolism observed in various tissues of diabetic subjects.

Impaired skeletal muscle responsiveness to the acute metabolic effects of insulin appears as both a predisposing factor for the development of type 2 diabetes, as well as a consequence of the diabetic milieu (Reaven, 1995; Cheatham and Kahn, 1996). Despite intensive investigative efforts, the exact mechanisms responsible for peripheral insulin resistance have not been fully defined. Endocrine and metabolic factors, such as the hyperglycemia (Yki Jarvinen, 1992; Rossetti, 1995), elevated levels of free fatty acids (Boden, 1997), hyperinsulinemia (Del *et al.*, 1994), as well as other potential mediators such as tumor necrosis factor (TNF) and leptin have been proposed to play a role (Hotamisligil and Spiegelman, 1994). Recently, the possibility that oxidative stress may play a role in the development or progression of insulin resistance has been raised (Paolisso and Giugliano, 1996). Clinical studies have demonstrated that several oxidative stress parameters correlate with the degree of metabolic derangement in diabetic patients (Paolisso *et al.*, 1994; Nourooz *et al.*, 1997). To establish a potential causative role for oxidative stress in the development of insulin resistance, we have used cell culture systems. In cell lines representing skeletal muscle and adipose tissue, we could demonstrate that exposure to oxidative stress manifested by decreased cellular glutathione (GSH) content and NF-κB activation resulted in altered cellular metabolism and in impaired metabolic response to insulin (Kozlovsky *et al.*, 1997; Rudich *et al.*, 1997). The cellular mechanisms underlying this effect included oxidation-induced decrease in the expression and translocation to the plasma membrane of the insulin-responsive glucose transporter GLUT4 (Rudich *et al.*, 1998; Tirosh *et al.*, 1999).

An alternative approach has been to improve insulin responsiveness using the antioxidant lipoic acid (LA). LA is a cofactor of multienzyme complexes such as pyruvate dehydrogenase (Packer, 1994), and is currently licensed in parts of Europe for the treatment of patients with painful diabetic neuropathy (Nagamatsu *et al.*, 1995; Ziegler *et al.*, 1995, 1997). Following treatment of cells, LA is transported across the cell membrane and reduced to the dihydrolipoic acid form. Effects of LA include free radical scavenging (Packer *et al.*, 1997; Bustamante *et al.*, 1998), inhibition of lipid peroxidation (Scholich *et al.*, 1989; Kagan *et al.*, 1992), increased GSH levels (Han *et al.*, 1995; Sen *et al.*, 1997; Obrosova *et al.*, 1998), and potential regeneration of other major intracellular antioxidants, such as ascorbate and vitamin E (Lykkesfeldt *et al.*, 1998; Scholich *et al.*, 1989). In addition, diverse effects of LA on glucose metabolism were described. LA was found to induce an increase in glucose transport in L6 muscle cells and in 3T3-L1 adipocytes, by stimulating translocation of the glucose transporter GLUT4 from internal membranes pools to the plasma membrane (Estrada *et al.*, 1996). Using lower concentrations of LA, we have been able to demonstrate a protective effect of LA against oxidation-induced insulin resistance, as well as against the depletion of low-molecular-weight reduced thiols and GSH in 3T3-L1 adipocytes (Rudich *et al.*, 1999). In addition, several animal and human studies demonstrated that LA treatment improved glucose metabolism by enhancing insulin-mediated glucose disposal (Jacob *et al.*, 1996; Henriksen *et al.*, 1997; Streeper *et al.*, 1997). In streptozotocin (STZ)-induced diabetic rats, we have previously demonstrated that 10 days of LA treatment reduced blood glucose, induced elevation in muscle GLUT4 protein content, and reversed the diabetes-induced impairment in insulin-dependent glucose uptake into isolated muscle (Khamaisi *et al.*, 1997). Despite these observations, it is cur-

rently unknown whether LA exerted its metabolic effects through its systemic and/or skeletal muscle antioxidant capacity, or instead through alternative mechanisms (Gohil *et al.*, 1999). The present study was conducted to evaluate whether the metabolic effects of LA can be attributed to its antioxidant property and to assess whether these effects can be potentiated by γ-linolenic acid (GLA), as shown in models of diabetic peripheral neuropathy (Cameron *et al.*, 1998). Our results demonstrate that LA and GLA-LA conjugate are capable of elevating skeletal muscle low-molecular-weight reduced thiols content in diabetic rats, and increasing the amount of GLUT4 protein in skeletal muscle membranes. GLA contributes to the metabolic action of the GLA-LA conjugate, resulting in a mild reduction in fasting blood glucose levels and improved insulin tolerance test through mechanisms seemingly distinct from those of LA.

MATERIALS AND METHODS

γ-Linolenic acid [*cis*-6,9,12-octadecatrienoic acid, (18:3ω6), GLA] and the 50% wt/wt conjugate of GLA and LA (GLA-LA) were from Scotia Pharmaceuticals (Carlisle, UK). The GLA-LA conjugate was kept in aliquots under nitrogen atmosphere at 4°C until used. LA (racemic mixture) was provided by ASTA Medica (Frankfurt, Germany). Recombinant human insulin was from Novo Nordisk (Bagsvaerda, Denmark). 2,2′-Azo-*bis*(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemical Industries Ltd (Osaka, Japan). Anti-GLUT4 antibodies were purchased from Chemicon International Inc. (Temecula, CA). Peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) were purchased from Amersham Life Science (Piscataway, New Jersey). All other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise stated in the text.

Animal treatment groups

All protocols were approved in advance by the Ben-Gurion University Animal Studies Committee. Male Sprague-Dawley rats were purchased from Harlan Laboratories (Jerusalem, Israel). They were kept at a 12-hr light-dark cycle at 23°C, housed 4 animals in a cage. Standard rat chow and tap water were provided *ad libitum* prior to the treatment period, and then as described below. Diabetes was induced at the age of 6–8 weeks after an overnight fast, by a single intraperitonial (i.p.) injection of a freshly prepared solution of STZ (65 mg/kg body weight) in 100 m*M* citrate buffer (pH 4.5). Diabetes was defined as nonfasting tail blood glucose concentration >400 mg/dl on two separate occasions (Glucometer Elite, Bayer Diagnostics, USA). Beginning 7 days following the induction of diabetes, animals were treated for 10 consecutive days by gavage with the following: Group 1, diabetic animals treated with 0.4 ml of corn oil (Diabetic vehicle group). Group 2, diabetic animals treated with 20 mg/kg body weight of the combined GLA-LA compound dissolved in 0.4 ml of corn oil (Diabetic GLA-LA group). This dose is equivalent to approximately 10 mg/kg per day GLA or LA. Group 3, diabetic animals treated with 10 mg/kg body weight of LA (Diabetic LA group). Group 4, diabetic animals treated with 10 mg/kg body weight of GLA dissolved in 0.4 ml of corn oil (Diabetic GLA group).

The GLA-LA conjugate is poorly absorbed when the stomach is empty. Thus, to ensure the administration of GLA-LA on a full stomach, all animals were food deprived overnight and allowed to eat at 7–8 AM each day, after which the various agents were administered by gavage between 10 AM and noon.

Twenty hours after the eighth treatment, fed diabetic animals were injected intravenously (i.v.) with 0.5 U/kg insulin. Tail blood samples were obtained after 0, 15, 30, 45, 60, and 90 min of insulin treatment to measure glucose levels. Results were expressed for each rat as percent of glucose level at time 0, and plotted as a function of time. The area under the curve (AUC) for each rat was calculated using the KaleidaGraph version 3.04 (Synergy Software, Reading, PA), and the mean value ± SE calculated for each group.

Twenty hours after the final (10th) treatment and 12 hr following an overnight fast, animals were weighed, anesthetized with phenobarbi-

tal (80 mg/kg, i.p.), and the gastrocnemius muscle and liver were rapidly dissected out. Gastrocnemius muscles were trimmed of fat and connective tissue, and livers were washed with saline; both tissues were frozen immediately in liquid nitrogen and kept at −70°C until used for further analyses. Blood was drawn by cardiac puncture into EDTA containing tubes, followed by centrifugation at 4°C. Plasma was stored frozen in aliquots at −70°C.

Muscle total membrane preparation and western blot analysis

Membranes were prepared as previously described (Ramlal *et al.*, 1989). A 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and western blot analysis were carried out as previously described (Khamaisi *et al.*, 1997), using a 1:2,000 dilution of anti-GLUT4. Blots were incubated with horseradish peroxidase-conjugated anti rabbit IgG. Luminescence was detected by electrochemiluminescence (ECL) (Pierce, Rockford, IL). Density values of the different bands were obtained after scanning the blots with video densitometry using the UVP-GDF 5000 system (UVP Inc., San Gabriel, CA), and thereafter quantitated using Image Quant, version 3.3 (Molecular Dynamics, Palo Alto, CA).

Total RNA isolation and northern blot analysis

Total cellular RNA was extracted from the gastrocnemius muscle, using isolation reagent (TRI REAGENT) and northern blot analysis was carried out as previously described (Chirgwin *et al.*, 1979). GLUT4 mRNA levels were detected using a random primer-labeled, full-length cDNA probe for GLUT4, under stringent hybridization conditions. Autoradiographs and densitometric analyses were performed with BIO-IMAGING analyzer BAS 2000 (FUJI Photo Film Co., Ltd., Japan).

Liver and muscle low-molecular-weight reduced thiols determinations

Frozen liver tissues were defrosted and homogenized in 5 volumes of 5% 5-sulfosalicylic acid per gram wet tissue. Powdered muscles tissues were homogenized in 4 volumes of 5% 5-sulfosalicylic acid per gram wet tissue. Liver and muscle homogenates were then centrifuged at 15,000 × *g* for 5 min at 4°C. Low-molecular-weight reduced thiols content was measured spectrophotometrically by Ellman's method (Ellman, 1959), using GSH as standard.

Plasma thiobarbituric acid-reactive substances

Thiobarbituric acid (TBA)-reactive substances (TBARS) were assayed using a procedure based on that of Yokode *et al.* (1988). Briefly, 20 μl of plasma was mixed with 1.5 ml of NaCl (150 mmol/liter), 0.5 ml of trichloracetic acid (20% wt/vol), and 0.5 ml of TBA reagent (0.67% TBA/glacial acetic acid, 1:1 vol/vol). The mixture was incubated at 95°C for 1 hr, cooled, and extracted with 4 ml of *n*-butanol. After centrifugation at 2,000 × *g* for 20 min, the supernatant was removed and analyzed fluorometrically (excitation 515 nm, emission 550 nm). Values were calculated by a standard curve with tetramethoxypropane, which is converted in 1:1 stoichiometry into malondialdehyde during the procedure (Yokode *et al.*, 1988).

Plasma lipid peroxidation

Plasma lipid peroxidation products were detected following oxidation with AAPH. Diluted plasma (1:10 with phosphate-buffered saline, 2 ml) was incubated for 2 hr at 37°C without (control) or with 100 mmol/liter AAPH. AAPH is a water-soluble azo compound that thermally decomposes and thus generates water-soluble peroxyl radicals at a constant rate (Frei *et al.*, 1988). Samples were then analyzed immediately for their oxidation state by measuring the amount of TBARS (Buege and Aust, 1978), and the amount of lipid peroxides, which were determined with a commercially available kit (cholesterol color reagent, CHOD iodide method; Diagnostica Merck, Darmstadt, Germany) (El-Saadani *et al.*, 1989). This assay is based on the oxidative activity of lipid peroxides that convert iodide to iodine and is measured spectrophotometrically at 365 nm.

Plasma vitamin E

Vitamin E (as α-tocopherol) was measured by high-performance liquid chromatography (HPLC) technique using the method of Catignani (1986). Tocopherol is lipid bound in plasma, and levels were therefore corrected for plasma cholesterol. Plasma total cholesterol levels were measured by standard enzymatic assay.

Other assays

Protein was measured using Bio-Rad protein assay (Bradford, 1976). Insulin concentration was measured using a rat insulin RIA kit from Linco Research, Inc. (St. Charles, MO).

Statistical analysis

All values are presented as mean ± SE. The data were analyzed using one-way analysis of variances (ANOVA) with Bonferroni post-hoc analysis. A p value less than 0.05 was regarded as indicating statistically significance.

RESULTS

Effects on blood glucose, insulin, and response to exogenous insulin

STZ-induced diabetic rats were treated for 10 consecutive days with either vehicle, 20 mg/kg per day GLA-LA conjugate, 10 mg/kg per day LA, or 10 mg/kg per day GLA. None of the treatments resulted in increased mortality or changes in body weight (Table 1). Animals treated with the GLA-LA conjugate exhibited a mild (10%), but statistically significant, decrease in fasting blood glucose levels as compared with vehicle-treated diabetic rats (375 ± 11 vs. 416 ± 16, $p = 0.03$, respectively). This effect on blood glucose levels could not be attributed to changes in plasma insulin, which was approximately 25% of the normal values for nondiabetic animals in our laboratory (1.08 ± 0.38 ng/ml). LA treatment alone at a concentration of 10 mg/kg had no effect on either 12 hr fasting blood glucose level or on plasma circulating insulin. GLA-treated animals (10 mg/kg) exhibited significantly elevated fasting blood glucose, which was associated with a significant reduction in plasma insulin (Table 1). Previous studies from our group and others (Jacob *et al.*, 1996) demonstrated that 30 mg/kg of lipoic acid treatment increased peripheral insulin sensitivity. To assess whether the GLA-LA conjugate treatment improved the response to exogenous insulin, an insulin tolerance test was performed on the eighth day of treatment. As presented by the percent reduction in blood glucose levels over time (Fig. 1A), and by the calculated AUC (Fig. 1B), GLA-LA, LA, and GLA treatments significantly improved the response to insulin by 23% ($p = 0.005$), 13% ($p < 0.04$), and 10% ($p < 0.04$), respectively, as compared to vehicle-treated diabetic rats. Despite the greater response observed with the GLA-LA conjugate

TABLE 1. CHARACTERISTICS OF THE DIFFERENT GROUPS

	Number	*Weight (g)*	*Fasting blood glucose (mg/dl)*	*Fasting plasma insulin (ng/ml)*
Diabetic vehicle-treated animals	8	175 ± 7	416 ± 16	0.25 ± 0.05
Diabetic GLA-LA-treated animals	7	183 ± 10	375 ± 11[a]	0.23 ± 0.03
Diabetic LA-treated animals	7	175 ± 5	422 ± 25	0.18 ± 0.04
Diabetic GLA-treated animals	7	178 ± 11	463 ± 24[b]	0.15 ± 0.01[b]

STZ-induced diabetic rats (7 days following induction of diabetes) were treated for 10 consecutive days by gavage with either vehicle (0.4 ml of corn oil), GLA-LA (20 mg/kg), LA (10 mg/kg), and GLA (10 mg/kg), as described in Materials and Methods. Following the final treatment, animals were fasted for 12 h, then weighed and anesthetized, after which blood samples were obtained. Blood glucose levels were determined using a Glucometer, and insulin concentrations were determined using a radioimmunoassay (RIA) kit, as described in Materials and Methods. Results are mean ± SE.

[a] $p = 0.03$ compared to vehicle-treated diabetic animals.

[b] $p = 0.05$ compared to vehicle-treated diabetic animals.

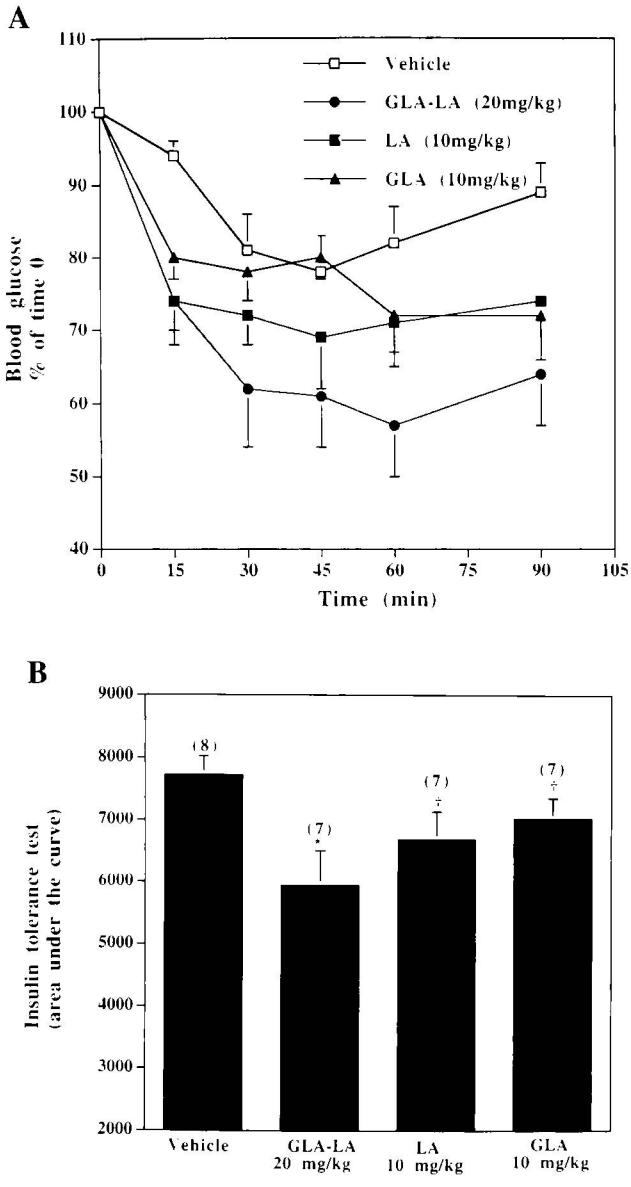

FIG. 1. Effect of LA, GLA, and GLA-LA conjugate treatments on glucose response during an intravenous insulin tolerance test in STZ diabetic rats. A. Insulin (0.5 U/kg body weight) was injected i.v. at the eighth day of treatment of diabetic rats with either vehicle (0.4 ml of corn oil, □), 20 mg/kg GLA-LA conjugate (●), 10 mg/kg LA (■), and 10 mg/kg GLA (▲), as described in Materials and Methods. Blood glucose levels were determined at the indicated times by Glucometer Elite (Bayer Diagnostic, Tarrytown, NY), and are presented as percent of glucose level at time 0. **B.** The area under the curve for each rat was calculated, and the mean value ± SE for each group is presented. Animal number for each group is presented in parentheses. *$p = 0.005$ versus vehicle-treated diabetic animals. †$p < 0.04$ versus vehicle treated diabetic animals.

as compared to the LA and GLA treatments alone, this difference did not reach statistical significance ($p = 0.16$ and $p = 0.2$, respectively).

Involvement of alterations in skeletal muscle GLUT4 content

In normal conditions, skeletal muscle accounts for 85% of insulin-stimulated glucose disposal, which depends on the expression and function of the insulin-sensitive glucose transporter GLUT4. To investigate whether changes in the expression of GLUT4 could contribute to the improvement in the insulin tolerance test, GLUT4 levels were determined in total membranes of gastrocnemius muscle. Consistent with previous observations in our laboratory, 17 days of STZ-induced diabetes caused a significant reduction in muscle GLUT4 protein content as compared to the levels observed in nondiabetic animals, with only a mild, nonsignificant reduction in the mRNA levels of GLUT4 (Khamaisi *et al.*, 1997). Ten days of GLA-LA conjugate or the LA treatments resulted in total membrane GLUT4 protein of 167 ± 26% ($p = 0.008$) and 153 ± 22% ($p = 0.009$) of the level observed in vehicle-treated diabetic animals, respectively (Fig. 2; Table 2). Rats treated with GLA alone did not display any significant change in gastrocnemiums GLUT4 content. As demonstrated in Table 2, these changes in GLUT4 protein in the GLA-LA- and LA-treated groups did not correlate with changes in gastrocnemius GLUT4 mRNA content.

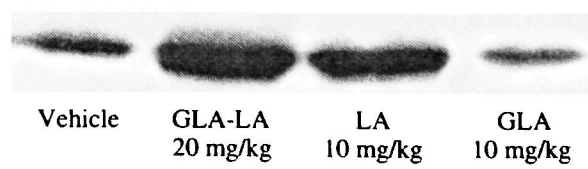

FIG. 2. GLUT4 protein gastrocnemius muscle of diabetic rats treated for 10 days with vehicle or with either GLA-LA, LA, or GLA. Total membranes were prepared from gastrocnemius muscle of diabetic rats treated as described in Materials and Methods. Membrane proteins were separated by SDS-PAGE on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoassayed using anti-GLUT4 antibody. Shown is a representative autoradiograph in which each lane represents an individual muscle preparation.

TABLE 2. DENSITOMETRY VALUES OF GLUT4 PROTEIN AND mRNA IN GASTROCNEMIUS MUSCLE

	GLUT4 protein arbitrary units	*GLUT4 mRNA arbitrary units*
Diabetic vehicle-treated animals	100 ± 6 (8)	100 ± 10 (6)
Diabetic GLA-LA-treated animals	167 ± 26[a] (7)	108 ± 21 (7)
Diabetic LA-treated animals	153 ± 22[b] (6)	88 ± 21 (3)
Diabetic GLA-treated animals	104 ± 22 (5)	110 ± 14 (3)

Densitometry analysis was performed for auto radiographs from western blots of total membrane GLUT4 protein and auto radiographs from northern blots of GLUT4 mRNA. Data are expressed as percent of the value obtained in vehicle-treated diabetic rats. Results are mean SE, of independent determinations whose number is presented in parenthesis.

[a]$p = 0.008$ versus vehicle-treated diabetic animals.

[b]$p = 0.009$ versus vehicle-treated diabetic animals.

Alterations in systemic and tissue markers for oxidative stress

LA has been documented in various experimental systems as a potent antioxidant (Packer *et al.*, 1997). To evaluate whether the effects observed with the various compounds are associated with changes in the oxidative status, systemic and tissue parameters of oxidative stress were evaluated. To evaluate systemic markers for antioxidant defense and oxidative damage, plasma levels of α-tocopherol, basal TBARS, as well as TBARS and peroxides levels after incubation with the water-soluble radical initiator AAPH were measured (Table 3). In accordance with previous reports (Pritchard *et al.*, 1986; Asayama *et al.*, 1994), cholesterol-standardized α-tocopherol levels in the STZ-induced diabetic rats was elevated as compared to laboratory values for normal rats (2.96 ± 0.18 mmol/mmol cholesterol). Nevertheless, as compared with vehicle-treated diabetic animals, both GLA-LA and LA treatments, but not GLA alone, resulted in an elevation in lipid corrected α-tocopherol (by 23.2% and 16.0%, respectively, $p < 0.03$ for both). Unsaturated fatty acid peroxidation was evaluated by measurements of basal malondialdehyde (MDA) as detected by its reaction with TBA. Consistent with previous reports, STZ-induced diabetes was associated with 23.4% elevation in plasma MDA as compared to the laboratory values

TABLE 3. PLASMA MARKERS OF OXIDATIVE STRESS

	α-Tocopherol/cholesterol (mmol/mmol cholesterol)	*Basal TBARS (μM)*	*Peroxides after oxidation with AAPH (mM)*	*TBARS after oxidation with AAPH (μM)*
Diabetic vehicle-treated animals	3.83 ± 0.21	4.27 ± 0.35	3.78 ± 0.13	64 ± 7
Diabetic GLA-LA-treated animals	4.72 ± 0.25[a]	3.40 ± 0.19[b]	3.20 ± 0.19[b]	49 ± 3[b]
Diabetic LA-treated animals	4.46 ± 0.25[a]	3.48 ± 0.24[b]	3.61 ± 0.27	62 ± 5
Diabetic GLA-treated animals	3.95 ± 0.24	3.45 ± 0.29[b]	3.44 ± 0.23	62 ± 6

STZ-induced diabetic rats were treated for 10 consecutive days as described in Fig 1. Twenty hours after the final treatment and 12 hr following an overnight fast, animals were anesthetized and blood was drawn by cardiac puncture. Plasma was analyzed for α-tocopherol, cholesterol, basal TBARS, and the AAPH-induced production of peroxides and TBARS as described in the Materials and Methods section. Results are mean ± SE, and the number of rats in each group is the same as in Table 1.

[a]$p < 0.03$ compared to vehicle-treated diabetic animals.

[b]$p < 0.05$ compared to vehicle-treated diabetic animals.

for normal animals (3.46 ± 0.30 μM). Ten days treatment with either GLA-LA conjugate, LA, or GLA compounds normalized plasma TBARS levels (Table 3). To evaluate the susceptibility of plasma to *in vitro* oxidation, plasma samples from the four groups were incubated with 100 mM AAPH, after which both lipid peroxides and TBARS were measured. Vehicle-treated diabetic animals displayed an increase in AAPH-induced TBARS (16.4%) and peroxides (23.5%) levels as compared to the normal values obtained in our lab for nondiabetic animals (55 ± 5 μM and 3.08 ± 0.38 mM, respectively). Only the GLA-LA conjugate was effective in significantly decreasing AAPH-induced lipid peroxides and TBARS formation (Table 3). To evaluate whether the systemic markers for an antioxidant effect of the various treatments as detected in plasma are associated with alteration in the redox state of tissues, muscle and liver low-molecular-weight reduced thiols content was measured (Fig. 3A,B, respectively). Diabetes was associated with a 20% and 17% decrease in muscle and liver low-molecular-weight reduced thiols content, respectively, as compared to values obtained in nondiabetic controls (0.59 ± 0.06 and 4.76 ± 0.16 μmol/gram of tissue, respectively). Treatment with either the GLA-LA conjugate or LA alone resulted in a similar degree of elevation in liver and muscle low-molecular-weight reduced thiols content, whereas GLA treatment alone did not affect this parameter. Taken together these data demonstrate the capacity of the GLA-LA conjugate to exhibit antioxidant properties in both plasma and tissues.

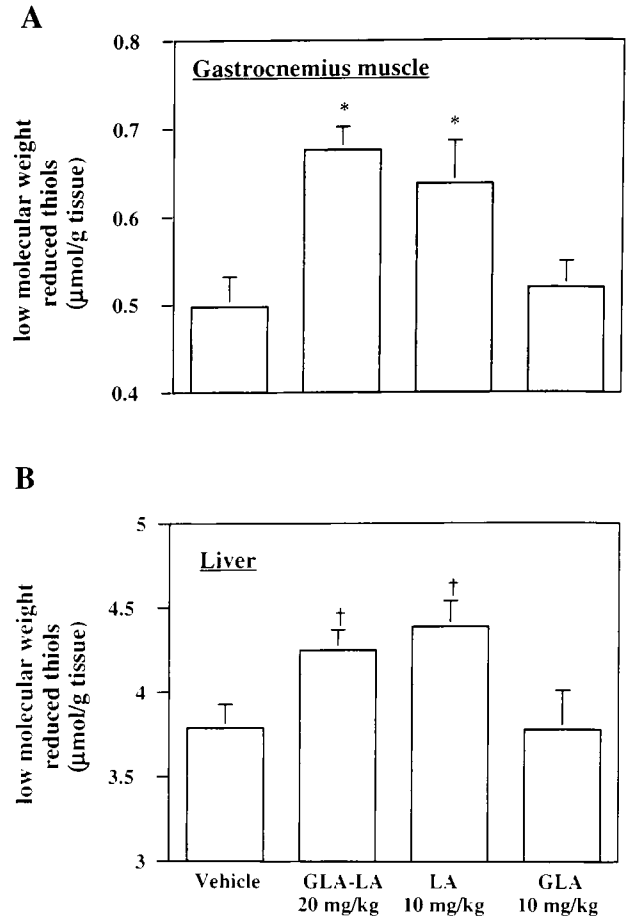

FIG. 3. Effect of LA, GLA, and GLA-LA conjugate treatment on muscle and liver low-molecular-weight reduced thiols levels. Low-molecular-weight reduced thiols were measured in powdered muscle **(A)** and homogenized liver **(B)** tissues by a reaction with 5,5-dithio*bis*-(2-nitrobenzoic acid), as described in Materials and Methods. Results are mean ± SE of 6 animals in each group. *$p < 0.01$ compared to vehicle-treated diabetic animals. †$p < 0.02$ compared to vehicle-treated diabetic animals.

DISCUSSION

The present study demonstrates that treatment of STZ-induced diabetic rats with LA, GLA, or a GLA-LA conjugate results in a larger reduction in blood glucose following exogenous insulin administration as compared to vehicle-treated animals (Fig. 1A,B), indicating improved peripheral insulin sensitivity. The effect achieved with the GLA-LA conjugate is superior to that observed following administration of an equivalent dose of each component alone, and is associated with a mild reduction in fasting blood glucose. The mode of action of the GLA-LA conjugate appears to represent the additive effects of LA and GLA, suggesting that each of these compounds improves insulin sensitivity by distinct mechanisms. Whereas LA displays systemic and tissue antioxidant effects that are associated with elevated skeletal muscle GLUT4 content, GLA appears to utilize other mechanisms.

Effects of the GLA-LA conjugate attributable to the LA component

Previous studies have demonstrated the capacity of LA to influence skeletal muscle glu-

cose utilization and peripheral insulin sensitivity in various models (Jacob *et al.*, 1995, 1996; Estrada *et al.*, 1996). In the STZ diabetic model, we have demonstrated that 30 mg/kg per day of LA resulted in reduced glucose levels, normalization of insulin-stimulated glucose transport activity in isolated soleus muscle preparations, and elevated levels of GLUT4 protein (Khamaisi *et al.*, 1997). In the present study, using a LA dose of 10 mg/kg per day, no effect on fasting blood glucose was observed (Table 1), but a significant 13% improvement in the hypoglycemic response to exogenous insulin could be demonstrated (Fig. 1), suggestive of improved insulin sensitivity at the skeletal muscle level. Moreover, skeletal muscle total membrane preparations from the LA-treated group were approximately 150% enriched in GLUT4 protein content (Fig. 2, Table 2). As in our previous report, this effect was not associated with alterations in the GLUT4 mRNA levels in the same muscle (Khamaisi *et al.*, 1997). Thus, the effects observed with the lower LA concentration (10 mg/kg) closely resembled the effects observed with 30 mg/kg, with an apparent dose dependency of effects demonstrated by fasting glucose levels.

The GLA-LA conjugate treatment also resulted in a significant elevation in skeletal muscle GLUT4 content as compared with the vehicle treated diabetic group. The degree of this elevation was not significantly different from the effect of LA treatment alone (162% versus 150%, respectively), whereas GLA treatment alone did not result in any elevation in skeletal muscle GLUT4 content (Fig. 2 and Table 2). These data suggest that the elevation in GLUT4 protein following treatment with the GLA-LA conjugate could be largely attributed to the LA component. Because hyperglycemia has been demonstrated to be a major factor in the development of skeletal muscle insulin resistance in the STZ diabetic rat, the small, nonsignificant difference between the LA alone and the GLA-LA conjugate may be attributed to the mild hypoglycemic effect of the latter (Table 1).

In this study, we assessed the effect of the various treatments on oxidative damage parameters and antioxidant markers. LA treatment enhanced tissue antioxidant capacity, as demonstrated by an elevated liver and skeletal muscle low-molecular-weight reduced thiols levels. This may represent elevation in intracellular dihydrolipoic acid content and/or increased cellular GSH. In support of the former, a recent study by Khanna *et al.* (1999) demonstrated that LA supplementation increased tissue lipoic acid content, and protected against oxidative lipid damage in red gastrocnemius muscle. The effects observed with the GLA-LA conjugate were equal to those observed with LA alone, whereas the GLA compound exhibited no effect on this parameter (Fig. 3). These data suggest again that the antioxidant effect of the GLA-LA compound in tissues can be largely attributed to its LA component. At the systemic level, the antioxidant properties of the GLA-LA conjugate may reflect the contribution of LA, and the interaction between the LA and GLA components, as discussed below.

Whether the effect of LA and the GLA-LA conjugate on skeletal muscle GLUT4 content depends on their ability to increase skeletal muscle GSH concentration is an intriguing question. Regression analysis revealed a moderate correlation ($r = 0.42$) between skeletal muscle low-molecular-weight reduced thiols and GLUT4 content among the various treatment groups (data not shown). Yet 92% depletion of skeletal muscle GSH content in nondiabetic rats by 10 days treatment with an inhibitor of glutathione synthesis (buthionine sulfoximine) did not significantly affect GLUT4 protein content (unpublished data). Thus, whether reduced skeletal muscle low-molecular-weight reduced thiols, GSH, and GLUT4 contents are independent alterations of skeletal muscle in the STZ diabetes model, or rather represent interrelated phenomena associated with the diabetic milieu, remains to be sorted out.

Effects of the GLA-LA conjugate attributable to the GLA component

GLA supplementation to humans has been demonstrated to be beneficial in the treatment of diabetic polyneuropathy (Keen *et al.*, 1993), and in STZ diabetic rats to improve nerve function (Cameron and Cotter, 1996; Cameron *et al.*, 1998). In addition, diet enriched with a long-chain polyunsaturated fatty acid such as

linolenic acid resulted in increased skeletal muscle membranes polyunsaturated fatty acid content (Field *et al.*, 1990). This correlated with improved peripheral insulin sensitivity (Storlien *et al.*, 1996). Various potential modes of action have been proposed, including alterations in cell membranes fluidity, and elevation in vasodilatatory cyclooxygenase-derived prostaglandins (Cameron *et al.*, 1991; Stevens *et al.*, 1993). In addition, GLA has been shown to potentiate the effect of antioxidants such as ascorbic acid and LA on peripheral nerve function in STZ diabetic rats (Cameron and Cotter, 1996; Hounsom *et al.*, 1998). Several mechanisms have been offered to explain this interaction, including alterations in the pharmacodynamics of the antioxidant component, and/or improved targeting of the antioxidant to lipophylic cellular and plasma components (Cotter *et al.*, 1995).

In the present study, GLA treatment alone was associated with a significant 10% elevation in the hypoglycemic response to exogenous insulin administration (Fig. 1) in a mechanism that did not involve elevation in skeletal muscle GLUT4 content (Fig. 2). The fact that the GLA-LA conjugate increased GLUT4 to the same extent as LA treatment alone, but improved the response to insulin by 23% as compared to 10% and 13% for the GLA alone and LA alone, respectively, suggests that GLA contributed to the insulin-sensitizing effect of the GLA-LA conjugate through a distinct mechanism. The interaction between the LA-derived mechanism and the GLA-derived mechanism appears to be additive rather than synergistic. Among the various potential modes of action, the vasodilatatory effect of GLA through the increased production of prostaglandin E1 is attractive (Manku *et al.*, 1979). Abnormalities in vasomotor function and abnormal transcapillary insulin delivery have been suggested to play a major role in the development of skeletal muscle insulin resistance (Laakso *et al.*, 1990). Thus, GLA-derived vasoactive substances, which may alter vascular tone and endothelial function, may improve insulin sensitivity through mechanisms independent of those exerted by LA, which affects directly skeletal muscle glucose disposal. In addition, GLA-mediated alteration in skeletal muscle membrane fluidity may have improved insulin-stimulated GLUT4 translocation, by affecting fusion processes of GLUT4-containing vesicles with the plasma membrane.

GLA-related improvement in the antioxidant capacity of the GLA-LA conjugate as compared with LA alone may be also suggested by the data presented here. Although GLA treatment alone did not increase plasma cholesterol-corrected vitamin E levels as LA did, it resulted in reduced basal TBARS levels to the same extent as LA. Furthermore, both LA alone and GLA alone did not provide any protection against AAPH induced oxidation of plasma, while the GLA-LA conjugate did (Table 3). These data suggest that, whereas at the tissue level it seems clear that the protective effect of the GLA-LA conjugate can be attributed to the LA component (Fig. 3), at the systemic level the interaction between LA and GLA appears to have contributed to the antioxidative potency of the conjugate. This may result from the potential ability of GLA to improve incorporation of the conjugate to lipophilic components, such as cell membranes and lipoproteins. Whether the metabolic effects of the GLA-LA conjugate can be attributed to its better systemic antioxidant potency requires further investigation.

In conclusion, in the STZ diabetic rat model, treatment with a GLA-LA conjugate improves peripheral insulin sensitivity, through the combination of mechanisms attributable to each of its components. These included elevated skeletal muscle GLUT4 content and may involve protection against diabetes-induced GSH depletion.

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ABBREVIATIONS

AAPH, 2,2′-azo-*bis*(2-amidinopropane) dihydrochloride; GLA, γ-linolenic acid [*cis*-6,9,12-octadecatrienoic acid, (18:3ω6)]; GLA-LA, γ-linolenic acid–α-lipoic acid conjugate; GLUT4, glucose transporter 4, the insulin-sensitive glucose transporter; GSH, reduced glutathione; LA, α-lipoic acid; MDA, malondialdehyde; ROS, reactive oxygen species; STZ, streptozotocin; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TNF, tumor necrosis factor.

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