



The Antidiabetic Effect of Garlic Oil is Associated with Ameliorated Oxidative Stress but Not Ameliorated Level of Pro-inflammatory Cytokines in Skeletal Muscle of Streptozotocin-induced Diabetic Rats

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Abstract

Oxidative stress and inflammatory condition has been broadly accepted being associated with the progression of diabetes. On the other hand, garlic (大蒜 *dà suàn*, bulb of *Allium sativum*) has been shown to possess both antioxidant and anti-inflammatory action in several clinical conditions. Our previous study demonstrated that treatment with garlic oil improves oral glucose tolerance and insulin tolerance and improves the insulin-stimulated utilization of glucose to synthesize glycogen in skeletal muscle in streptozotocin (STZ)-induced diabetes, *in vivo* and *ex vivo*, respectively. The aim of the present study is to investigate the antioxidant and anti-inflammatory effects of garlic oil (GO) in the skeletal muscle of diabetic rats. Rats with STZ-induced diabetes received GO (10, 50, or 100 mg/kg body weight) or corn oil by gavage every other day for 3 weeks. Control rats received corn oil only. GO dose-dependently improved insulin sensitivity, as assessed by the insulin tolerance test, and oral glucose tolerance. GO significantly elevated total glutathione and glutathione peroxidase activity and lowered the nitrate/nitrite content in skeletal muscle at 50 and 100 mg/kg and significantly elevated glutathione reductase activity and lowered lipid peroxidation at 100 mg/kg. By contrast, GO did not reverse diabetes-induced elevation of IL-1 β and TNF- α in skeletal muscle at any tested dose. On the other hand, GO elevated the expression of GLUT4 in skeletal muscle along with glycogen content as observed with PAS staining. In conclusion, the antidiabetic effect of garlic oil is associated with ameliorated oxidative stress in skeletal muscle.

Key words: Antidiabetic; Antioxidant, Garlic oil, Skeletal muscle, Streptozotocin

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Introduction

Diabetes mellitus is a metabolic disturbance that results from insufficient insulin secretion and insulin resistance with the feature of persistent hyperglycemia, which eventually results in specific complications. Poorly controlled hyperglycemia has been implicated in the progression of diabetes. Patients with chronic diabetes are reported to have lowered antioxidant capacity accompanied by elevated indices of oxidative stress (Ceriello et al., 1997; Ramakrishna & Jaikhani, 2008). Both acute and chronic hyperglycemia have been shown to cause elevated concentrations of reactive oxygen species and lowered enzymatic and nonenzymatic cell antioxidant defenses in the normal and diabetic conditions (Ceriello et al., 1997; Bonnefont-Rousselot et al., 2000; Catherwood et al., 2002). Studies at the molecular level have demonstrated that oxidative stress activates a family of serine/threonine kinases, thus interfering with the insulin signaling pathway (Rains & Jain, 2011). GLUT4 expression was also reported to be suppressed by elevated oxidative stress (Pessler et al., 2001; Pessler-Cohen et al., 2006), which affects the insulin-regulated cellular utilization of glucose.

In other lines of investigation, previous studies have suggested that hyperglycemia may be a proinflammatory state (Pickup & Crook, 1998; Festa et al., 2000). Elevations in proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) have been observed in the plasma of persons with uncontrolled diabetes and of healthy volunteers who underwent a hyperglycemic clamp procedure (Esposito et al., 2002), and in isolated human monocytes challenged with a high concentration of glucose (Shanmugam et al., 2003; Shanmugam et al., 2004). The production of reactive oxygen species as a result of acute hyperglycemia subsequently leads to elevated peripheral levels of interleukin-1 (IL-1), TNF- α , and derivatives of nitric oxide (NO) via the activation of transcription factors such as nuclear factor kappa B (NF κ B) or activator protein-1 (AP-1) in a variety of cell types (Esposito et al., 2002; Ling et al., 2003; Maritim et al., 2003). In addition, chronic exposure to hyperglycemia results in the accumulation of glycated products such as advanced glycation end products, which also contribute to the elevation of peripheral proinflammatory cytokines through the stimulation of macrophages (El-Seweid et al., 2002). In the IL-1 β /TNF- α -induced acute inflammatory response, skeletal muscle uptake of glucose is suppressed, which

suggests an inhibitory effect of these proinflammatory cytokines on insulin sensitivity (Ling et al., 1994; Kapur et al., 1997).

Garlic (大蒜 dà suàn, bulb of *Allium sativum*) has a long history of medicinal use for various health problems, including infection, certain type of cancers, cardiovascular disease, and diabetes (Liu et al., 2007). Mathew and Augusti (1973) and Chang & Johnson (1980) suggested that the most probable mechanism of action of the antidiabetic effect of garlic is via direct effects as an insulin secretagogue. We previously demonstrated that treatment with garlic oil improves oral glucose tolerance and insulin tolerance and improves the insulin-stimulated utilization of glucose to synthesize glycogen in skeletal muscle in streptozotocin (STZ)-induced diabetes, *in vivo* and *ex vivo*, respectively (Liu et al., 2005). In accordance with the findings of a study by us on the antioxidant effects of garlic oil in nondiabetic animals *in vivo* (Chen et al., 2003) and the findings of a study by our colleagues on the anti-inflammatory effect of the sulfur-containing compounds of garlic oil in cultured macrophages *in vitro* (Liu et al., 2006), we were interested in investigating whether the action of garlic oil on glycemic control in diabetes is via its antioxidant and anti-inflammatory effects. Under the action of insulin, skeletal muscle mass is the main consumer of glucose in the body, and thus the primary site responsible for the reduced insulin-induced glucose utilization in the diabetic states. To date, however, no investigation has directly assessed the antioxidant and anti-inflammatory effects of garlic oil on mammalian skeletal muscle in the diabetic condition. Therefore, the present study aimed to investigate the effects of garlic oil on the antioxidant defense system and inflammatory condition in skeletal muscle and its association with glycemic control in diabetes.

Materials and Methods

Garlic oil preparation

Garlic oil was prepared from the same batch used in a previous study by steam distillation (Ou et al., 2010) and was composed mainly of 40.83% diallyl disulfide, 38.93% diallyl trisulfide, 7.17% methyl allyl trisulfide, 3.77% diallyl sulfide, 2.75% methyl allyl disulfide, and minor amounts of many other volatile compounds.

Animals and experimental procedure

Four-week-old weanling male Wistar rats were purchased from the National Animal Breeding and

Research Center (Taipei, Taiwan). The animals were kept under a 12-h light-dark cycle at an ambient temperature of 23°C and were given free access to water and standard rat feed (Rodent Diet 5001; Purina Mills, Richmond, IN). All animals were allowed to adapt to the environment for 1 week after their arrival before the experiment started. Diabetes was induced by the injection of STZ (65 mg/kg body weight in citrate buffer, pH 4.5) into a lateral tail vein. The control rats were injected with the same volume of vehicle. Three days after the injection, the diabetic animals were randomly assigned to 4 groups and received by gavage 10, 50, or 100 mg/kg body weight of garlic oil or the vehicle (corn oil; 2 ml/kg body weight) every other day for 3 weeks. The control rats received corn oil by gavage (2 mL/kg body weight). During the 3 weeks of treatment, the animals were housed in metabolic cages and were given free access to water and a powdered diet (Rat Diet 5012; Purina Mills, Richmond, IN). Food and water intakes and urine excretion were measured.

An insulin-tolerance test was carried out on day 10 after the injection. After they were allowed to recover from the insulin-tolerance test for 1 week, the rats were starved overnight and an oral-glucose-tolerance test was carried out on day 17 after the injection. The animals were then starved overnight before they were killed on day 24 after the injection for blood collection and skeletal muscle isolation. Blood samples were prepared for plasma and stored at -20°C until analyzed. Measurements of glucose and insulin in plasma were carried out within 2 weeks. Muscle samples were frozen in liquid nitrogen or fixed in formalin immediately after collection and were used for analysis of markers of inflammation and oxidative stress or for histological analysis, respectively. Housing conditions and experimental procedures were performed according to the *NIH Guide for the Care and Use of Laboratory Animals*, and all protocols were approved by the ethical committee for animal experimentation of Chung Shan Medical University, Taichung, Taiwan.

Insulin-tolerance test and oral-glucose-tolerance test

To test insulin tolerance, an insulin bolus (1 U/kg body weight human regular insulin; Eli Lilly, Indianapolis, IN) was administered by intraperitoneal injection. Samples of blood were withdrawn from the lateral tail vein immediately before and 5, 10, 15, 30, 60, 90, and 120 min after the injection. Plasma prepared from heparin-containing blood samples was frozen at -20°C until analyzed for glucose. The first-order rate

constant for the disappearance rate of glucose (K_{ITT}) was calculated as described previously (Liu *et al.*, 2005).

The oral-glucose-tolerance test was performed by orally administering by gavage a solution of 10% (w/v) glucose (1 g/kg body weight). Blood samples were withdrawn from the lateral tail vein immediately before and 15, 30, 60, 90, and 120 min after the bolus glucose loading. Heparin-containing blood samples were immediately centrifuged, and the plasma was separated and frozen at -20°C until analyzed for glucose. The area under curve (AUC) of the blood glucose response to oral glucose loading was calculated by the trapezoidal rule.

Biochemical analysis of blood and muscle samples

For the analysis of glucose, plasma was deproteinized with 5% (v/v) HClO₄ and then neutralized with 0.5 M triethanolamine/2 M KOH. Universal pH indicator was added to ensure that the deproteinized samples were properly neutralized. Glucose concentrations were analyzed enzymatically by the method of Bergmeyer (Bergmeyer, 1974). The dilution factor during the deproteinization procedure was adjusted for the concentration of glucose in each sample. Plasma concentrations of insulin were determined spectrophotometrically with a rat insulin ELISA kit (Mercodia, Uppsala, Sweden), and contents of TNF- α and interleukin IL-1 β or nitrate/nitrite in the gastrocnemius muscle were measured spectrophotometrically with the use of the rat TNF- α and IL-1 β ELISA kit (Biosource International, Inc., Camarillo, CA) or the nitrate/nitrite kit (Roche Diagnostics, Mannheim, Germany), respectively, according to the manufacturer's instructions and were analyzed with a micro-plate reader (VersaMax; Molecular Devices Ltd., Sunnyvale, CA).

Markers of oxidative stress were determined with gastrocnemius muscle tissue. Lipid peroxidation was determined by measuring thiobarbituric acid-reactive substrates (TBARS) in a fluorescence spectrophotometer (Hitachi F4500, Tokyo, Japan) for the estimation of malondialdehyde content according to the procedure of Fraga *et al.* (Fraga *et al.*, 1988). The total glutathione (GSH) level was assessed by the GSH reductase-DTNB (5.5 dithiobis-2-nitrobenzoic acid) recycling procedure and was analyzed spectrophotometrically at 410 nm as described by Gherghel *et al.* (Gherghel *et al.*, 2005). GSH peroxidase activity was determined spectrophotometrically with a coupled procedure by using H₂O₂ as the substrate (Lawrence and Burk, 1976).

GSH reductase activity was measured by the method of Bellomo *et al.* (Bellomo *et al.*, 1987). Superoxide dismutase (SOD) activity was determined with the Randox SOD kit according to the manufacturer's instructions (Randox Laboratories Ltd, Antrim, UK) and was calculated by the degree of inhibition of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride reaction with superoxide anion generated by xanthine-xanthine oxidase.

Western blot analysis for GLUT4 expression

Samples were prepared by homogenizing gastrocnemius muscle in 250 mM sucrose containing 20 mM HEPES and 1 mM EDTA, pH 7.4. After centrifugation of homogenates for 10 min at 14,000 × g, protein concentrations in the samples were measured with a bicinchoninic acid protein assay reagent kit (Pierce, IL). Equal amounts of muscle protein of each animal in each group were electrophoresed on SDS-PAGE (12%) gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated with nonfat dried milk (50 g/L in 15 mM Tris 150 mM NaCl buffer, pH 7.4) at 4°C overnight to block nonspecific binding. Then, membranes were incubated with polyclonal rabbit anti-GLUT4 antibody (Abcam, Cambridge, UK) at 37°C for 1 h followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (Abcam, Cambridge, UK). Alpha-tubulin was used as internal control with mouse monoclonal antibody to alpha-tubulin (Abcam, Cambridge, UK) as the primary antibody and horseradish peroxidase-conjugated sheep polyclonal antibody to mouse immunoglobulin G as the second antibody. Antibody-bound transporter protein was visualized by using enhanced chemiluminescence substrate, Western Lightning® Plus-ECL (PerkinElmer, Boston, MA) according to the manufacturer's specifications. Films were scanned by using a Luminescent Image Analyzer (FUJIFILM LAS-1000; FujiFilm, Tokyo, Japan). Protein molecular weight marker (Sigma, St. Louis, MO) was used to identify bands of GLUT4 at 45 kDa. The relative intensity of bands of GLUT4 to alpha-tubulin was calculated and the data were expressed as % relative intensity to that of the controls.

Histological analysis of skeletal muscle

Soleus muscle tissue fixed in 10% neutral buffered formalin was embedded in paraffin, sectioned at 5 µm, and stained by periodic acid Schiff (PAS) staining for glycogen in muscle tissue and counterstained with

hematoxylin. Positive PAS staining appears as purple.

The formalin-fixed soleus muscle tissue was otherwise used for immunostaining of 4-hydroxynonenal (HNE)-modified protein with polyclonal rabbit anti-HNE antibody (Calbiochem, San Diego, CA) as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (Abcam, Cambridge, UK) as the secondary antibody. Color was developed by use of a DAB (3,3'-diaminobenzidine) peroxidase substrate kit (Vector Laboratories, Burlingame, CA) and the samples were counterstained with hematoxylin. Positive staining for HNE appeared as brown.

Statistical analysis

The data are expressed as mean ± SD and were analyzed by one-way analysis of variance. Student's *t*-test was used to detect differences in means between the control group and the group of diabetic rats. Duncan's multiple-comparison test was used to detect differences among the means of the STZ-injected groups. *P* values < 0.05 were considered significant. All statistical analyses were performed with commercially available software (SPSS 12 for WINDOWS; SPSS Inc., Chicago, IL).

Results

Animal characteristics

STZ-induced diabetes in rats caused dramatic and significant elevations in food and water intake and urine excretion ($P < 0.05$; Table 1). Fasting blood glucose was more than 2-fold higher, whereas the insulin level was more than 13-fold lower, in the diabetic rats than in the controls ($P < 0.05$). Thus, insufficiency of insulin secretion was directly indicated as the major cause of the diabetes in these animals. The diabetic rats showed significantly less body weight gain along with a significant loss of extensor digitorum longus (EDL) muscle and gastrocnemius muscle weight relative to body weight, which are features of an insufficient anabolic effect from insulin ($P < 0.05$; Table 1). Treatment with garlic oil improved the plasma insulin level in a dose-dependent manner and such effect was significant at the highest dose (100 mg/kg), but marginally improved the fasting glucose level in diabetic rats. Similarly, the elevated volume of urine excretion in the diabetic rats was ameliorated by garlic oil in a dose-dependent manner.

Table 1. Effect of garlic oil on body weight gain, food and water intake, urine excretion, plasma concentrations of glucose and insulin, and skeletal muscle wt/body wt ratio of streptozotocin-induced diabetic rats^x

	Control ^y	DM	DM + GO10 ^z	DM + GO50 ^z	DM + GO100 ^z
Body weight gain (g)	130.9 ± 27.4	80.7 ± 26.0 *	57.4 ± 38.3	40.2 ± 44.0	61.1 ± 29.5
Food intake (g/24h)	26.8 ± 4.6	32.7 ± 2.8 *	33.5 ± 3.1	31.0 ± 6.6	27.5 ± 3.7
Water intake (mL/24h)	49.5 ± 12.1	120.1 ± 13.9 *	135.4 ± 29.8	133.5 ± 25.7	99.1 ± 26.2
Urine excretion (mL/24h)	16.4 ± 3.5	107.0 ± 15.4 ^{*a}	113.3 ± 27.7 ^a	105.5 ± 17.4 ^a	73.5 ± 19.7 ^b
Plasma glucose (mg/dL)	105 ± 12	222 ± 18 *	203 ± 16	215 ± 21	201 ± 13
Plasma insulin (ng/mL)	1.625 ± 0.351	0.112 ± 0.026 ^{*a}	0.104 ± 0.025 ^a	0.120 ± 0.046 ^a	0.377 ± 0.103 ^b
Muscle weight/body weight x 100 (%)					
Soleus	0.051 ± 0.003	0.050 ± 0.008	0.050 ± 0.005	0.049 ± 0.004	0.051 ± 0.005
EDL	0.049 ± 0.003	0.041 ± 0.004 ^{*a}	0.045 ± 0.003 ^b	0.043 ± 0.006 ^{ab}	0.044 ± 0.006 ^{ab}
Gastrocnemius	0.610 ± 0.025	0.502 ± 0.112 *	0.556 ± 0.041	0.524 ± 0.075	0.535 ± 0.081

^x Values are the mean V SD for six rats per group.

^y Rats injected with citrate buffer and treated with vehicle (corn oil).

^z GO10, GO50, or GO100 represent 10, 50, or 100 mg/kg garlic oil, respectively.

* Significant difference between the control group and the DM group ($P < 0.05$).

^{ab} DM groups that do not share a letter are significantly different ($P < 0.05$).

Oral-glucose-tolerance test and insulin-tolerance test

The integral values of the blood glucose response after oral glucose loading are given in Figure 1. Glucose tolerance deteriorated in the vehicle-treated diabetic animals: the area under the glucose tolerance curve was significantly greater in the diabetic group than in the control group. However, this deteriorated glucose tolerance in diabetes was attenuated by treatment with garlic oil in a dose-dependent manner (Figure 1). K_{ITT} was calculated in accordance with the initial blood glucose response after the injection of insulin. As shown in Figure 2, K_{ITT} was significantly slower in the diabetic rats, which suggested the development of insulin resistance in these animals ($P < 0.05$). In addition, the K_{ITT} for the insulin-induced plasma glucose decay was significantly improved in the garlic oil-treated diabetic groups compared with the vehicle-treated diabetic group ($P < 0.05$).

Muscular GLUT4 expression

The induction of diabetes by STZ caused a significant reduction of GLUT4 expression in skeletal muscle by 30.5% compared with that of the controls, which was reversed by treatment with garlic oil in a dose-dependent manner. The highest dose (100 mg/kg) of garlic oil elevated the expression of this glucose transporter by 26.3% compared with that of the vehicle-treated group (Figure 3). These results suggested that less GLUT4 is available in STZ-induced diabetes when under the stimulation of insulin but that this reduction can be reversed by treatment with garlic oil.

Muscular oxidative stress and inflammation indices

Compared with the levels in controls rats, lipid peroxidation (as measured by levels of TBARS) was significantly higher in diabetic rats, while total GSH

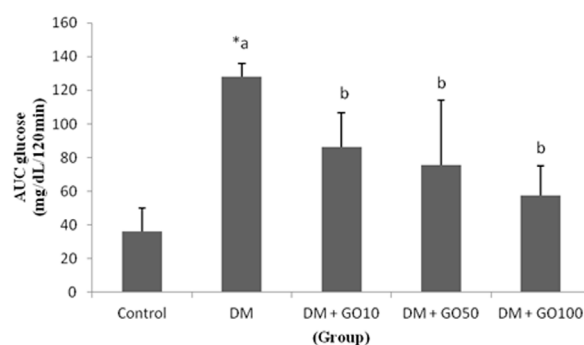


Figure 1. Effect of garlic oil on the area under the curve of the plasma glucose response to an oral glucose bolus. A glucose bolus (1 g/kg body weight) was administered orally at 17 days after the induction of diabetes. Diabetic rats received by gavage 10, 50, or 100 mg/kg garlic oil (GO10, GO50, or GO100, respectively) or the vehicle (DM). Control rats were treated with vehicle only. Data are means ± SD for six rats per group. *Significant difference between the control group and the DM group ($P < 0.05$). ^{a,b}DM groups not sharing the same superscript letter are significantly different ($P < 0.05$).

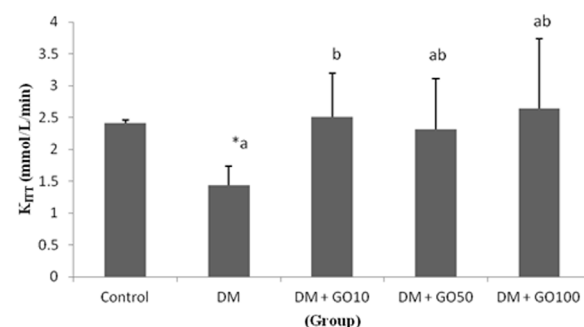


Figure 2. Effect of garlic oil on the first order rate constant for the disappearance rate of glucose in plasma (K_{ITT}) after the insulin bolus. An insulin bolus (1 U/kg body weight) was administered intraperitoneally on day 10 after the induction of diabetes. Diabetic rats received by gavage 10, 50, or 100 mg/kg garlic oil (GO10, GO50, or GO100, respectively), or the vehicle (DM). Control rats were treated with vehicle only. Data are means ± SD for six rats per group. *Significant difference between the control group and the DM group ($P < 0.05$). ^{a,b}Groups not sharing the same superscript letter are significantly different ($P < 0.05$).

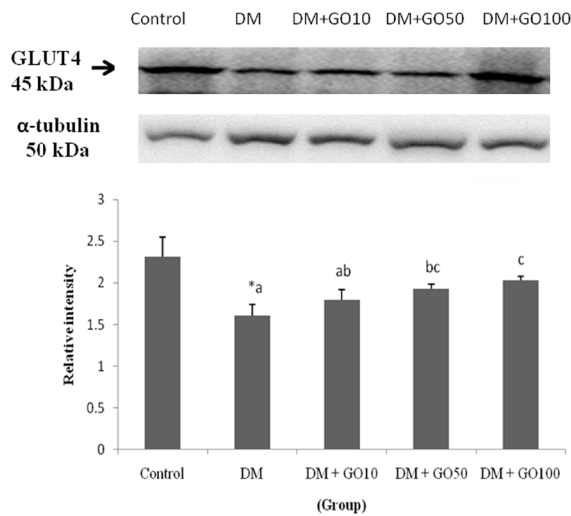


Figure 3. Effect of treatment with garlic oil on muscular GLUT4 expression. Diabetic rats received by gavage 10, 50, or 100 mg/kg garlic oil (GO10, GO50, or GO100, respectively) or the vehicle (DM) for 3 weeks. Control rats were treated with vehicle only. Data are means D SD for six rats per group. *Significant difference between the control group and the DM group ($P < 0.05$). ^{a,b}Groups not sharing the same superscript letter are significantly different ($P < 0.05$).

content and the activity of SOD were significantly lower in skeletal muscle ($p < 0.05$). Activities of GSH peroxidase and GSH reductase were marginally lowered in diabetic rats compared with control rats (Table 2). Treatment with garlic oil significantly reversed the elevated lipid peroxidation and elevated total GSH content in skeletal muscle in a dose-dependent manner. In addition, the activities of GSH peroxidase and GSH reductase in skeletal muscle of diabetic rats were significantly improved by treatment with garlic oil in a dose-dependent manner (Table 2). The investigation of levels of proinflammatory cytokines in the skeletal muscle showed that the contents of both IL-1I and TNF-T were significantly higher in diabetic rats

than in the controls ($P < 0.05$). Treatment with garlic oil only marginally reversed the elevation of these proinflammatory cytokines compared with the vehicle-treated diabetic animals, however, and without statistical significance (Table 2). On the other hand, STZ-induced diabetes significantly elevated the nitrate/nitrite content in skeletal muscle ($P < 0.05$; Table 2), which was significantly suppressed by treatment with garlic oil in a dose-dependent manner (Table 2).

Histological analysis

The results of the HNE immunostaining of the cross-section of soleus muscle showed an apparently greater positively stained area (dark-brown color) in the diabetic group than in the controls (Figure 4). Treatment with garlic oil appeared to decrease the level of the positively stained area, which suggested that garlic oil ameliorated the level of the prooxidant in the skeletal muscle of diabetic rats.

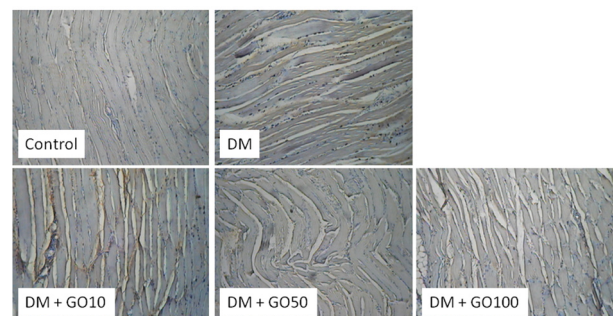


Figure 4. Immunostaining of the soleus muscle with anti-HNE (4-hydroxynonenal) antibody. Diabetic rats received by gavage 10, 50, or 100 mg/kg garlic oil (GO10, GO50, or GO100, respectively) or the vehicle (corn oil) for 3 weeks. Control rats were treated with vehicle only. Original magnification x 100.

Table 2. Effect of garlic oil on contents of IL-1T, TNF-alpha, Nitrate/nitrite, and GSH; activity of GSH peroxidase, GSH reductase, and SOD; and lipid peroxidation in skeletal muscle of streptozotocin-induced diabetic rats^x

	Control ^y	DM	DM + GO10 ^z	DM + GO50 ^z	DM + GO100 ^z
GSH (nmol/mg protein)	5.34 ± 1.53	1.46 ± 0.90 ^{a,*}	1.91 ± 0.59 ^a	3.39 ± 0.98 ^b	5.05 ± 1.33 ^c
GSH reductase (nmol NADPH/min/mg protein)	10.36 ± 5.12	6.06 ± 2.47 ^a	7.44 ± 0.82 ^a	10.47 ± 4.89 ^{ab}	13.63 ± 5.41 ^b
GSH peroxidase (nmol NADPH/min/mg protein)	30.5 ± 7.9	22.8 ± 6.0 ^a	28.2 ± 4.4 ^{ab}	33.6 ± 9.4 ^b	36.5 ± 6.6 ^b
SOD (U/mg protein)	0.423 ± 0.116	0.201 ± 0.042 ^{a,*}	0.190 ± 0.044	0.325 ± 0.126	0.325 ± 0.121
TBARS (nmol/mg protein)	0.261 ± 0.112	0.627 ± 0.192 ^{b,*}	0.565 ± 0.370 ^b	0.421 ± 0.104 ^{ab}	0.230 ± 0.115 ^a
IL-1β (ng/mg protein)	1.284 ± 0.577	3.249 ± 1.787 [*]	1.999 ± 0.909	2.091 ± 0.306	2.268 ± 0.644
TNF-α (ng/mg protein)	0.349 ± 0.053	1.192 ± 0.676 [*]	1.100 ± 0.563	0.821 ± 0.556	0.689 ± 0.270
Nitrate/nitrite (nmol/mg protein)	2.49 ± 1.97	5.73 ± 1.77 ^{b,*}	3.15 ± 1.93 ^{ab}	2.73 ± 1.71 ^a	2.23 ± 0.79 ^a

^x Values are the mean V SD for six rats per group.

^y Rats injected with citrate buffer and treated with vehicle (corn oil).

^z GO10, GO50, or GO100 represent 10, 50, or 100 mg/kg garlic oil, respectively.

* Significant difference between the control group and the DM group ($P < 0.05$).

^{abc} DM groups that do not share a letter are significantly different ($P < 0.05$).

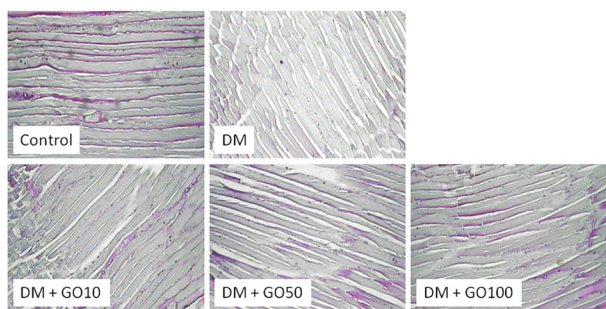


Figure 5. Cross-sections of soleus muscle stained with periodic acid-Schiff (PAS). Diabetic rats received by gavage 10, 50, or 100 mg/kg garlic oil (GO10, GO50, or GO100, respectively) or the vehicle (corn oil) for 3 weeks. Control rats were treated with vehicle only. Original magnification x 100.

Results of the PAS staining of the cross-section of soleus muscle showed an apparently greater positively stained area (purple color) in the controls than in the diabetic rats (Figure 5). Treatment with garlic oil appeared to elevate the level of positive staining, thus suggesting improved glycogen storage in the skeletal muscle of the diabetic rats (Figure 5).

Discussion

We previously reported that glucose intolerance in STZ-induced diabetic rats is associated with both decreased insulin secretion and the development of insulin resistance along with lowered *ex vivo* utilization of glucose stimulated by insulin in skeletal muscle. Furthermore, we reported that treatment with garlic oil improved glycemic control *in vivo* along with the glucose utilization rate stimulated by insulin in skeletal muscle *ex vivo* (Liu *et al.*, 2005). The results of the present study demonstrated that the lowered insulin sensitivity of rats with STZ-induced diabetes is at least partly due to lowered GLUT4 expression in skeletal muscle. This result is consistent with a report by Muñoz *et al.* (1996), who found lowered protein content of GLUT4 in both red and white skeletal muscle of STZ-induced diabetic animals. Gaster *et al.* (2001) demonstrated the reduction of GLUT4 expression in slow fibers in subjects with type 2 diabetes and proposed that this may well reduce the insulin-sensitive GLUT4 pool in diabetes and contribute to skeletal muscle insulin resistance. On the other hand, the present study demonstrated that the hypoglycemic effect of garlic oil in diabetes is at least partly via the improvement of GLUT4 expression in skeletal muscle and is associated with ameliorated local oxidative stress in skeletal muscle. In addition, consistent with the results of our previous study showing that garlic oil reversed the

lowered insulin-stimulated glycogen synthesis from glucose in diabetic skeletal muscle *ex vivo* (Liu *et al.*, 2005), the present study showed reversed glycogen storage in diabetic skeletal muscle with garlic oil treatment.

Investigation of diabetic subjects has found increased protein oxidation, lipid peroxidation, and NO levels and decreased levels of enzymatic and nonenzymatic antioxidants in peripheral plasma or in erythrocytes (Ramakrishna & Jaikhani, 2008). Similarly, in STZ-induced diabetic rats, increased oxidative stress is accompanied by decreased antioxidant power in plasma and erythrocytes, pancreas, and kidney (Kakkar *et al.*, 1998; Anwar & Meki, 2003; Nakhaee *et al.*, 2009; Ali & Agha, 2009). In the skeletal muscle of STZ-induced diabetic rats, prooxidant compounds, such as hydrogen peroxide and HNE, are found to increase whereas antioxidant levels fall (Aragno *et al.*, 2004). Bravard *et al.* (2011) proposed that mitochondria and xanthin oxidase were the major sources of the hyperglycemia-induced production of reactive oxygen species in skeletal muscle. Consistent with these previous findings, the present study reported a significant reduction of total GSH content and activities of GSH peroxidase, GSH reductase, and SOD in skeletal muscle of diabetic rats. The elevated lipid peroxidation and levels of HNE in skeletal muscle in the present study confirm the existence of elevated oxidative stress in this tissue as a consequence of the exhausting of antioxidant mechanisms in the diabetic condition. In another study, low-grade oxidant stress was demonstrated to lower insulin sensitivity in isolated rat skeletal muscle (Dokken *et al.*, 2008). It was proposed that direct exposure of mammalian skeletal muscle to an oxidant stress disturbs the insulin signaling pathway via the stimulation of p38 MAPK (Henriksen *et al.*, 2011). Increased oxidative stress has also been reported to repress the expression of GLUT4 in adipocytes and is associated with impaired binding of nuclear proteins to the insulin-responsive element in the GLUT4 promoter (Pessler *et al.*, 2001; Pessler-Cohen *et al.*, 2006).

Garlic has been known for its antioxidant effects in a variety of pathophysiological conditions (Liu *et al.*, 2007); however, these effects of garlic have been demonstrated to differ in different tissues and organs. For example, in STZ-induced diabetic rats, although garlic oil elevates the level of total thiols and ameliorates lipid peroxidation in plasma and erythrocyte, these two indexes of oxidative stress are not affected in the kidneys of these animals (Anwar and

Meki, 2003). Furthermore, it was recently demonstrated that methanolic-garlic extract attenuates oxidative stress in hepatic and intestinal tissues of STZ-induced diabetic rats (Rajani Kanth *et al.*, 2008) and that fresh garlic homogenate ameliorates renal oxidative stress and nitric oxide production in STZ-induced diabetic rats (Mariee *et al.*, 2009). To our knowledge, however, no studies have reported the effect of garlic oil on local redox status in the skeletal muscle or its association with the capability of this tissue to utilize glucose. Our data showed that garlic oil improved the antioxidant mechanisms in skeletal muscle in a dose-dependent manner in addition to lowering the level of the prooxidant HNE and lowering the level of lipid peroxidation in this tissue. We interpret our findings to mean that garlic oil has antioxidant activity in diabetic skeletal muscle, thus improving GLUT4 expression and reversing insulin resistance, which was reflected by the ameliorated glycogen content in the skeletal muscle.

Inflammatory mechanisms have been recognized a cause of insulin resistance, and the identified inflammatory factors involved include molecules such as TNF- α , IL-1 β , and NO (Jager *et al.*, 2007; Tilg & Moschen, 2008; Nov *et al.*, 2010). A hyperglycemia-related inflammatory condition has been proposed, at least partly via the elevated oxidative stress that directly activates certain transcription factors, such as NF- κ B and AP-1. Activation of these transcription factors in turn increases the production of proinflammatory cytokines from various cell types along with increased expression of inducible nitric oxide synthase and the production of NO (Maritim *et al.*, 2003), the latter of which could further worsen oxidative stress. The accumulation of advanced glycation end products as the result of chronic hyperglycemia was also proposed to activate cells of the immune system to generate these molecules (El-Seweidy *et al.*, 2002). Elevated contents of TNF- α , IL-1 β , and NO are found in skeletal muscle of type 2 diabetes patients (Torres *et al.*, 2004; Wellen & Hotamisligil, 2005), which is consistent with the findings of the present study with STZ-induced diabetic rats. Mingrone *et al.* (2002) reported that the amount of TNF- α mRNA in skeletal muscle correlates inversely with GLUT4 mRNA and the glucose uptake level. In addition, an *in vitro* study demonstrated that proinflammatory cytokines lowering GLUT4 expression in skeletal muscle by increasing NO production (Bédard *et al.*, 1997). Both may explain at least in part our finding that the elevated levels of TNF-T and NO occurred concomitantly with lowered GLUT4

expression in skeletal muscle of diabetic rats.

Garlic and its components have been reported to inhibit the production of TNF- α , IL-1 β , and NO in a variety of cell types, at least partly due to the inhibition of NF κ B activity (Aggarwal & Shishodia, 2004). Although it has been shown that two major S-containing compounds of garlic oil, diallyl sulfide and diallyl disulfide, can suppress bacterial infection-induced elevation of TNF- α in STZ-DM rats, to our knowledge, data are lacking on the effect of garlic oil in the elevated proinflammatory condition associated with diabetes or hyperglycemia in humans or in experimental animals. The present study demonstrated that oral administration of garlic oil only marginally ameliorated the skeletal muscle content of TNF- α and IL-1 β of diabetic rats and thus may not be the major cause of the reversed GLUT4 expression and glycogen storage found in these animals. Nevertheless, the present study found that the hypoglycemic effect of garlic oil is associated with a lowered level of NO in skeletal muscle. This finding is consistent with the finding of a recent study by Padiya *et al.* (2011), who showed that in diet-induced metabolic syndromes in rats, treatment with raw garlic homogenate effectively improves insulin sensitivity and normalizes serum levels of nitric oxide. The present study further confirmed that the inhibitory effect of garlic oil on NO is associated with improved GLUT4 expression.

Garlic has been used as a folk remedy for diabetes in many areas of the world; however, the mechanisms by which garlic improves glycemic control remain to be clarified. In a model of STZ-induced diabetes in rats, the present study demonstrated that garlic oil can improve glucose tolerance and insulin sensitivity via the elevation of GLUT4 expression, which may be explained in part by an improved antioxidant defense system and lowered NO production in skeletal muscle. Thus, the results of this study provide a basis for the use of garlic oil to improve insulin sensitivity in diabetes.

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