Mol Cell Biochem (2016) 414:105–113
DOI 10.1007/s11010-016-2664-7

L-Cysteine supplementation increases adiponectin synthesis and secretion, and GLUT4 and glucose utilization by upregulating disulfide bond A-like protein expression mediated by MCP-1 inhibition in 3T3-L1 adipocytes exposed to high glucose

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Received: 12 November 2015 / Accepted: 11 February 2016 / Published online: 20 February 2016

Abstract Adiponectin is an anti-diabetic and anti-atherogenic adipokine; its plasma levels are decreased in obesity, insulin resistance, and type 2 diabetes. An adiponectin-interacting protein named disulfide bond A-like protein (DsbA-L) plays an important role in the assembly of adiponectin. This study examined the hypothesis that L-cysteine (LC) regulates glucose homeostasis through the DsbA-L upregulation and synthesis and secretion of adiponectin in diabetes. 3T3L1 adipocytes were treated with LC (250 and 500 μM, 2 h) and high glucose (HG, 25 mM, 20 h). Results showed that LC supplementation significantly ($p < 0.05$) upregulated the DsbA-L, adiponectin, and GLUT-4 protein expression and glucose utilization in HG-treated adipocytes. LC supplementation significantly ($p < 0.05$) promoted the secretion of total and HMW adiponectin secretion in HG-treated adipocytes. In addition, LC significantly ($p < 0.05$) decreased ROS production and MCP-1 secretion in HG-treated cells. We further investigated whether MCP-1 has any role of LC on DsbA-L expression and adiponectin levels in 3T3-L1 cells. Treatment with LC prevented the decrease in DsbA-L, adiponectin, and GLUT-4 expression in 3T3L1 adipocyte cells exposed to MCP-1. Thus, this study demonstrates that DsbA-L and adiponectin upregulation mediates the beneficial effects of LC on glucose utilization by inhibiting MCP-1 secretion in adipocytes and provides a novel mechanism by which LC supplementation can improve insulin sensitivity in diabetes.

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Keywords Adiponectin · Adipocytes · L-cysteine · Diabetes · Disulfide bond A-like protein

Introduction

Adiponectin is a major adipocyte-secreted protein and is downregulated in obesity and diabetes [1]. Epidemiological evidence has indicated that circulating adiponectin levels are reduced in patients with insulin resistance, type 2 diabetes, obesity, or cardiovascular disease [2–4]. Adiponectin is considered a potent modulator of lipid and glucose metabolism with anti-diabetic, anti-atherogenic, and anti-inflammatory properties, and it plays an important role in the pathogenesis of metabolic diseases. Circulating adiponectin exists in three oligomeric forms: trimer, hexamer, and high molecular weight (HMW), the latter being the major bioactive isoform. DsbA-L protein was recently identified as a chaperone protein that catalyzes two trimers into a disulfide-linked hexamer, which further helps assemble the HMW form of adiponectin; [5]. The level of DsbA-L is significantly reduced in obese mice and human subjects [5]. Studies showed that fat-specific overexpression of DsbA-L promoted adiponectin multimerization and protected mice against diet-induced obesity and insulin resistance [6] suggesting a potential mechanism by which DsbA-L promotes adiponectin biosynthesis and function. DsbA-L has been identified as a key regulator for adiponectin multimerization and secretion in adipocytes. Thus, targeting adiponectin synthesis and secretion has been regarded as an important therapeutic tool for preventing insulin resistance associated with obesity and type 2 diabetes.

Diabetic patients have lower blood levels of L-cysteine and altered cysteine homeostasis [7–10]. Supplementation

with cysteine-rich proteins (whey protein and α-lactalbumin) [11–14], L-cysteine (LC) or N-acetylcysteine [15–23], or the cysteinate form of different compounds is better than the non-cysteinate form [24–27] at increasing glucose utilization and lowering glycemia in diabetic animal studies. There is epidemiological evidence suggesting that overweight subjects with a high intake of milk and dairy products are at lower risk of developing diseases related to the insulin resistance syndrome [14]. Recognition of L-cysteine deficiency has led to many supplementation studies using N-acetylcysteine (NAC) as a source to replenish cysteine levels in diabetes. Recent reviews have discussed various studies that provide evidence for the benefits of LC supplementation in lowering oxidative stress and insulin resistance biomarkers in type 2 diabetic patients and diabetic animals [7, 28]. There is no study that has examined whether DsbA-L plays any role in the beneficial effect of L-cysteine on improved glucose metabolism in diabetes.

This study has examined the hypothesis that L-cysteine upregulates the DsbA-L and therefore could promote synthesis and secretion of adiponectin in adipocytes through DsbA-L upregulation mediated by monocyte chemoattractant protein-1 inhibition in 3T3-L1 adipocytes exposed to high glucose.

Materials and methods

3T3L1 cell culture and differentiation

The murine 3T3L1 fibroblast cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in high-glucose (HG) DMEM containing 10 % (v/v) FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in a humidified atmosphere containing 5 % (v/v) CO_2. Three days after achieving confluence, to allow for differentiation into adipocytes, cells were incubated in HG DMEM containing 10 % (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin supplemented with 100 milliunits/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 250 nM dexamethasone for 2 days. The cells were then placed in the same medium containing insulin but lacking any other supplements for an additional 2 days. The media were replaced every 2 days thereafter until >85 % of the cells contained lipid droplets. Seven to 10 days after the induction of differentiation, 3T3L1 adipocytes were ready to be used in experiments [29]. The cells were incubated with serum-free low-glucose DMEM during the experimental incubation period.

Treatment with high glucose (HG) and L-cysteine (LC)

Cells were treated with normal glucose (5.5 mM) and HG (25 mM) with and without LC. In this study, cells were exposed to 25 mM HG. Many previous studies have reported that glucose concentrations as high as 50 mM have been found in the blood of patients with uncontrolled diabetes [30]. It is true that blood glucose levels in patients are not likely to stay as high as 25 mM for 24 h. However, tissue damage in diabetic patients occurs over many years of countless hyperglycemic episodes. Thus, the glucose concentration of 25 mM used in this cell-culture study does not seem unreasonable. Cells were pretreated with two different concentrations (250 and 500 μM) of LC for 2 h, followed by HG exposure for the next 20 h. After treatment, cells were lysed in RIPA supplemented with protease and phosphatase inhibitors. Lysates were cleared by centrifugation and total protein concentrations were determined using a BCA assay kit (Pierce/Thermo Scientific, Rockford, IL, USA).

MCP-1 treatment of adipocytes

3T3-L1 cells were treated with recombinant human MCP-1 (SRP3109, Sigma-Aldrich, St. Louis, MO, USA) dissolved in phosphate buffered saline solution containing 1 mg/mL bovine serum albumin at a concentration of 1 and 2.5 ng/ml for 24 h under serum-free conditions. After treatment, cells were lysed in RIPA supplemented with protease and phosphatase inhibitors. Lysates were cleared by centrifugation and total protein concentrations were determined using a BCA assay kit (Pierce/Thermo Scientific, Rockford, IL, USA).

Glucose utilization and cytokine secretion

Glucose assays were done at 0 h and at the end of the experiments. The glucose utilization level was determined by subtracting glucose values at the end of the experiments (leftover glucose) from the 0 h glucose level. All assays were done in duplicate at each time point. Bayer Contour Next EZ Glucose Meter (Bayer HealthCare LLC, Mishawaka, IN, USA) was used for the glucose utilization assay. The MCP-1 (monocyte chemoattractant protein-1) level in the supernatants of treated cells was determined by the sandwich ELISA method using a commercially available kit from R&D Systems (Minneapolis, MN, USA). Adiponectin levels were determined using a kit and reagents from ALPCO Diagnostics (Salem, NH). All appropriate controls and standards as specified by each

manufacturer's kit were used. In the cytokine assay, control samples were analyzed each time to check the variation from plate to plate on different days of analyses.

ROS assay in adipocytes

For the ROS measurement, cells were pretreated with two different concentrations (250 and 500 μM) of LC for 2 h, followed by HG exposure for the next 20 h. After treatment, cells were labeled with 2′, 7′-dichlorofluorescein diacetate (H_2DCFDA, Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 20 μM and incubated at 37 °C for 30 min using a plate reader, and the fluorescence was determined at filter settings of 485 nm excitation and 528 nm emission.

Immunoblotting

All samples contained approximately the same amount of protein (∼15–20 μg) and were run on 10 % SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked at room temperature for 2 h in a blocking buffer containing 1 % BSA to prevent non-specific binding and then incubated with anti-DsbA-L (1:2000 dilution), anti-adiponectin (1:1000), and anti-GLUT4 (1:1000) primary antibodies at 4 °C overnight. The membranes were washed in TBS-T (50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 0.1 % Tween 20) for 30 min and incubated with the appropriate HRP-conjugated secondary antibody (1:5000 dilution) for 2 h at room temperature and developed using the ultrasensitive ECL substrate (Millipore, MA). The intensity of each immunoblotting band was measured using the histogram tool of Adobe Photoshop CS5. Antibodies against DsbA-L, adiponectin, GLUT-4, and β-actin were purchased from Abcam, Inc. (Cambridge, MA, USA). All other chemicals were purchased from Sigma unless otherwise mentioned.

Statistical analysis

Results are expressed as mean ± SE. Data were analyzed using Student's t test or ANOVA followed by Duncan's multiple range test (DMRT with SPSS version 15.00.; SPSS Inc., Chicago, IL, USA). In all cases, $p < 0.05$ was considered to be statistically significant. In all tests, $p < 0.05$ was considered significant.

Results

Figure 1 illustrates the effect of LC supplementation on DsbA-L, adiponectin levels, and total and HMW adiponectin secretion in HG-treated 3T3L1 adipocytes. It shows that HG exposure caused a reduction in the expression of both DsbA-L (Fig. 1a) as well as adiponectin (Fig. 1b). In addition, HG exposure also caused a significant decrease in total (Fig. 1d) and HMW adiponectin secretion (Fig. 1e) in adipocytes. Supplementation with LC caused the upregulation of DsbA-L and adiponectin protein expression, and increased the secretion of total and HMW adiponectin in HG-treated adipocytes. LC did not have any effect in control glucose-treated adipocytes.

GLUT4 is a major player in the regulation of glucose metabolism. To determine whether upregulation of adiponectin by LC also upregulates the glucose metabolism, we investigated the effect of LC supplementation on GLUT4 and glucose utilization in adipocytes exposed to high glucose. Figure 1c, f shows the effect of LC supplementation on GLUT4 total protein expression and glucose utilization in HG-treated 3T3L1 adipocytes. HG treatment caused a significant decrease in GLUT4 protein expression (Fig. 1c) in adipocytes. Treatment with LC upregulated the GLUT4 total protein expression and glucose utilization in HG-treated adipocytes. The beneficial effect on glucose utilization level (Fig. 1f) was greater with higher concentration of LC.

ROS levels are elevated in diabetes and in high-glucose-treated cell and are implicated to play a role in impaired glucose metabolism [31, 32]. Figure 2 demonstrates the effect of LC supplementation on ROS production and MCP-1 secretion in HG-treated 3T3L1 adipocytes. We also observed that HG was capable of producing more ROS in adipocytes and LC pretreatment of cells resulted in significantly lower levels of ROS than those seen in the cells treated with only HG (Fig. 2a). Similarly, HG stimulated the secretion of MCP-1 and pretreatment with LC significantly lowered the secretion of MCP-1 in HG-treated adipocytes (Fig. 2b).

Since HG has increased the secretion of MCP-1, we analyzed whether HG affects adiponectin synthesis and secretion and DsbA-L and GLUT-4 expression are mediated through MCP-1. Figure 3 illustrates the effect of MCP-1 on adipocytes at two different concentrations (1.0 and 2.5 ng/ml). Treating 3T3-L1 adipocytes with MCP-1 significantly and concentration dependently inhibited the expression of DsbA-L (Fig. 3a), adiponectin (Fig. 3b), and GLUT-4 (Fig. 3c) expression in adipocytes.

To determine whether LC has the potential to overcome MCP-1 effect on adiponectin synthesis and secretion and GLUT-4 expression in adipocytes, we pretreated the cells with LC for 2 h before treatment with MCP-1 (2.5 ng/ml) for next 24 h. Figure 4 shows that adipocytes exposed to MCP-1 significantly attenuated DsbA-L (Fig. 4a) expression and reduction in adiponectin (Fig. 4b) expression. In addition, MCP-1 exposure also caused a significant decrease in total (Fig. 4d) and HMW adiponectin secretion

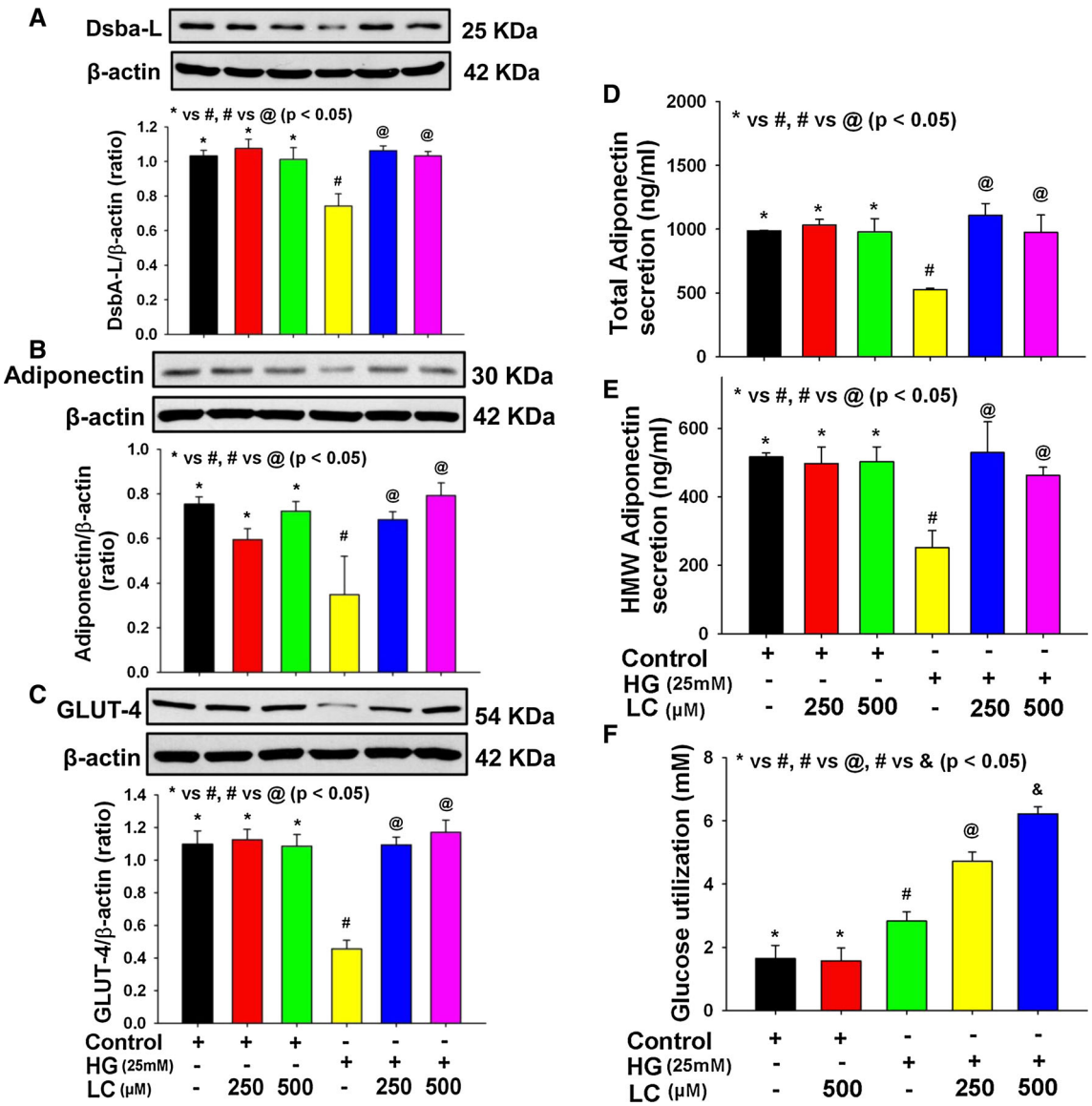

Fig. 1 Effect of LC supplementation on DsbA-L, adiponectin, GLUT-4 protein expression, total and HMW adiponectin secretion, and glucose utilization in 3T3L1 adipocytes exposed to high glucose. **a** DsbA-L protein expression, **b** adiponectin protein expression, **c** GLUT-4 protein expression, **d** total adiponectin secretion, **e** HMW adiponectin secretion, and **f** glucose utilization. Cells were pretreated with LC for 2 h followed by high-glucose (25 mM) exposure for the next 20 h. Values are mean ± SE ($n = 3$)

(Fig. 4e) in adipocytes. Pretreatment with LC prevented the deleterious effects on the DsbA-L and adiponectin protein expression and the secretion of total and HMW adiponectin in MCP-1 treated adipocytes. Similarly, pretreatment with LC prevented the inhibition of GLUT4 (Fig. 4c) in adipocytes exposed to MCP-1.

Discussion

The present study demonstrates that LC upregulates the synthesis and secretion of adiponectin in 3T3-L1 adipocytes exposed to high glucose. L-cysteine supplementation also caused inhibition of MCP-1 secretion in high-glucose-exposed adipocytes. The expression of DsbA-L and GLUT-4 and glucose utilization in HG-treated adipocytes were also increased by the LC supplementation. In addition, exogenous MCP-1 treatment inhibited the adiponectin synthesis and secretion and LC restores the synthesis of adiponectin in MCP-1-treated adipocytes.

A strong correlation between adiponectin and systemic insulin sensitivity has been well established both in vivo and in vitro in mice, other animals, and humans [33–42]. Insulin-sensitizing thiazolidinedione (TZD) class of compounds are known to improve lower plasma levels of adiponectin associated with type 2 diabetes [43–45].

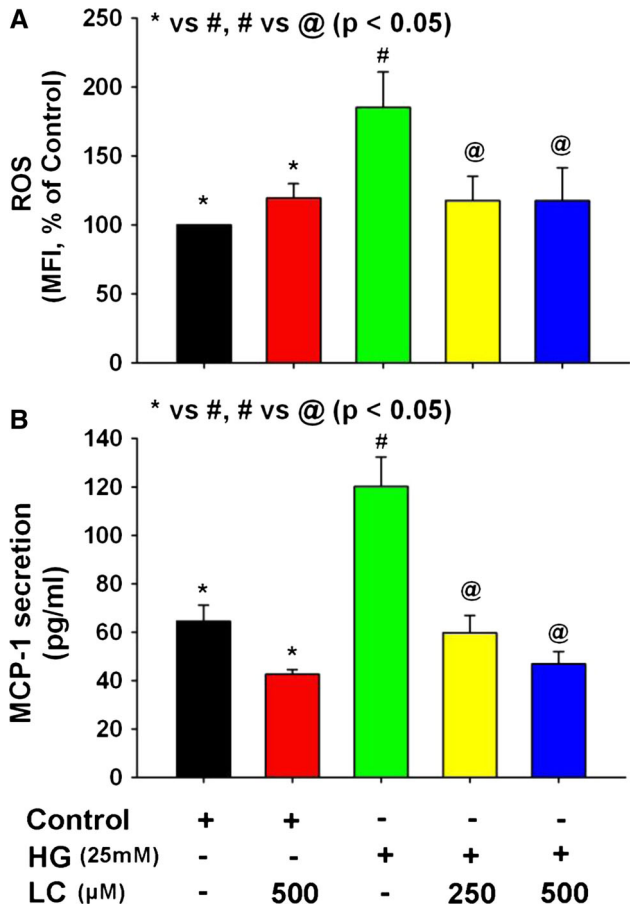

Fig. 2 Effect of LC supplementation on ROS levels and MCP-1 secretion in 3T3L1 adipocytes exposed to high glucose. **a** ROS levels and **b** MCP-1 secretion. Cells were pretreated with LC for 2 h followed by high-glucose (25 mM) exposure for the next 20 h. Values are mean ± SE ($n = 3$)

Epidemiological studies demonstrate a strong correlation between insulin sensitivity and circulating adiponectin levels [46, 47]. Clinical studies suggest that high-molecular weight form of adiponectin is a better correlate for insulin sensitivity in general than total adiponectin [48, 49]. This study demonstrates that LC supplementation upregulates the DsbA-L expression and increases the synthesis and secretion of total and HMW adiponectin. LC supplementation increased GLUT-4 protein expression and glucose utilization in adipocytes exposed to high glucose. This suggests that LC upregulation of DsbA-L may have a key role in increasing adiponectin level, thereby increasing the glucose utilization and metabolism in adipocytes.

MCP-1 levels are increased in obese subjects and diabetic patients. MCP-1 is an important factor involved in inflammatory processes and induces the inflammation of adipocytes and microvasculature [50]. Our results show that HG significantly increased secretion of MCP-1 and treatment with LC effectively inhibited the MCP-1

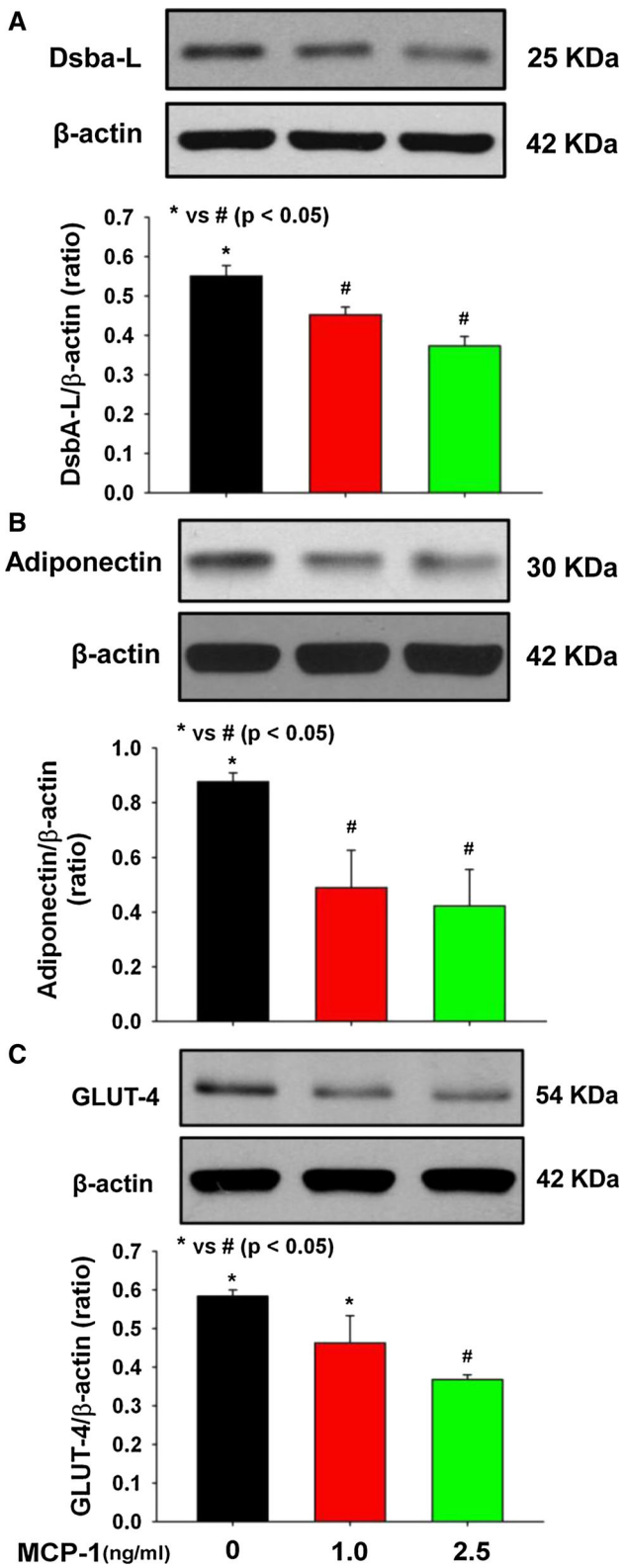

Fig. 3 Effect of MCP-1 treatment on DsbA-L, adiponectin, and GLUT-4 protein expression in 3T3L1 adipocytes. **a** DsbA-L protein expression, **b** adiponectin protein expression, and **c** GLUT-4 protein expression. Cells were pretreated with LC for 2 h followed by MCP-1 exposure for the next 24 h. Values are mean ± SE ($n = 3$)

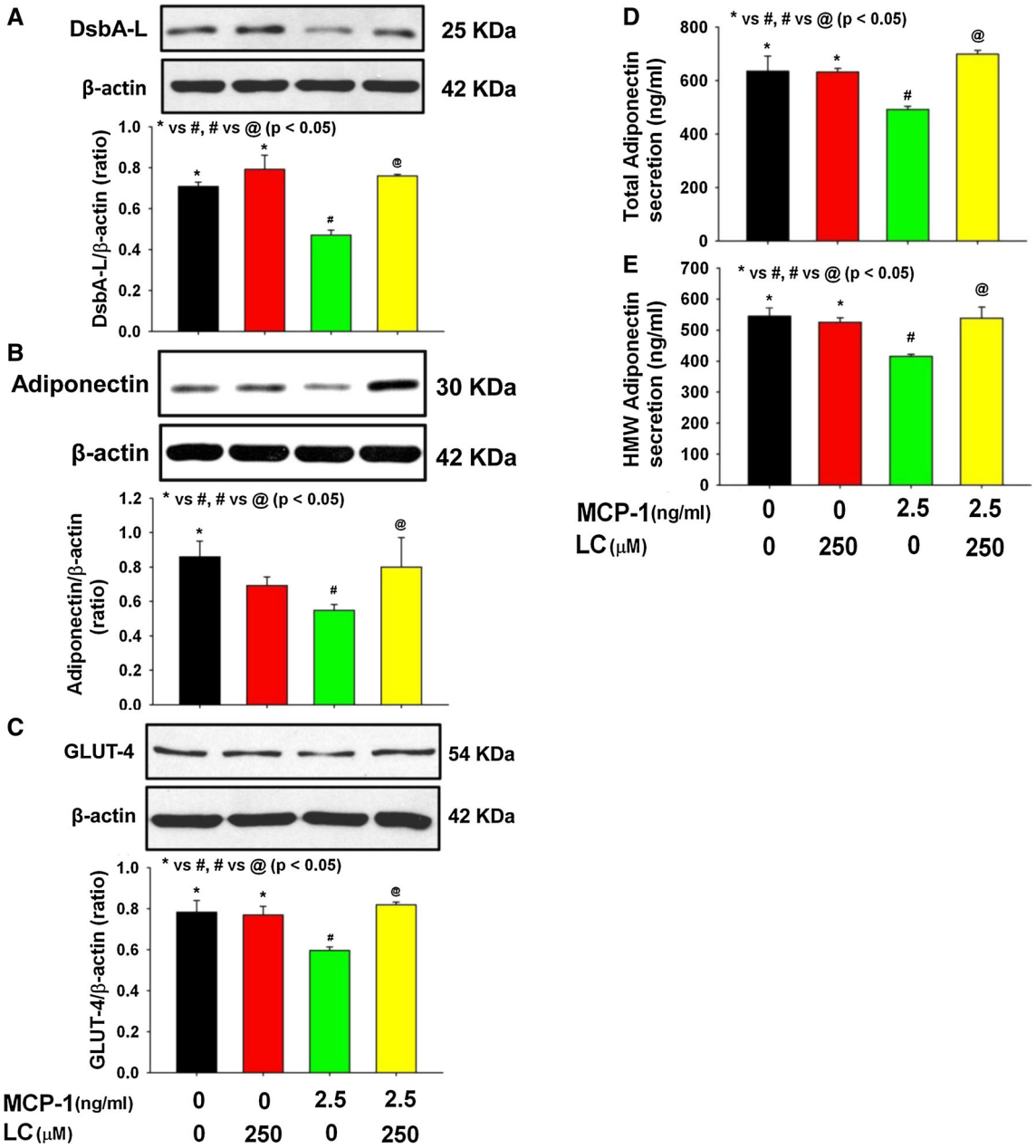

Fig. 4 Effect of LC supplementation on DsbA-L, adiponectin, and GLUT-4 protein expression and total and HMW adiponectin secretion in 3T3L1 adipocytes exposed to MCP-1. **a** DsbA-L protein expression, **b** adiponectin protein expression, **c** GLUT-4 protein expression, **d** total adiponectin secretion, and **e** HMW adiponectin secretion. Cells were pretreated with LC for 2 h followed by MCP-1 exposure for the next 24 h. Values are mean ± SE ($n = 3$). Data in Fig. 4b were analyzed by using Student's t test

secretion in HG-treated adipocytes. On the other hand, treatment with MCP-1 significantly reduces adiponectin synthesis and secretion and DsbA-L and GLUT-4 expression. This study also demonstrates that LC effectively attenuated the MCP-1-mediated decrease in adiponectin synthesis and secretion in 3T3-L1 adipocytes. Furthermore, the MCP-1-induced reduction in the DsbA-L and GLUT-4 protein expression was also prevented by LC. MCP-1 concentration used (2.5 ng/ml) is similar to that reported in the blood of patients [51, 52]. Hence, the concentration of MCP-1 used to treat adipocytes is within a physiological range. The effect of LC on MCP-1 receptors, CCR2, and CCR4 expression levels in cells is not known. These results indicate that LC supplementation can reverse the MCP-1-mediated changes in the synthesis and secretion of adiponectin at DsbA-L level in 3T3-L1 adipocytes. We believe that MCP-1 is not the only contributing factor in the harmful effect of high glucose on adiponectin secretion in

adipocytes; factor(s) other than MCP-1 may also contribute to the inhibition of adiponectin secretion by high glucose.

Oxidative stress plays an important role in the pathogenesis of IR [53–58]. High-glucose treatment is known to produce ROS which in turn activates the transcription factor NF-κB and increased expression and secretion of MCP-1 [59], suggesting that ROS generation played a substantial role in the MCP-1 secretion, thereby contributing to impaired glucose metabolism. Other studies also suggest that MCP-1 via MCP-1-induced protein (MCPIP) can enhance ROS generation in cardiomyocytes [60]. This suggests that excess ROS can increase MCP-1 secretion which can cause further increase in ROS and impaired glucose metabolism. Activation of GLUT4 by the insulin-dependent and/or insulin independent signaling pathway plays a critical role in the glucose metabolism and has been suggested as a therapeutic target for pharmacological strategies to control hyperglycemia [61]. The present study shows that LC prevented the ROS accumulation induced by the HG indicating the beneficial effect of LC on DsbA-L and GLUT4 expression and glucose utilization may be related to the inhibition of intracellular oxidative stress.

Adiponectin appears to be a major modulator of insulin action and its levels are reduced in type 2 diabetes, and thus targeting adiponectin synthesis has been regarded as an important therapeutic tool for preventing the insulin resistance associated with type 2 diabetes. Figure 5 shows a schematic representation of the mechanism by which LC

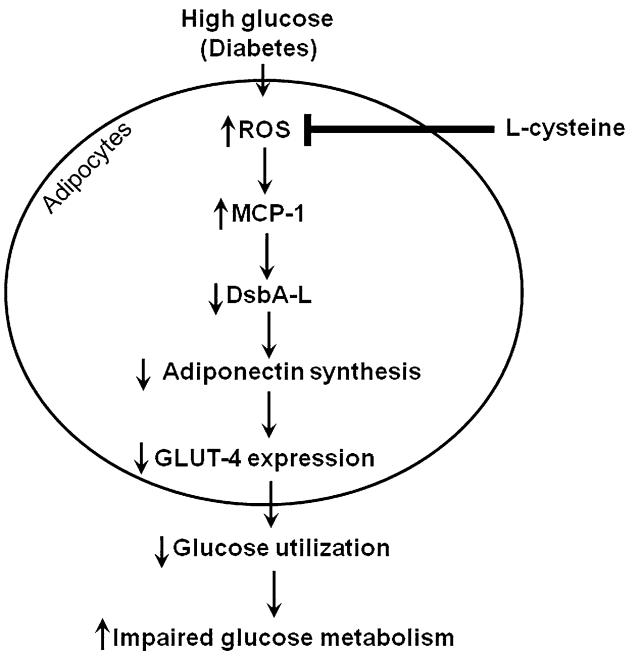

Fig. 5 Schematic diagram of the proposed mechanism of LC on adiponectin synthesis and glucose utilization in 3T3L1 adipocytes exposed to high glucose

supplementation can inhibit the ROS and MCP-1. The inhibition of MCP-1 can prevent the downregulation of DsbA-L and the impairment in adiponectin synthesis and insulin resistance in diabetes. Our study provides a novel biochemical mechanism and the ability of LC to upregulate total and HMW adiponectin synthesis and glucose utilization in adipocyte cell model. Future studies are needed to confirm whether LC supplementation increases total and high-molecular weight adiponectin synthesis and glucose metabolism using type 2 diabetic patients. These studies will provide the biochemical mechanism by which LC or novel molecules containing cysteinyl moiety can be used as an adjuvant therapy to lower glycemia and prevent insulin resistance in diabetes.

Acknowledgments The authors are supported by grants from NCCAM of the National Institutes of Health (RO1 AT007442), the Malcolm Feist Endowed Chair in Diabetes from LSUHSC, Shreveport. The authors thank Georgia Morgan for excellent editing of this manuscript.

Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

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