RESEARCH ARTICLE

L-cysteine supplementation upregulates glutathione (GSH) and vitamin D binding protein (VDBP) in hepatocytes cultured in high glucose and in vivo in liver, and increases blood levels of GSH, VDBP, and 25-hydroxy-vitamin D in Zucker diabetic fatty rats

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Scope: Vitamin D binding protein (VDBP) status has an effect on and can potentially improve the status of 25(OH) vitamin D and increase the metabolic actions of 25(OH) vitamin D under physiological and pathological conditions. Diabetes is associated with lower levels of glutathione (GSH) and 25(OH) vitamin D. This study examined the hypothesis that upregulation of GSH will also upregulate blood levels of VDBP and 25(OH) vitamin D in type 2 diabetic rats.

Methods and results: L-cysteine (LC) supplementation was used to upregulate GSH status in a FL83B hepatocyte cell culture model and in vivo using Zucker diabetic fatty (ZDF) rats. Results show that LC supplementation upregulates both protein and mRNA expression of VDBP and vitamin D receptor (VDR) and GSH status in hepatocytes exposed to high glucose, and that GSH deficiency, induced by glutamate cysteine ligase knockdown, resulted in the downregulation of GSH, VDBP, and VDR and an increase in oxidative stress levels in hepatocytes. In vivo, LC supplementation increased GSH and protein and mRNA expression of VDBP and vitamin D 25-hydroxylase (CYP2R1) in the liver, and simultaneously resulted in elevated blood levels of LC and GSH, as well as increases in VDBP and 25(OH) vitamin D levels, and decreased inflammatory biomarkers in ZDF rats compared with those in placebo-supplemented ZDF rats consuming a similar diet.

Conclusion: LC supplementation may provide a novel approach by which to raise blood levels of VDBP and 25(OH) vitamin D in type 2 diabetes.

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1 Introduction

The deficiency of 25-hydroxy-vitamin D has become a worldwide epidemic. Epidimiological studies demonstrate that higher rates of deficiency of 25-hydroxy-vitamin D could be linked to the prevalence of several chronic diseases, including diabetes [1–4]. Previous studies have reported an association between lower blood levels of 25(OH) vitamin D, VDBP, and GSH in diabetic patients, and in African-American subjects [5–11].

Cholecalciferol is also called vitamin D_3 (ingested from vitamin D_3 rich foods or formed in the skin from 7-dehydrocholesterol and ambient UV exposure), and vitamin D_2 (from fortified foods, mushrooms, some vegetables). Vitamin D_2 and vitamin D_3 function as prohormones (with no biological effect). These two forms have different side chains but are theoretically used by the body in an identical manner [2, 12]. The conversion of vitamin D_2 or vitamin D_3 into active compounds (irrespective of source) requires a two-step

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enzymatic hydroxylation process to occur. 25-hydroxy-vitamin D [25(OH) vitamin D] is formed by the hydroxylation of cholecalciferol by vitamin D 25-hydroxylase (CYP2R1) in the liver [12, 13]. 25(OH) vitamin D is transported in the circulation bound to VDBP [14–19]. The complex is taken up by renal proximal tubule epithelial cells through receptor mediated endocytosis [15, 16]. The kidney is the second site where 1-α-hydroxylase converts 25(OH) vitamin D to 1,25(OH)$_2$D$_2$ or 1,25(OH)$_2$D$_3$ [14]. Renal 25(OH) vitamin D metabolism is initiated in part by the internalization of the 25 (OH) vitamin D/VDBP complexes following the binding of VDBP to megalin [15, 16]. Thus, VDBP is important for handling 25(OH) vitamin D and the synthesis of 1,25(OH)$_2$ vitamin D$_3$ [15–22]. 1,25(OH)$_2$D$_3$ is the active form of vitamin D [2,12]. The levels of 1,25(OH)$_2$ vitamin D$_3$ (calcitriol) are under homeostatic control because of its dependence upon circulating parathyroid hormone concentrations [2]. The known systematic effect of 1,25(OH)$_2$ vitamin D or calcitriol appears to be the maintenance of serum calcium and phosphate concentrations [1–3]. 1,25(OH)$_2$D$_3$ also has anti-inflammatory properties and its higher blood levels are associated with better health outcomes [1–4]. 25(OH) vitamin D is considered a comprehensive and stable indicator of 1,25(OH)$_2$ vitamin D status [23]. Risk factors for 25(OH) vitamin D deficiency include race (darker pigmented skin tones), higher body mass index (BMI), winter season, higher geographic latitudes, and diet. These associations have been reported previously and derive from observations of decreased synthesis of vitamin D in skin due to darker skin pigmentation, lack of sunlight exposure, and/or exercise during the winter months, and among obese people [1–4].

Circulating and tissue levels of glutathione (GSH) decrease with age, and in chronic diseases such as diabetes [24–27]. GSH is a co-factor of many enzymes, a potent antioxidant, and plays an important role as a scavenger of toxic oxygen radicals, which helps to maintain normal cell functions [25–27]. Vitamin D binding protein (VDBP) is a main transporter of vitamin D and 25(OH) vitamin D [17–20]. Recent studies suggest that blood concentrations of VDBP are positively related to the half-life of circulating 25(OH) vitamin D [20], and that blood levels of GSH have a positive relationship with those of VDBP and 25(OH) vitamin D in the blood of African-American type 2 diabetic patients [10]. L-cysteine is a rate limiting precursor of GSH biosynthesis [25].

The aim of this study is to investigate whether GSH status has any effect on VDBP and/or 25(OH) vitamin D levels in type 2 diabetes. This study tested the hypothesis that the upregulation of GSH helps raise blood levels of VDBP and 25(OH) vitamin D. This study reports that LC supplementation upregulates both protein and mRNA expression of VDBP in hepatocytes exposed to high glucose and in vivo in the liver of type 2 ZDF rats. In addition, in vivo studies also found upregulation of vitamin D 25-hydroxylase in liver, elevated blood levels of VDBP and 25(OH) vitamin D, and lower levels of inflammation

biomarkers in type 2 ZDF rats supplemented with LC but not with placebo. This study reports a novel approach to increase blood levels of VDBP and 25(OH) vitamin D in type 2 diabetes.

2 Materials and methods

2.1 Cell culture, L-cysteine, and high glucose (HG) treatment of hepatocytes

The FL83B hepatocyte cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). FL83B hepatocytes were cultured and maintained in F-12K complete medium. Pretreatment of the cells, maintained at a concentration of 1×10^6/mL of media, was done for 2 h with LC (0-300 μM), followed by treatment for 22 h with HG (25 mM). Control cells were treated with mannitol, which is considered an osmolarity control in some experiments. After 24 h, complete media with serum was added and the cells were then treated as described in the figures.

2.2 Animal studies

The animal protocol was approved by the institutional Animal Welfare Committee (P-15-006). All procedures performed were in accordance with the ethical standards of the institution. Male Zucker diabetic rats at an age of 5 weeks and weighing about 200–220 g were purchased from Charles River (Wilmington, MA, USA). The animals were allowed to acclimate to the environmental and handling conditions for 2 days. Computer generated randomization was used to divide rats into two groups, after which they were housed and labelled in individual cages. After overnight fasting they were weighed and tested for hyperglycemia by measuring their blood glucose concentration. Blood was collected via tail incision and the blood glucose levels were measured using an Advantage Accu-chek glucometer (Boehringer Mannheim Corp., Indianapolis, IN, USA). One group of rats was labeled as diabetic controls and gavaged with saline alone. Rats in the other group were labelled as the L-cysteine (LC) group and supplemented with 1 mg LC/kg body weight daily by oral gavage. A third group included in the study, called the control group, consisted of male Sprague Dawley (SD) rats who were also gavaged daily with saline. Rats in all groups were given an equal volume of saline vehicle or LC daily for 8 weeks by oral gavage using 20G feeding needles (Popper and Sons, New Hyde Park, NY, USA). Blood glucose and body weight were monitored weekly in all rats. Based on any change in their weights the LC supplementation dose was adjusted accordingly every week to maintain a similar dose per Kg BW over the entire period of the study.

All ZDF rats were fed a high calorie Purina 5008 lab chow diet (Charles River, Wilmington, MA, USA). This diet

contained 3.4 IU vitamin D_3 per gram diet. SD rats were fed a standard 8640 lab chow diet, which contained 3 IU vitamin D_3 per gram diet (Harlan, Indianapolis, IN, USA); both ZDF and SD rats were maintained at $22 \pm 2°C$ with 12:12-h light/dark cycles. Food intake of all rats was monitored. The control group contained six rats. There were also six rats each in the placebo and LC-supplemented diabetic rat groups. At the end of 8 weeks the rats were fasted overnight and euthanized by exposing them to halothane (2-bromo-2-chloro-1,1,1-trifluoroethane). Blood was drawn with a 19½ gauge needle via cardiac puncture into vacutainer tubes. Aliquots of blood collected from all rats were sent to the clinical laboratory of LSUHSC-Shreveport for blood chemistry profiles, including liver and renal function and red blood cell counts. Plasma was isolated in a 4°C centrifuge at 3000 rpm for 10 min from blood collected into EDTA tubes. Rat livers were perfused using cold saline. Once extracted, they were labeled appropriately, and stored at −80°C.

2.3 Quantitative PCR in rat liver lysates

About 50 mg of liver tissue was weighed and homogenized in 1 mL TRIzol reagent (Invitrogen, Grand Island, NY, USA). The RNA extraction was performed according to the instructions provided. The concentration and quality of the extracted RNA were determined on a NanoDrop spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). A High Capacity RNA-To-cDNA kit (Invitrogen) was used to synthesize cDNA. QPCR was performed using a 7900HT Real Time PCR system and software (Applied Biosystems, Grand Island, NY, USA) using the FAM-labeled primer/probe set *Rn00561256_m1 for VDBP (also called GC), Rn00690616_m1 for VDR, Rn01754615_ml for CYP2R1, and Rn01775763_g1 for GAPDH* (Invitrogen), respectively. The relative fold change of mRNA was calculated using the relative quantification (ΔΔCT) method.

2.4 25(OH) vitamin D, VDBP, and cytokine assays

Plasma levels of 25(OH) vitamin D were determined using an ELISA kit (Eagle Biosciences, Nashua, NH, USA). Plasma VDBP quantification was carried out using a kit purchased from Alpco (Salem, NH, USA). The kit includes polyclonal antibodies that detect total VDBP levels. The cytokines MCP-1 (R&D Systems Inc., Minneapolis, MN, USA) and CRP (Alpco) were determined using the sandwich ELISA method and commercially available kits. In the cytokine assay, control samples were analyzed each time to check the variation from plate to plate on different days of analysis. Protocols as given in the manufacturer's kit were followed using appropriate controls and standards.

2.5 GSH, LC, cell viability assays, and immunoblotting

Levels of GSH and LC were determined using HPLC [28]. In cell culture studies, the whole cell suspension was processed for the GSH assay [29]. Cell viability was determined using the Alamar Blue method (Alamar Biosciences, Sacramento, CA, USA). Details of immunoblotting are similar to those given in our previous publications [8, 30]. The antibodies for VDBP (52 KD), β-Actin (40 KD), GCLC (75 KD), and VDR (48 KD) were purchased from Abcam (Cambridge, MA, USA). The intensity of each immunoblotting band was measured using the histogram tool of Adobe Photoshop CS5.

2.6 ROS assay

Hepatocytes were lysed after treatment and reactive oxygen species were measured in the cell lysates using 20 μM of the oxidant-sensitive probe dicholorodihydrofluorescein diacetate (H_2DCFDA, Sigma Chemical Co., St. Louis, MO, USA, USA). Liver lysate aliquots (20 μg) were used to measure ROS with H_2DCFDA. Details of the ROS assay are as given before [29].

2.7 GCLC siRNA transection

GCLC siRNA was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), Catalogue number, sc-41979. The γ-GCLC siRNA used is a pool of three different siRNA duplexes. Sequences of siRNA oligonucleotides used is given in Supporting Information Material. Different concentrations of siRNA (0–200 nM) were used to knockdown GCLC using transfection reagent (lipofectamine, Invitrogen) as described previously [30].

2.8 Quantitative PCR in hepatocytes

mRNA extraction and cDNA synthesis were done as described in the previous section. The following primers were used for hepatocytes: VDBP (also called GC) (*Mm04243540_m1*), *VDR (Mm00437297_m1), GCLC (Mm00802655_m1), and GAPDH (Mm03302249_g1)* and were purchased from Invitrogen. Sequence of oligonucleotides of primers used is given in Supporting Information Material. The relative fold change of mRNA was calculated using the relative quantification (ΔΔCT) method and then normalized to % control for representation.

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise mentioned. Data from cell culture and rat studies were analyzed using ANOVA with Sigma Stat software (SPSS, Chicago, IL, USA). A *p* value of less than 0.05 for a statistical test was considered significant.
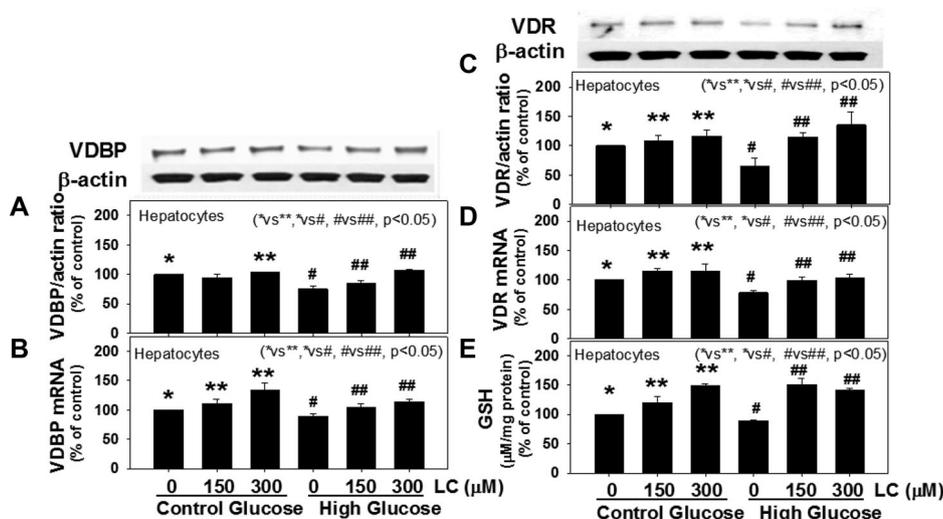
Figure 1. Effect of L-cysteine supplementation in hepatocytes treated with control and high glucose. Details of treatment, euthanasia of rats, and blood and tissue analyses are given in the Materials and methods. Levels of VDBP (A, B), VDR (C, D) and GSH (E) in hepatocytes cultured with high glucose. Values are mean ± SE, $n = 4$.

3 Results

3.1 Effect of LC supplementation on VDBP and VDR expression in hepatocytes

Figure 1 illustrates the effect of LC supplementation on GSH and VDBP and VDR in normal and high glucose (HG)-treated hepatocytes. High glucose treatment caused a significant decrease in both the protein expression and mRNA levels of VDBP and VDR in hepatocytes; L-cysteine supplementation resulted in upregulation of both protein expres-

sion and mRNA levels of VDBP (A, B) and VDR (C, D) in hepatocytes cultured with high glucose. There was a significant decrease in GSH levels (E) after high glucose treatment, which was prevented by supplementation with LC, a precursor of GSH, in hepatocytes. GSH deficiency was induced by knocking down GCLC using antisense-mRNA to determine whether GSH has a direct effect on VDBP and VDR expression in hepatocytes. Figure 2 illustrates the effect of GCLC-antisense on GCLC knockdown (A, B), decrease in GSH (C), and an increase in ROS (D) levels, as well as a decrease in the VDBP (E,F) and VDR (G,H) levels. This suggests that GSH
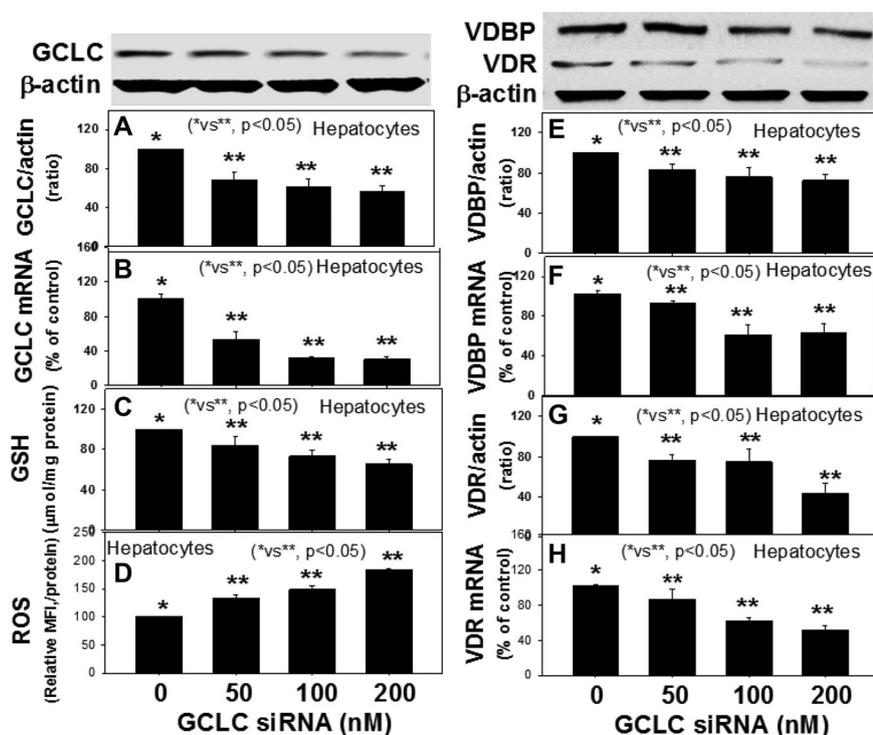

Figure 2. Effect of GSH deficiency on GCLC-KD (A, B), GSH (C), ROS (D), VDBP (E, F), and VDR (G, H) levels in FL83B hepatocytes treated with different concentrations of GCLC siRNA (Mean ± SE, $n = 4$).
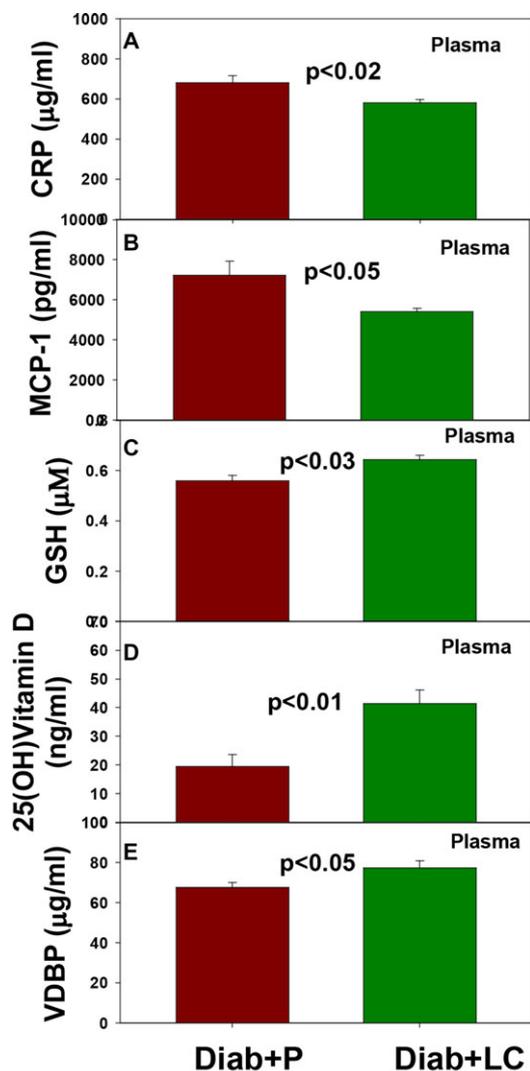
Figure 3. Effect of LC supplementation on levels of VDBP (E), 25(OH) vitamin D (D), GSH (C), MCP-1 (B), and CRP (A) levels in the plasma of diabetic control (D+P, $n = 6$) and LC-supplemented (D+LC, $n = 6$) ZDF rats. Rats were supplemented with placebo (saline) or L-cysteine (1 mg/kg BW) via daily oral gavage for 8 weeks. Values are mean $\pm$ SE.

Figure 4. Effect of LC supplementation on protein and mRNA expression of VDBP (A, B), CYP2R1 (D, E), ROS (F), and GSH (C) levels in the livers of diabetic control (D+P, $n = 6$) and LC-supplemented (D+LC, $n = 6$) ZDF rats. Blots shown here are from three rats each. Rats were supplemented with placebo or L-cysteine (1 mg/kg BW) via daily oral gavage for 8 weeks. Values are mean $\pm$ SE.

deficiency can impair the VDBP and VDR protein and mRNA expression in hepatocytes.

3.2 Effect of LC supplementation on VDBP, 25(OH) vitamin D, GSH, MCP-1, and CRP levels in Zucker diabetic rats

Cell culture studies suggest that LC upregulates the VDBP and VDR protein expression mediated by the GSH status in monocytes. This led to our hypothesis that supplementation with LC, a precursor of GSH, may boost the blood levels of VDBP and 25(OH) vitamin D, and its metabolic actions
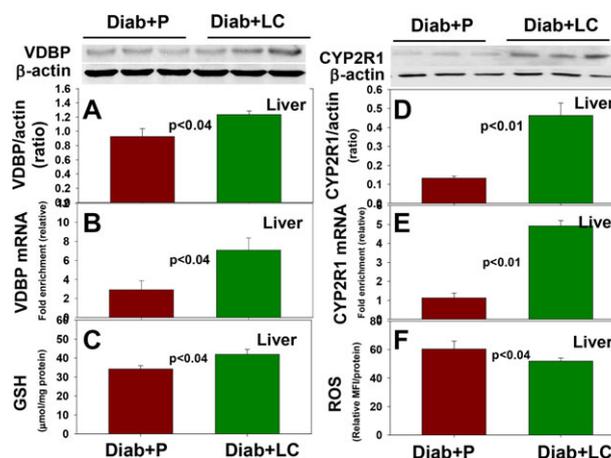
in vivo in diabetic rats. Figure 3 shows the effect of LC or placebo supplementation on blood levels of VDBP, 25(OH) vitamin D, GSH, and biomarkers of inflammation in ZDF rats. Blood levels of both VDBP (E) and 25(OH) vitamin D (D) were significantly higher in LC-supplemented compared with placebo-supplemented diabetic rats. LC-supplemented diabetic rats also showed an increase in plasma GSH (C) and a decrease in MCP-1 (B) and CRP (A). Figure 4 shows a significantly higher protein and mRNA expression of VDBP (A, B) and vitamin D 25-hydroxylase (CYP2R1) [D, E] levels in the livers of rats supplemented with LC compared to those supplemented with placebo; GSH levels (C) were significantly higher and ROS (F) levels were significantly lower in the livers of LC-supplemented rats. The livers of LC-supplemented rats showed higher levels of expression of protein ($p = 0.17$) and mRNA ($p = 0.30$) for VDR, but these values were not statistically significant in comparison to those of placebo-supplemented rats. This suggests that LC supplementation can increase blood levels of VDBP and 25(OH) vitamin D, and also decrease inflammation biomarkers in vivo in T2D rats.

Table 1 shows no change in the blood levels of biomarkers of liver and kidney function tests, including blood cell counts or serum calcium, between LC and placebo groups, suggesting that LC supplementation is safe. There was no difference in daily food intake between LC-supplemented and placebo-supplemented diabetic rats. There were significantly lower body weights and an increase in plasma L-cysteine levels among LC-supplemented rats compared with those in the placebo group. Total cholesterol and triglyceride levels also did not differ between LC-supplemented and

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Table 1. The effect of L-cysteine supplementation on body weight, plasma lipids, and liver and kidney function tests in Zucker diabetic rats. Rats were supplemented with placebo (saline) or L-cysteine (1 mg/kg BW) daily gavage for 8 weeks. Values are mean ± SE. Values marked * are significantly different ($p < 0.05$)

	Diabetic+P	Diabetic+LC
N	6	6
Body weight (g)	371 ± 3.9	346 ± 6.3*
Food intake (g/day)	38 ± 1.2	37.1 ± 1.0
AST (IU/L)	173 ± 32	181 ± 19
ALT (IU/L)	99.6 ± 6.4	95.3 ± 13.1
ALP (IU/L)	24.0 ± 4.9	22.5 ± 3.1
Creatinine (mg/dL)	0.4 ± 0.0	0.4 ± 0.0
RBC ($10^6/\mu$L)	9.5 ± 0.15	9.04 ± 0.07
Hemoglobin (g/dL)	16.0 ± 0.23	15.42 ± 0.14
L-cysteine (μM)	173.4 ± 14.2	218.8 ± 7.7*
Calcium (mg/dL)	10.1 ± 0.3	9.9 ± 0.4
Total cholesterol (mg/dL)	188.9 ± 7.0	171 ± 11.5
Triglycerides (mg/dL)	571 ± 29	509 ± 55

placebo-supplemented rats. This suggests that no negative side effects result from LC supplementation.

3.3 VDBP, VDR, and CYP2R1 status in liver of diabetic rats

We investigated VDBP and VDR status in the liver to investigate whether hyperglycemia in vivo also has any effect on the VDBP and VDR downregulation similar to that observed in high glucose-treated cultured hepatocytes. Figure 5 illustrates that protein and mRNA expression of VDBP (A, B) and VDR (C, D) is significantly lower, and ROS levels are significantly higher (G), in the livers of ZDF rats compared with those of age-matched normal rats. Figure 5 also shows lower levels of both protein and mRNA expression of CYP2R1 (E, F) in type 2 diabetic rats compared with those in normal rats. Rats in each group were 14 weeks old; blood glucose levels were significantly higher (349 ± 19 mg%) in diabetic rats compared with those in normal rats (162 ± 8 mg%). This suggests that level of VDBP and VDR expression were significantly reduced both in hepatocytes treated with high glucose (Fig. 1) and in the liver of diabetic rats. This suggests that uncontrolled glycemia in diabetes may play a role in the previously reported lower blood levels of VDBP seen in diabetic rats [31, 32].

4 Discussion

Epidemiological studies suggest a positive association between better health outcomes and higher blood levels of 25(OH) vitamin D [1–4]. This study reports that LC supplementation can boost circulating levels of 25(OH) vitamin D and its efficacy, mediated by an increase in GSH and VDBP levels in Zucker diabetic fatty rats, a model of type 2 diabetes.
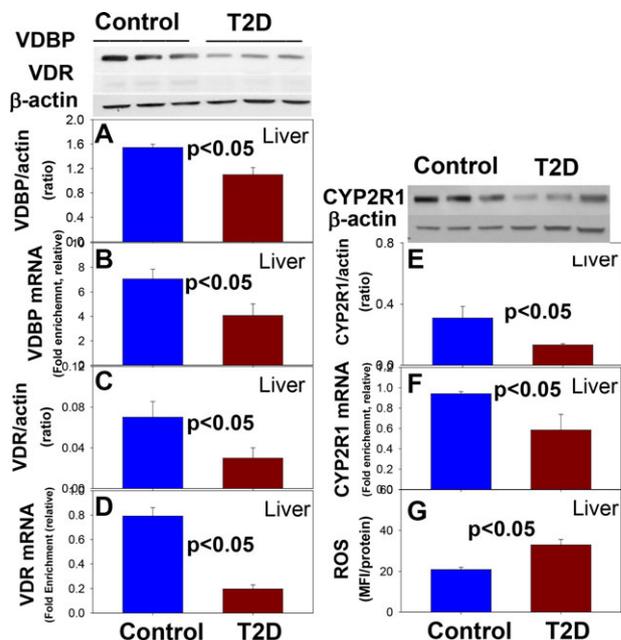

Figure 5. VDBP (A, B), VDR (C, D), and CYP2R1 (E, F) expression and ROS (G) levels in the livers of ZDF ($n = 6$) and age-matched normal rats ($n = 6$). Values are mean ± SE. Details of treatment, euthanasia of rats, and blood and tissue analyses are given in the Material and methods.

VDBP is primarily synthesized and secreted by the liver [20–22]. VDBP, also called GC, is a 52–59 kDa monomeric glycoprotein with a short half-life of 2.5–3 days compared with the 1–2 month half-life of 25(OH) vitamin D [2]. Studies of humans with a genetic mutation for VDBP and VDBP-knockdown mice demonstrate low plasma levels of 25(OH) vitamin D [18, 19]. Genetic variations in VDBP are known to influence 25(OH) vitamin D blood levels in response to vitamin D supplementation [21, 23]. VDBP plays a major role in transporting vitamin D and 25(OH) vitamin D to various tissues and participating in their conversion to 1,25(OH)$_2$ vitamin D$_3$, an active form of vitamin D. Recent studies suggest that blood concentrations of VDBP are positively related to the half-life of circulating 25(OH) vitamin D [20]. These studies suggest that increasing VDBP availability can potentially increase 1,25(OH)$_2$ vitamin D$_3$ levels and the metabolic actions of 25(OH) vitamin D. All cells in the body, including hepatocytes, have receptors known as VDR [15, 33–35]. The biological response to 1,25(OH)$_2$ vitamin D$_3$ is directly related to the VDR content of target tissues and VDR expression is regulated by physiological factors including calcium, 1,25(OH)$_2$ vitamin D$_3$, and VDBP [2]. This suggests that the higher circulating levels of 25(OH) vitamin D could be due to the upregulation of VDBP and vitamin D 25 hydroxylase, as seen in the liver and blood of LC-supplemented ZDF rats compared with those of placebo-supplemented rats consuming a similar diet, could be due to the upregulation of VDBP status. The decrease in inflammatory biomarkers could be due

to improved metabolic actions or efficacy of 25(OH) vitamin D mediated by the upregulation of VDR in LC-supplemented rats.

Previous studies in the literature report lower blood levels of VDBP, 25(OH) vitamin D, LC, and GSH in diabetes [5–11]. GSH is a physiological antioxidant, a co-factor of many enzymes, and plays an important role in a multitude of cellular processes. LC can also have a direct effect on post-translational modification or S-glutathionylation of proteins, which can cause modification of structure and function and thereby provide protection against oxidative signaling events [24–26]. Elevated oxidative stress in various tissues from uncontrolled hyperglycemia may cause increased LC utilization and the lower levels seen in diabetes. GSH is formed from LC by the enzymatic action of glutamate-cysteine ligase (GCLC). Studies indicate that LC supplementation and an improvement in GSH status is potentially useful for prevention of oxidative stress and insulin resistance [36–39]. Renal dysfunction and increased urinary excretion of VDBP and 25(OH) vitamin D are implicated in the 25 (OH) vitamin D deficiencies associated with diabetes [16, 31, 32]. GSH deficiency, induced by GCLC knockdown, resulted in the downregulation of VDBP and VDR in hepatocytes, which suggests that an improvement in the VDBP and 25 (OH) vitamin D statuses may be associated with an improvement in the GSH levels in LC-supplemented rats. The beneficial effect of LC on biomarkers of vascular inflammation in diabetic rats may result from a beneficial effect on lowered oxidative stress [36–39]. The increases in blood levels of GSH, VDBP, and 25(OH) vitamin D in LC-supplemented diabetic rats, along with data from cell culture studies, demonstrate a potential link between GSH status and those of VDBP and 25(OH) vitamin D blood levels in diabetes.

Powe et al. [9] suggested that 25(OH) vitamin D that is not bound to the VDBP or the free form of 25(OH) vitamin D is important for the biological actions of 25(OH) vitamin D. However, other investigators have subsequently challenged this view. 25(OH) vitamin D bound to the VDBP is, in fact, the form that participates in $1,25(OH)_2$ vitamin D_3 synthesis and in the regulation of parathyroid hormone. It is possible that both 25(OH) vitamin D bound to VDBP and free 25(OH) vitamin D play important roles in the metabolic actions and efficacy of circulating 25(OH) vitamin D under different physiological and pathological conditions [4, 17–19].

Whether LC supplementation increases reabsorption of VDBP and megalin/Dab2 in proximal tubules and thereby contributes to increased blood levels of VDBP in LC-supplemented rats is not known. One limitation of this study is that diabetic rats were not maintained on a vitamin D deficient diet to create a vitamin D deficiency and then examined to determine the efficacy of L-cysteine in improving VDBP and 25(OH) vitamin D status. Another limitation is that blood levels of $1,25(OH)_2$ vitamin D_3 was not investigated due to insufficient plasma. Nevertheless, this study has demonstrated a potential link between the status of 25(OH) vitamin D and VDBP, and that of GSH and LC, using a cell culture model and in diabetic rats.

Deficiency in GSH and GSH generating enzymes is linked to obesity in murine and human studies [40, 41]. Decreased GSH accelerates adipogensis in adipocytes. A recent study reported a positive effect on insulin sensitivity and body composition in older HIV-infected patients as the result of increasing glutathione with cysteine supplementation [26]. Our study also observed an increase in GSH levels and a decrease in body weight gain in LC-supplemented diabetic rats compared with placebo. Reduction in oxidative stress can mediate the upregulation of both the insulin dependent and insulin independent signaling cascades, GLUT4, and glucose metabolism, and lower insulin resistance [42], thereby contributing to reduction in weight gain in LC-supplemented diabetic rats. This indicates that an improvement in GSH status is a potentially useful therapeutic target for prevention of oxidative stress and weight gain. Thus, LC supplementation appears to regulate GSH and energy expenditure and weight gain. This study also shows that levels protein and mRNA expression of VDBP, VDR, and vitamin D 25-hydroxylase were significantly reduced in liver of diabetic rats. This suggests that uncontrolled glycemia may also contribute to the lower blood levels of VDBP observed in addition to the excess urinary loss of VDBP previously reported in diabetic rats [31, 32]. The changes seen in VDBP and VDR in livers of diabetic rats are likely to be from the hyperglycemia, because a similar effect, lower expression of VDBP and VDR, was seen in high-glucose treated hepatocytes compared with control cells.

This study suggest a potential link between lower blood levels of VDBP and 25 (OH) vitamin D with those of lower GSH, and that increases in GSH levels as the result of LC supplementation can upregulate VDBP and 25(OH)vitamin D status. It appears that supplementation with LC increases blood levels of GSH and prevents the excess oxidative stress (ROS) associated with T2D. The upregulation of GSH and decrease in ROS results in upregulation of VDBP, vitamin D 25-hydroxylase and thereby increased blood levels of 25(OH) vitamin D, and its metabolic actions. The potential mechanism for the increased blood level of 25(OH) vitamin D could be normalization of the status of VDBP, resulting in the better transport of cholcalciferol by the VDBP for its hydroxylation. Another potential mechanism could involve the up-regulation of protein expression and activity of 25-vitamin D hydroxylase by either increased levels of GSH or lower oxidative stress in LC-supplemented diabetic rats.

5   Conclusion

25(OH) vitamin D deficiency and an excessive rate of diabetes have both become epidemic worldwide. Vitamin D supplementation is widely recommended, but higher doses of vitamin D supplementation are needed to achieve normal circulating 25(OH) vitamin D levels, and some patients do not respond well to supplementation [43–45]. This study

reports that LC supplementation can boost circulating levels of 25(OH)vitamin D and its efficacy, mediated by an increase in GSH, VDBP, and vitamin D 25-hydroxylase levels in ZDF rats. Future validation of animal studies in humans could have a tremendous impact on the practice of medicine and could lead to the novel approach that combined vitamin D and L-cysteine supplementation be used, instead of supplementation with vitamin D alone, to optimize 25(OH) vitamin D levels, thus helping to address and possibly prevent the health hazards associated with diabetes.

The authors have declared no conflict of interest.

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