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Vitamin D upregulates glutamate cysteine ligase and glutathione reductase, and GSH formation, and decreases ROS and MCP-1 and IL-8 secretion in high-glucose exposed U937 monocytes

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ABSTRACT

Introduction: Glutathione is a major endogenous antioxidant and its deficiency is implicated in the etiology and progression of a number of human diseases. Vitamin D is important for the prevention of osteoporosis, cardiovascular disease, diabetes, autoimmune diseases, and some cancers. Using a monocyte cell model, this study examined the hypothesis that vitamin D upregulates glutamate cysteine ligase (GCLC) and glutathione reductase (GR), which catalyzes GSH biosynthesis.

Methods: U937 monocytes were pretreated with and without 1,25 $(OH)_2$ vitamin D (10–25 nM) for 24 h and then exposed to control and high glucose (HG, 25 mM) for 4 h. Levels of GSH were determined using HPLC; GR activity by oxidation of NADPH; GCLC protein, MCP-1, and IL-8 using ELISA kits.

Results: 1,25 $(OH)_2$ vitamin D supplementation significantly upregulated expression of GCLC and GR, levels of GCLC protein and GR activity, and formation of GSH in control and HG-treated monocytes. 1,25 $(OH)_2$ vitamin D caused significantly ($p < 0.05$) lower secretion of IL-8 and MCP-1, and lower ROS levels in monocytes exposed to control and HG-treated monocytes.

Conclusions: This study demonstrates a positive link between vitamin D and GSH levels, and that some beneficial effects of vitamin D supplementation may be mediated by an improvement in the cellular GSH levels and a decrease in ROS and pro-inflammatory cytokines.

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1. Introduction

Glutathione (GSH) plays an important role in a multitude of cellular processes and its deficiency is implicated in the etiology and progression of a number of human diseases including cardiovascular, immune, diseases of aging, and diabetes [1–7]. GSH is a cofactor of many enzymes that are involved in the detoxification of oxygen radicals and the detoxification of drugs. GSH deficiency is implicated in the progression of chronic diseases, including insulin resistance and diabetes [1,2,5,6]. Recently, epidemiological studies have demonstrated an association between vitamin D deficiency and the outcome of several chronic diseases, including diabetes [8–11]. However, evaluation of both the basic research and clinical evidence related to the role of vitamin D supplementation in the prevention and treatment of chronic non-skeletal diseases remains to be done. The blood levels of GSH are lower in diabetes [5,12,13]. This study demonstrates that vitamin D can upregulate GCLC and GR, and that GSH can form in cultured monocytes. In addition, this study reports that the effect of vitamin D on GSH formation was accompanied by inhibition of ROS, and IL-8 and MCP-1 secretion in monocytes treated with control and high glucose levels. This study suggests that upregulation of cellular GSH by vitamin D provides evidence for a novel mechanism by which vitamin D supplementation may reduce oxidative stress and thereby provide lower vascular inflammation and associated complications in diabetes.

2. Materials and methods

2.1. Human pro-monocytic cell line

The U937 monocyte cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells were maintained at 37 °C in RPMI 1640 medium containing 7 mM glucose, 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 12 mM sodium carbonate, 12 mM HEPES and 2 mM glutamine in a humidified atmosphere containing 5% (v/v)

Abbreviations: GSH, glutathione; ROS, reactive oxygen species; GCLC, glutamate cysteine ligase catalytic unit; GCLM, glutamate cysteine ligase modulatory unit; GR, glutathione reductase.

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CO_2. For treatments, cells were washed once in plain RPMI 1640 before being suspended in fresh medium (complete) containing serum and other supplements [14,15].

2.2. Treatment with high glucose (HG) and vitamin D

Cells (10^6/ml) were pretreated with three different concentrations of 1,25 $(OH)_2$ vitamin D (0 nM, 10 nM, 25 nM) for 24 h and followed by HG (25 mM) exposure for the next 4 h. 1,25 $(OH)_2$ vitamin D is an active form of vitamin D. In this study, control cells were exposed with media having 7 mM glucose. In the body, glucose is continuously degraded and formed to maintain a 5 mM blood glucose level. However, in cell culture studies, we observed that incubating cells with media having a 5 mM glucose concentration for 24 h caused a decrease in glucose concentration to levels lower than 2 mM. In cell culture studies, glucose gets metabolized but not replaced. For this reason, our experience shows that a 7 mM glucose concentration does not lead to a glucose deficiency at 24 h incubation. In high glucose studies, cells were exposed to a high glucose concentration of 25 mM. 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. Many previous studies have reported that glucose concentrations as high as 50 mM have been found in the blood of patients with uncontrolled diabetes. Thus, the glucose concentration of 25 mM used in this cell culture study does not seem unreasonable. In some experiments, cells were instead exposed to 18 mM mannitol (control) since the media contains 7 mM glucose. After treatment, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM EDTA, 10 mM NaF, and 1 mM $NaVO_4$). Lysates were cleared by centrifugation and total protein concentrations were determined using BCA assay (Pierce/Thermo Scientific, Rockford, IL).

2.3. GSH, GR activity and GCLC protein assays

GSH was determined by HPLC method [16]. Whole cell suspension was processed as described before [16]. GSH concentration is expressed per volume of cell suspension. Glutathione reductase (GR) together with its cofactor, NADPH, catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH). The oxidation of NADPH to NADP is monitored as a decrease in absorbance at 340 nm. The rate of decrease in (ΔA_{340}) is directly proportional to the glutathione reductase activity in the sample because the enzyme is present in rate limiting concentrations. GR activity was determined using protocol described by Beutler [17]. The GR activity was expressed as a rate of decrease in absorbance at 340 nm/min due to the oxidation of NADPH by GR, and was normalized/mg protein. GCLC total protein level in the cell lysate was determined by ELISA kit (Catalog #MBS 704124, MyBiosource, San Diego, CA). All appropriate controls and standards as specified by manufacturer's kit were used each time. Level of GCLC protein was expressed as total protein.

2.4. Cell viability, ROS and cytokine and immunoblotting studies

Cell viability was determined using the Alamar Blue reduction bioassay (Alamar Biosciences, Sacramento, CA). This method is based upon Alamar Blue dye reduction by live cells. Intracellular reactive oxygen species (ROS) levels were measured in treated cells using the fluorescent dye, H_2DCFDA (2′,7′dichlorofluorescein diacetate) [17]. After treatment, cells were washed once with PBS and then loaded with 5 μM H_2DCFDA in PBS with 4% FBS. The cells were incubated at 37 °C for 30 min in the dark and subsequently washed with PBS, harvested in PBS with 0.5% Triton X-100, centrifuged at 12,000 × *g* for 10 min at 37 °C, and the supernatant collected. The intensity of DCF fluorescence in the supernatant was read at excitation and emission wavelengths of 488 nm and 530 nm, respectively, using a multidetection microplate reader (Synergy HT, BIOTEK). The change in intracellular ROS level was plotted as mean fluorescence intensity (MFI). The oxidative stress sensitive dye DCFH-DA diffuses passively through the cellular membrane. Intracellular esterase activity causes the formation of DCFH, a nonfluorescent compound, which emits fluorescence when it is oxidized to DCF [18]. Although ROS measurement by DCFH-DA is nonspecific, this dye has been widely used to measure the formation of overall intracellular reactive intermediates. All appropriate controls and standards as specified by each manufacturer's kit were used for IL-8 and MCP-1 assay using ELISA kits (R and D Systems, Minneapolis, MN). In the cytokine assay, control samples were analyzed each time to check the variation from plate to plate on different days of analysis. Details of immunoblotting are similar to as given in our previous publications [15,33]. The antibodies for GCLC (73 kDa), GCLM (31 kDa) and GR (58 kDa) were purchased from Abcam (Cambridge, MA). The intensity of each immunoblotting

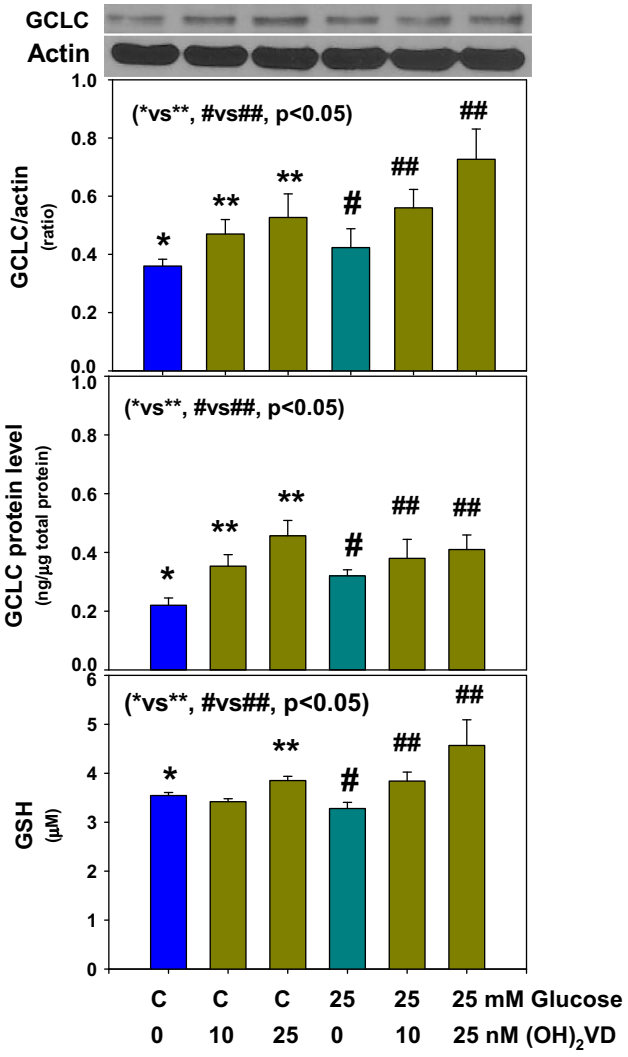

Fig. 1. Effect of 1,25 $(OH)_2$ vitamin D supplementation on upregulation of glutamate cysteine ligase (GCLC) expression, GCLC protein and GSH levels in U937 monocytes cultured without and with high glucose. Values are mean ± SE (n = 4).

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