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Original article

Role of NF-E2-related factor 2 in neuroprotective effect of L-carnitine against high glucose-induced oxidative stress in the retinal ganglion cells

Yu Cao [a,1], Xin Li [a,1], Chen-Jing Wang [a], Ping Li [a], Bo Yang [a], Chun-Bo Wang [b,*], Le-Xin Wang [c,**]

[a] The Affiliated Hospital of Qingdao University, Qingdao 266003, China
[b] Department of Pharmacology, Medical College, Qingdao University, Qingdao 266071, China
[c] School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW 2650, Australia

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ABSTRACT

L-Carnitine (LC) has protective effects on high glucose-induced oxidative stress in the retinal ganglion cells (RGCs). The aim of this study was to investigate the role of NF-E2-related factor 2 (Nrf2), Kelch like-ECH-associated protein 1 (Keap1), haemoxygenase-1 (HO-1) and γ-glutamyl cysteine synthetase (γ-GCS) in the protective effect of LC on RGCs. RGCs were first processed with high concentrations of glucose. LC treatment at three concentrations (50 μM, 100 μM and 200 μM) was applied to high glucose stimulated RGCs. The expression of Nrf2, Keap1, haemoxygenase-1 (HO-1) and γ-glutamyl cysteine synthetase (γ-GCS) was quantified by Western blot in the treatment and control (high glucose stimulation) groups. In the three LC groups (50 μM, 100 μM and 200 μM), Nrf-2 (0.71 ± 0.04, 0.89 ± 0.05, 1.24 ± 0.05 vs 0.56 ± 0.03, $p < 0.05$), HO-1 (0.58 ± 0.04, 0.76 ± 0.06, 0.89 ± 0.07 vs 0.25 ± 0.03, $p < 0.01$), and γ-GCS protein expression (0.66 ± 0.03, 0.79 ± 0.05, 0.84 ± 0.08 vs 0.84 ± 0.08, $p < 0.01$) was higher than in the control group. The levels of Keap1 protein were in the LC groups were lower than in the control group (0.50 ± 0.03, 0.45 ± 0.02, 0.53 ± 0.03 vs 0.86 ± 0.05, $p < 0.01$). In conclusion, in high glucose stimulated RGCs, LC treatment was associated with an increased level of Nrf2, HO-1and γ-GCS. LC treatment was also associated with a reduced expression of Keap1 protein. These results suggest that the protective effect of LC treatment on RGCs may be related to Nrf2-Keap1 pathway.

1. Introduction

Reactive oxidative stress (ROS) is associated with diabetic retinopathy in the retina and capillary cells. NF-E2-related factor 2 (Nrf2) is a redox sensitive factor that provides defenses against the cytotoxic ROS [1]. Nrf2 is constitutively expressed in all tissues. It is retained in the cytosol by binding to a cluster of proteins, including cytosolic inhibitor, Kelch like-ECH-associated protein 1 (Keap1) [1]. Under oxidative stress, Nrf2 dissociates from Keap1 and moves to the nucleus to bind with the antioxidant-response element (ARE) to regulate the production of Nrf2-dependent Phase 2 enzymes, such as haemoxygenase-1 (HO-1) and γ-glutamyl cysteine synthetase (γ-GCS), suggesting that Nrf2 plays an important role in protecting organs from diabetes [2,3].

L-Carnitine (LC) plays an important physiological role in shuttling the long-chain fatty acids across the inner mitochondrial membrane for oxidation and ATP production. LC is derived from both dietary sources (75%) and endogenous biosynthesis (25%) in human body. Experimental and clinical data support the notion that LC treatment exerts beneficial effects on several disorders related to oxidative stress [4].

Previous studies in our laboratory have demonstrated the effects of LC on high glucose-induced oxidative stress in the retinal ganglion cells [5]. The present work was designed to test the hypothesis that LC protects the retinal ganglion cells against high glucose-induced oxidative damage through Nrf2-Keap1-ARE signal transduction pathway.

* Corresponding author. Tel.: +86 532 83780029; fax: +86 532 83780029.
** Corresponding author. Tel.: +61 2 69332905; fax: +61 2 69 332587.
E-mail addresses: cbwang666@126.com (C.-B. Wang), lwang@csu.edu.au (L.-X. Wang).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Cell culture, treatment, purification and identification

Twenty-five mm^2 culture flasks and plates were incubated with 100 μg/ml polylysine at 37 °C overnight, and were washed three times with PBS. Briefly, retinas from 1- to 3-day-old Wistar rats were incubated at 37 °C for 10–15 min in 0.125% trypsinase solution [4,5]. To yield a suspension of single cells, the tissue was triturated sequentially through a narrow-bore Pasteur pipette in DMEM/F12 solution containing 10% BSA. After centrifugation at 1000 rpm for 5 min, the cells were rewashed in another DMEM/F12 solution, and cell density was adjusted to 1×10^6 ml^{-1} [6,7].

The retinal suspension was incubated in the flasks at 37 °C in 5% CO_2 incubator for 24 h. The coverslips were washed in PBS three times, and were fixed for an additional 4 h in 4% paraformaldehyde. The sections were blocked with 5% (w/v) bovine serum albumin (BSA) and 0.1% Triton X-100 for 30 min at 37 °C, and washed in PBS three times. The slips were incubated with both polyclonal antibodies specific for Thy1.1 (1:100, abcam) and Map2 (1:200, abcam), for 12–24 h at 40 °C. After washed in PBS three times, the secondary antibodies (TRITC-Goat-anti-Mouse and FITC-Goat-anti-Rabbit, Beijing Zhong Shan-Golden Bridge Biol Tech, Beijing, China) were added in dark room and incubated for 1 h at 37 °C. The coverslips were washed in PBS three times and sealed with buffer glycerol. The images were captured by a Leica fluorescence microscope. The purity of the nerve cells of the retina is determined by dividing the number of double positive cells by the total number of cells in the corresponding field. The purity was calculated from five fields of vision in each experiment.

2.2. High glucose stimulation

High glucose (HG) conditions are defined as 30 mM glucose. This glucose level is commonly used to induce glucose-related dysfunction while maintaining cell viability. The cultured cells were randomized as following: 100 μM L-carnitine, 30 mM HG, 30 mM HG + 50 μM L-carnitine, 30 mM HG + 100 μM L-carnitine, 30 mM HG + 200 μM L-carnitine.

2.3. Preparation of total cell lysates, cytosolic and mitochondria fractions

Cells were harvested and centrifuged at 800 × *g* at 4 °C for 10 min, cell suspension was then taken into a glass homogenizer and homogenized for 30 strokes using a tight pestle on ice. Homogenates were centrifuged at 800 × *g* at 4 °C for 10 min to collect the supernatant. The resulting supernatants were further centrifuged at 10,000 × *g* at 4 °C for 20 min to obtain the cytosol (supernatant) and mitochondria (deposition) fraction. Protein concentrations were determined using the BCA Protein Assay Kit.

2.4. Western blotting

This analysis was performed on 40 mg of protein from each cell lysate. Proteins were electroblotted onto a PVDF membrane after fractionated by SDS-PAGE. The membranes were incubated overnight at 4 °C to probe possible cross-contamination in cytosolic and mitochondria fractions. Primary antibodies were Nrf2 (1:1000), Keap-1 (1:1000). HO-1 (1:1000), γ-GCS (1:1000), β-actin (1:1000), PCNA (1:1000). Secondary antibodies (Peroxidase-Conjugated AffiniPure Goat Anti-Rabbit lgG, 1:4000) were diluted in blocking solution and incubated with the membranes, followed by blocking with 5% non-fat dried milk. Excess antibody was washed off with 20 mM TBST (20 mM Tris, 150 mM NaCl, pH 7.5 and 0.1% Tween 20) before incubation in ECL advance. The bands were scanned and densitometrically analyzed using an automatic image analysis system (Alpha Innotech Corporation, San Leandro, CA, USA). These quantitative analyses were normalized to β-actin and PCNA (after stripping).

2.5. Statistical analysis

Data are expressed as mean ± SE. Statistical comparisons were made using one-way ANOVA. Student–Newman–Keuls was used as a post hoc test. *p*-Values of less than 0.05 were considered to represent statistical significance.

3. Results

3.1. Morphological Identification of retinal neurons

Cells with connected neurites were increased after 96 h of culture. High-purity RGCs were obtained. The details were showed in the previous studies of our laboratory [5].

3.2. Effect of LC on high glucose-induced Nrf2 and Keap1 expression in RGCs

Nuclear Nrf2 protein expression was slightly increased in the RGCs in the high glucose group compared with the control group. After treatment with LC at 50 μM, 100 μM and 200 μM, nuclear

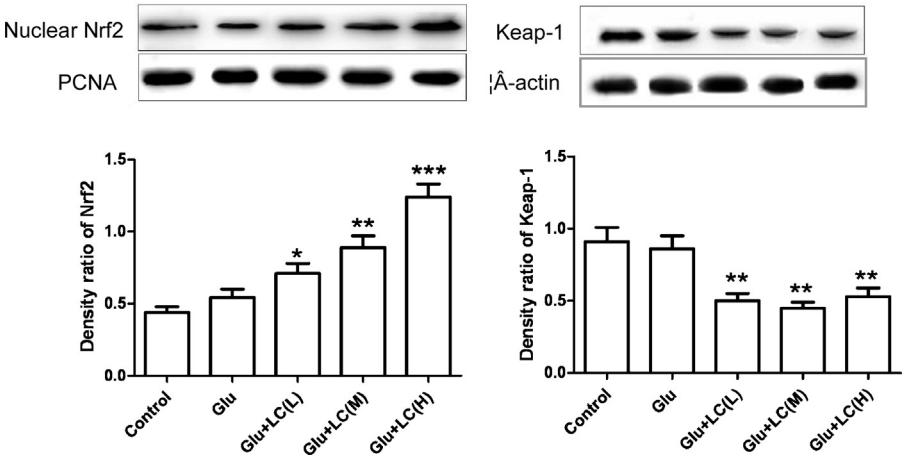

Fig. 1. Comparison of expression of NF-E2-related factor 2 (Nrf2) and Kelch like-ECH-associated protein 1 (Keap1) proteins in the control, high glucose stimulation (GLU) and L-carnitine (LC) treatment groups. L, M ad H: low (50 μM), medium (100 μM) and high dose (200 μM), respectively.

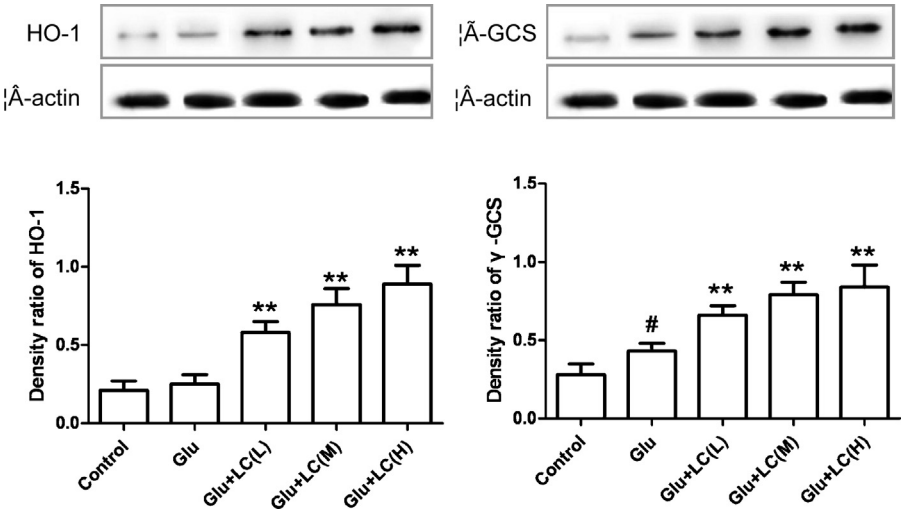

Fig. 2. Comparison of expression of haemoxygenase-1 (HO-1) and γ-glutamyl cysteine synthetase (γ-GCS) proteins in the control, high glucose stimulation (GLU) and L-carnitine (LC) treatment groups. L, M ad H: low (50 μM), medium (100 μM) and high dose (200 μM), respectively.

Nrf2 protein was higher than in the high glucose group (0.71 ± 0.04, 0.89 ± 0.05, 1.24 ± 0.05 vs 0.56 ± 0.03, $p < 0.05$).

As shown in Fig. 1, protein expression of Keap1 was slightly decreased after treatment with high glucose. However, the levels of Keap1 protein were in the LC groups; 50 μM, 100 μM and 200 μM, were lower than in the high glucose group (0.50 ± 0.03, 0.45 ± 0.02, 0.53 ± 0.03 vs 0.86 ± 0.05, $p < 0.01$).

3.3. Effect of LC on high glucose-induced HO-1 and γ-GCS expression in RGCs

The protein expression of HO-1 remained unchanged after treatment with high glucose. The level of HO-1 protein in the LC group was higher than in the high glucose group (0.58 ± 0.04, 0.76 ± 0.06, 0.89 ± 0.07 vs 0.25 ± 0.03, $p < 0.01$).

Furthermore, the level of γ-GCS protein in the LC groups was higher than in the high glucose group (0.66 ± 0.03, 0.79 ± 0.05, 0.84 ± 0.08 vs 0.84 ± 0.08, $p < 0.01$) (Fig. 2).

4. Discussion

Type 2 diabetes is an age-related disease associated with vascular pathologies, including severe blindness, renal failure, atherosclerosis, and stroke. ROS, especially mitochondrial ROS, plays a key role in regulating the cellular redox status. Cells have evolved endogenous defense mechanisms against sustained oxidative stress such as the redox-sensitive transcription factor Nrf2 [8]. Dissociation of Nrf2 with Keap1 and translocation to nucleus are contributing factors for the increased ARE-mediated expression of OH-1and γ-GCS. Thus, regulation of Nrf2-Keap1 by pharmacological means could serve as a potential therapy to attenuate oxidative stress in diabetic retinopathy. LC is primarily known for its participation in mitochondrial fatty acid oxidation. Treatment with LC attenuates neuronal damage prevents apoptosis, and improves energy status in high glucose stress through less understood mechanisms [5]. Because mitochondrial biogenesis could be a possible mechanism for LC-induced improvement in bioenergetics in neurons [9], the present study aimed at exploring signaling pathways of LC-induced neuroprotection in the high glucose stress.

Nrf2 is a master redox regulator of antioxidant defense genes, including NAD(P)H quinone oxidoreductase-1 (NQO1), glutathione S-transferase, and HO-1. Under normal conditions, Nrf2 is bound to Keap-1 as a complex. Antioxidant activities through the Nrf2 pathway are induced by conditions of oxidative stress, and the Nrf2/Keap-1 complex becomes disrupted and Nrf2 is translocated to the nucleus where it binds to ARE sequences [10]. The results about expression of Nrf2 and Keap1 changed slightly and probably suggested that natural oxidative stress was initiated by high glucose. It is supported by the published research, in which levels of SOD, GPx, CAT and T-AOC in the L-carnitine groups were lower in the high-glucose group than in the controls [5]. These results can also rule out the role of LC acting as an oxidative stress inducer. Here we showed that the expression of Keap1 in RGCs was decreased, but the nuclear levels of Nrf2 were increased after LC treatment. However, the binding of Nrf2 to Keap1 was not shown in this study. This disparity could attribute to the maintenance of redox homeostasis, which was also seen in liver damage [11]. This hypothesis is supported by another study where inhibition of IκBα phosphorylation and NF-κB-p65 nuclear translocation, which resulted in accelerated Keap-1 protein degradation, enhanced Nrf-2 nuclear translocation, and increased HO-1 protein expression in cardiac regeneration [12].

HO-1 has emerged as a particularly attractive tool for the prevention and management of a broad range of human and animal diseases characterized by elevated levels of reactive oxygen-containing molecules [13]. In a rat model of transient global cerebral ischemia, sevoflurane post-conditioning increases nuclear factor erythroid 2-related factor and HO-1 expression [14]. In our study, the level of HO-1 protein in the LC group was higher than in the high glucose group, suggesting a role of HO-1 in adaptive responses to oxidative stress.

γ-GCS, which is the rate-limiting enzyme of glutathione (GSH) biosynthesis and an important scavenger of ROS, is considered as a potential therapeutic target for many cancers [15]. The level of γ-GCS protein in the LC group was higher than in the high glucose group in the present study. Collectively, these results indicate that LC treatment upregulates γ-GCS in RGCs by inducing ROS-mediated Nrf2 gain.

In summary, in high glucose treated RGCs, LC treatment was associated with an increased level of Nrf2, HO-1and γ-GCS. LC treatment was also associated with a reduced expression of Keap1 protein. These results suggest that the antioxidant effect of LC treatment may be related to Nrf2-Keap1 pathway.

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