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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

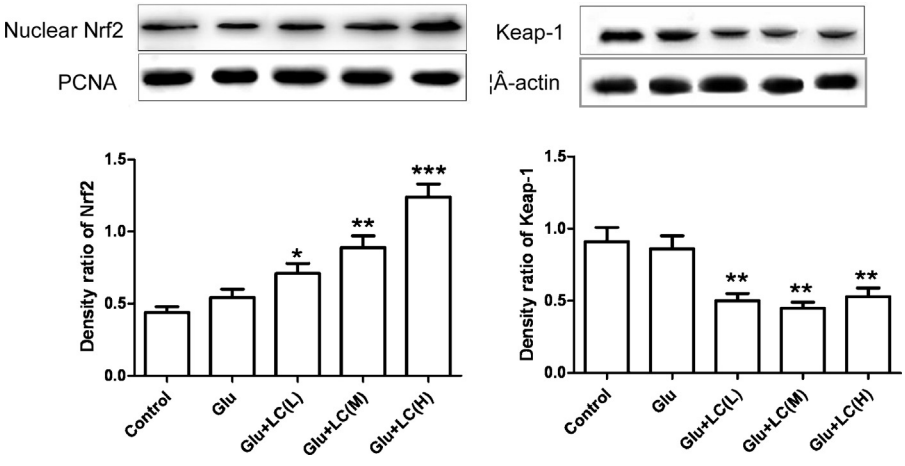

Fig. 1. Comparison of expression of NF-E2-related factor 2 (Nrf2) and Kelch like-ECH-associated protein 1 (Keap1) proteins in the control, high glucose stimulation (GLU) and L-carnitine (LC) treatment groups. L, M ad H: low (50 μM), medium (100 μM) and high dose (200 μM), respectively.