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Original article

Protective effect of L-carnitine against bilirubin-induced neuronal cell death

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Abstract

There is growing evidence that glutamate receptor-mediated injury plays a crucial role in bilirubin neurotoxicity. L-carnitine (LC) has been shown to prevent glutamate-induced toxicity in neuronal cell culture. The purpose of this study is to assess whether LC is able to prevent bilirubin neurotoxicity. Unconjugated bilirubin at different concentrations was administered to cerebellar granular cell cultures prepared from 1-day-old Sprague–Dawley rats. The neuroprotective effect of LC was examined. LC at doses of 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M was applied to culture flasks. LC at a dose of 10^{-4} M significantly blocked bilirubin neurotoxicity. On the other hand, LC significantly increased bilirubin toxicity at a higher dose (10^{-3} M). LC at the doses of 10^{-5} and 10^{-6} M was found to be ineffective. 10^{-4} M LC decreased bilirubin-induced neuronal cell death from 47.72 ± 3.68 to $27.23 \pm 5.14\%$, ($P=0.003$). The present study demonstrates, for the first time, that LC protects against bilirubin neurotoxicity in a dose-dependent manner in cerebellar granular cell culture of rats. Further research is needed to confirm our findings and to clarify the mechanisms responsible for the protective effect of LC.
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Keywords: Unconjugated bilirubin; L-carnitine; Cerebellum; Cell culture; Neuroprotection

1. Introduction

Bilirubin neurotoxicity remains a significant problem in neonatal period despite recent advances in the care of jaundiced neonates. Bilirubin encephalopathy has a vast clinical spectrum ranging from sensorineural hearing loss and choreoathetoid cerebral palsy, seizures or death from kernicterus, to mild mental retardation and subtle cognitive disturbances [1]. Despite intensive investigations for many years, the exact cellular and molecular mechanisms underlying bilirubin-induced neurotoxicity are still unclear. Although several mechanisms have been thought to implicate in bilirubin neurotoxicity, it has recently been proposed that glutamate receptor-mediated injury may play a major role [2,3]. This hypothesis has been based on the fact that bilirubin encephalopathy is synergistically worsened by hypoxia/ischemia that also shares an excitotoxic mechanism of neuronal injury [4,5]. It has been shown in in vitro studies that exposure of astrocytes to unconjugated bilirubin (UCB) decreases the uptake of glutamate and lead to overstimulation of *N*-methyl-D-aspartate (NMDA) receptors and ultimately cell death [6–9].

LC is a natural compound that facilitates the transport of fatty acids into mitochondria for β-oxidation and has many biological functions. It has been shown in limited studies that LC prevents glutamate- and/or kainic acid (KA)-induced neurotoxicity in neuronal cell culture [10–12]. LC was also suggested to prevent NMDA neurotoxicity in in vitro studies [10,13]. In this study, we therefore aimed to investigate whether LC is able to prevent bilirubin-induced neurotoxicity in cerebellar granular cell cultures of newborn rats.

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2. Materials and methods

Primary cultures of cerebellar granular cells were prepared from 1-day-old Sprague–Dawley rats as in previous studies [14]. We studied on granular cells of the cerebellum because of the fact that they were reported to be far more sensitive to bilirubin toxicity compared to cortical or hippocampal neurons [15]. Briefly, one newborn rat was decapitated for each of experimental groups, and the cerebellum was dissected out. It was suspended in 5 ml of Dulbecco's minimal essential medium (DMEM) containing 2 ml of trypsin–EDTA (0.25% trypsin, 0.02% EDTA) at 37 °C for 20 min. Trypsin digestion was ended by 10 ml of DMEM containing deoxyribonuclease type 1 (120 units per ml). After 3 min of centrifugation at 800 rpm, the pellet was suspended. Bases of the 25 cm^2 polypropylene tissue culture flasks were covered with poly-D-lysine. 0.1 mg of poly-D-lysine (MW 30,000–70,000), was dissolved in phosphate buffer solution and the flask bases were filled. After 5 min at room temperature, the solution was vacuumed and left to dry on a laminar flow bench overnight. Cell suspensions (0.2 ml) were plated in 2.5 ml of medium containing 10% fetal calf serum and DMEM, without antibiotics. After a period of 30 min, media were changed to eliminate non-adherent cells, and fresh DMEM was added to the flasks. The culture dishes were kept at 37 °C in humidified 95% air and 5% CO_2. After 24–48 h, 10 μM cytosine arabinoside (cytosine 1-β-D-arabinofuranoside) was added to the culture medium to prevent the replication of non-neuronal cells. Culture media were changed twice a week and neurons were used for neurotoxicity experiments after 8 days in vitro. Each of the experimental groups was tested in five culture media ($n=5$) and at least 15 microscopic areas were counted for each medium tested. LC was diluted with distilled water while UCB was dissolved in 0.1 N NaOH. UCB was tested in cell culture flasks in 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M concentrations for 4 h. 10^{-4} M UCB was accepted as the most suitable threshold concentration for our neurotoxicity model as in previous studies [16]. Forty-five minutes before the addition of UCB, LC was applied into the culture flasks in 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M concentrations for the 10^{-4} M UCB toxicity experiments. Then, UCB was added to the media for 4 h. Following this process, cultures were washed once with 5 ml DMEM and then 2.5 ml of the same solution was added. They were further incubated at 37 °C in humidified 95% air and 5% CO_2 for an additional 16 h. Cultures were dyed with 1.5 ml 0.4% trypan blue and neuronal cell death was assessed over 5–15 min by a blind examination with an inverted light microscope. Cell death population was calculated as the percentage of stained (non-viable cells), relative to the total number of cells counted. Data were expressed as means ± SEM. The statistical significance of the differences was determined by one-way ANOVA test using the SPSS 10.0 program. Values less than 0.05 were assumed significant. The study was approved by the Animal Research Ethics Committee of our university.

3. Results

The dead cell percentage was found to be 2.14 ± 0.32 in the control group while it was 89.22 ± 2.43, 47.72 ± 3.68, 47.38 ± 5.06, 45.70 ± 2.13, 43.08 ± 4.75, and 37.50 ± 3.27 in the 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M bilirubin groups, respectively. The dead cell percentage was significantly higher in all bilirubin concentrations compared to the control group ($P<0.001$). The dose-response curve was shown in Fig. 1.

LC at concentrations of 10^{-6} and 10^{-5} M had no effect on bilirubin toxicity (dead cell percentage: 33.00 ± 4.61 and 49.80 ± 1.90, respectively; $P>0.05$). On the other hand, effective dose of LC in bilirubin toxicity was found to be 10^{-4} M (dead cell percentage: 27.23 ± 5.14, $P=0.003$). LC at concentrations of 10^{-4} M decreased cell death 43%. LC in higher dose (10^{-3} M) increased cell death significantly (dead cell percentage: 78.17 ± 2.48, $P<0.001$). Detailed information was given in Fig. 2.

4. Discussion

The results demonstrate, for the first time, that in neuronal cell culture LC has a protective effect against

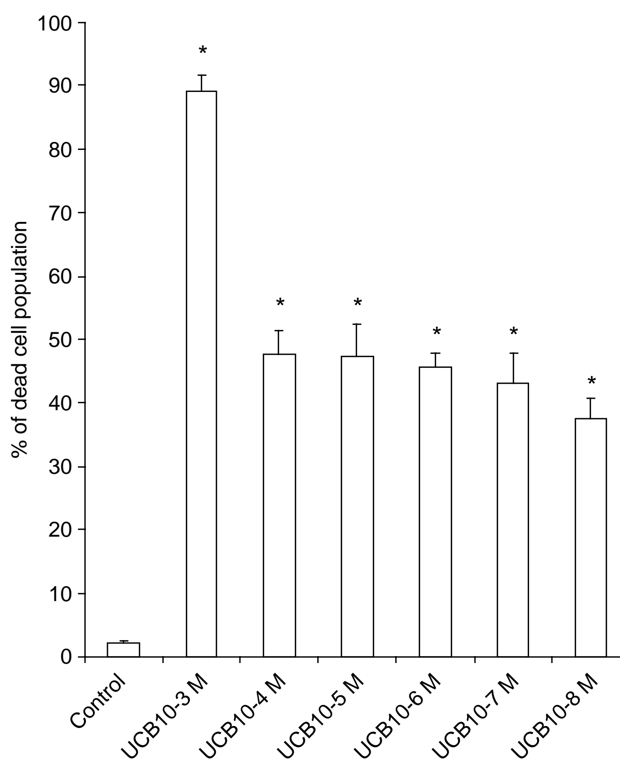

Fig. 1. Dose-response curve of UCB. *$P<0.001$ with respect to the control group.

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