L-Carnitine protects against apoptosis of murine MC3T3-E1 osteoblastic cells

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Summary. L-Carnitine (LC), an amino acid with a major role in cellular energy metabolism, has positive effects on bone metabolism. However, the effect of LC on apoptosis of osteoblast in vitro has not been reported. The aim of this study was to investigate the action of LC on apoptosis of mouse osteoblastic cell line MC3T3-E1. Cell apoptosis was measured by sandwich-enzyme-immunoassay. Release of cytochrome c from mitochondria into cytosol and Bcl-2, Bax protein levels were determined by Western blot analysis. The enzyme substrate was used to assess the activation of caspase-3 and caspase-9. LC inhibited MC3T3-E1 cell apoptosis induced by serum deprivation. Our study also shows that LC decreased cytochrome c release and caspase-3 and caspase-9 activation in serum-deprived MC3T3-E1 cells. Furthermore, LC protected against MC3T3-E1 cell apoptosis induced by the glucocorticoid (GC) dexamethasone (Dex).

Keywords: L-Carnitine – Osteoblast – Apoptosis – Glucocorticoid

Introduction

L-Carnitine (LC) is widely distributed among tissues including nervous system, skeletal muscle, heart, liver, bone, etc. (Winter and Zorn, 1990; Gatti et al., 1998; Chiu et al., 1999). LC is known to be a conditionally amino acid compound as a cofactor for the transport of long-chain acyl CoA through the inner mitochondrial membrane for beta-oxidation (Fritz, 1963). Previous studies have confirmed that oral intake of LC increased the bone mineral density (BMD) and concluded that LC has a beneficial effect on bone mineralization (Benvenga et al., 2001). It has recently been demonstrated that LC increased metabolic activity and protein production of porcine osteoblast-like cells in vitro (Chiu et al., 1999). Colucci et al. (2005) reported that LC enhanced human osteoblast proliferation and the expression of collagen type I (COLLI), bone sialoproteins (BSPs), osteopontin (OPN) and insulin-like growth factor binding protein (IGFBP)-3 and increased the formation of mineralized nodules. These findings indicate that LC is beneficial for bone formation. However, no data have been published about the effect of LC on osteoblast apoptosis.

Recent evidence indicates that apoptosis represents the most common fate of osteoblasts during physiologic bone remodeling (Jilka et al., 1998, 1999; Plotkin et al., 1999). The majority of osteoblasts die by apoptosis. The frequency of osteoblast apoptosis could have a significant impact on the number of osteoblasts present at the site of bone formation (Fritz, 1963). Pharmacologic GCs induce osteoporosis. Studies in mice and humans, as well as in vitro experiments, strongly suggest that the deleterious effects of GCs on the skeleton are partially caused by proapoptotic effects on osteoblasts (Weinstein et al., 1998; Gohel et al., 1999; Canalis and Delany, 2002). Osteoblast apoptosis may play an important role in the GC-induced osteoporosis (Manolagas, 2000).

The present study was undertaken to determine whether LC regulates serum deprivation or the GC dexamethasone (Dex)-induced apoptosis of osteoblastic MC3T3-E1 cells, and to examine the mechanisms by which LC act on cell apoptosis.

Materials and methods

Reagents

Anti-Bcl-2, Bax, cytochrome c antibodies, anti-mouse, and rabbit IgG peroxidase conjugate antibodies were purchased from Santa Cruz Biotechnology Inc. (Waltham, MA, USA). Substrates for caspase-3-like proteins and the ELISA kit for caspases 3 and 9 were purchased from R&D Systems (Minneapolis, MN, USA). Substrates for caspase-3 and caspase-9 were from Cell Signaling Technology (Beverly, MA, USA). DNA ladders for detection of DNA fragmentation were from Promega (Madison, WI, USA). BSA, bovine serum albumin, was from Sigma-Aldrich (St. Louis, MO, USA). Bicinchoninic acid protein assay kit was from Pierce (Rockford, IL, USA). A rabbit IgG peroxidase conjugate was from Dako (Carpinteria, CA, USA). Osteogenic medium was from In VitroCell (Lancaster, PA, USA). Medium 199 was from Biological Industries (Kibbutz Beit Haemek, Israel). DMEM was from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was from Hyclone (Logan, UT, USA).

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proteinase (Ac-DEVD-MCA) and caspase-9-like proteinase (Ac-LEHD-AFC), LC, Dex and anti-β-actin polyclonal antibody were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Mouse MC3T3-E1 cell culture
The mouse osteoblastic cell line MC3T3-E1 was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in α-MEM (Gibco BRL, Gaithersburg, MD), supplemented with 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml ascorbic acid. Cells were maintained in a humidified, 95% air, 5% CO2 atmosphere at 37°C. The medium was changed twice a week and the cells were subcultured using 0.05% trypsin with 0.01% EDTA.

Cell apoptosis measurement
Apoptosis was assayed directly by measurement of cytoplasmic nucleosomes (i.e., DNA complexed with histone in the cytoplasm) using a Cell Death Detection ELISA Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany), according to the kit protocol. The Cell Death Detection ELISA Kit allows the specific determination of mono- and oligo-nucleosomes in the cytoplasmatic fraction of cell lysates. Briefly, cells were plated at a density of 1 x 10⁴ cells/well in 24-well plates for 1 day followed by culture in serum-free medium for 48 h in the absence or presence of 10⁻² to 10⁻⁴ M LC. One percentage FBS treatment was used to observe basal levels of apoptosis. The cell layers were rinsed with phosphate-buffered saline (PBS) and extracted with 0.5 ml of lysis buffer after a 30 min incubation at 4°C. The cell lysates were then centrifuged for 10 min at 15,000 rpm, and the aliquots of aqueous supernatant were tested for apoptosis using the Cell Death Detection Kit.

To study the effect of LC on Dex-induced MC3T3-E1 cells apoptosis. Cells were cultured in the absence or presence of 10⁻² M Dex and simultaneously treated with vehicle or 10⁻⁴ to 10⁻² M LC for 6 h.

Detection of Bcl-2 and Bax expression by Western blot analysis
For investigating the effect of LC on Bcl-2 and Bax protein expression in MC3T3-E1 cells, Western blot was performed. Cells were plated at a density of 1 x 10⁴ cells/well in 24-well plates and grown to confluence in 6-well plates and then treated with 10⁻⁴ to 10⁻² M LC in α-MEM for 24 h. Cell monolayers were lysed with Triton lysis buffer (50 mM Tris–HCl, pH 8.0 containing 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide, 10 mM EDTA, 10 μg/ml aprotinin, and 1 μg/ml aminophenylbenzenesulfonyl fluoride). Protein concentrations were determined by Bradford assay using BSA as a standard.

Statistical analyses
Data are presented as the mean ± SD. Comparisons were made using a one-way ANOVA. All experiments were repeated at least three times, and representative experiments are shown.

Results
LC protected MC3T3-E1 cells against serum deprivation-induced apoptosis
Figure 1 shows that MC3T3-E1 cells in 1% FBS medium had basal levels of apoptosis (0.22 ± 0.04 ELISA absorbance units). 10⁻⁴ to 10⁻² M LC protects MC3T3-E1 cells against serum deprivation-induced apoptosis. After