Amino Acids (2008) 35: 419–423
DOI 10.1007/s00726-007-0598-9
Printed in The Netherlands

Amino Acids

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

H. Xie[*,1], **S.-Y. Tang**[*,2], **H. Li**[1], **X.-H. Luo**[1], **L.-Q. Yuan**[1], **D. Wang**[1], and **E.-Y. Liao**[1]

[1] Institute of Endocrinology and Metabolism, The Second XiangYa Hospital of Central South University, Changsha, Hunan, China
[2] School of Nursing, Central South University, Changsha, Hunan, China

Received July 20, 2007
Accepted August 12, 2007
Published online October 2, 2007;

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 nervers 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

* The first two authors contributed equally to this work

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% CO_2 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 assessed 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 1×10^4 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^{-5} to 10^{-2} 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^{-6} 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 1×10^5 cells/well 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 aminoethylbenzenesulfonyl fluoride). Protein concentrations were determined by Bradford assay. One hundred micrograms of protein from each cell layer were loaded onto a 7.5% polyacrylamide gel. After electrophoresis, the SDS-PAGE separated proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was incubated with anti-Bcl-2 monoclonal antibody or anti-Bax monoclonal antibody or β-actin polyclonal antibody at 1:500 in PBS for 2 h. Then, the membrane was re-probed with peroxidase-conjugated secondary antibodies at 1:1000 in PBS for 1 h. Blots were processed using an ECL Kit (Santa Cruz) and exposed to X-ray film.

Analysis of cytochrome c *release*

Release of cytochrome *c* from mitochondria into cytosol was measured by Western blot. Briefly, cells were treated with or without 10^{-4} to 10^{-2} M LC for 24 h, and homogenated with Triton lysis buffer as described above. The cell lysates were centrifuged at 100,000 *g* for 30 min to yield soluble cytosolic fraction (supernatant). Supernatants were then subjected to Western blot analysis as described above with anti-mouse cytochrome *c* antibody.

Assays for caspase-3 and caspase-9

MC3T3-E1 cells were grown in the absence or presence of 0.1% FBS in α-MEM for 1.5 h and serum-deprived cells were also treated with vehicle or 10^{-4} to 10^{-2} M LC for 1.5 h. This is the time point at which profound activation of caspase-3 and caspase-9 can be detected (Mogi et al., 2004; Tang et al., 2007). Cells were lysed with 400 μl of lysis buffer (10 mM HEPES, pH 7.5 containing 0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, and 2 mM phenylmethylsulphonylfluoride). Aliquots (50 μl) of the extracts were incubated for 1 h at 37 °C with 50 μM enzyme substrate (Ac-DEVD-MCA for caspase-3-like proteinase and Ac-LEHD-AFC for caspase-9-like proteinase) in 10 mM HEPES, pH 7.5, containing 50 mM NaCl, 2.5 mM dithiothreitol in a 100 μl reaction mixture. The fluorescence of the released AMC was measured with a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. AFC release was monitored at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Protein concentrations of extracts were estimated by conducting a Bradford protein 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

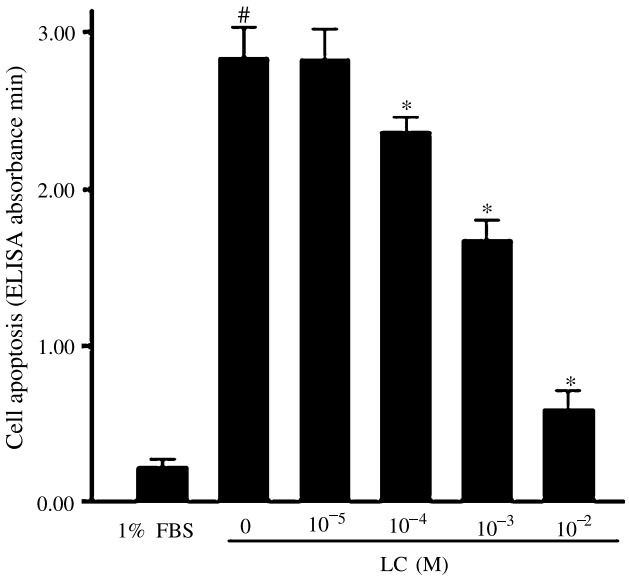

Fig. 1. Effects of LC on serum deprivation-induced MC3T3-E1 cells apoptosis by Cell Death ELISA Detection. MC3T3-E1 cells were grown in the absence or presence of 1% FBS in α-MEM for 48 h and serum-deprived cells were also treated with vehicle or 10^{-5} to 10^{-2} M LC for 48 h. Apoptosis was assessed using a Cell Death Detection Kit, and expressed as ELISA absorbance units. The bars represent the mean ± SD ($n = 6$). $^{\#}P < 0.05$ vs. 1% FBS treated control; $^{*}P < 0.05$ vs. serum-deprived cells with vehicle treatment

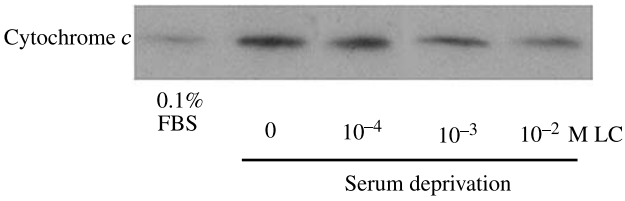

Fig. 2. Effects of LC on cytochrome *c* release in MC3T3-E1 cells. MC3T3-E1 cells were grown in the absence or presence of 0.1% FBS in α-MEM for 24 h and serum-deprived cells were also treated with vehicle or 10^{-4} to 10^{-2} M LC for 24 h. Western blot analysis was performed using anti-cytochrome *c* antibody

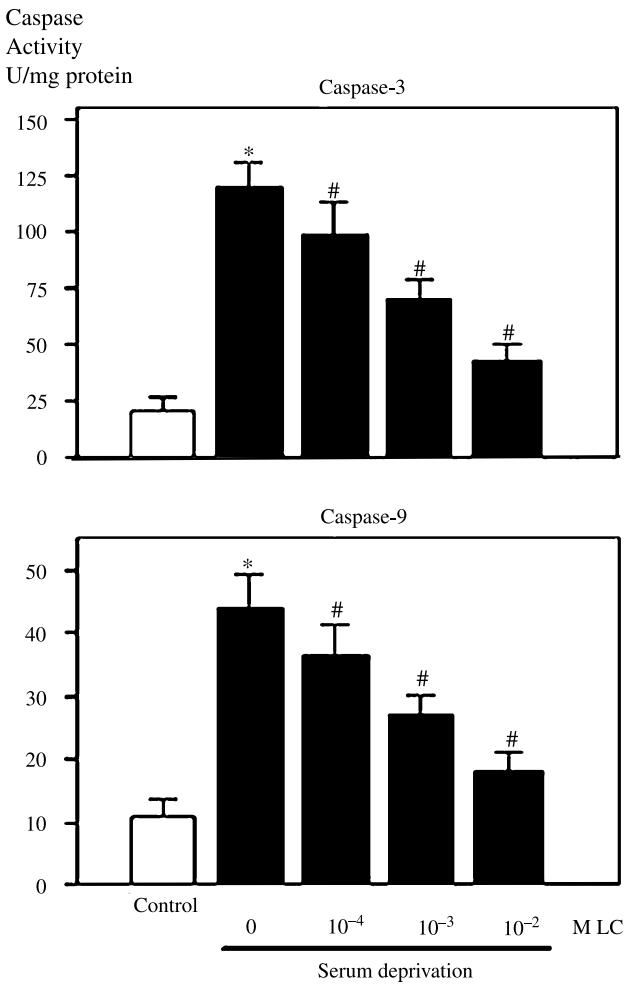

Fig. 3. Effect of LC on serum deprivation-induced caspase-3 and caspase-9 activation. MC3T3-E1 cells were grown in the absence or presence of 0.1% FBS in α-MEM for 1.5 h and serum-deprived cells were also treated with vehicle or 10^{-4} to 10^{-2} M LC for 1.5 h. Caspase-3 and caspase-9 activities were determined by the use of specific fluorosubstrates. Data (Caspase activity: U/mg protein) are expressed as the mean ± SD of three experiments. One unit was defined as the amount of the enzyme required to release 1 μM AMC or AFC per hour at 37 °C. $^{*}P<0.05$ vs. 0.1% FBS treated control; $^{\#}P<0.05$ vs. serum-deprived activity of caspase

48 h of starvation, apoptotic cells at 10^{-4} M (2.37 ± 0.09 ELISA absorbance units), 10^{-3} M (1.67 ± 0.14 ELISA absorbance units) and 10^{-2} M (0.59 ± 0.12 ELISA absorbance units) LC were less than that of vehicle-treated group (2.84 ± 0.19 ELISA absorbance units, all $P<0.05$), showing a maximal anti-apoptotic effect at 10^{-2} M.

Effects of LC on cytochrome c *release and caspases activation in serum-deprived MC3T3-E1 cells*

Western blot was performed to examine Bcl-2 and Bax protein expression in MC3T3-E1 cells. We only detected Bax but not Bcl-2 protein expression in MC3T3-E1 cells (data not shown). Serum deprivation or LC treatment did not alter Bax protein expression in MC3T3-E1 cells (data not shown).

Cytochrome *c* was released into cytoplasm in the serum-free culture; however, release was inhibited by 10^{-4} to 10^{-2} M LC (Fig. 2). Similar results were presented in Fig. 3, the serum-deprivation activated caspase-3 and caspase-9 were also markedly decreased in 10^{-4} to 10^{-2} M LC-treated cells.

LC inhibits Dex-induced apoptosis of MC3T3-E1 cells

Figure 4 shows the inhibitory effect of LC on Dex-induced MC3T3-E1 cells apoptosis determined by Cell Death

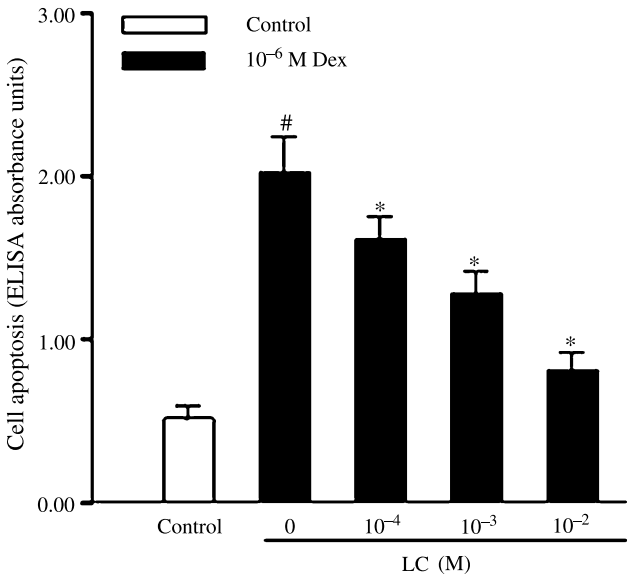

Fig. 4. Effect of LC on Dex-induced apoptosis in MC3T3-E1 cells determined by Cell Death ELISA. Cells were cultured in the presence of 10^{-6} M Dex with or without 10^{-4} to 10^{-2} M LC for 6 h. Apoptosis was assessed using a Cell Death Detection Kit, and expressed as ELISA absorbance units. The bars represent the mean ± SD ($n=6$). $^{\#}P<0.05$, compared with vehicle treated group; $^{*}P<0.05$, compared with Dex alone

ELISA. Treatment with the 10^{-6} M Dex alone for 6 h significantly increased the cell apoptosis. Treatment with LC inhibited apoptosis induced by dexamethasone, with effective concentrations between 10^{-4} and 10^{-2} M.

Discussion

In the present study we showed that LC protected MC3T3-E1 cells against serum deprivation-induced apoptosis and this anti-apoptotic effect involved the regulation of cytochrome *c* release and caspase-3 and caspase-9 activation. We also found that LC prevented the synthetic GC Dex-induced apoptosis of MC3T3-E1 cells.

The rate of bone formation and resorption is largely determined by the number of bone-forming (osteoblast) and bone-resorbing (osteoclast) cells present in the basic multicellular units responsible for the regeneration of the adult skeleton (Manolagas and Jilka, 1995). Similarly to other regenerating tissues, the number of bone cells is controlled by changes not only in the production of mature cells but also in their survival. 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).

LC has positive effects on bone metabolism. In a clinical trial, Benvenga et al. (2001) reported that 2 or 4 g/d oral LC were able to prevent/minimize hyperthyroidism-related bone loss, they confirmed that oral intake of LC increased the bone mineral density (BMD) of hyperthyroidism patients and concluded that LC has a beneficial effect on bone mineralization. In in vitro experiments, LC positively affected osteoblast proliferation and differentiation (Chiu et al., 1999; Colucci et al., 2005). LC has been found to halt or reduce apoptosis of rat bone marrow cell cultures (BMC), lymphocyte, skeletal muscle myocytes, cardiac myocytes and primary cultured neurons (Moretti et al., 1998; Andrieu-Abadie et al., 1999; Ishii et al., 2000; Vescovo et al., 2002; Abd-Allah et al., 2005). However, the effect of LC on apoptosis of osteoblast remains unknown. Here, administration of LC suppressed serum deprivation-induced apoptosis of osteoblastic MC3T3-E1 cells, indicating an anti-apoptotic role for LC in addition to its positive effects on proliferation and differentiation in osteoblasts.

Apoptosis is a tightly regulated physiological process (Adams and Cory, 1998). Bax/Bcl-2 ratio is correlated with cell apoptosis (Adams and Cory, 1998). In this study, we only detected Bax but not Bcl-2 protein expression in MC3T3-E1 cells. This is consistent with previous study (Beck et al., 2001; Chua et al., 2003). Furthermore, we found that neither serum deprivation nor LC can alter Bax protein level in MC3T3-E1 cells. Caspases, cysteine proteases with aspartate specificity, are important mediators of apoptosis. Caspase-9 is an initiator caspase that is involved in mitochondrial damage (Duan et al., 1996). Caspase-3 is effector caspase that is responsible for cleaving nucleases in addition to cellular substrates (Orth et al., 1996; Li and Darzynkiewicz, 1998). Cytochrome *c* is known to bind Apaf-1 and permit the recruitment of procaspase-9 (Li et al., 1997). Cytochrome *c* release is a marker for mitochondria-related apoptosis (Li et al., 1997). Our previous study has demonstrated that serum deprivation induces caspase-9 and caspase-3 activation and cytochrome *c* release from mitochondria in MC3T3-E1 cells (Tang et al., 2007). And, there have been reports indicating that LC is capable of suppressing apoptosis through the mitochondrial pathway in some cell types (Kashiwagi et al., 2001; Qi et al., 2003; Pillich et al., 2005). In the present study, with the treatment of LC, however, the serum deprivation-induced release of cytochrome *c* from the mitochondria was inhibited and the activation of the caspase-3 and caspase-9 was also suppressed. Therefore, it would appear that LC suppresses MC3T3-E1 cells apoptosis through the mitochondrial pathway – inhibiting the release of cytochrome *c* and activation of caspase-9 and caspase-3.

GCs are used in the treatment of autoimmune, pulmonary, and gastrointestinal disorders, as well as in transplantation. A frequent side effect of long-term GC therapy is reduction in bone density. It is the third most prevalent form of osteoporosis after postmenopausal and senile osteoporosis (Weinstein et al., 1998). An increase in osteoblast apoptosis has been demonstrated in patients with GC-induced osteoporosis (Weinstein et al., 2000). Mice administered with GCs also show higher number of apoptotic osteoblasts that appear to be responsible for the decreased bone formation (O'Brien et al., 2004). In vitro studies also demonstrate that GCs can induce apoptosis of osteoblasts (Gohel et al., 1999; Liu et al., 2004). These findings indicate that increased osteoblast apoptosis is responsible for the GCs-induced bone loss or osteoporosis. This study demonstrates that LC inhibits the GC Dex-induced apoptosis of MC3T3-E1 cells. It will be possible that LC in the future as a new regimen of benefit for prevention or treatment of GC-induced osteoporosis.

In conclusion, our study provides the evidence that LC protects serum deprived MC3T3-E1 cells from apoptosis by inhibiting of cytochrome *c* release and caspase-3 and caspase-9 activation. We also illustrate that LC inhibits

the GC Dex-induced apoptosis of MC3T3-E1 cells. The next step is to use animal model to investigate the direct effects of LC on bone.

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Authors' address: Er-Yuan Liao, Institute of Endocrinology and Metabolism, The Second XiangYa Hospital of Central South University, Changsha, Hunan 410011, China,
E-mail: eyliao2003@21cn.com