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L-Carnitine and Isovaleryl L-Carnitine Fumarate Positively Affect Human Osteoblast Proliferation and Differentiation *In Vitro*

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Abstract. Age-related bone loss is characterized by decreased osteoblast activity, possibly related to the reduction of energy production. Carnitine promotes energy availability and its concentration declines with age; Therefore, two Carnitine derivatives, L-carnitine fumarate (LC) and isovaleryl L-carnitine fumarate (Iso-V-LC), have been tested on several parameters of human osteoblasts *in vitro*. Both compounds significantly increased osteoblast activity, but the new compound Iso-V-LC was more efficient than LC at lower concentrations. They both significantly enhanced cell proliferation, [^{3}H]-proline incorporation and the expression of collagen type I (COLLI), and the bone sialoproteins (BSPs) and osteopontin (OPN). The percentage of alkaline phosphatase (ALP)–positive cells and the secretion of osteocalcin were not modified by LC and Iso-V-LC. Both molecules increased the formation of mineralized nodules, but Iso-V-LC reached the maximum effect at a concentration 10-fold lower than that of LC. Furthermore, we showed that insulin-like growth factor (IGF)-I and IGF-II mRNA levels were not modified by the treatment. However, the two compounds induced an increase of insulin-like growth factor binding protein (IGFBP)-3 and a decrease of IGFBP-5 in both osteoblast lysates and the extracellular matrix (ECM). In conclusion these data suggest that carnitine and, in particular, its new derivative, Iso-V-LC supplementation in the elderly may stimulate osteoblast activity and decrease age-related bone loss.

Key words: Carnitine — Carnitine derivatives — Osteoblast — Proliferation — Differentiation

A large number of bone diseases result from a negative balance between bone resorption and formation. Two main mechanisms directly contribute to bone loss: postmenopausal osteoporosis, frequently characterized by increased osteoclast activity, and aging processes, prevalently resulting from decreased osteoblast activity. Osteoblasts have a high rate of energy consumption during bone formation and bone protein synthesis; therefore, it is possible that the decreased energy production that accompanies aging could contribute to reduce osteoblast activity.

Although some studies suggest that glucose is the major fuel substrate for bone [1, 2], more recent studies indicate that cells of the osteoblastic lineage generate 40% to 80% of the energy demands through fatty acid oxidation [3]. This suggests that the modulation of fatty acid oxidation, which requires a large number of factors, may regulate the amount of energy available for protein synthesis in osteoblasts. The carnitine system, comprehensive of carnitine and its derivatives, is necessary for glucose and lipid metabolism in cells. Carnitine is an essential cofactor for the transport of long-chain fatty acids across the inner mitochondrial membrane into the mitochondrial matrix for β-oxidation, which is the most efficient metabolic pathway for energy production [4]. Thus, carnitine facilitates energy availability, and is especially important for those tissues with high energy requirements. Carnitine levels decrease with age [5] and its deficiency can lead to cardiomyopathy and skeletal muscle weakness [6, 7]. This loss of energy availability could also compromise osteoblast activity and bone production in an age-related manner.

It has recently been demonstrated that carnitine increased metabolic activity and protein production of porcine osteoblast-like cells *in vitro* [8]. However, no data have been published about the effect of carnitine and/or its derivatives on human osteoblasts. Moreover, several *in vivo* and *in vitro* studies suggest that proliferating and differentiating factors affecting osteoblast activity exert their role through the involvement of insulin-like growth factor (IGF) expression. IGF-I and -II are the most abundant of the growth factors produced and are stored in the bone matrix; they stimulate proliferation and differentiation of osteoblasts [9–13]. However, the IGF bioavailability in bone is determined not only by bone cell expression but also by the actions of insulin-like growth factors binding proteins (IGFBPs). Six high-affinity IGFBPs have been character-

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ized [14], and they are produced by bone cells and modulate IGF action on bone. In particular, IGFBP-2 seems to inhibit the action of IGFs on osteoblast replication and matrix synthesis [15]; IGFBP-3 exerts both inhibitory and stimulatory effects on bone cells, but it has been shown that the accumulation of endogenous IGFBP-3 correlates with enhanced IGF-I activity in osteoblasts [16]. IGFBP-4 inhibits IGF-stimulated effect in a variety of bone cell models [17–20], and IGFBP-5, like IGFBP-3, has both inhibitory and stimulatory effects on bone. When intact and in solution, endogenous or exogenous IGFBP-5 inhibits IGF-stimulated bone cell growth [19, 21].

In the present study we investigated whether the new carnitine derivatives, the Iso-V-LC, as well as the LC, could modulate proliferation and differentiation of human osteoblasts *in vitro* through the involvement of IGFs and IGFBPs. Results showed that both carnitine derivatives affect osteoblast activity, increasing both cell proliferation and matrix secretion, and modulate the expression of IGFBP-3 and -5. Furthermore, we demonstrated that Iso-V-LC was already effective at concentrations lower than those of LC.

Materials and Methods

Human Osteoblasts

Trabecular bone specimens, obtained after written informed consent from patients in the department of orthopaedics, were cleaned of soft tissues, reduced to small fragments, and digested with 0.5 mg/mL *Clostridium histolyticum* neutral collagenase (Sigma Chemical Co, St Louis, MO, USA) in phosphate-buffered saline (PBS) with gentle agitation for 30 minutes at 37°C [22].

Bone fragments were then washed (three times) with minimum essential medium (α-MEM) (Gibco Ltd, Uxbridge, UK) containing 3.024 g/L sodium bicarbonate, and cultured in medium supplemented with 10% FCS (fetal calf serum; Gibco Ltd), 100 IU/mL, penicillin, 100 mg/mL streptomycin, 2.5 mg/mL amphotericin B, and 50 IU/mL mycostatin, at 37°C in a water-saturated atmosphere containing 5% CO_2. Cells were fed by medium replacement every 3 to 4 days. In these conditions, the osteoblast resident in the- explant proliferated and migrated to the culture substrate, reaching confluence within 3 to 4 weeks. Cells were then trypsinized and transferred to appropriate culture dishes for characterization and experiments.

Cell Proliferation

Human osteoblasts were seeded in 96-well plates at density of 15,000/cm^2 and cultured in the presence of increasing concentrations of LC and Iso-V-LC, ranging from 10^{-5} M to 10^{-3} M. The experiments were performed in a-α-MEM containing 10% FCS, and the cells cultured without the drugs represented the control. The effect of the two molecules on osteoblast proliferation was evaluated by Titer-tek technique every 24 hours for 4 days. The cells were fixed with 20% methanol for 10 minutes at room temperature, after which they were rinsed with PBS, air-dried, and stained with 0.5%-crystal violet for 15 minutes and then rinsed extensively. The dye was released from the cells by the addition of 0.1 M sodium citrate in 50% ethanol. The optical density of the released stain solution was read in a Titer-tek colorimeter (Microtech Bio-Rad, Hercules, CA, USA) at a wavelength of 540 nm. Results represent the averages of three experiments and are expressed as the absorbance ± standard error (SE) of each treatment performed in quadruplicate.

Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) activity was evaluated by histochemical and fluorimetric methods every 24 hours for 4 days on human osteoblasts cultured in α-MEM supplemented with 10% of FCS, in the presence or in the absence of LC and Iso-V-LC ranging in concentration from 10^{-5} to 10^{-3} M. Histochemical staining was performed with an appropriate Sigma Kit used on semiconfluent osteoblasts treated or not with the two compounds for 96 hours. Results are expressed as percentage of ALP-positive osteoblasts treated with LC and Iso-V-LC versus the total number of the cells present in the well. Each treatment was performed in quadruplicate.

ALP activity was measured by a fluorimetric method, with 4-methyl-umbelliferyl-phosphate used as substrate. Monolayers were solubilized in 0.1% sodium dodecyl-sulphate (SDS). The cell lysates were incubated at pH 10.3 in the presence of 200 μmol/L substrate at 37°C for 30 minutes. The 4-methyl-umbelliferone produced by the enzyme was detected by monitoring its fluorescence at 369-nm excitation and 448-nm emission wavelengths. The rate of production was converted into nmol/minute from standard curves. Results were normalized per milligram of cell protein. Protein content was measured by Bicinchromimic Aciol (BCA) reagent kit (Pierce Biotechnology, Rockford, MN, USA).

Preparation of Extracellular Matrix

Human osteoblasts were grown for 7 to 10 days until semiconfluence in α-MEM supplemented with 10% FCS, 50 μg/mL ascorbic acid, and 10 mM β-glycerophosphate, and then cultured for 24 and 48 hours in the presence or in the absence of increasing concentrations of LC and Iso-V-LC. Extracellular matrix (ECM) was prepared as described by Knudsen et al. [23], keeping the plates on ice and using ice-cold solutions. The cells were rinsed twice in PBS and the cell membranes were removed by incubation for 10 minutes in 0.5% Triton X-100 in PBS, pH 7.4. The nuclei and cytoskeletons were removed by incubation for 5 minutes in 25 nM ammonium acetate, pH 9.0. The ECM was rinsed twice in PBS and scraped from the culture plates. Aliquots of ECM extract were mixed with Laemmli sample buffer and subjected to Western blot analysis to detect IGFBP expression.

Proline incorporation

^{3}[H]-Proline incorporation was performed in semiconfluent monolayers of human osteoblasts seeded on 24-well dishes and cultured in the presence of increasing concentrations of LC and Iso-V-LC (from 10^{-5} to 10^{-3} M) for 48 hours. Cells cultured without the two compounds were utilized as controls. Osteoblasts were pulsed with 1μCi proline for 2 hours, to avoid cell secretion of ^{3}[H]-hydroxyproline with tropocollagen fibers. Cells were then washed (three times) with PBS, solubilized in 1 mL of 0.1% SDS containing 10 μg/mL^{-1} bovine serum albumin (BSA) and precipitated by addition of 100% trichloroacetic acid (TCA). After incubation for 30 minutes at 4°C, trichloroacetic acid (TCA)-precipitable material was pelleted by centrifugation at 955 g, redissolved in 500 μL of 0.1% SDS, and counted in a Beckman 600 scintillation spectrophotometer after the addition of 500 μL of distillated water and 9 mL of scintillation fluid. Results represent the mean ± SE of three experiments performed in quadruplicate.

Table 1. Primer sequences, annealing temperatures, cycle number and product size

Gene	Sense primer	Antisense primer	Annealing temp. (°C)	Cycles	Product size (bp)
CollI	5′-tgacgagaccaagaactg-3′	5′-ccatccaaaccactgaaacc-3′	55	25	600
IGF-I	5′-atgctcttcagttcgtgtgt-3′	5′-agctgacttggcaggcttga-3′	65	35	185
IGF-II	5′-ctgtgctacccccgccaagt-3′	5′-acgtttggcctccctgaacg-3′	65	35	213
IGFBP-2	5′-cacgtggacagcaccatgaacatg-3′	5′-gtagaagagatgacactcggggtc-3′	65	35	446
IGFBP-3	5′-gctctgcgtcaacgctagtg-3′	5′-gcttcctgcctttggaaggg-3′	65	35	439
IGFBP-4	5′-ccatccaggaaagcctgca-3′	5′-tggaagttgccgttgcggt-3′	65	40	310
IGFBP-5	5′-ttgcctcaacgaaaagagc-3′	5′-agaatcctttgcggtcaca-3′	63	35	377
GAPDH	5′-gtgaaggtcggagtgaacggattt-3′	5′-cacagtcttctgggtggcagtgat-3′	65	35	555
GAPDH	5′-ggagtcaacggatttggt-3′	5′-gtgatgggatttccattgat-3′	63	35	209

Western Blot Analysis

Human osteoblasts, cultured in 6-wells dishes for 2 weeks in α-MEM supplemented with 10% FCS, 50 μg/ml ascorbic acid, and 10^{-8} M dexamethasone, were stimulated with increasing concentrations of LC and Iso-V-LC, ranging from 10^{-5} to 10^{-3} M, for 48 hours, to investigate the *BSP*, *OPN*, *COLL I* and IGFBP expression in osteoblast lysates. Osteoblasts cultured without the two compounds were utilized as controls. The cells were washed three times with PBS and solubilized with lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L ethylene diamine tetraacetic acid (EDTA), 1% NP40, and 1 mmol/L phenylmethyl sulfonyl fluoride]. Cell lysates were centrifuged at 14,000 rpm at 4°C for 10 minutes and pellets were discarded. Protein content was measured using Micro BCA protein kit (Pierce); 30 μg of cell proteins were subjected to SDS-PAGE gel and subsequently transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia, London, UK), as described elsewhere [24], probed with primary antibodies directed against: BSP and OPN (LF83 and LF 123, respectively, both kindly given by L.W. Fischer, NIDCR, Bethesda, MD, USA), COLL I (Chemicon International Inc., Temecule, California, USA), IGFBP-2-3-4-5 (Santa Cruz, California-USA), and β actin. After incubation with appropriate peroxidase-conjugated secondary antibodies, specific reactions were revealed with the ECL detection kit and visualized on Hyperfilm™ (Amersham Pharmacia, UK). Moreover, the IGFBP expression was also studied in ECM as described previously.

Osteocalcin Assay

Osteocalcin secretion was measured in cell culture supernatants collected from confluent osteoblasts plated in 24-well dishes and cultured with α-MEM, 10% FCS, 50 μg/mL ascorbic acid, 10^{-8} M dexamethasone, and 10^{-8} M vitamin D_3 for up to 14 days. During the last week of culture, the cells were cultured in the presence or in the absence of LC and Iso-V-LC at concentrations ranging from 10^{-3}M to 10^{-7} M, and the medium was changed every 3 days. Media were collected, centrifuged at 1300 rpm for 5 minutes, and tested by using a human osteocalcin enzyme-linked immunosorbent assay (ELISA) kit, according to manufacturer's instructions (DRG Diagnostics, Germany). Osteocalcin levels were expressed as nanograms per milligrams of cell protein and each treatment was performed in quadruplicate.

Mineralized Bone Nodule Formation

Human osteoblasts were seeded in 6-well plates and cultured in α-MEM supplemented with 10% FCS, 50 μg/mL, of ascorbic acid, 10mM of β-glycerophosphate, 10^{-8} M dexamethasone, and increasing concentrations of LC and Iso-V-LC, ranging from 10^{-7} to 10^{-3} M. Human osteoblasts cultured without the two compounds were utilized as control. The medium was changed every 3 days for 8 weeks and the mineralized matrix nodules were detected with von Kossa staining. The cells were fixed with 3% para-formaldehyde for 10 minutes, stained with silver nitrate ($AgNO_3$), rinsed with distilled water, exposed for 1 hour to bright light, and finally observed. The photomicrographs of mineral nodules formed in cultures were obtained using a Nikon Eclipse E400 microscope equipped with a Nikon plan fluor 10x/030 DICL. The microscope was connected to a Nikon digital camera (DXM 1200); the acquisition and the quantitative analysis of the mineralized surface were accomplished by using the software LUCIA G version 4.61 (BUILD 0.64) for Nikon Italy. The percentage of mineral nodules was represented by the histogram (Fig. 5).

RNA Isolation and Reverse-Transcriptase (RT)-PCR Amplification

The osteoblasts were subjected to mRNA extraction using spin columns (RNeasy, Qiagen, Hilden, Germany), according to the manufacturer's instructions, to detect the expression of IGFI-, -II and IGFBP-2,-3,-4, and -5. Before RNA extraction, osteoblasts were cultured in the presence of 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate, to induce the osteoblastic phenotype. Briefly for the first strand cDNA synthesis (Superscript First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbard, CA, USA), an RT mixture containing 1 μg total RNA, dNTPs, Oligo(dT), RT buffer, $MgCl_2$, DTT, RNaseOUT, Superscript II RT, and diethyl pyrocarbonate (DEPC)-treated water to a final volume 100 μL was prepared according to the manufacturer's instructions. Two microliters of diluted cDNA were transferred into a 50 μL PCR reaction mixture containing dNTPs, $MgCl_2$, primers, autoclaved distilled water, and platinum *Taq* DNA polymerase (Invitrogen).

Amplification reactions specific for the cDNAs of IGFI-, -II, IGFBP-2,-3,-4, and -5 and the housekeeping gene glyceraldehyde phosphate dehydrogenase (*GAPDH*) were carried out using *Taq*/polymerase (Invitrogen). The primers and RT-PCR conditions are reported in Table 1. PCR products were analyzed by 1.5% agarose gel electrophoresis containing 0.01% ethidium bromide, and the resulting bands were detected by a light sensitive CCD video system (BioDocAnalyze, Whatman Biometra, Germany).

Statistical Analyses

Overall group differences were assessed by using analysis of variance (ANOVA) Differences were considered significant for $P < 0.05$.

Results

We evaluated whether LC and Iso-V-LC could have an effect on osteolblast proliferation and differentiation. Therefore, we first evaluated proliferation every 24

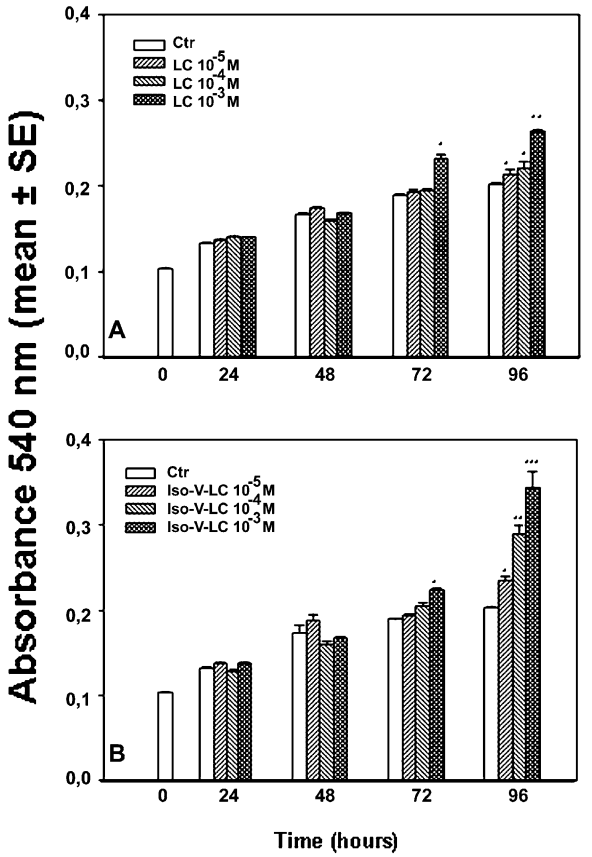

Fig. 1. *Proliferation of human osteoblasts*. Cell proliferation has been evaluated by the Titer-tek technique on human osteoblasts plated on 96-well microtiter plates in the presence of increasing concentrations of LC (**A**) and Iso-V-LC (**B**). The experiments have been performed in the presence of 10% fetal calf serum (FCS), and the cells cultured without the drugs represent the controls. The effect of the molecules was evaluated every 24 hours for 4 days. Results are expressed as absorbance read at 540 nm. Values represent the average ± standard error (SE) of each treatment performed in quadruplicate for three independent experiments. $*P < 0.03$, $**P < 0.004$, $***P < 0.0001$ vs. control.

hours for 4 days in the presence of increasing concentrations of the two carnitine derivatives with respect to untreated cultures as controls. As shown in Figure 1, no effect was found after 24 and 48 hours, whereas the higher concentration (10^{-3} M) of both LC and Iso-V-LC positively affected osteoblast proliferation after 72 hours of treatment. The increase in cell growth was persistent and dose-dependent after 96 hours; however, all concentrations of Iso-V-LC tested were more effective than LC in inducing osteoblast proliferation (Figs. 1A, B). We next examined whether the increased proliferation could be associated with a dedifferentiation state of the osteoblast toward the fibroblast-like phenotype. To that end the well-established osteoblastic parameters such as *ALP*, *COLLI*, *BSE*, and *OPN* expression; osteocalcin secretion; and mineralized nodule formation were examined. Although the carnitine derivatives increased the total cell number, the percentage of ALP-positive

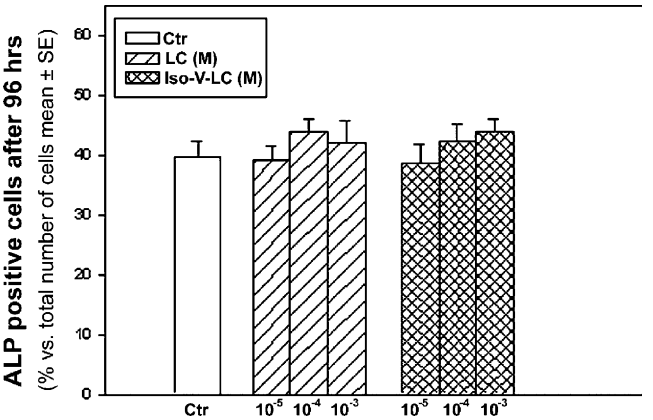

Fig. 2. *Alkaline phosphatase (ALP) activity*. ALP evaluated by histochemical methods on human osteoblasts cultured in α-MEM supplemented by 10% of FCS, in the presence or absence of LC and Iso-V-LC ranging in concentration from 10^{-5} to 10^{-3} M. Results are expressed as percentage of ALP-positive cells treated with the two molecules versus the total number of cells present in the well. Values represent the average ± SE of each treatment performed in quadruplicate for three independent experiments.

cells, evaluated histochemically every 24 hours for 4 days, was not changed in all the conditions tested with respect to the controls. Figure 2 shows the results obtained after 96 hours of treatment. This result was further confirmed by ALP biochemical assay (not shown). Moreover, to evaluate the synthesis of newly formed procollagen, osteoblasts were treated with LC and Iso-V-LC for 48 hours and labeled with ^{3}[H]-proline for 2 hours. In this condition, we showed an increase in ^{3}[H]-proline incorporation, and the maximum effect was reached by LC at 10^{-3} M and by Iso-V-LC at 10^{-5} M (Fig. 3A). We also demonstrated a dose-dependent increase of COLL type I at mRNA and protein levels in osteoblasts stimulated with the two compounds for 48 hours (Fig. 3B). In addition, by using Western blot analysis, we demonstrated the increase of *BSP* and *OPN* expression after 48 hours of treatment with LC and Iso-V-LC (Fig. 4). In contrast, by ELISA , we did not find any effect on osteocalcin secretion in the media collected from osteoblasts cultured for 48 hours in the presence of both carnitine derivatives (not shown). Furthermore, by quantitative analysis of Von Kossa staining we demonstrated that LC and, more efficiently, Iso-V-LC, positively affected the mineralized nodule formation, and the effect of the agents on the mineralization was already evident at the lowest concentrations tested.

Finally, we investigated if the biologic effects induced by LC and Iso-V-LC on osteoblast proliferation and differentiation could involve IGF-mediated pathway. By using RT-PCR we showed that the IGF-I and -II mRNA levels in osteoblasts stimulated that were with different concentrations of carnitine derivatives from 2 to 48 hours were not affected by the treatment (Fig. 6). In the same experimental conditions, we evaluated the

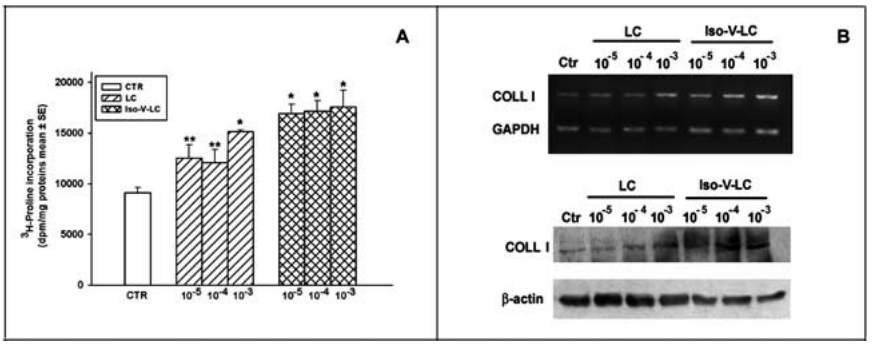

Fig. 3. *[^{3}H]-Proline incorporation and collagen expression, by human osteoblasts.* The effect of increasing concentrations of LC and Iso-V-LC on the uptake of [^{3}H]-proline in human osteoblasts measured by counting the released radioactivity with a β-scintillation counter. The cells cultured without the two molecules represent the controls. Results represent the average ± SE of three independent experiments $*P < 0.001$, $**P < 0.02$ vs. control (**A**). mRNA and protein levels analyzed by RT-PCR and Western blot, respectively for collagen type I (COLLI) in human osteoblasts treated for 48 hours with the indicated concentrations of LC and Iso-V-LC (**B**). The figure shows one of three independent experiments performed.

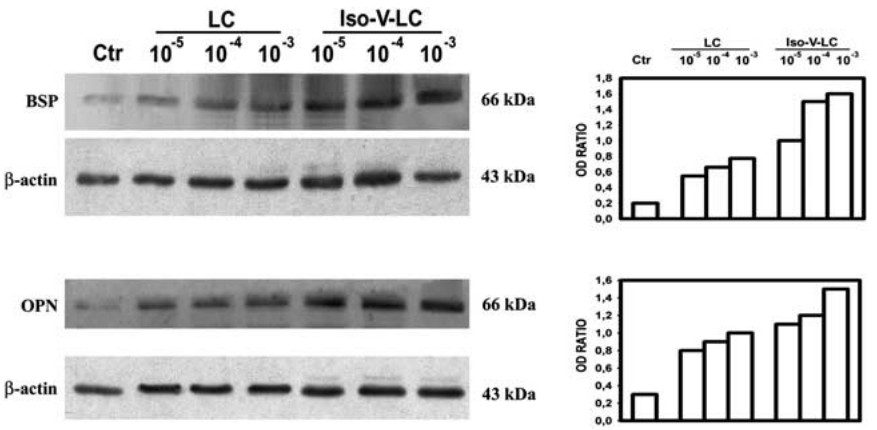

Fig. 4. *OPN and BSP expression by human osteoblasts.* Confluent human osteoblasts were cultured in the absence (ctr, control), or in the presence of different concentrations of LC and Iso-V-LC for 48 hours. For each sample 30-μg proteins were electrophoresed in a 10% SDS-PAGE, blotted to nitrocellulose filter paper, probed with LF 83 (anti-BSP Ab) and LF 123 (anti-OPN Ab). The intensity of the bands was quantified by densitometry (histograms) and normalized to β-actin. The figure shows one of three independent experiments performed.

mRNA levels of IGFBPs, demonstrating that LC and Iso-V-LC induced an increase of IGFBP-3 starting from 6 up to 48 hours, and a decrease of IGFBP-5 starting from 2 hours of treatment (Fig. 6). We also demonstrated that LC and Iso-V-LC induced the enhancement of IGFBP-3 and the reduction of IGFBP-5 expression at protein levels in the ECM secreted by the osteoblasts after 24 and 48 hours of treatment. As shown in the Figure 7, these effects were even more evident after 48 hours of treatment, and were exerted more efficiently by Iso-V-LC than by LC at all the concentration tested. In contrast, the expression of both IGFBP-2 and -4 were not affected by the treatment at both mRNA and protein levels (data not shown). Moreover, the expression of IGFBPs in cell lysates, as we identified by Western blot analysis, had similar patterns to those secreted by the osteoblasts (data not shown).

Discussion

In the present study we showed that two carnitine derivatives, LC and Iso-V-LC, promoted human osteoblast proliferation and differentiation *in vitro* and that Iso-V-LC exerted its activity at lower concentration than those of LC.

It is well known that carnitine facilitates energy availability and is important for tissues with high energy requirements. Carnitine deficiency that occurs with age affects some tissues, such as cardiac and skeletal muscles [6, 7], where energy is used for mechanical work; in fact, carnitine is already utilized as a dietary supplement to increase the performance of these tissues. The deficiency of carnitine and the associated loss of energy could also compromise osteoblast activity and bone matrix synthesis in an age-related manner, thereby leading to skeletal disease such as osteoporosis. However, its effects on bone tissues are at present poorly investigated. Therefore, we investigated whether carnitine treatment could be successfully utilized to improve human osteoblast activity. To this end, we tested the effects of the two carnitine derivatives, LC and Iso-V-LC, on several osteoblast parameters such as proliferation, protein matrix secretion, mineralized nodule formation, and IGF/IGFBP expression by using an *in vitro* model consisting of human osteoblasts obtained from trabecular bone specimens.

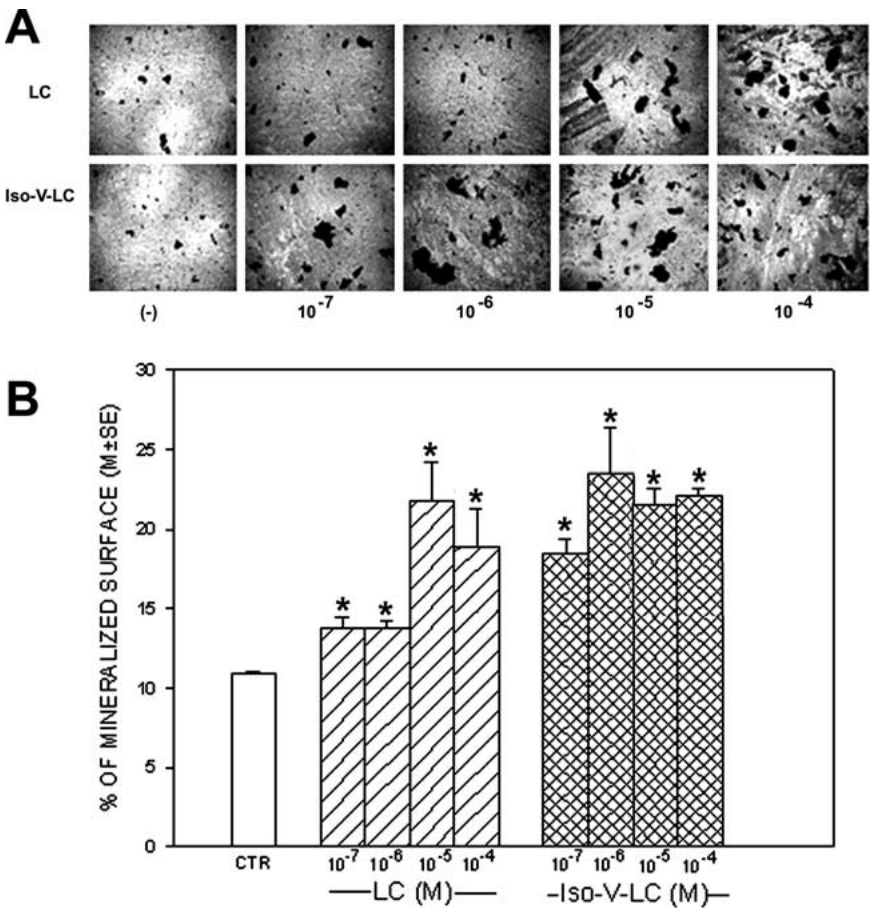

Fig. 5. *Van Kossa staining for mineralized nodule formation by human osteoblasts.* Confluent human osteoblasts, cultured with ascorbic acid and β-glycerolphosphate, were treated or not with increasing concentrations of LC and Iso-V-LC for 8 weeks. The formation of mineralized nodules was detected on fixed cells by von Kossa staining. Photomicrographs were obtained by a digital Nikon camera connected to the microscope (**A**). Quantitative analysis of mineralized nodules was performed by scanning the surface of mineralization areas on a computer-assisted image analyzer. Values are expressed as percentage of mineralized surface versus the total area (**B**). Results represent the average ± SE of three independent experiments $*P < 0.01$ vs. control.

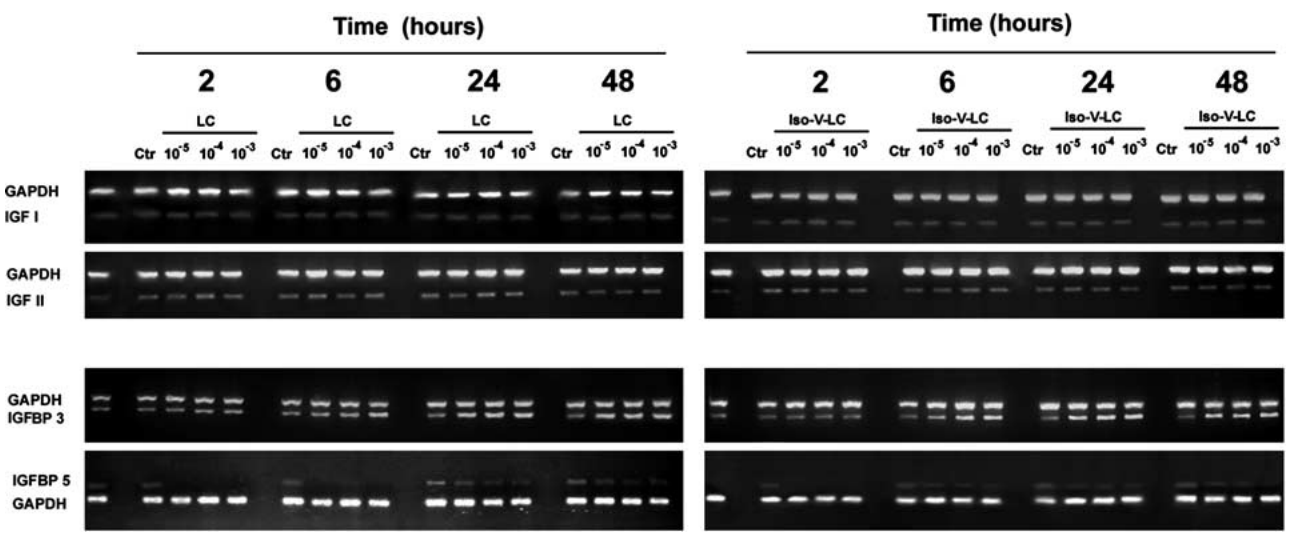

Fig. 6. *mRNA levels of IGFs and IGFBPs in human osteoblasts.* Time course of the mRNA levels analyzed by RT-PCR for IGF-I and -II, and IGFBP-3 and -5 in human osteoblasts treated with increasing concentrations of LC and Iso-V-LC. Both compounds induced an increase of IGFBP-3 expression starting from 6 hours, and a decrease of IGFBP-5 expression starting from 2 hours of treatment. The figure shows one of three independent experiments performed.

The results of this study provide evidence that both molecules were active in osteoblast proliferation; however, their maximum effect occured at different concentrations. The efficiency of Iso-V-LC, never tested before on osteoblastic cells, was demonstrated in that the highest activity of this drug on osteoblastic proliferation was displayed at a concentration 10-fold less than that of LC. The effects on cell growth were not dependent

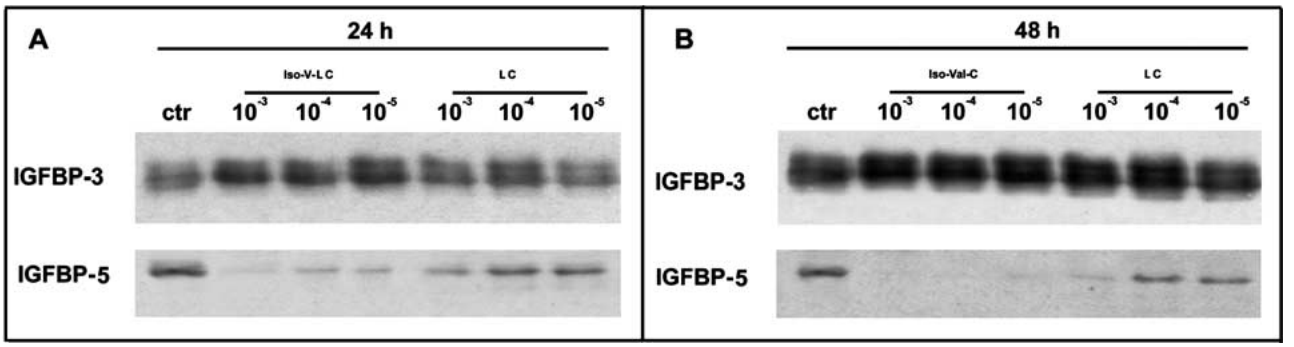

Fig. 7. *IGFBP-3 and IGFBP-5 accumulation in the extracellular matrix of human osteoblasts.* Confluent osteoblasts cultured with ascorbic acid and β-glycerolphosphate were treated or not with LC and Iso-V-LC at the indicated concentrations for 24 and 48 hours. The extracellular matrix (ECM) was extracted as described in the Materials and Methods section. ECM samples were separated on 10% SDS-PAGE and the proteins were transferred onto a nitrocellulose membrane. IGFBP-3 and IGFBP-5 were detected using, respectively, anti-IGFBP-3 and anti-IGFBP-5 antibodies and a chemiluminescence detection system.

upon the osteoblast dedifferentiation toward the fibroblast like phenotype; in fact, the percentage of ALP-positive cells and the secretion of osteocalcin were not changed by the treatment with respect to the controls. These findings indicate that in our system a constant percentage of cells continuously differentiate toward the osteoblastic phenotype, as occurs in physiologic conditions.

Moreover, matrix protein production was positively modulated by both compounds, as demonstrated by the increase of [^{3}H]-proline incorporation, an indicator of newly formed procollagen [25], as well as by the upregulation of COLLI and the two bone sialoproteins; BSP and OPN. Again Iso-V-LC demonstrated greater efficiency in terms of dose on the expression of the above-mentioned proteins.

We also showed that long exposure of osteoblasts to both compounds (8 weeks) induced an increased formation of mineralized nodules relative to controls with a different scenario in terms of active concentrations. The lowest concentration of Iso-V-LC was sufficient to induce a significant increase in mineralization rate, and its maximum effect was reached at a concentration 10-fold less than that of LC. With respect to the effects of LC, our findings are consistent with those in the literature, which show that this compound stimulates the metabolic activity of porcine bone marrow osteoblasts [8]. However, herein we demonstrated for the first time the positive effects of the new carnitine derivative, Iso-V-LC, on human osteoblast activity.

Results of several *in vivo* and *in vitro* studies suggest that proliferating and differentiating factors that affect osteoblast activity, exert their effects through the involvement of IGF-I and -II. However, although IGF-I and -II mRNA levels were not affected by both compounds in our system, we cannot exclude their involvement in LC- and Iso-V-LC-mediated effects. It has been shown that the IGF bioavailability in bone is determined not only by IGF bone cell expression but also by the actions of IGFBPs. Therefore, the increased IGFBP-3 expression, induced by both carnitine derivatives, demonstrated in the present work could correlate with an enhanced IGF-I activity in inducing osteoblast proliferation, in agreement with literature data obtained in rat osteoblasts [16]. Moreover, our findings that demonstrated the decreased expression of IGFBP-5 known to inhibit IGF-stimulated bone cell growth [19, 21], could also contribute to the enhanced osteoblast proliferation we found. Consequently, we postulate that the increase in levels of IGFBP-3 and the reduction in levels of IGFBP-5 induced by LC and Iso-V-LC could be in turn responsible for the proliferative and differentiative effects (by increasing the availability of IGF-I and II in our culture system) on human osteoblasts.

In conclusion, our data demonstrated that both carnitine derivatives, LC and Iso-V-LC, are able to positively affect osteoblast proliferation and differentiation *in vitro*; secondly, our results marked the difference between the two drugs in terms of active dose on osteoblast activity. Exposure for 96 hours of osteoblasts to higher concentrations of Iso-V-LC significantly increased their proliferation, whereas long exposures (8 weeks) to low concentrations of this drug efficiently activated the mineralization process.

These findings could indicate that these compounds, although not tissue specific, are useful as dietary supplementation for stimulating bone formation in elderly persons.

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