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REGULAR ARTICLE

Evaluating effects of L-carnitine on human bone-marrow-derived mesenchymal stem cells

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Abstract Mesenchymal stem cells (MSCs) are multipotent cells showing potential for use in regenerative medicine. Culture techniques that are more stable and methods for the more efficient production of MSCs with therapeutic efficacy are needed. We evaluate the effects of growing bone marrow (Bm)-derived MSCs in the presence of L-carnitine, which is believed to promote lipid metabolism and to suppress apoptosis. The presence of L-carnitine decreased the degree of drug-induced apoptosis and suppressed adipogenic differentiation. Metabolomic analysis by means of the exhaustive investigation of metabolic products showed that, in addition to increased β-oxidation and the expression of all carnitine derivatives other than deoxycarnitine (an intermediate in carnitine synthesis), polysaturated and polyunsaturated acids were down-regulated. An integrated analysis incorporating both serial analysis of gene expression and metabolomics revealed increases in cell survival, suggesting the utility of carnitine. The addition of carnitine elevated the oxygen consumption rate by BmMSCs that had been cultured for only a few generations and those that had become senescent following repeated replication indicating that mitochondrial activation occurred. Our exhaustive analysis of the effects of various carnitine metabolites thus suggests that the addition of L-carnitine to BmMSCs during expansion enables efficient cell production.

Keywords Metabolomics · Microarray · Fatty acid · Mitochondria · Apoptosis

Abbreviations

ADSCs	Adipose-derived stem cells
BmMSC	Bone-marrow-derived mesenchymal stem cell
MSCs	Mesenchymal stem cells
SAGE	Serial analysis of gene expression
SREBF	Sterol regulatory element binding transcription factor
VDAC	Voltage-dependent anion channel

Introduction

L-carnitine is a compound with a molecular weight of 161.21 and is known to influence lipid metabolism. It is synthesized in the liver from lysine and methionine through a process that also requires vitamin C, iron, niacin and other molecules. It is also absorbed in large quantities from the diet and accumulates mainly in the muscles. Carnitine is administered to treat congenital metabolic abnormalities, such as carnitine transporter defects and acquired carnitine deficiency caused by hemodialysis and other factors. Carnitine is widely taken as a supplement, even in the absence of any deficiency, although the value of its overconsumption is debatable.

Carnitine is taken up into cells via the carnitine transporter SLC22A5 in the cell membrane, after which it acts as a necessary component for the passage of long-chain fatty acids through the mitochondrial membrane (Grube et al. 2005).

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The inner mitochondrial membrane does not permit the direct passage of acyl-CoA and carnitine therefore acts as a fatty acid acyl transporter. Fatty acid acyl-CoA binds temporarily to carnitine, forming fatty acid acylcarnitine. This reaction is catalyzed by carnitine acyltransferase I, which is embedded in the outer mitochondrial membrane. Fatty acid acylcarnitine is passed through the inner membrane by the acyl-carnitine/carnitine transporter and is transferred into the matrix. The fatty acid acyl group is then transferred from carnitine to coenzyme A inside the mitochondrion in a reaction catalyzed by the enzyme carnitine acyltransferase II, which is localized on the inner surface, thus regenerating fatty acid acyl-CoA. Free carnitine is then transferred back to the intermembrane space by the acyl-carnitine/carnitine transporter. This pathway for the transport of fatty acid acyl-CoA is known as the "carnitine shuttle." Fatty acid acyl-CoA that enters the mitochondria is known to undergo oxidation by enzymes in the matrix.

Carnitine has previously been shown to be useful for treating fatty liver because it promotes fatty acid β-oxidation (Ishikawa et al. 2014; Malaguarnera et al. 2010). It is also thought to be a valuable antioxidant and can inhibit oxidative stress associated with cardiomyopathy, the mitochondria membrane-permeability transition and pro-apoptotic proteins (Chao et al. 2011).

Mesenchymal stem cells (MSCs) can differentiate into fat cells, myocytes, chondrocytes and other types of mesenchymal cells and show great potential for use in regenerative medicine. Cultured human MSCs (hMSCs) exhibit multipotentiality and are used in a variety of treatments for their capacity to suppress inflammation, among other abilities (Huang et al. 2016). In the liver, for example, hMSCs are effective in treating hepatic cirrhosis and the ability of MSCs to ameliorate thioacetamide-induced oxidative stress in hepatocytes has been demonstrated, both in vivo and in vitro (Quintanilha et al. 2014). The quality of cultured MSCs, however, varies according to their culture conditions which is believed to affect their therapeutic efficacy. The oxygen concentration in bone marrow is approximately 2–5 % and culturing bone-marrow-derived MSCs (BmMSCs) in 20 % oxygen subjects them to stress, which may decrease their ability to maintain genetic stability. When passaged repeatedly in culture, they also undergo senescence, leading to increasing numbers of aneuploid cells and declining stemness maintenance (Estrada et al. 2012, 2013). Little is known, however, about the mechanisms whereby these issues arise and basic studies to investigate matters such as culture conditions and drug treatment are required to enable the efficient culture of MSCs in such a way as to ensure their therapeutic efficacy.

The results of previous studies of carnitine and MSCs have shown that carnitine suppresses transforming growth factor-β (TGF-β)-induced cell senescence and that carnitine and acetylcarnitine modulate mesenchymal differentiation in adipose-derived stem cells (ADSCs; Wu et al. 2014). Changes in the expression of lipid-associated genes are also believed to inhibit adipogenic differentiation. A previous study addressed the modulation of mesenchymal differentiation or lipid-associated gene expression in BmMSCs (Menssen et al. 2011) but much remains unknown about the mechanism of action of carnitine on BmMSCs. Furthermore, no study has as yet used an exhaustive analysis of metabolites to investigate the effect of carnitine administration on MSCs. In this study, we used metabolomics and serial analysis of gene expression (SAGE) to investigate the effect of the addition of carnitine to BmMSCs. Our findings should facilitate applications in regenerative medicine in future studies.

Materials and methods

Cells and cell culture

The studied BmMSCs were bone marrow cells purchased from Allcells and were continuously cultured and used at passage 4 and passage 16. Flow cytometry results confirmed that the BmMSCs were CD45(−), CD11b(−), CD90(+), CD73(+) and CD105(+). Mononuclear cells derived from bone marrow were also purchased (Lonza, Basel, Switzerland). Cells were resuspended in Dulbecco's modified Eagle medium, low glucose (Gibco, Gaithersburg, Md., USA), with 10 % fetal bovine serum. MSCs were cultured in humidified incubators with 5 % CO_2 and initially allowed to adhere for 72 h, followed by media changes every 3 to 4 days. When cultures reached approximately 90 % confluence, adherent cells were detached and replated (passaged).

Proliferation assay

Aliquots of 3000 BmMSCs were seeded in individual wells of 96-well plates, after which L-carnitine was added at various concentrations. Proliferation was measured by using an IncuCyte HD imaging system (Essen BioScience). L-carnitine was purchased from Wako (Osaka, Japan).

Apoptosis assay

Aliquots of 3×10^3 cells were seeded in 96-well plates, L-carnitine was added and the cells were cultured for 5 days. Doxorubicin was then added and a CyQuant assay was performed to measure the number of cells 24 h later. A Caspase 3 Assay Kit (Promega) was then employed to measure caspase 3 activity and the activity per cell was calculated. The experiments were performed in triplicate ($n = 6$).

Adipogenic differentiation assay

The Human Mesenchymal Stem Cell Functional Identification Kit was purchased from R&D Systems. Cell differentiation was performed following the manufacturer's instructions. Briefly, cells were seeded at a density of 2×10^4 cells/cm^2. When the cells were 100 % confluent, the media in each well were replaced with 0.5 ml adipogenic differentiation medium to induce adipogenesis. The media were replaced with fresh adipogenic differentiation medium every 3–4 days. After 5–7 days, we confirmed that lipid vacuoles had started to appear in the induced cells. After 14 days, the cells were stained with Oil Red O. Total cell number and adipogenic cell number were counted in a ×20 field for each carnitine concentration ($n = 6$).

Western blot analysis

Protein lysates were obtained by homogenizing tissues or cell pellets in sample buffer containing 62.5 mM TRIS–HCl (pH 6.8), 4 % SDS, 200 mM dithiothreitol, 10 % glycerol and 0.001 % bromophenol blue at a ratio of 1:10 (w/v), followed by boiling. Western blot analysis was performed with purified polyclonal anti-human rabbit IgG. Antibodies against beta-actin were purchased from Sigma.

Metabolome analysis

Metabolomic and statistical analyses were conducted at Metabolon as described previously (http://www.ncbi.nlm.nih.gov/pubmed/?term=24816252>). Briefly, cell pellets were subjected to methanol extraction and then split into aliquots for analysis by ultrahigh performance liquid chromatography/mass spectrometry (UHPLC/MS) in the positive, negative, or polar ion mode and by gas chromatography/mass spectrometry (GC/MS). Metabolites were identified by automated comparison of ion features to a reference library of chemical standards followed by visual inspection for quality control. For statistical analyses and data display, any missing values were assumed to be below the limits of detection; these values were imputed with the compound minimum (minimum value imputation). To determine statistical significance, Welsh's two-factor t-tests were performed in ArrayStudio (Omicsoft) or "R" to compare protein-normalized data between experimental groups; $P < 0.05$ was considered significant.

Total RNA isolation

Total RNA was isolated from the cerebellum of each individual animal by using TRIzol Reagent (Life Technology) and purified by using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. RNA samples were quantified by an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del., USA) and the quality was confirmed with the Experion System (Bio-Rad Laboratories, Hercules, Calif., USA).

SAGE protocol

The Ion Ampliseq Transcriptome Human Gene Expression Kit (Life Technologies) was used for library creation. An Ion Proton next-generation sequencer library of analysis beads was created and an Ion PI IC 200 Kit (Life Technologies) and an Ion PI Chip Kit v2 BC were used for sequencing by using an Ion Proton next-generation sequencer. The results of metabolomic analysis and SAGE were integrated by Ingenuity Pathways Analysis (IPA).

Measurements of oxygen consumption rate

Oxygen consumption rate (OCR) measurements were performed by using a Seahorse Biosciences XF96 Extracellular Flux Analyzer. Cells were seeded at 6000 cells/well in XF96 microplates (Seahorse Biosciences). After a 24-h incubation, the growth media were exchanged for XF Assay Medium (Seahorse Biosciences) supplemented with 25 mM glucose (Sigma-Aldrich). OCR measurements were made over 5-min periods following a 3-min mix period. Cells were treated with a sequential addition of 1 μg/ml oligomycin (Sigma-Aldrich), 300 nM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; Sigma-Aldrich) and 2 μM rotenone (MP Biomedicals). The spare respiratory capacity and coupling efficiency were calculated according to Seahorse Bioscience instructions and the basal OCR was normalized to the cell number.

Statistical analysis

The results were analyzed by either the Student's *t*-test or Welsh's two-factor t-tests and the data are presented as the means ± standard deviation, with significance levels being established at $P < 0.05$.

Results

Effects of carnitine on proliferation and apoptosis

To investigate the effect of carnitine on proliferation, we added it to BmMSC cultures at a concentration of 0 mM, 1 mM, 3 mM, or 10 mM. Proliferation was not significantly affected by the presence of L-carnitine at concentrations from 1 to 10 mM (Fig. 1a). We then evaluated the effect of carnitine on suppressing apoptosis. BmMSCs were treated with doxorubicin, which efficiently induces apoptosis and caspase-3

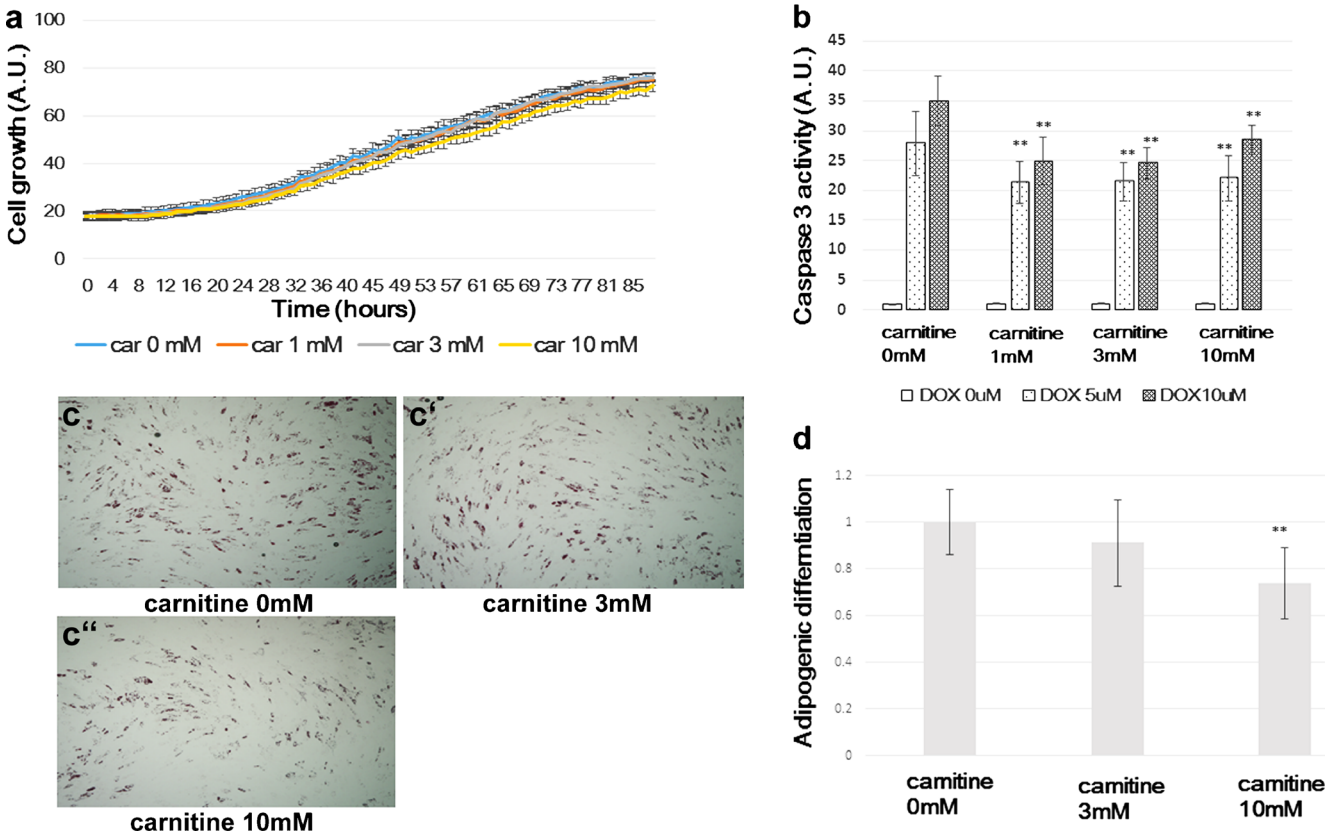

Fig. 1 Evaluation of effects of L-carnitine on proliferation, apoptosis and differentiation of BmMSCs. **a** Measurement of effect of L-carnitine on proliferation by using the IncuCyte system. Data are presented as averages ± standard error (*A.U.* arbitrary units). **b** Effect of L-carnitine in suppressing doxorubicin-induced apoptosis. L-carnitine was added 2 days before addition of doxorubicin and its effects were evaluated by measuring caspase-3/7 activities at 24 h after doxorubicin was added (*DOX* doxorubicin). **c–c"** Evaluation of adipogenic differentiation by Oil Red O staining. **d** Graphical representation of the data from **c** for adipogenic differentiation

activity was measured after 24 h. When doxorubicin (10 μM) was added to BmMSCs that were also exposed to carnitine (1 mM, 3 mM, or 10 mM), caspase-3 activity decreased, confirming the action of carnitine in suppressing apoptosis in BmMSCs (Fig. 1b). Next, we evaluated the effect of long-term carnitine administration on cell growth and apoptosis. BmMSCs were cultured with carnitine from mononuclear cells to passage 1. No positive effects of long-term carnitine administration were seen on cell proliferation (Supplemental Fig. 1) and anti-apoptosis (Supplemental Fig. 2).

Effect of carnitine on differentiation potential

Previous data have shown that the addition of carnitine to ADSCs inhibits adipogenic differentiation. As the lipid metabolism of ADSCs may differ from that of BmMSCs, however, we evaluated the effect of carnitine treatment on the differentiation potential of BmMSCs. Adipogenic differentiation decreased by 25 % after the addition of carnitine at a concentration of 10 mM (Fig. 1c–c"). No noticeable change in osteogenic differentiation was observed after the addition of carnitine (data not shown).

Metabolomics

We then used capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) analysis to investigate metabolic changes attributable to the addition of carnitine. Principal component analysis (PCA) revealed a clear distinction between the carnitine-treated cells and control cells (Fig. 2a). Hierarchical clustering showed that the abundance ratios of metabolites in carnitine-treated and control cells were reversed (Fig. 2b).

Effect on acylcarnitines

Next, we investigated the production of carnitine metabolites. Carnitine was 38.3 times more abundant in carnitine-treated cells than in control cells and significant increases in numerous different acylcarnitines were observed (Table 1). Particularly large changes were seen for the acylcarnitines decanoylcarnitine (ratio = 183.2), laurylcarnitine (ratio = 155.7) and myristoleoylcarnitine (ratio = 128.4), as shown in Table 1. The only carnitine metabolite that decreased was deoxycarnitine (ratio = 0.36).

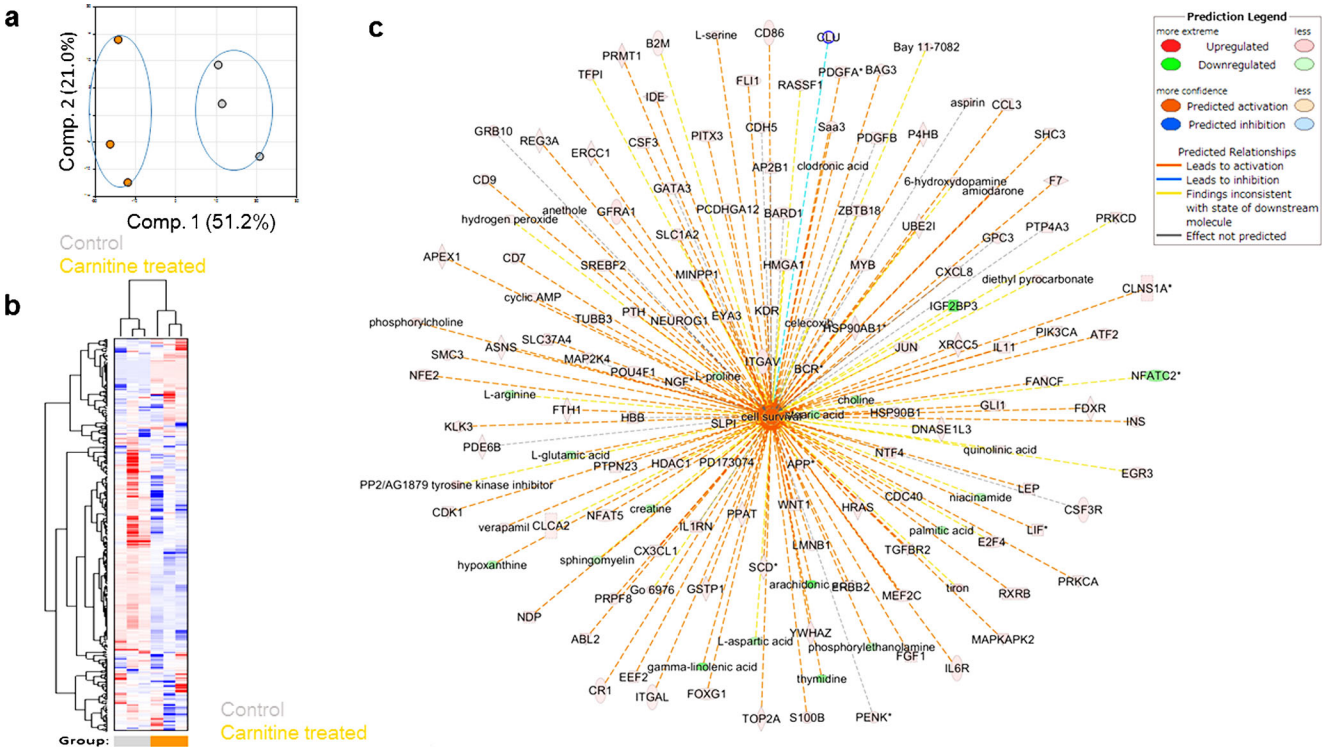

Fig. 2 Metabolomics and serial analysis of gene expression (SAGE) analysis. **a** Principal component analysis (PCA)-normalized metabolic data obtained from BmMSCs and control cells at 48 h after the addition of 10 mM L-carnitine. Percentage values indicated on the axes represent the contribution rate of the first (*Comp. 1*) and second (*Comp. 2*) principal components to the total amount of variation. **b** Heat map of the hierarchical cluster analysis (*columns* BmMSCs with L-carnitine added and control cells, *rows* normalized levels of each metabolite). The dendrogram for each heatmap shows the relatedness of the normalized metabolite level patterns. **c** Enhancement of cell survival. Functional annotation identified by an integrated Ingenuity Pathways Analysis (IPA) of the results of metabolomics and gene expression profiles for genes that had read counts of 50 or more and that either increased significantly to at least 1.5 times greater or decreased to less than 0.75 times lower. *Colors* are used to indicate both the direction (*red* up-regulation, *green* downregulation) and the magnitude (*color intensity*) of the expression changes of the target metabolites

Effect on fatty acids

While investigating changes in long-chain fatty acids, we found a significant decrease in the content of fatty acids containing 15–18 carbon atoms, including pentadecanoate (ratio = 0.76), palmitate (ratio = 0.65), margarate (ratio = 0.54) and stearate (ratio = 0.64). Fatty acids containing 20 or more carbon atoms, such as arachidate (20:0; ratio = 0.86) and eicosenoate (20:1; ratio = 0.62) also decreased but not to a statistically significant extent (Table 2). The production of many polyunsaturated fatty acids, including docosapentaenoate (22:5n3; ratio = 0.51), eicosapentaenoate (20:5n3; ratio = 0.44) and arachidonate (20:4n6; ratio = 0.45), significantly decreased in carnitine-treated cells (Table 3).

Effects on energy metabolism

Decreases were evident in the production of metabolites of the glycolysis, gluconeogenesis and pyruvate metabolism pathways, including pyruvate (ratio = 0.78), lactate (ratio = 0.83) and glycerate (ratio = 0.68), with the only metabolite to increase being 2,3-diphosphoglycerate (ratio = 1.74). In the

Table 1 Changes in carnitine metabolites resulting from addition of L-carnitine. Metabolites that were significantly up-regulated are shown in *italic* ($P < 0.05$) and those that were significantly down-regulated are shown in **bold** ($P < 0.05$). The ratio represents the induction of metabolites by carnitine (LC treated/non-treated). Data were analyzed from three carnitine-treated mesenchymal stem cells (MSC) groups vs three control MSC groups ($n = 3$)

Biochemical name of metabolite	Ratio	*P*-value
Carnitine	*38.32*	0.0003
Acetylcarnitine	*31.29*	1.04E-06
Hydroxybutyrylcarnitine	*35.28*	1.49E-05
Valerylcarnitine	*38.24*	6.72E-06
Hexanoylcarnitine	*40.72*	1.84E-05
Octanoylcarnitine	*68.08*	7.14E-05
Decanoylcarnitine	*183.02*	0.0005
Cis-4-decenoyl carnitine	*168.98*	0.0003
Laurylcarnitine	*155.07*	0.0009
Myristoylcarnitine	*20.63*	0.0006
Palmitoylcarnitine	*7.14*	0.0138
Stearoylcarnitine	*6.42*	0.0231
Linoleoylcarnitine	*18.78*	0.001
Oleoylcarnitine	*12.9*	0.0049
Myristoleoylcarnitine	*128.46*	0.0014
Deoxycarnitine	**0.36**	0.0006

Table 2 Changes in long-chain fatty acids resulting from addition of L-carnitine (*bold* significant decrease)

Biochemical name of fatty acid	Ratio	*P*-value
Pentadecanoate (15:0)	**0.76**	0.002
Palmitate (16:0)	**0.65**	0.002
Palmitoleate (16:1n7)	**0.59**	0.026
Margarate (17:0)	**0.54**	0.030
10-Heptadecenoate (17:1n7)	**0.58**	0.034
Stearate (18:0)	**0.64**	0.004
Nonadecanoate (19:0)	**0.52**	0.024
10-Nonadecenoate (19:1n9)	0.58	0.057
Arachidate (20:0)	0.86	0.342
Eicosenoate (20:1)	0.62	0.065
Erucate (22:1n9)	0.87	0.411
Oleate/vaccenate (18:1)	**0.59**	0.018

pentose phosphate pathway, significant decreases were evident for 5-phosphoribosyl diphosphate (ratio = 0.45) and ribulose/xylulose 5-phosphate (ratio = 0.19). In the tricarboxylic acid cycle, the production levels of alpha-ketoglutarate (ratio = 0.80), succinate (ratio = 0.84) and fumarate (ratio = 0.63) all decreased significantly (Table 4).

Functional annotation analysis integrating SAGE and metabolomics

We used SAGE to analyze changes in gene expression following the addition of carnitine. The gene *sod2* was significantly up-regulated (as previously reported) and the expression of *srebf1* and *srebf2* also increased, as did that of *vdac*, which forms small pores in the outer mitochondrial membrane

Table 3 Changes in polyunsaturated fatty acids (n3 and n6) resulting from addition of L-carnitine (*bold* significant decrease)

Biochemical name of fatty acid	Ratio	*P*-value
Stearidonate (18:4n3)	0.88	0.459
Eicosapentaenoate (EPA; 20:5n3)	**0.44**	0.022
Docosapentaenoate (n3 DPA; 22:5n3)	**0.51**	0.037
Docosahexaenoate (DHA; 22:6n3)	0.77	0.125
Docosatrienoate (22:3n3)	**0.55**	0.049
Linoleate (18:2n6)	**0.6**	0.034
Linolenate [alpha or gamma; (18:3n3 or 6)]	**0.49**	0.021
Dihomo-linolenate (20:3n3 or n6)	**0.41**	0.009
Arachidonate (20:4n6)	**0.45**	0.030
Adrenate (22:4n6)	0.44	0.081
docosapentaenoate (n6 DPA; 22:5n6)	**0.59**	0.027
Docosadienoate (22:2n6)	**0.53**	0.043
Dihomo-linoleate (20:2n6)	**0.51**	0.040
Mead acid (20:3n9)	0.59	0.078

Table 4 Effect of L-carnitine addition on energy metabolism (*italic* significant increase, *bold* significant decrease)

Pathway	Biochemical name of metabolite	Ratio	*P*-value
Glycolysis, gluconeogenesis and pyruvate metabolism	Glucose	1.17	0.495
	Glucose 6-phosphate	1.33	0.871
	Fructose-6-phosphate	0.78	0.416
	Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate, myo-inositol 1,4 or 1,3-diphosphate	0.2	0.056
	2,3-Diphosphoglycerate	*1.74*	0.009
	Dihydroxyacetone phosphate (DHAP)	**0.53**	0.020
	3-Phosphoglycerate	0.77	0.184
	Phosphoenolpyruvate (PEP)	0.78	0.390
	Pyruvate	**0.78**	0.025
	Lactate	**0.83**	0.080
	Glycerate	**0.68**	0.022
Pentose phosphate pathway	6-Phosphogluconate	0.75	0.448
	Ribose 1-phosphate	0.85	0.431
	5-Phosphoribosyl diphosphate (PRPP)	**0.45**	0.002
	Sedoheptulose-7-phosphate	0.89	0.461
	Ribulose/xylulose 5-phosphate	**0.19**	0.010
	Arabonate/xylonate	0.89	0.588

(Table 5). We performed an integrated analysis of the results of metabolome analysis and of those genes with read counts ≥ 50 that either showed ≥ 1.5 times greater expression or ≤0.75 times the expression level in control cells. Functional annotation revealed enhanced cell survival, cell viability and migration of cells and reduced organismal death in carnitine-treated BmMSCs (Table 6). Next, we focused on the enhancement of cell survival. Up-regulation of the *myb*, *erbb2* and leukemia inhibitory factor (*lif*) genes and others contributed to enhanced cell survival (Fig. 2c).

Effect of carnitine on OCR

Acetylcarnitine has previously been reported to restore mitochondrial functions in senescent cells. We therefore evaluated the OCR in non-senescent passage 4 BmMSCs (MSC-P4) and

Table 5 Effect of carnitine on gene expression determined by serial analysis of gene expression (SAGE)

Gene	Ratio (control/carnitine)	*P*-value
SOD1	1.16	0.105
SOD2	1.21	0.015
SOD3	1.16	0.458
VDAC1	1.20	0.035
SREBF1	1.26	0.025
SREBF2	1.50	0.018

Table 6 Disease or functional annotations exhibiting a significant change resulting from addition of L-carnitine

Categories	Disease or function annotation	*P*-value	Predicted activation state	Activation z-score
Organismal development	Size of body	5.08E-09	Increased	7.490
Cell death and survival	Cell viability	6.00E-12	Increased	6.806
Cell death and survival	Cell survival	3.65E-12	Increased	6.775
Cellular movement	Cell movement	3.87E-21	Increased	6.245
Cellular growth and proliferation, tissue development	Generation of cells	7.37E-19	Increased	6.181
Cellular movement	Migration of cells	2.77E-21	Increased	6.025
Cellular growth and proliferation	Proliferation of cells	3.25E-31	Increased	5.852
Gene expression	Expression of RNA	2.32E-11	Increased	5.697
Gene expression	Transcription	8.51E-11	Increased	5.428
Infectious diseases	Viral infection	8.39E-09	Increased	5.390
Cardiovascular disease, hematological disease	Anemia	1.51E-05	Decreased	−3.427
Organismal injury and abnormalities	Bleeding	1.40E-07	Decreased	−4.364
Developmental disorder	Hypoplasia of organ	6.46E-07	Decreased	−5.703
Developmental disorder	Growth failure	4.15E-06	Decreased	−6.016
Developmental disorder	Dysgenesis	1.40E-07	Decreased	−6.036
Developmental disorder	Hypoplasia	1.64E-07	Decreased	−6.036
Organismal survival	Neonatal death	3.39E-07	Decreased	−6.618
Organismal survival	Perinatal death	3.17E-09	Decreased	−7.749
Organismal survival	Morbidity or mortality	7.31E-25	Decreased	−11.534
Organismal survival	Organismal death	1.49E-24	Decreased	−11.611

senescent passage 17 BmMSCs (MSC-P17) that had become senescent after repeated replication. Measurements of the OCR at 48 h after the addition of carnitine at various concentrations revealed that it was elevated in both MSC-P4 (Fig. 3a) and MSC-P17 (Fig. 3d) cells as a result of carnitine addition. Basal respiration and maximal respiration were

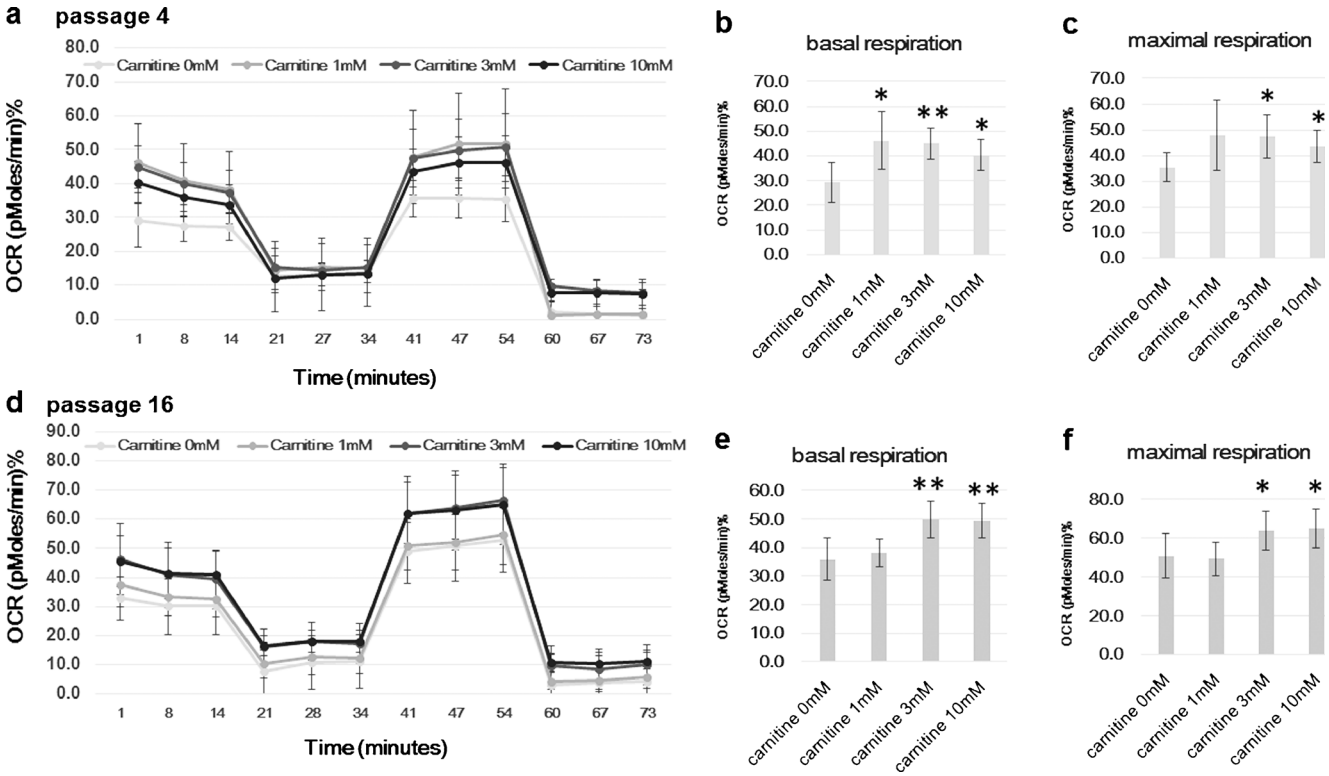

Fig. 3 Effect of L-carnitine on non-senescent passage 4 BmMSCs (MSC-P4) and senescent passage-16 BmMSCs (MSC-P16). **a–c** MSC-P4. **d–f** MSC-P16. **a**, **d** Effect of L-carnitine on oxygen consumption rate (OCR), which was measured by using a flux analyzer before and after administration of oligomycin, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), antimycin A and rotenone. **b**, **e** Basal respiration at 1 min. **c**, **f** Maximal respiration at 41 min. $^{*}P < 0.05$, $^{**}P < 0.01$

increased by the addition of L-carnitine in both MSC-P4 (Fig. 3b, c) and MSC-P17 (Fig. 3e, f) cells.

Discussion

L-carnitine plays an important role in fatty acid transport and lipid catabolism in the mitochondria. In this study, we found that carnitine does not affect cell proliferation. Carnitine is known to suppress vascular smooth muscle cell proliferation and osteoblastic differentiation (Orlandi et al. 2007; Xie et al. 2010). Carnitine has also been shown to attenuate the angiotensin-II-induced proliferation of cardiac fibroblasts (Chao et al. 2011). However, L-carnitine can also promote cell proliferation and ATP synthesis in human primary chondrocytes (Stoppoloni et al. 2013) and may thus either suppress or enhance proliferation depending on the cell type.

L-carnitine may suppress apoptosis by elevating ATP because of the increased β-oxidation of fatty acids and reduced fatty acid toxicity. When we incubated BmMSCs with L-carnitine and/or doxorubicin, which induces apoptosis in a wide variety of cell types, L-carnitine clearly suppressed apoptosis. The stability of the inner mitochondrial membrane and changes in the membrane potential have been associated with the anti-apoptotic effect of L-carnitine (Oyanagi et al. 2008). PGI2 and the PPARα-signaling pathway have been shown to be important in the mechanism, whereby L-carnitine prevents the doxorubicin-induced apoptosis of cardiomyocytes (Chao et al. 2011); in this study, we also observed an elevated protective effect of L-carnitine in doxorubicin-treated BmMSCs.

Our investigation of the effect of L-carnitine on BmMSC differentiation showed that it inhibited adipogenic differentiation. Adipogenic differentiation has been reported to be suppressed by the presence of L-carnitine (Lu et al. 2015). Adipogenic and osteogenic differentiation have a reciprocal relationship and miR-22 up-regulation has been reported to promote osteogenic differentiation and to inhibit adipogenic differentiation of human ADSCs by repressing HDAC6 protein expression. L-carnitine suppresses osteoblastic differentiation and ectopic mineralization in NIH3T3 cells (Ge et al. 2015). The results of other studies, however, have shown that L-carnitine promotes osteoblastic differentiation; thus, further investigations are required.

Although studies have been conducted to investigate the effect of L-carnitine administration on BmMSCs, no studies have addressed its effect by metabolomics analysis. Here, we used metabolomics to investigate the effect of L-carnitine on BmMSCs. The addition of L-carnitine resulted in elevated levels of L-carnitine and its metabolites. The only type of carnitine that decreased was deoxycarnitine, which is the immediate precursor of carnitine in the carnitine synthesis pathway that begins with L-lysine and L-methionine and passes through 6-N-trimethyllysine, 3-hydroxy-6-N-trimethyllysine and deoxycarnitine aldehyde. These data suggested that the carnitine-synthesis system was down-regulated. Focusing on L-carnitine, its level increased by 38.3-fold in cells exposed to 10 mM L-carnitine, compared with its level detected in control cells. This change represents an increase of ~10-fold over the L-carnitine level previously reported in hepatocytes from mice fed oral L-carnitine, suggesting that our culture system generates a much greater excess of L-carnitine compared with the situation in vivo. In vivo, excess L-carnitine is excreted in the urine, whereas in cultured cells, its concentration might easily rise. The concentrations of L-carnitine used in this study were higher than those found in vivo, although the number of differentially expressed genes was comparatively small, suggesting that the administration of high concentrations of L-carnitine is safe. With the objective of more efficient BmMSC preparation in vitro, higher concentrations than those in vivo measured can probably be used with few problems, although further research on considerations such as safety will be required in the future.

Long-chain fatty acids containing 16 or more carbon atoms are first converted to acyl-CoA in cytoplasmic microsomes or on the mitochondrial outer membrane, after which the acyl-CoA acyl group binds to L-carnitine on the cytosol side of the inner mitochondrial membrane to form acylcarnitine and passes through the membrane. Medium-chain and short-chain fatty acids, on the other hand, can pass through the inner membrane without binding to L-carnitine. In this study, the addition of L-carnitine significantly decreased the levels of many saturated and unsaturated long-chain fatty acids. The presence of L-carnitine may have resulted in the formation of large quantities of the various types of acylcarnitine and β-oxidation inside the mitochondria may have reduced the levels of intracellular long-chain fatty acids and unsaturated fatty acids.

The levels of intermediates involved in the glycolysis pathway also decreased, including pyruvate (ratio = 0.78), lactate (ratio = 0.83) and glycerate (ratio = 0.68). We hypothesize that glycolysis may have been down-regulated by the shift to ATP synthesis caused by β-oxidation. In the presence of excess carnitine, the amount of CoA is limited and if the β-oxidation of fatty acids is underway at maximum speed, then CoA is converted into its bound forms (acyl-CoA and acetyl-CoA) and the level of CoA is insufficient, thereby suppressing β-oxidation and making the CoA/acyl-CoA ratio important. If insufficient free CoA is present inside the mitochondrion, acetyl-CoA is hydrolyzed by acetyl-CoA hydrolase (EC 3.1.2.1) to generate free CoA. Acetyl-CoA hydrolase activity is inhibited by free CoA. Metabolomic analysis was performed at 48 h after the addition of carnitine and possibly, although β-oxidation was enhanced after the addition of L-carnitine, the amount of CoA gradually decreased, which would tend to decrease β-oxidation.

The use of SAGE to analyze changes in gene expression after the addition of L-carnitine revealed several changes in

gene expression. The expression of *sod2*, which is a verified biological defense mechanism against oxidative stress, is known to increase with L-carnitine administration and L-carnitine is also known to intensify SOD activity (Sener et al. 2004), suggesting an association between carnitine homeostasis and SOD gene regulation (Aydogdu et al. 2006). We considered that the up-regulation of *srebf1* and *srebf2*, which are genes known to regulate lipid synthesis, was a complementary mechanism to increase intracellular fatty acid levels, which had rapidly decreased as a result of L-carnitine addition. Voltage-dependent anion channels (VDACs) are found in the outer mitochondrial membrane, where they form small pores and have been shown to be involved in the transport of ATP and ADP and in the regulation of apoptosis.

When we performed further analysis by integrating SAGE and metabolomics, the function annotations obtained interestingly included the enhancement of cell survival. Elevated expression of genes, including *c-myb*, *erbb2* and *lif*, contributed to enhanced cell survival (Fig. 2c). Gene *c-myb* can increase the survival of Jurkat cells in cell culture (Zhou et al. 2011), *errb2* increases the viability of MCF7 cells (Knuefermann et al. 2003) and *lif* is involved in the survival of cells (Pesce et al. 1993). These data demonstrate that the pretreatment of BmMSCs with L-carnitine is effective for cell survival but further in-depth studies are required.

L-carnitine administration has previously been associated with the restoration of mitochondria and the suppression of the induction of senescence by TGF-β, suggesting that L-carnitine is involved in mitochondrial activation and senescence (Kerner et al. 2015; Rosca et al. 2009). In this study, we found that L-carnitine induced an increase in OCR, even in MSC-P17 cells, which had become senescent after repeated passages in culture, indicating that L-carnitine causes mitochondrial activation, even in senescent cells. With respect to the relationship between L-carnitine and senescence, previous data have shown that acetyl-L-carnitine suppresses apoptosis and aging in yeast by inhibiting mitochondrial fission (Palermo et al. 2010) and an evaluation of the dynamics of mitochondrial fission/fusion is therefore required.

In this investigation, we confirmed that L-carnitine suppresses apoptosis in BmMSCs. We also conducted a detailed metabolomic analysis of the effect of L-carnitine on BmMSCs. The data from this study may provide important information for the preparation of the MSCs needed to perform regenerative medical procedures in the future.

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Compliance with ethical standards

Contribution K.F., T.T. and I.S. conceived and designed the experiments. K.F. performed the experiments. T.M., N.Y. and L.Q. analyzed the data. Y.F and T.T. contributed reagents/materials/analysis tools. K.F. and T.T. wrote the paper.

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