ONCOLOGY REPORTS 15: 119-122, 2006

SPANDIDOS PUBLICATIONS

L-carnitine inhibits apoptotic DNA fragmentation induced by a new spin-labeled derivative of podophyllotoxin via caspase-3 in Raji cells

SHE-NING QI, ZHI-FENG ZHANG[1], ZHEN-YUN WANG[1], AKIRA YOSHIDA[2] and TAKANORI UEDA[2]

Department of Histology and Embryology, School of Basic Medicine, Lanzhou University, Lanzhou 730000, P.R. China

Received June 8, 2005; Accepted August 15, 2005

Abstract. L-carnitine (ß-hydroxy-trimethylaminobutyric acid) plays an essential metabolic role that consists of transferring the long chain fatty acids through the mitochondrial barrier, thus allowing their energy-yielding oxidation. GP7 (4-[4''-(2'', 2'', 6'', 6''-tetramethyl-l''-piperidinyloxy) amino] -4'-demethyl-epipodophyllotoxin) is a new spin-labeled derivative of podophyllotoxin semi-synthesized by our university. In this study, we examined the activity of L-carnitine in GP7-induced apoptosis in Burkitt's lymphoma cell line, Raji. GP7 induced time- and dose-dependent apoptotic DNA fragmentation accompanied by caspase-3 activation in Raji cells, and the kinetics of caspase-3 activation induced by GP7 was well correlated with that of apoptotic DNA fragmentation. L-carnitine treatment prevented GP7-induced caspase-3 activation, suppressed caspase-3 cleavage and abrogated GP7-induced apoptotic DNA fragmentation in Raji cells. Our findings suggest that L-carnitine is a potent anti-apoptotic agent to human lymphoma cells and may exert its anti-apoptotic effect via inhibition of caspase-3 activity in GP7-treated Raji cells.

Introduction

L-carnitine (ß-hydroxy-trimethylaminobutyric acid) plays an essential metabolic role that consists of transferring the long chain fatty acids through the mitochondrial barrier, thus allowing their energy-yielding oxidation (reviewed in ref. 1). L-carnitine has therapeutic effects on AIDS (2), Alzheimer's disease (3) and ischemic injury (4,5). Recent studies suggest that L-carnitine has caspases and anti-apoptotic regulating activity (6-10).

GP7 (4-[4''-(2'', 2'', 6'', 6''-tetramethyl-l''-piperidinyloxy)-amino]-4'-demethyl- epipodophyllotoxin) is a new spin-labeled derivative of podophyllotoxin semi-synthesized by our university (11). GP7 has many similarities to clinically used etoposide but has lower toxicity and higher total chemical yield (based on podophyllotoxin) when compared with etoposide and thus is a promising anticancer drug of the podophyllotoxin class (11).

In the present study, we examined the activity of L-carnitine in GP7-induced apoptosis in Burkitt's lymphoma cell line, Raji. L-carnitine treatment prevented GP7-induced caspase-3 activation, suppressed caspase-3 cleavage and abrogated GP7-induced apoptotic DNA fragmentation in Raji cells. Our findings suggest that L-carnitine is a potent anti-apoptotic agent to human lymphoma cells and inhibition of caspase-3 activity is one of its anti-apoptotic mechanisms in GP7-treated lymphoma cells.

Correspondence to: Dr She-Ning Qi, Department of Histology and Embryology, School of Basic Medicine, Lanzhou University, Lanzhou 730000, P.R. China
E-mail: qishening@hotmail.com

Present addresses: [1]Qingyang Municipal Hospital, Qingyang 745000, P.R. China; [2]First Department of Internal Medicine, Faculty of Medicine, University of Fukui, Matsuoka, 910-1193 Fukui, Japan

Key words: L-carnitine, apoptosis, DNA fragmentation, podophyllotoxin, caspase-3

Materials and methods

Materials. L-carnitine was purchased from ICN Biomedicals (Irvine, CA, USA). GP7 (purity ≥98%) was kindly provided by Professor Tian Xuan (School of Chemistry and Chemical Engineering, Lanzhou University) and dissolved in Me_2SO to achieve a stock solution of 10 mM and stored at -20˚C. Ac-DEVD-MCA was purchased from Peptide Institute (Osaka, Japan). Monoclonal antibody CPP32 was purchased from Immunotech (Marseille Cedex, France). During the experiment, all samples, including controls, contained 0.1% Me_2SO that had no observed effect on any of the assays performed.

Cell culture. Burkitt's lymphoma cell line, Raji, was obtained from ATCC (Rockville, MD, USA). Cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS) (Sigma, St. Louis, MO, USA) at 37˚C under 5% CO_2 in a humidified atmosphere.

DNA extraction and agarose gel electrophoresis. After GP7 treatment, the cells were lysed in a solution containing 500 mM Tris-HCl (pH 8.0), 20 mM EDTA, 10 mM NaCl, 1% (w/v) SDS and 100 μg/ml proteinase K at 37˚C for 48 h. DNA was extracted by standard phenol-chloroform-isoamyl alcohol

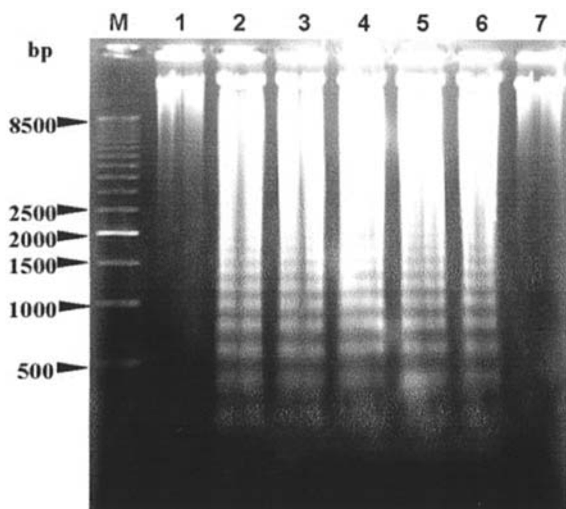

Figure 1. Agarose gel electrophoresis of DNA in Raji cells treated with GP7. Raji cells were untreated (lane 1) or treated with 18 μM GP7 for 6, 12 or 24 h (lanes 2-4, respectively), or treated with 9 or 4.5 μM GP7 (lanes 5 and 6) for 24 h, or treated with 18 μM GP7 in the presence of 5 mM L-carnitine for 24 h (lane 7). Left lane (M) shows the migration of 500-bp DNA size markers. Cellular DNA was extracted and subjected to agarose gel electrophoresis as described in Materials and methods. L-carnitine was added 60 min prior to GP7 administration.

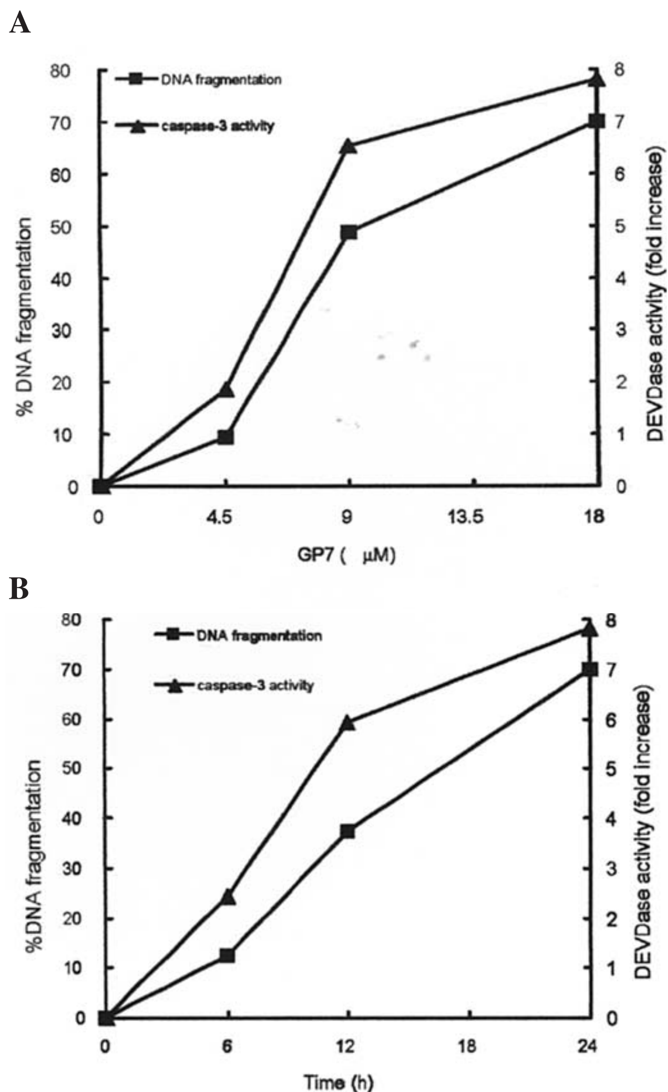

Figure 2. GP7 induces apoptotic DNA fragmentation and caspase-3 activation in a dose- and time-dependent manner in Raji cells. A, dose-response curve of GP7-induced DNA fragmentation and caspase-3 activation in Raji cells. Raji cells were incubated with different doses of GP7 for 24 h. B, time courses of GP7-induced DNA fragmentation and caspase-3 activation in Raji cells. Raji cells were treated with 18 μM GP7 for the indicated times. Cellular DNA of Raji was extracted and subjected to agarose gel electrophoresis. The percentage of DNA fragmentation was determined from a densitometric scan as described in Materials and methods. Aliquots of Raji cells were collected and assayed for caspase-3 activity with Ac-DEVD-MCA as substrate. Assays were performed as described in Materials and methods. Results are mean values from three separate experiments.

extraction procedures as described previously (6), and treated with 100 μg/ml of RNase A at 37˚C for 30 min. DNA samples were electrophoretically separated on 1.2% agarose gels. After electrophoresis, the gels were stained with ethidium bromide.

DNA fragmentation assay. DNA fragmentation was quantified by scanning photographic negatives with a densitograph gel documentation system (ATTO-densitograph, Osaka, Japan) and integrating the area under the curves. Integrated areas were divided at a 2000-bp molecular weight marker position into high-and low-molecular weight DNA, and the percentage of DNA fragmentation was determined by dividing the area of low-molecular weight DNA by the total area.

Preparation of cell lysates. Cell lysates were prepared by washing cells three times in ice-cold phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) and then incubating the cells for 15 min on ice at a density of $1x10^8$/ml in TKM buffer [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl] containing 0.25% Triton X-100. The cell lysates were centrifuged (10,000 g for 15 min at 4˚C) and the pellets were discarded. The supernatants were aliquoted and frozen at -80˚C.

Measurement of caspase-3 activity. Caspase-3 activity was analyzed by cleavage of the fluorometric substrates, Ac-DEVD-MCA. Enzyme reactions were performed in 96-well plates (Dainippon Seiyaku, Osaka, Japan) with 180 μl of cell lysates prepared as described above, and Ac-DEVD-MCA (final concentration, 1 μM). Each sample was seeded in triplicate. After incubation at 37˚C for 90 min, AMC (7-amino-4-methyl coumarin) released from the substrates was measured at excitation and emission wavelengths of 355 and 460 nm using a fluorescence microplate reader (Fluoroscan Ascent, Dainippon Seiyaku).

Western blot analysis of caspase-3 cleavage. Cells ($4x10^7$) were washed and solubilized in Laemmli buffer. Equivalent amounts of proteins were mixed with an equal volume of 2 x ME buffer, boiled, and resolved by electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The proteins were transferred from the gel to a nitrocellulose membrane using electroblotting apparatus (Bio-Rad) (12V, 60 min). Membranes were incubated in PBST (PBS with 0.1% Tween-20) containing 5% nonfat dried milk overnight to inhibit nonspecific binding. The membranes were then incubated with monoclonal antibody, CPP32, for 60 min. After washing in PBST for 30 min, membranes were incubated for

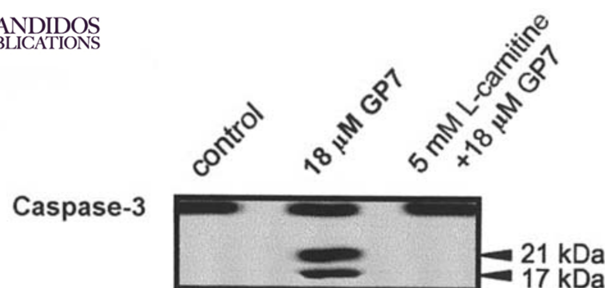

Figure 3. Western blot analysis of caspase-3 cleavage in Raji cells. Cells were untreated or treated with 18 μM GP7, or treated with 18 μM GP7 in the presence of 5 mM L-carnitine for 24 h. Western blot was performed as described in Materials and methods. L-carnitine was added 60 min prior to GP7 administration.

another 60 min with sheep anti-mouse Ig-HRP-linked whole antibody (Amersham Pharmacia Biotech, NJ, USA). The membranes were then washed and developed with enhanced chemiluminescence (ECL) (Pierce, Rockford, USA).

Results

GP7 induces DNA fragmentation in a time- and dose-dependent manner in Raji cells. To examine the effect of GP7 on DNA integrity, Raji cells were incubated with 18 μM GP7 for varying lengths of time. Treatment of Raji cells with 18 μM GP7 for 6, 12 or 24 h induced internucleosomal DNA fragmentation and DNA ladder was observed (Fig. 1, lanes 2-4). When Raji cells were incubated with different doses of GP7 for 24 h, the degree of DNA fragmentation increased as doses increased (Fig. 1, lanes 4-6). The amounts of DNA fragmentation induced by GP7 were quantified by a densitograph gel documentation system. The results show that GP7 induced DNA fragmentation in a dose- and time-dependent manner (Fig. 2).

GP7 treatment activates caspase-3 in Raji cells. Ac-DEVD-MCA, a fluorogenic substrate that corresponds to the cleavage site found in numerous executioner caspase-3 targets, was used to measure the activity of caspase-3 in GP7-induced DNA fragmentation. Treatment with GP7 induced a dose- and time-dependent activation of caspase-3 in Raji cells (Fig. 2).

Caspase-3 is cleaved into its active forms in Raji cells after GP7 treatment. We analyzed the cleavage of caspase-3 after GP7 treatment. Western blot showed that caspase-3 was cleaved into its active forms in Raji cells after GP7 treatment (Fig. 3).

L-carnitine inhibits GP7-induced caspase-3 activation in Raji cells. We investigated whether L-carnitine might inhibit GP7-induced caspase-3 activity. Raji cells were pretreated with L-carnitine for 60 min and then treated with 18 μM GP7 for 24 h, L-carnitine inhibited GP7-induced caspase-3 activation in a dose-dependent manner (Fig. 4).

L-carnitine inhibits GP7-induced caspase-3 cleavage in Raji cells. We investigated whether L-carnitine might inhibit GP7-induced caspase-3 cleavage. Raji cells were pretreated with L-carnitine for 60 min and then treated with 18 μM GP7 for 24 h, L-carnitine inhibited GP7-induced caspase-3 cleavage (Fig. 3).

L-carnitine inhibits GP7-induced apoptotic DNA fragmentation in Raji cells. GP7 induced apoptotic DNA fragmentation in

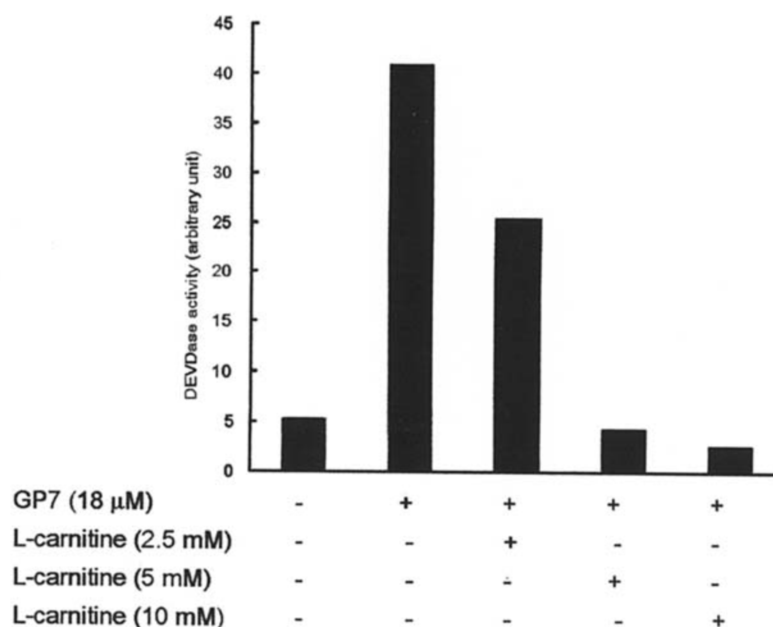

Figure 4. Effects of L-carnitine on GP7-induced caspase-3 activation in Raji cells. Raji cells were treated with 18 μM GP7 in the absence or presence of L-carnitine (2.5, 5 or 10 mM) for 24 h. L-carnitine was added 60 min prior to GP7 administration. After washing cells three times with PBS, cell lysates were prepared and assayed for caspase-3 activity with Ac-DEVD-MCA as substrate. Assays were performed as described in Materials and methods. Results are mean values from three separate experiments.

Raji cells in a time- and dose-dependent manner. The kinetics of this apoptotic DNA fragmentation was well correlated with that of caspase-3 activation induced by GP7. L-carnitine treatment prevented GP7-induced caspase-3 activation in Raji cells. So we further investigated the effects of L-carnitine on GP7-induced internucleosomal DNA fragmentation. Pretreatment of Raji cells with 5 mM L-carnitine for 60 min inhibited GP7-induced DNA fragmentation in Raji cells (Fig. 1, lane 7).

Discussion

Apoptosis is the mechanism of cell death activated in mammalian cells following exposure to a wide variety of stimuli including anticancer agents. A hallmark of apoptosis is the fragmentation of nuclear DNA. In this study, GP7 induced apoptotic DNA fragmentation in Raji cells (Fig. 1), and the kinetics of GP7-induced DNA fragmentation were in a time- and dose-dependent manner (Fig. 2), indicating that induction of apoptosis is one of the anticancer mechanisms of GP7.

Studies have demonstrated that caspase-3 plays a crucial role in mediating apoptotic DNA fragmentation during apoptosis (12-16). In the present study, treatment with GP7 induced a time- and dose-dependent activation of caspase-3 in Raji cells (Fig. 2), and caspase-3 activation was well correlated with the kinetics of internucleosomal DNA fragmentation (Fig. 2). Western blot showed that caspase-3 was cleaved into its active forms in Raji cells after GP7 treatment (Fig. 3). These data indicate that caspase-3 is closely involved in GP7-induced apoptotic DNA fragmentation in Raji cells.

Our previous studies found that L-carnitine can directly inhibit the activity of recombinant caspase-3 *in vitro* in a dose-dependent manner, and abrogate idarubicin-induced activation of caspase-3 in HL-60 and Jurkat cells (6). However, inhibition of caspase-3 activity does not necessarily mean the ability to inhibit apoptotic DNA fragmentation, as we have found in idarubicin-treated HL-60 and Jurkat cells (6). In this study, pretreatment of Raji cells with various doses of L-carnitine for 60 min inhibited GP7-induced caspase-3 activation in a dose-dependent manner (Fig. 4). Five mM L-carnitine prevented caspase-3 cleavage (Fig. 3), and abrogated GP7-induced apoptotic DNA fragmentation (Fig. 1). These data indicate that L-carnitine is an effective anti-apoptotic agent which may exert its anti-apoptotic effect by inhibition of caspase-3 activity in GP7-treated lymphoma cells. These data also suggest that activation of caspase-3 is indispensable for GP7-induced internucleosomal DNA fragmentation during apoptosis in Raji cells and GP7-induced DNA fragmentation in Raji cells is caspase-3-dependent.

L-carnitine has already demonstrated its therapeutic effects on AIDS (2), Alzheimer's disease (3) and ischemic injury (4,5). The use of L-carnitine as an anti-apoptotic agent is very promising since it is likely to be less toxic compared with synthetic inhibitors (7).

Acknowledgements

This study was supported by Scientific Research Foundation from Gansu Science and Technology Bureau.

References

1. Matera M, Bellinghieri G, Costantino G, Santoro D, Calvani M and Savica V: History of L-carnitine: implications for renal disease. J Ren Nutr 13: 2-14, 2003.
2. Gelbard HA, James HJ, Sharer LR, Perry SW, Saito Y, Kazee AM, Blumberg BM and Epstein LG: Apoptotic neurons in brain from paediatric patients with HIV-1 encephalitis and progressive encephalopathy. Neuropathol Appl Neurobiol 21: 208-217, 1995.
3. Cotman CW and Anderson AJ: A potential role for apoptosis in neurodegeneration and Alzheimer's disease. Mol Neurobiol 10: 19-45, 1995.
4. Linnik MD, Zobrist RH and Hatfield MD: Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke 24: 2002-2008, 1993.
5. Gottlieb RA, Burleson KO, Kloner RA, Babior BM and Engler RL: Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 94: 1621-1628, 1994.
6. Qi SN, Yoshida A and Ueda T: Activation of caspases-3/7 is dispensable for idarubicin-induced apoptotic DNA fragmentation in human leukemia cells. Int J Oncol 22: 1123-1128, 2003.
7. Mutomba MC, Yuan H, Konyavko M, Adachi S, Yokoyama CB, Esser V, McGarry JD, Babior BM and Gottlieb RA: Regulation of the activity of caspases by L-carnitine and palmitoylcarnitine. FEBS Lett 478: 19-25, 2000.
8. Moretti S, Famularo G, Marcellini S, Boschini A, Santini G, Trinchieri V, Lucci L, Alesse E and De Simone C: L-carnitine reduces lymphocyte apoptosis and oxidant stress in HIV-1-infected subjects treated with zidovudine and didanosine. Antioxid Redox Signal 4: 391-403, 2002.
9. Vescovo G, Ravara B, Gobbo V, Sandri M, Angelini A, Della Barbera M, Dona M, Peluso G, Calvani M, Mosconi L and Dalla Libera L: L-carnitine: a potential treatment for blocking apoptosis and preventing skeletal muscle myopathy in heart failure. Am J Physiol Cell Physiol 283: C802-C810, 2002.
10. Andrieu-Abadie N, Jaffrezou JP, Hatem S, Laurent G, Levade T and Mercaider JJ: L-carnitine prevents doxorubicin-induced apoptosis of cardiac myocytes: role of inhibition of ceramide generation. FASEB J 13: 1501-1510, 1999.
11. Chen YZ, Wang YG, Li JX, Tian X, Jia ZP and Zhang PY: Anticancer drugs. II. Synthesis and biological evaluation of spin labeled derivatives of podophyllotoxin. Life Sci 45: 2569-2575, 1989.
12. Qi SN, Yoshida A, Wang ZR and Ueda T: GP7 can induce apoptotic DNA fragmentation of human leukemia cells through caspase-3-dependent and -independent pathways. Int J Mol Med 13: 163-167, 2004.
13. Wolf BB, Schuler M, Echeverri F and Green DR: Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. J Biol Chem 274: 30651-30656, 1999.
14. Jiang D, Jha N, Boonplueang R and Andersen JK: Caspase 3 inhibition attenuates hydrogen peroxide-induced DNA fragmentation but not cell death in neuronal PC12 cells. J Neurochem 76: 1745-1755, 2001.
15. Kim HS, Rhim H, Jeong SW, Kim JW and Kim IK: Induction of apoptosis dependent on caspase activities and growth arrest in HL-60 cells by PGA2. Prostaglandins Other Lipid Mediat 70: 169-183, 2002.
16. Anuradha CD, Kanno S and Hirano S: RGD peptide-induced apoptosis in human leukemia HL-60 cells requires caspase-3 activation. Cell Biol Toxicol 16: 275-283, 2000.