Regulation of the activity of caspases by L-carnitine and palmitoylcarnitine

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Abstract L-Carnitine facilitates the transport of fatty acids into the mitochondrial matrix where they are used for energy production. Recent studies have shown that L-carnitine is capable of protecting the heart against ischemia/reperfusion injury and has beneficial effects against Alzheimer's disease and AIDS. The mechanism of action, however, is not yet understood. In the present study, we found that in Jurkat cells, L-carnitine inhibited apoptosis induced by Fas ligation. In addition, 5 mM carnitine potently inhibited the activity of recombinant caspases 3, 7 and 8, whereas its long-chain fatty acid derivative palmitoylcarnitine stimulated the activity of all the caspases. Palmitoylcarnitine reversed the inhibition mediated by carnitine. Levels of carnitine and palmitoyl-CoA decreased significantly during Fas-mediated apoptosis, while palmitoylcarnitine formation increased. These alterations may be due to inactivation of β-oxidation or to an increase in the activity of the enzyme that converts carnitine to palmitoylcarnitine, carnitine palmitoyltransferase I (CPT I). In support of the latter possibility, fibroblasts deficient in CPT I activity were relatively resistant to staurosporine-induced apoptosis. These observations suggest that caspase activity may be regulated in part by the balance of carnitine and palmitoylcarnitine.

Key words: Apoptosis; L-Carnitine; Palmitoylcarnitine; Caspase; Carnitine palmitoyltransferase I

1. Introduction

Apoptosis is a form of cell death characterized by a sequence of events which result in the elimination of the cell without release of its contents into the extracellular space [1]. This form of cell death is mediated by a group of highly specific cysteine proteases known as cysteine aspartic acid specific proteases (caspases). Under normal circumstances, caspases exist as dormant zymogens, but when a cell receives a death signal, caspases are activated through limited proteolysis. Once activated, effector caspases both activate other caspases and cleave a host of cellular substrates, leading to the characteristic morphological changes associated with apoptosis, as for example DNA fragmentation and condensation of cellular organelles.

Proteolysis is an irreversible event and thus there must be strict control of caspase activation in order to prevent inappropriate cell death. Indeed, it has been shown that caspase activity is regulated by a number of interacting proteins, including IAPs (inhibitors of apoptosis proteins) [2–4] and Flips (FADD-like ICE inhibitory proteins) [5]. Caspases have also been shown to be affected by small molecules, notably nitric oxide, which acts at the critical thiol residue in the catalytic center [26].

Apoptosis has been implicated in the etiology of several human diseases, including Alzheimer's [6], AIDS [7] and ischemic injury [8,9]. Interestingly, a number of studies and clinical trials have shown that L-carnitine has therapeutic effects in these conditions [10–12], raising the possibility that it may be acting as an anti-apoptotic agent. Carnitine is a naturally occurring quaternary amine zwitterion that facilitates the transport of fatty acids into the mitochondria for β-oxidation. Its concentration varies from 0.1 to a few mM, depending on the tissue [13]. Highest concentrations of carnitine are found in cardiac and skeletal muscle, concentrations exceeding 3 mM having been reported in human skeletal muscle [14]. Of the total carnitine, over 90% is found in the cytosol, with only a small fraction found within the mitochondrial pool [15].

In this study, we investigated the effects of carnitine and palmitoylcarnitine on caspase activity. We show that the levels of these key components of fatty acid metabolism are altered during apoptosis and that they can modulate caspase activity.

In this study, we investigated whether the beneficial effects of L-carnitine were a result of its anti-apoptotic activity. Our results showed that L-carnitine protected Jurkat cells against Fas-mediated apoptosis and inhibited the activity of recombinant caspases. Its anti-caspase activity could be a mechanism for its anti-apoptotic effect.

2. Materials and methods

2.1. Materials

L-Carnitine, D-carnitine, betaine, taurine, trimethyllysine, acetylcarnitine, choline, phosphorylcholine chloride and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) were ob-

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Abbreviations: Caspase, cysteine aspartic acid specific protease; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CPT, carnitine palmitoyltransferase; PARP, recombinant poly-(ADP-ribose) polymerase; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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tained from Sigma (St. Louis, MO, USA). Palmitoylcarnitine was obtained from Fluka (Milwaukee, WI, USA). The compounds were used at the indicated concentrations in the assays of recombinant caspase activity. Where noted, L-carnitine was added to whole cells at different concentrations, 1 h prior to addition of anti-Fas antibody.

2.2. Cell culture and induction of apoptosis

Jurkat cells were obtained from ATCC and maintained in RPMI 1640 with 5% fetal bovine serum and 2 mM L-glutamine. The cells were used when they had grown to 1×10^6 cells/ml. To induce apoptosis, the cells were incubated with 500 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company, WA, USA) in serum-free medium at a cell density of 4×10^7 cells/ml. Apoptosis was assessed by acridine orange staining and scoring of nuclei showing chromatin condensation and/or nuclear fragmentation. Between 150 and 200 cells were scored for each condition. Fibroblasts from controls and carnitine palmitoyltransferase (CPT) I deficient patients were obtained from Dr. Jean-Marie Saudubray of the Department of Pediatrics, Hopital des Enfants Malades, Paris, France [16,17]. Both cell lines were maintained in DMEM with 10% fetal bovine serum. For induction of apoptosis, cells were seeded in a 96-well plate with 10 000 cells/well in 100 μl of medium. The cells were left overnight at 37°C to attach, then treated with different concentrations of staurosporine for varying periods of time. Percentage of cell death was detected using the cell impermeant DNA dye YOPRO-1 (Molecular Probes, Eugene, OR, USA) as described [18], using a Microplate Fluorescence Reader (Bio-tek Instruments, Inc., Winooski, VT, USA), Ex_{max}/Em_{max} (nm) = 480/520.

2.3. Preparation of cell fractions

Whole cell lysates were prepared from cells (5×10^7 for each assay) incubated with anti-Fas antibody for the required time and then washed once in phosphate-buffered saline (PBS: 137 mM NaCl, 8 mM KCl, 10 mM Na_2HPO_4, 2 mM K_2PO_4, pH 7.4). Cells were resuspended in 250 μl MF buffer (20 mM PIPES, pH 6.8, 100 mM sucrose, 1 mM EGTA, 1 mM EDTA, 10 mM dithiothreitol (DTT), 25 mM KCl, 10 mM NaCl, 1 g/l bovine serum albumin and 5% Percoll), supplemented with 25 μg/ml leupeptin, 20 μg/ml pepstatin and 1 mM PMSF. Cells were then disrupted by sonication and the lysate cleared by centrifugation at $14\,000\times g$ for 30 min at 4°C. Supernatants were used for assays.

For making fractions, after anti-Fas treatment, cells (1×10^9) were washed in 10 ml MA buffer (100 mM sucrose, 1 mM EGTA, 20 mM MOPS pH 7.4, 1 g/l bovine serum albumin), then resuspended in 6 ml ME buffer (20 mM PIPES, pH 6.8, 100 mM sucrose, 1 mM EGTA, 1 mM EDTA, 10 mM DTT, 1 g/l bovine serum albumin, 5% Percoll), supplemented with leupeptin, pepstatin and PMSF. The cells were disrupted by nitrogen cavitation as previously described [19], and nuclei and unbroken cells removed by two centrifugations at $1200\times g$. The postnuclear supernatant was centrifuged at $14\,000\times g$ for 30 min at 4°C and both the postmitochondrial supernatant and mitochondrial pellet recovered. S100 cytosol was prepared by ultracentrifugation of the postmitochondrial supernatant at $100\,000\times g$ for 30 min at 4°C. The mitochondrial pellet was washed in two changes of 0.5 ml sucrose wash buffer (0.25 M sucrose, 10 mM Tris–HCl pH 7.4, 1 mM EGTA), then resuspended in sucrose buffer (0.3 M sucrose, 10 mM Tris–HCl pH 7.4 and 1 mM EGTA), with protease inhibitors.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western analysis

One-dimensional SDS–PAGE was carried out using a 4–20% gradient running gel (Novex, San Diego, CA, USA). Western blotting was performed using 30 μg protein for each sample or as noted in the figure legends. Caspase 2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), caspase 3 antibody was from PharMingen (San Diego, CA, USA) and caspase 9 antibody was a gift from Dr. Guy Salvesen (Burnham Institute, La Jolla, CA, USA). The antibody against poly(ADP-ribose) polymerase (PARP) was from Boehringer Mannheim (Germany).

2.5. Caspase activity assays

Caspase activity was measured at room temperature using a method adapted from Stennicke and Salvesen [20]. Briefly, in a Corning 96-well plate, 20 ng of recombinant caspase 3 or 7, or 200 ng recombinant caspase 8 (PharMingen, San Diego, CA, USA) was added to caspase assay buffer (20 mM PIPES, pH 6.8, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 10% sucrose) and 100 μM of the substrate *N*-acetyl-DEVD *p*-nitroanilide (DEVD-pNA) in a final volume of 120 μl. The plate was placed in a Molecular Devices UVmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA, USA) and left to equilibrate for 5–10 min at room temperature. Hydrolysis of the substrate was followed at 405 nm for 60 min. Caspase activity in cell lysates was measured in the same way using 100 μg protein in a final volume of 150 μl.

2.6. Caspase cleavage of endogenous substrates

To determine the effect of carnitine or palmitoylcarnitine on the cleavage of endogenous substrates by the recombinant caspases, 100 μg of S100 cytosols from non-apoptotic cells was incubated with 20 ng of either recombinant caspase 3 or 8 for 2 h at 37°C in the presence of increasing concentrations of either compound. For autoactivation of caspase 9, S100 cytosols (100 μg) were incubated with 10 μg/ml horse heart cytochrome *c* and 0.1 μM dATP for 2 h at 37°C in the presence or absence of carnitine and palmitoylcarnitine. Samples (30 μg protein) were subjected to SDS–PAGE and Western analysis, then the blots were immunostained with anti-caspase antibodies. Alternatively, 20 ng of recombinant caspase 3 was preincubated with palmitoylcarnitine in caspase assay buffer for 10 min at room temperature, before addition of increasing concentrations of either carnitine or palmitoyl-CoA. 2 μg of human recombinant PARP (Trevigen, Gaithersburg, MD, USA) was added in a final volume of 25 μl and the mixtures incubated at 37°C for 25 min. The samples were then subjected to SDS–PAGE and Western analysis.

2.7. CPT and hydrolase assays

To measure CPT I activity in fibroblasts, we used the method of McMillin [21]. This distinguishes between CPT I and CPT II activities, based on the fact that CPT I is located in the outer mitochondrial membrane and so can interact with substrates after digitonin permeabilization of whole cells, while CPT II is within the mitochondrial inner membrane and cannot utilize substrates until the membrane is solubilized with detergent (Triton X-100), which also inactivates CPT I. We confirmed that CPT I activity revealed by digitonin permeabilization could be inhibited to greater than 95% by 100 μM malonyl-CoA (data not shown). Cells were resuspended in permeabilization buffer (140 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.56 mM $CaCl_2$, 40 mM HEPES pH 7.0, 0.1% bovine serum albumin, 5 mM ATP, 5 μM KCN) containing 10 μM digitonin. Cells were incubated at 37°C for 10 min, then resuspended in buffer without digitonin and 300 μM of unlabeled carnitine containing 1 μCi [^{3}H]carnitine (Sigma, St. Louis, MO, USA) as tracer was added. Samples were incubated for 10 min at 37°C and 30 μM palmitoyl-CoA was added to initiate the reaction. Samples were incubated for another 20 min and the reaction stopped by the addition of 1.5 ml butanol-saturated 0.73 M HCl. [^{3}H]Palmitoylcarnitine was extracted with 1 ml *n*-butanol, while [^{3}H]carnitine remained in the aqueous phase. [^{3}H]Palmitoylcarnitine was quantified by scintillation counting.

2.8. Palmitoyl-CoA measurements in whole cells

Jurkat cells (3×10^8) were induced to undergo apoptosis with 500 ng/ml anti-Fas IgM or incubated in parallel, then washed once in ice-cold PBS before being added dropwise to liquid nitrogen. The frozen cell pellets were ground with a teflon pestle, then resuspended in 1 ml isopropanol for lipid extraction. 5 nCi of ^{14}C-labeled palmitoyl-CoA was added in order to monitor efficiency of recovery during the subsequent steps. Samples were extracted as described [22] and analyzed by high performance liquid chromatography using a Nucleosil 100 C18 column (5 micron, 150×4.6 mm; Alltech Associates, Inc., Deerfield, IL, USA) and a gradient system. The two mobile phase solvents were 25 mM KH_2PO_4 (pH 4.9) and 100% acetonitrile. The long-chain acyl-CoA esters were eluted by a linear gradient [23]. Palmitoyl-CoA was identified using a palmitoyl-CoA standard. Samples with the appropriate retention time were recovered and analyzed by scintillation counting. Values for palmitoyl-CoA were corrected for differences in recovery of the radiolabeled internal control. Cell number and cellular protein were not different between control and Fas-treated groups.

2.9. Carnitine measurements in whole cells

Carnitine content in Jurkat cells was determined using the radioisotopic assay of McGarry [24] and McMillin [25] with modification.

Briefly, 0.5 ml cell suspension containing 2.5–3.0×10^8 cells was precipitated in 0.3 ml 10% $HClO_4$. After centrifugation, pellets were washed in 0.3 ml 10% $HClO_4$ by vortexing. Two fractions were combined and buffered with Tris (final concentration 115 mM), neutralized with KOH to pH 7.8 and placed on ice for 60 min to precipitate $KClO_4$. After centrifugation, the $KClO_4$ pellets were washed in 0.2 ml 115 mM ice-cold Tris buffer. Two supernatants were combined and aliquoted into triplicate samples. Each sample had an equivalent of 2.5–4.0 mg of cell protein. Reaction was started by adding 3 U carnitine acetyltransferase (Boehringer Mannheim) to the mixture containing 0.05 μCi [^{3}H]acetyl-CoA (specific activity (S.A.) 200 mCi/mmol, American Radiolabeled Chemicals, Inc.), 100 μM acetyl-CoA. Samples were incubated at room temperature for 2 h. The reaction was stopped by adding 500 μl stirred slurry of AG1-X8 resin (chloride form, Bio-Rad). The mixture was vortexed and placed on ice. This procedure was repeated three times at 5 min intervals. All samples were centrifuged at 4°C. A 0.1 ml supernatant was used for scintillation counting. An enzyme blank was included to correct for nonspecific radioactivity in the supernatants. A carnitine standard curve (2.5, 5.0, 10 μM) was included with each experiment.

2.10. Detection of palmitoylcarnitine formation during apoptosis

A 2 ml culture of Jurkat cells at a density of 1×10^6 cells/ml was transferred into each well of a culture plate, then 10 μCi [^{3}H]carnitine (S.A. 80 Ci/mmol, American Radiolabeled Chemicals, Inc.) was added. After 3 h of labeling, anti-Fas antibody (500 ng/ml) was added to one well and incubated for 1 h to induce apoptosis. At this time, both control and apoptotic cells were collected and washed once in PBS. After centrifugation, cells were resuspended in 0.2 ml PBS. To extract palmitoylcarnitine, 0.4 ml water-saturated *n*-butanol was added to each sample and vortexed for 5 min. Samples were centrifuged to separate the butanol and aqueous phases. Butanol fractions were concentrated to approximately 50 μl, loaded on a thin layer chromatography (TLC) plate (Silica Gel 60, 200 micron, Fisher Scientific) and resolved in chloroform:methanol:water (65:25:4). Results were detected by autoradiography.

3. Results

3.1. Effect of L-carnitine on Fas-induced apoptosis in Jurkat cells

To determine if L-carnitine could protect against Fas-mediated apoptosis, Jurkat cells were induced to undergo apoptosis with Fas ligation in the presence or absence of increasing concentrations of L-carnitine. After 4 h, cells were harvested and apoptosis was scored by changes in nuclear morphology. L-Carnitine suppressed apoptosis in a dose-dependent fashion (Table 1) consistent with inhibition of activated DEVD-pNA-cleaving caspases (Fig. 1A). In addition, Western analysis using caspase 2 as a marker for apoptosis showed that carnitine inhibited caspase 2 processing (Fig. 1B), suggesting that L-carnitine is acting upstream of caspase 2 cleavage.

3.2. L-Carnitine inhibits the activity of recombinant caspase 3

We wondered whether the inhibition of caspase 2 cleavage by L-carnitine resulted from the inhibition of an upstream caspase 2 activator. It has been reported that caspase 2 may be cleaved by caspase 3 [26] and as a result, we tested whether carnitine was acting at this level. Carnitine inhibited the ability of recombinant caspase 3 to cleave the native protein substrate caspase 2 in cell lysates (Fig. 1C). We compared L-carnitine to other biologically relevant quaternary amines, including D-carnitine, betaine and trimethyllysine. We found that L-carnitine was a potent inhibitor of recombinant caspase 3, followed by trimethyllysine and D-carnitine (Fig. 1D).

Table 1
Nuclear morphology after Fas ligation

[Carnitine] (mM)	Apoptotic cells (%)	
	Control	Fas
0	4	45
5	10	30
10	12	14

Jurkat cells were subjected to Fas ligation as described in the legend to Fig. 1, and aliquots were analyzed for nuclear fragmentation after acridine orange staining. Similar results were obtained in two independent experiments.

3.3. Effect of palmitoylcarnitine on the activity of recombinant caspases 3 and 7

Caspase activity is enhanced by CHAPS, which is a quaternary amine zwitterion with a long hydrophobic side chain. Palmitoylcarnitine is also a quaternary amine zwitterion with a hydrophobic side chain. We evaluated whether a carnitine ester of either a short-chain or a long-chain fatty acid (acetate and palmitate, respectively) would enhance caspase activity. The inhibitory effects of carnitine revealed a steep dose–response curve, with minimal inhibition at 1 mM, slight inhibition at 3 mM and nearly full inhibition at 5 mM. We found that palmitoylcarnitine, but not acetylcarnitine, increased the activity of recombinant caspase 3 (Fig. 2A) and caspase 7 (data not shown), showing a maximal effect at 50 μM.

3.4. L-Carnitine and palmitoylcarnitine have antagonistic effects

The opposing effects of carnitine and palmitoylcarnitine led us to examine the ability of palmitoylcarnitine to reverse inhibition by carnitine of recombinant caspase 3. Palmitoylcarnitine was able to reverse this inhibition at a concentration of 10 μM (Fig. 2B). Furthermore, caspase 3 activity was partially protected when the enzyme was preincubated with either palmitoylcarnitine then carnitine added (Fig. 2B) or preincubated with carnitine then palmitoylcarnitine added (not shown), suggesting an antagonistic interaction.

We next examined the effect of these compounds on cleavage of endogenous caspase substrates. The addition of palmitoylcarnitine to Jurkat cell lysates was not sufficient to cause spontaneous activation of procaspases (data not shown). Immunoblot analysis of substrate cleavage in cell lysates measures an endpoint rather than enzymatic rate, and so we could not detect an effect of palmitoylcarnitine on the rate of activated caspases to process protein substrates. To further evaluate the antagonistic effects of carnitine and palmitoylcarnitine, we looked at cleavage of human recombinant PARP by recombinant caspase 3. As shown in Fig. 2C, 10 mM carnitine inhibited the generation of the 85 kDa PARP fragment and this was partially reversed by 50 μM palmitoylcarnitine (Fig. 3C).

3.5. Regulation of the activity of caspase 8 by L-carnitine and palmitoylcarnitine

The preceding data showed that carnitine and palmitoylcarnitine regulated the activity of effector caspases 3 and 7, implying that in vivo they could regulate cleavage of endogenous caspase substrates. We tested whether these compounds had an effect on the activity of an initiator caspase, caspase 8. Fig. 3A shows that carnitine at 5 mM inhibited the activity of caspase 8, whereas palmitoylcarnitine stimulated the activity of the enzyme. Since caspase 8 directly cleaves caspases 3 and 9 [27], we tested whether this would be affected by incubation

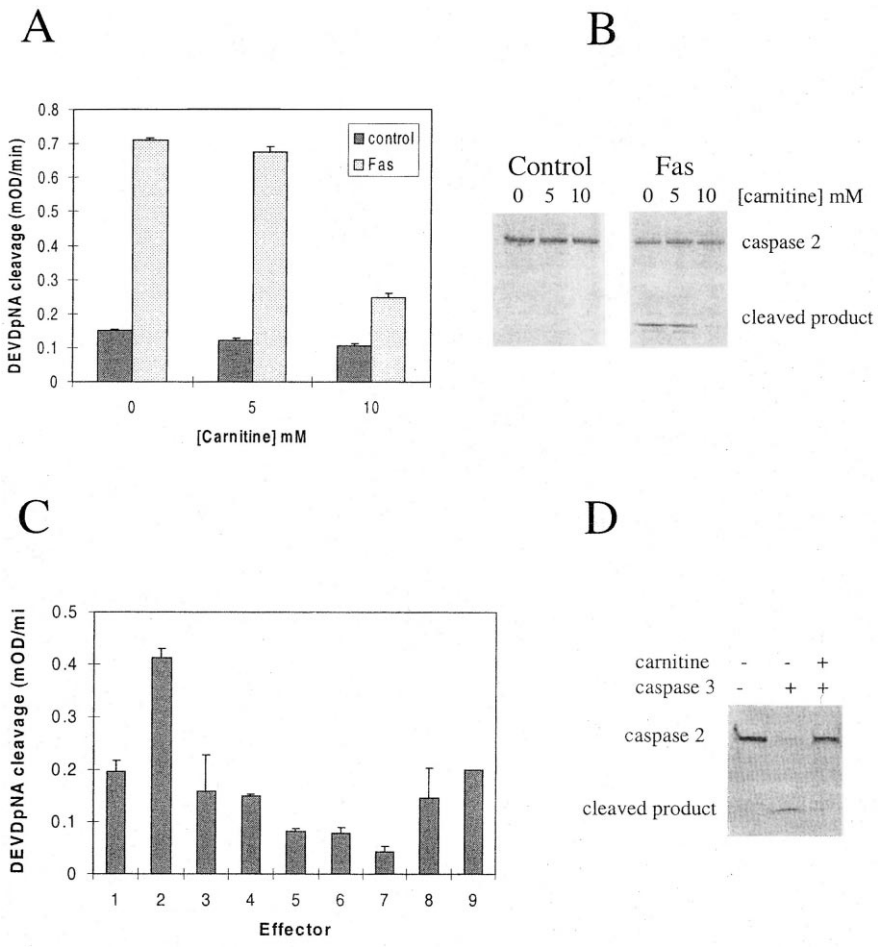

Fig. 1. Effect of L-carnitine on Fas-induced apoptosis in Jurkat cells. Jurkat cells were treated with anti-Fas antibody in the presence or absence of increasing concentrations of L-carnitine and incubated at 37°C for 4 h. The cells were lysed and whole cell lysates prepared as described in Section 2. (A) Activation of DEVD-specific caspases in whole cell lysates (100 μg) was measured by cleavage of the synthetic peptide substrate DEVD-pNA. The data shown are mean ± S.D. from a triplicate experiment representative of two different experiments. (B) 100 μg proteins were subjected to SDS–PAGE and Western analysis using anti-caspase 2 antibodies. The results shown are representative of two independent experiments. (C) Inhibition of recombinant caspase 3 activity by L-carnitine. Different naturally occurring compounds (at a final concentration of 5 mM) were preincubated for 15 min with 20 ng recombinant caspase 3 and then enzyme activity measured by DEVD-pNA cleavage. 1, none; 2, CHAPS; 3, betaine; 4, taurine; 5, trimethyllysine; 6, D-carnitine; 7, L-carnitine; 8, choline; 9, phosphorylcholine chloride. The data shown are mean ± S.D. from a triplicate experiment representative of three independent experiments. (D) Jurkat cells were lysed by nitrogen cavitation and S100 cytosols prepared as described in Section 2. 100 μg protein samples were incubated with 20 ng of recombinant caspase 3 for 2 h at 37°C in the presence or absence of 5 mM L-carnitine. 30 μg protein was analyzed by immunoblotting with antibody to caspase 2. The results shown are from one experiment representative of two independent experiments.

with either carnitine or palmitoylcarnitine. Fig. 3B shows that 5 mM carnitine inhibited the processing of caspase 3 and caspase 9 by caspase 8, and that 10 μM palmitoylcarnitine reversed the inhibition. Carnitine was also able to inhibit proteolytic activation of caspase 9 mediated by Apaf-1, cytochrome *c* and dATP (not shown).

3.6. Carnitine and palmitoyl-CoA levels during apoptosis

The foregoing observations demonstrated that carnitine and palmitoylcarnitine could regulate caspase activity in vitro, and that high levels of exogenous carnitine could attenuate caspase 2 processing and apoptosis. In order to determine if the balance of carnitine and palmitoylcarnitine is altered during apoptosis, we measured carnitine levels in Jurkat cells undergoing Fas-mediated apoptosis. We found that the carnitine level decreased by 30% within 2 h after Fas ligation (Table 2).

Palmitoyl-CoA can be converted by CPT I to form palmitoylcarnitine, which we have shown favors caspase activity. We measured palmitoyl-CoA levels and found them to be significantly reduced during Fas-mediated apoptosis (Table 2). This observation is consistent with the notion that alterations in fatty acid metabolism occur during apoptosis and may modulate caspase activity.

3.7. Palmitoylcarnitine increases during apoptosis

It could be argued that the observed reductions in palmitoyl-CoA and free carnitine could represent losses from dying cells. If, however, the reductions in carnitine and palmitoyl-CoA are due to increased activity of CPT I, we would be able to detect increased palmitoylcarnitine formation. We loaded cells with [^{3}H]carnitine and then subjected them to Fas ligation for 1 h before lysing them and extracting palmitoylcarnitine for TLC. As shown in Fig. 4, an increase in the formation of palmitoylcarnitine was evident as early as 60 min after Fas ligation, consistent with the idea that there is a shift in the ratio of carnitine to palmitoylcarnitine during apoptosis.

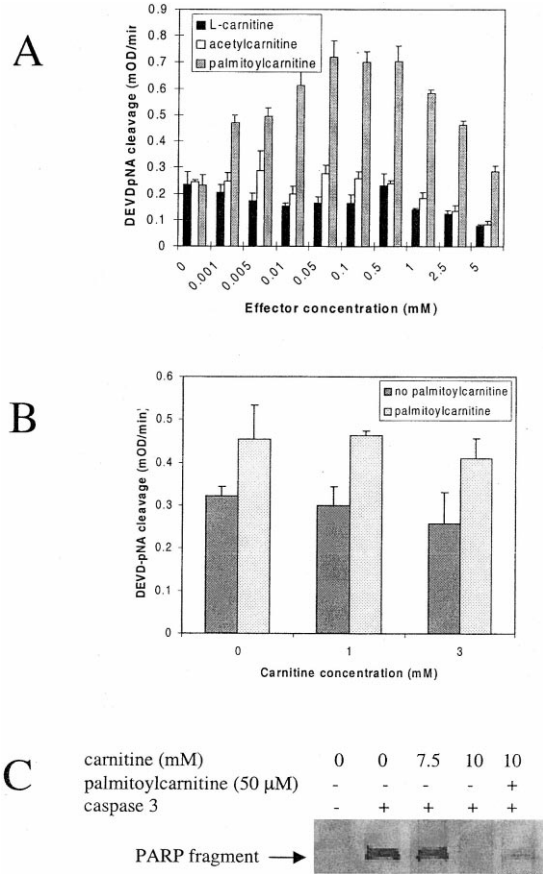

Fig. 2. Effect of L-carnitine and its acyl derivatives on the activities of recombinant caspases. Different concentrations of L-carnitine, acetylcarnitine and palmitoylcarnitine were added to 20 ng of (A) recombinant caspase 3. Enzyme activities were then assayed as already described. (B) 20 ng of recombinant caspase 3 was incubated with 50 μM palmitoylcarnitine for 10 min at room temperature then increasing concentrations of carnitine added and enzyme activities determined. The data shown are mean ± S.D. from triplicate experiments done at least two different times. (C) Recombinant caspase 3 (20 ng) was preincubated with or without 50 μM palmitoylcarnitine before addition of increasing concentrations of carnitine and 2 μg PARP. The mixtures were incubated at 37°C for 25 min then the samples subjected to SDS–PAGE and Western analysis. The results shown are representative of three independent experiments.

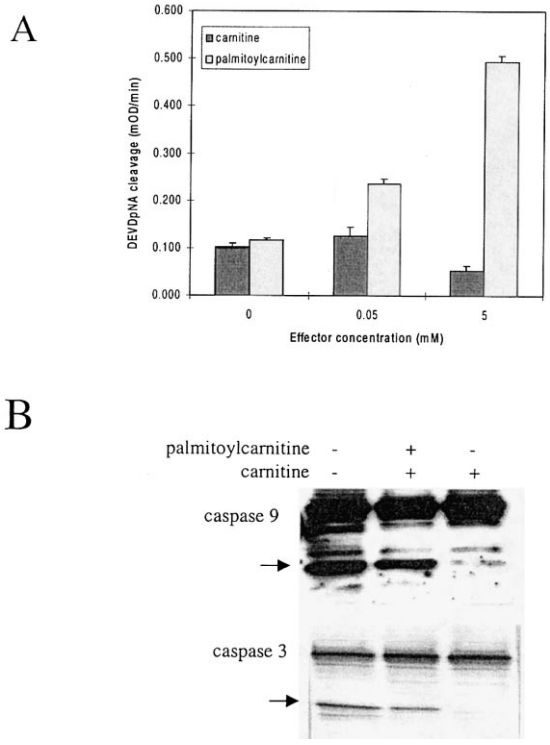

Fig. 3. Effect of L-carnitine and palmitoylcarnitine on the activity of recombinant caspase 8. (A) Different concentrations of L-carnitine and palmitoylcarnitine were added to 200 ng of recombinant caspase 8 and enzyme activities assayed as already described. The data shown are mean ± S.D. from a triplicate experiment. (B) S100 cytosols (100 μg) were incubated with 20 ng of recombinant caspase 8 for 2 h at 37°C in the presence or absence of 5 mM L-carnitine and 10 μM palmitoylcarnitine. 30 μg proteins were analyzed by Western blotting with anti-caspase 9 and anti-caspase 3 antibodies. 30 μg of protein was analyzed by Western blotting with anti-caspase 9 and anti-caspase 3 antibodies. Arrows indicate cleaved products.

Table 2
Alterations in the levels of carnitine and palmitoyl-CoA during Fas-mediated apoptosis

	Control (%) (± S.E.M.)	*P* value
Carnitine ($n=4$)	70 ± 9	< 0.05
Palmitoyl-CoA ($n=6$)	58 ± 7	< 0.05

Carnitine and palmitoyl-CoA levels were determined in Jurkat cells after 2 h incubation with anti-Fas antibody, or after 1–2 h incubation without antibody. Results are reported as percent of control values. Control values for carnitine were 0.42 ± 0.03 nmol/mg of cellular protein. Control values for palmitoyl-CoA were 198 ± 90 ng/10^8 cells. Apoptosis was determined, and only those experiments in which apoptosis was less than 5% in control cells and greater than 20% in Fas-ligated cells were included. Statistical significance was determined by paired Wilcoxon rank-sum for palmitoyl-CoA and by one-sample *t*-test for carnitine.

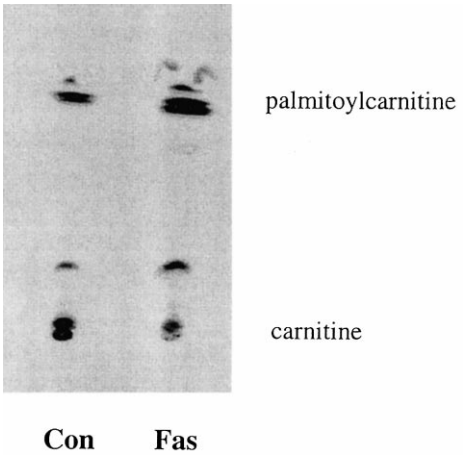

Fig. 4. Effect of Fas ligation on palmitoylcarnitine formation. Jurkat cells were loaded with [^{3}H]carnitine for 3 h then resuspended in fresh media with or without anti-Fas antibody. After 1 h, palmitoylcarnitine was extracted in butanol and resolved by TLC and detected by autoradiography. **Con**: control; **Fas**: Fas-treated. This experiment is representative of three. Palmitoylcarnitine (upper spots) is increased after Fas ligation. Free carnitine remains at the origin, and although butanol extraction does not quantitatively recover free carnitine, relatively more free carnitine is detected in the control extracts than in the Fas-ligated cells, an observation which supports the direct measurements of carnitine reported in Table 2.

3.8. CPT I deficient fibroblasts show resistance to staurosporine-induced apoptosis

To test the possibility that CPT I may play an active role in promoting caspase activation during apoptosis, we examined the susceptibility to apoptosis in a fibroblast cell line derived from a patient with CPT I deficiency, compared to a normal fibroblast line. Treatment of the fibroblast cell lines with anti-Fas antibody did not lead to apoptosis, as measured by cell detachment, uptake of the YOPRO-1 dye and cleavage of caspases. On the other hand, another inducer of apoptosis, staurosporine, led to an increase in cell death as measured by the same parameters. Incubation of wild type fibroblasts with 5 and 10 μM staurosporine for 12 h led to an increase in the percentage of cell death, and this was significantly reduced in CPT I deficient fibroblasts (49% of wild type, $n = 3$, $P < 0.001$ by ANOVA), consistent with our hypothesis that conversion of carnitine to palmitoylcarnitine may enhance apoptosis.

4. Discussion

The results from this study suggest that caspases are subject to regulation by non-peptide metabolites. Our data show that carnitine can inhibit caspase activity at various points in the Fas ligation pathway. It can inhibit the activity of an initiator caspase, caspase 8, as well as the processing of caspase 9, thereby effectively inhibiting cleavage and activation of downstream caspases. Furthermore, carnitine also inhibited cleavage of caspase substrates by downstream caspases, making it a general caspase inhibitor, at least blocking one aspect of apoptosis. The concentration of carnitine needed to inhibit caspases is at the upper limits of levels seen in skeletal muscle and probably exceeds the normal concentration in lymphocytes. However, the finding that carnitine levels decrease during Fas-mediated apoptosis suggests that the endogenous carnitine may play a regulatory role. Palmitoylcarnitine reversed the inhibition by carnitine, and palmitoylcarnitine levels rose early in apoptosis. The mechanism by which carnitine and palmitoylcarnitine regulate caspase activity remains to be determined. Although a number of quaternary amines have been shown to be non-specific inhibitors of various enzymes, the ability of palmitoylcarnitine at a concentration of 10 μM to reverse this inhibition suggests that the interaction is biologically relevant. Palmitoylcarnitine increases caspase activity at concentrations as low as 1 μM, well below its critical micellar concentration.

These findings suggest that CPT I, which converts the substrates carnitine and palmitoyl-CoA into palmitoylcarnitine, may be involved in regulation of apoptosis. Our results show that levels of carnitine and palmitoyl-CoA decreased, while palmitoylcarnitine formation was increased. Additionally, palmitoylcarnitine reversed the inhibition of caspase activity by carnitine, which may suggest that under physiological conditions, the changes in the ratios of these metabolites may be important in regulating caspase activity. Accumulation of long-chain acyl carnitines has been reported in cardiac tissue during ischemia [28,29], most likely due to a cessation of β-oxidation. We suggest that this shift in the balance of carnitine and its acyl derivatives may contribute to apoptosis.

The possible involvement of CPT I in apoptosis was initially discovered based on the enhanced expression of its mRNA in the murine hematopoietic cell line, LyD9, when deprived of interleukin-3 [30], and also indirectly by its association with Bcl-2 [31]. Paumen et al. used etomoxir to inhibit CPT I, which they found increased apoptosis [31]. However, they used a concentration of etomoxir (200 μM) that would also inhibit CPT II, thereby preventing conversion of acylcarnitines back to acyl-CoA for β-oxidation [32,33]. Since they did not measure the relevant metabolites, it is impossible to conclude whether palmitoylcarnitine levels decreased or remained unchanged in their model. It was recently reported that carnitine inhibits an acid sphingomyelinase, thereby preventing ceramide generation [34,35]. Further investigation of fatty acid metabolism changes during apoptosis is needed to clarify the role of CPT I in regulating apoptosis.

The use of a simple metabolite like carnitine as an anti-apoptotic agent is very attractive since it is likely to be relatively non-toxic compared to synthetic inhibitors. The chemotherapeutic effects of L-carnitine have already been demonstrated in AIDS and Alzheimer's disease and ischemic injury [10–12]. It is worthwhile understanding how palmitoylcarnitine and carnitine may regulate apoptotic events under physiological conditions and in pathophysiologic states. For instance, during myocardial ischemia, palmitoylcarnitine levels increase greatly. We suggest that this would tend to favor caspase activation, leading to apoptosis. The use of CPT I inhibitors such as etomoxir should be re-evaluated in view of alteration of the ratio of carnitine to palmitoylcarnitine and consequent modulation of caspase activity. This may explain one beneficial aspect of etomoxir therapy for heart failure [36].

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