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Acetyl-L-carnitine suppresses thyroid hormone-induced and spontaneous anuran tadpole tail shortening

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Mitochondrial membrane permeability transition (MPT) plays a crucial role in apoptotic tail shortening during anuran metamorphosis. L-carnitine is known to shuttle free fatty acids (FFAs) from the cytosol into mitochondria matrix for β-oxidation and energy production, and in a previous study we found that treatment with L-carnitine suppresses 3, 3′, 5-triiodothyronine (T_3) and FFA-induced MPT by reducing the level of FFAs. In the present study we focus on acetyl-L-carnitine, which is also involved in fatty acid oxidation, to determine its effect on T_3-induced tail regression in *Rana rugosa* tadpoles and spontaneous tail regression in *Xenopus laevis* tadpoles.

The ladder-like DNA profile and increases in caspase-3 and caspase-9 indicative of apoptosis in the tails of T_3-treated tadpoles were found to be suppressed by the addition of acetyl-L-carnitine. Likewise, acetyl-L-carnitine was found to inhibit thyroid hormone regulated spontaneous metamorphosis in *X. laevis* tadpoles, accompanied by decreases in caspase and phospholipase A_2 activity, as well as non-ladder-like DNA profiles.

These findings support our previous conclusion that elevated levels of FFAs initiate MPT and activate the signaling pathway controlling apoptotic cell death in tadpole tails during anuran metamorphosis.

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Apoptosis, often called programmed cell death, is a genetically controlled response for eliminating unwanted cells during development as well as for the maintenance of tissue homeostasis (Kerr et al. 1974; Mignotte and Vayssiere 1998). Cell deletion in tadpole tail atrophy during anuran metamorphosis depends upon programmed cell death (Kerr et al. 1974). Mitochondria are thought to have an important role in the mechanism of apoptotic cell death in various organs as well as the shortening of tadpole tails (Kashiwagi et al. 2000, 2001; Kowaltowski et al. 2001; Kanno et al. 2004). Opening of the MPT pore is prerequisite for releasing cytochrome *c* (Cyt. *c*) to form apoptosomes, leading to the activation of the caspase-cascade (Li et al. 1997; Raff 1998; Hanada et al. 2003; Kanno et al. 2004).

Acetyl-L-carnitine (ALC) is an acetylated derivative of L-carnitine (LC) and plays an important role in carrying fatty acids across the inner mitochondrial membrane (Bremer 1983; Traina 2011). ALC suppresses the MPT, and so does cyclosporin A (CsA), which is a known inhibitor of the MPT (Di Lisa et al. 1985; Paradies et al. 1999; Kashiwagi et al. 2001; Nishimura et al. 2008). In addition, ALC was also found to suppress the serum deprivation-induced apoptosis of cultured neuronal cells (Furuno et al. 2001; Pillich et al. 2005; Xie et al. 2008). Since LC successfully inhibits oxidative damage to mitochondria (Nikula et al. 1985; Dhitavat et al. 2005; Virmani et al. 2005), suppression of the MPT coupled with fatty acid oxidation has been postulated to be responsible for the

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protective effect of ALC (Duan and Karmazyn 1990; Pastorino et al. 1993).

Thyroid hormones (THs), such as T_3 and thyroxine (T_4), are believed to trigger and control the entire process of amphibian metamorphosis, including tadpole tail shortening (Dodd and Dodd 1976). We have previously shown that catalase activity decreased in tails undergoing regression during spontaneous and T_4-induced metamorphosis (Kashiwagi 1995), while D-amino acid oxidase and urate oxidase activity increased, leading to accumulation of H_2O_2, resulting in tail apoptosis. We also found that T_4-enhanced shortening of the tadpole tail tip in a tissue culture system was accompanied by the activation of caspase-3 like protease, fragmentation and ladder formation of DNA (Kashiwagi et al. 1999). Su et al. (1997a, 1997b) found that T_3 stimulated apoptosis in cultured *X. laevis* tadpole intestinal epithelium cells in a CsA-inhibitable mechanism. Moreover, CsA was shown to suppress T_3-induced tail regression in tadpoles of the bullfrog *Rana catesbeiana* (Little and Flores 1992). We analyzed the suppression mechanism of CsA on T_3-induced tadpole tail shortening, and found that CsA suppressed tail shortening concomitant with the suppression of caspase-3 and -9 like protease, DNA fragmentation and DNA ladder formation (Hanada et al. 2003). In this context, we observed that thyroxine-induced mitochondrial MPT was suppressed not only by CsA but also by LC (Kashiwagi et al. 2001). These findings suggest that reactive oxygen species (ROS), mitochondrial MPT and caspase cascade are involved in the mechanism of apoptotic tadpole tail shortening.

The purpose of the present study is to further investigate the role of MPT and ALC in tail shortening.

MATERIAL AND METHODS

Chemicals

T_3 and proteinase K were obtained from Sigma Chemical Co. (St. Louis, MO, USA). ALC was provided by Lonza Japan (Tokyo, Japan). All other chemicals were of analytical grade and were obtained from Nacalai Tesque (Kyoto, Japan).

Animals

Animals were treated according to the basic principles expressed in International Guiding Principles for Biomedical Research Involving Animals (1985), as well as Policies and Procedures/Best Practices for Laboratory Animal Care by the Stanford University School of Medicine (< http://med.stanford.edu/compmed/animal_care/amphibians.html >).

Two amphibian species, the wrinkled frog *Rana rugosa* and the African clawed frog *Xenopus laevis*, were used in the present study.

Adult specimens of *R. rugosa* were raised in the laboratory. Ovulation was induced by injecting *R. catesbeiana* (bullfrog) pituitaries into the body cavity of mature females. Eggs from a single female were artificially fertilized with a chlorine-free water suspension of crushed male testes. Tadpoles were raised in chlorine free tap water at 26–27°C with changes of water every other day. They were fed on boiled spinach and staged according to the criteria of Taylor and Kollros (1946).

Adult *X. laevis* were derived from standard strains maintained by the Hiroshima University Institute for Amphibian Biology. Mating was induced by injecting human chorionic gonadotropin (hCG; Sigma) into the dorsal lymph sac (males 125 U, females 500–600 U). Matings were carried out by placing individual male-female pairs into separate tanks with 5–10 cm of chlorine free tap water at 19°C for 10–12 h. Tadpoles were maintained at 19–22°C, fed SERA Micron (Sera Heinsberg, Germany), and staged according to Nieuwkoop and Faber (1956).

Extraction of DNA and analysis of ladder formation

Tails were homogenized in a lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% Triton X-100), and analysis of DNA ladder formation was performed according to the techniques described previously (Kashiwagi et al. 1999; Hanada et al. 2003). DNA was extracted by incubating lysates in 0.3 mg ml^{-1} of proteinase K at 50°C for 30 min, and subsequently in 0.7 mg ml^{-1} RNase at 50°C for 30 min. The resultant DNA samples were subjected to 2% agarose gel electrophoresis at 100 V using a running buffer containing 45 mM Tris-HCl (pH 8.0), 45 mM boric acid, 5 µg ml^{-1} ethidium bromide and 1 mM EDTA. After electrophoresis agarose gels were observed under ultraviolet light.

Assay for caspase-3 and -9 like protease activity

The activity of caspase-3 and -9 like proteases in tadpole tails was assayed as described previously (Kashiwagi et al. 1999). Briefly, T_3-treated and untreated tails were lysed in 200 µl of buffer (50 mM Tris–HCl, pH 7.5, 0.5% Nonidet P-40, 0.5 mM EDTA, and 150 mM NaCl) on ice for 30 min, and then centrifuged at 15 000× g for 10 min. Supernatants were removed and incubated at 37°C for 1 h in 200 µl of reaction buffer (20 mM HEPES, pH 7.5, 0.1 M NaCl, and 5 mM dithiothreitol) containing 10 µM fluorogenic peptide substrate, either Ac-DEVD-MCA for caspase-3, or Ac-LEHD-MCA for caspase-9. Reactions were stopped by adding 0.2 M Gly-HCl buffer (pH 2.8). Fluorescence intensity of released 7-amino-4-methylcoumarin (AMC) was measured with a Hitachi 650-10 LC fluorospectrophotometer. Wave lengths were 355 nm for excitation and 460 nm for emission. One unit

of activity was defined as the amount of enzyme required for the formation of 1 µM AMC per h. Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Assay for phospholipase activity

To prepare cytosol and membrane fractions for assay purposes, tails were first homogenized in ice-cold homogenization buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM DTT, 1 mM PMSF, 2 µg ml^{-1} aprotinin and 2 µg ml^{-1} leupeptin). Homogenates were sonicated on ice using a Branson sonicator (15 s for 4 cycles at 30% output) and then centrifuged at 1000× g for 5 min at 4°C to remove unbroken cells and debris. The supernatant was further centrifuged at 100 000× g for 1 h at 4°C.

The resultant ultracentrifuged supernatant was the cytosol fraction, while the pellet, suspended in 50 µl of ice-cold homogenization buffer and sonicated on ice for four 10-s cycles, was the membrane fraction (Mitnaul et al. 2007).

Cytosol and membrane fraction phospholipase A_2 (PLA_2) was determined using an EnzChek phospholipase A_2 assay kit (Molecular Probes, Invitrogen) according to manufacture's instructions. PLA_2 activity was determined according to increase in fluorescence intensity using a microplate reader (Ex. 485 nm; Em. 538 nm).

Statistical analyses

Results are expressed as the mean ± SE. Data were analyzed by Student's *t*-test and χ^2-test. *p*-values below 0.05 are considered significant.

RESULTS

Effect of ALC on T_3-induced Rana rugosa tadpole tail shortening

Figure 1 shows the effect of ALC on tail shortening in T_3-treated and untreated tadpoles. TK stage X *Rana rugosa* tadpoles were raised at a population density of one individual per 50 ml in trays containing various solutions as follows: group-1 tadpoles were raised in Cl-free tap water, and group-2 and -3 tadpoles were raised in Cl-free tap water containing 1 mM ALC. After six days, 5×10^{-8} M of T_3 was added to the water of group-1 and -3, and treatment was continued for one day. At the end of T_3 treatment, group-1 tadpoles were returned to water only, while group-3 tadpoles to the ALC solution, and both groups were further kept for another five days. Group-2 tadpoles continued treatment for all 12 days with no addition of T_3. Control tadpoles were raised in water and not exposed to ALC or T_3. The results show that T_3 induces tail regression, and that this is suppressed by ALC. Group-2 (ALC only) showed no difference from untreated controls.

Effect of ALC on T_3 -induced R. rugosa tadpole tail apoptosis

Figure 2 shows the effect of ALC on DNA ladder formation in tails of T_3-treated and untreated *R. rugosa* tadpoles. For T_3-treated group-1 tadpoles, tail length decreased to approximately 40% of the untreated control level by the end of 3 days after completion of T_3-treatment, or day 10 of the experiment (Fig. 2A). This change in tail length parallels the observed marked increase in DNA

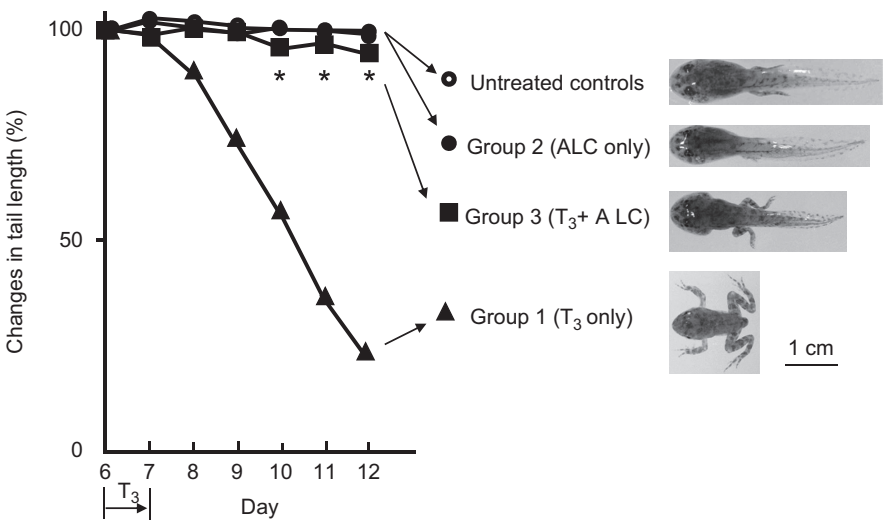

Fig. 1. Effect of acetyl-L-carnitine (ALC) on tail shortening in T_3-treated and untreated *R. rugosa* tadpoles. Each measurement was carried out using 3 individual tadpoles, and values given represent the mean. *Significantly greater ($P < 0.01$) than corresponding values for control tadpoles.

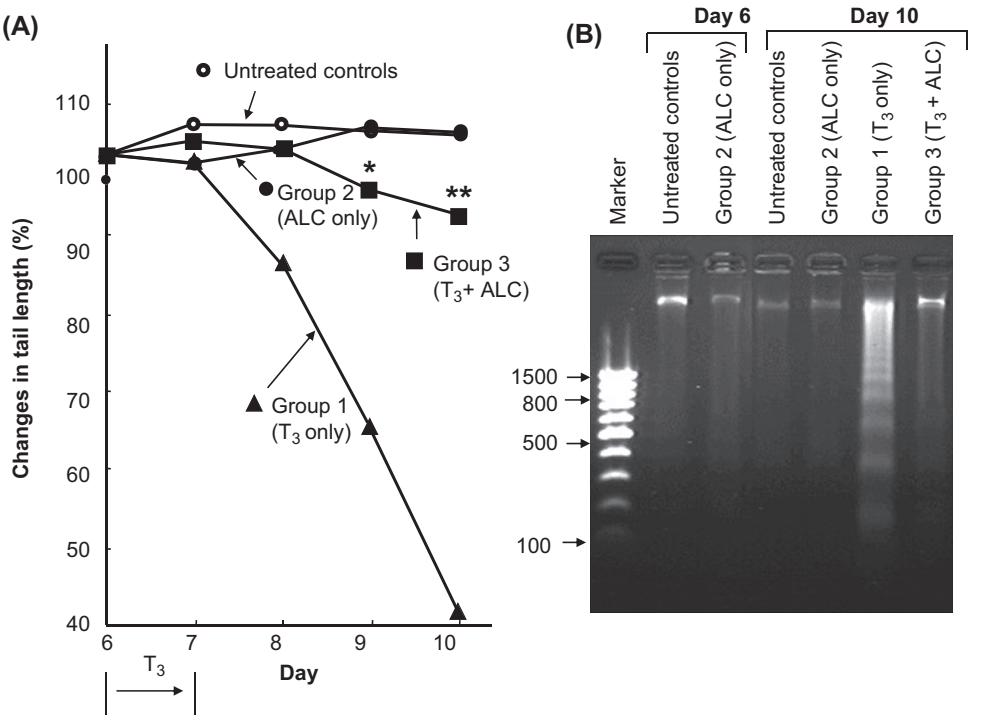

Fig. 2. Effect of acetyl-L-carnitine (ALC) on DNA ladder formation in tails of T_3-treated and untreated *R. rugosa* tadpoles. (**A**) DNA was extracted from the tail of each group shown in this figure. *Significantly greater ($P < 0.05$) than corresponding values for group-1 tadpoles. **Significantly greater ($P < 0.01$) than corresponding values for group-1 tadpoles. (**B**) Ladder formation. Experimental conditions were the same as described in Fig. 1. Each measurement was carried out using six individual tadpoles, and values given represent the mean.

laddering (Fig. 2B). Such changes were not observed in group-2 (ALC only), group-3 (T_3 + ALC) or untreated control tadpoles.

Effect of ALC on caspase-3 and -9 activity in tails of T_3-induced R. rugosa tadpoles

Caspase-3 and -9 like protease activity values were measured on the third day after completion of T_3-treatment (day 10). For T_3-treated group-1 tadpoles, caspase-3 activity showed a six-fold increase over the untreated control level, and caspase-9 activity a two-fold increase (Fig. 3A–B). In group-3 (T_3 + ALC) tadpoles, however, these changes were absent, indicating a suppression of the effects of T_3 by ALC.

Effect of ALC on spontaneous metamorphosis in X. laevis tadpoles

Xenopus laevis has been used as the animal of choice in cell biological and developmental studies. In tadpoles undergoing spontaneous metamorphosis (Fig. 4) endogenous plasma thyroid hormone levels have been shown to be low at prometamorphic stages 55–57, but increase rapidly at midclimax stages 61–62 at the onset of rapid tail regression (Leloup and Buscaglia 1977; Atkinson 1981).

Three NF stage 55 *X. laevis* tadpoles were maintained in trays containing 600 ml of 1 mM ALC solution. Tadpoles kept in Cl-free tap water served as untreated controls. The experiments were repeated three times. Figure 4 shows that ALC suppressed spontaneous metamorphosis.

Effect of ALC on tail shortening and DNA ladder formation in X. laevis tadpoles

Experimental conditions were the same as described above. Length of developing and regressing tails is defined as distance from the body terminus to the absolute tail tip, ranging from premetamorphic tadpole stage to the end of metamorphic climax. Figure 5 shows that ALC suppressed tadpole tail shortening and DNA ladder formation.

Effect of ALC on caspase-3 and -9 activity in spontaneously regressing tails of X. laevis tadpoles

Experimental conditions were the same as described above. Figure 6 shows changes in caspase-3 (A) and -9 (B) activity for tadpole tails of ALC-treated and untreated control tadpoles on day 0 and 14 of the experiment. Activity in control tails increased markedly between

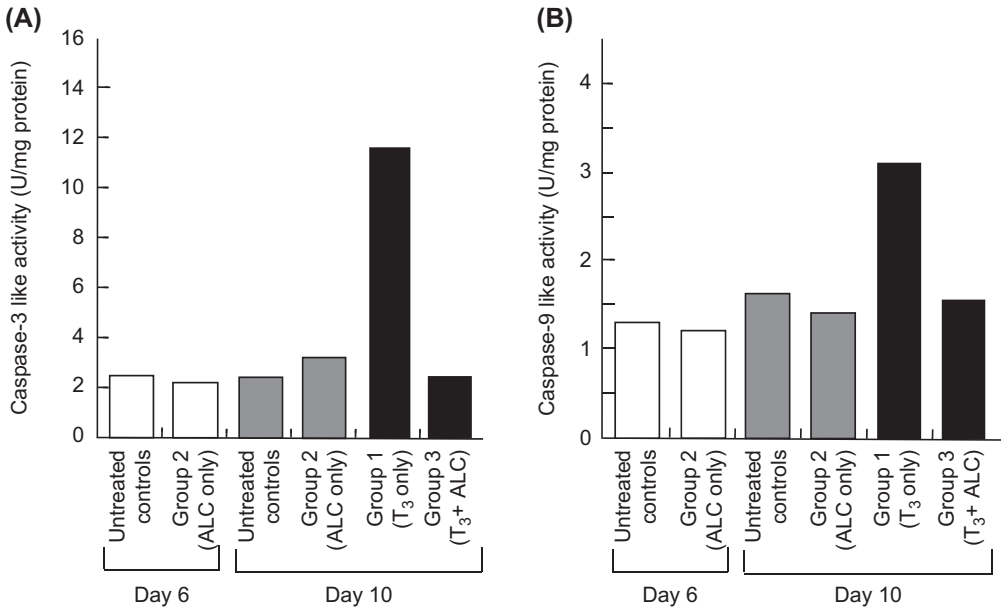

Fig. 3. Effect of acetyl-L-carnitine (ALC) on (**A**) caspase-3 and (**B**) -9 activity in tails of T_3-treated and untreated *R. rugosa* tadpoles. Experimental conditions were the same as described in Fig. 1. Caspase activity was measured using synthetic peptide as substrate. Each measurement was carried out using four individuals.

day 0 and 14, but was suppressed in the presence of ALC.

Effect of ALC on phospholipase A_2 activity in tails of X. laevis tadpoles

LC forms acyl-esters with amphipathic long chain fatty acids and plays an important role in the maintenance of mitochondrial function through β-oxidation-dependent ATP generation. This suggests that the toxic effects of high concentrations of FFAs generated by phospholipase A_2 (PLA_2) might be decreased by LC and / or ALC (Sagar and Das 1995). In order to test this possibility, PLA_2 activity was measured in the tails of ALC-treated and untreated control groups (Fig. 7). Experimental conditions were the same as described above. In control tadpole tails, activity increased slowly between premetamorphosis (stages 53) and middle metamorphic climax

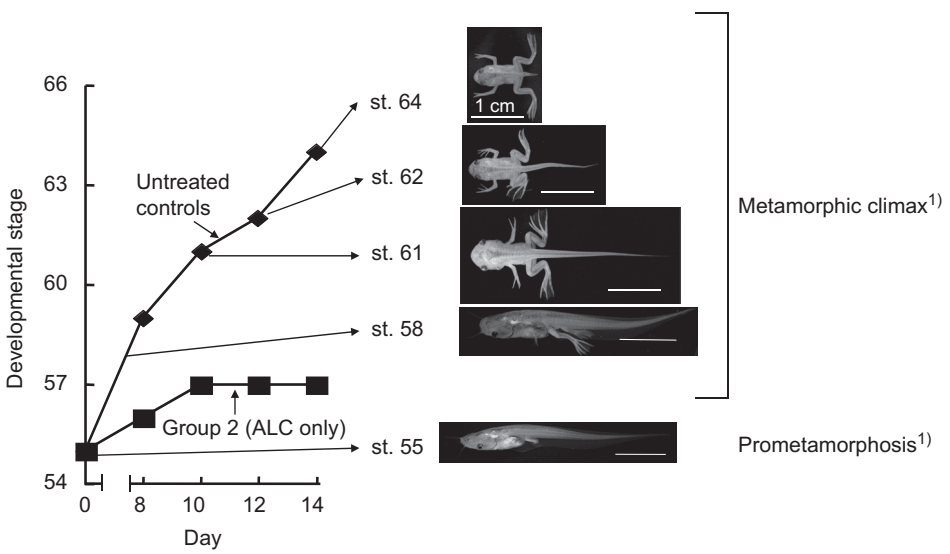

Fig. 4. Changes in tail length of *Xenopus laevis* tadpoles undergoing spontaneous metamorphosis and the suppressive effect of acetyl-L-carnitine (ALC). Staging was done according to Nieuwkoop and Faber (1956). We started experiments using prometamorphic stage 55 tadpoles. 1) Staging according to Dodd and Dodd (1976). Scale bar: 1 cm. Each measurement was carried out using four individuals.

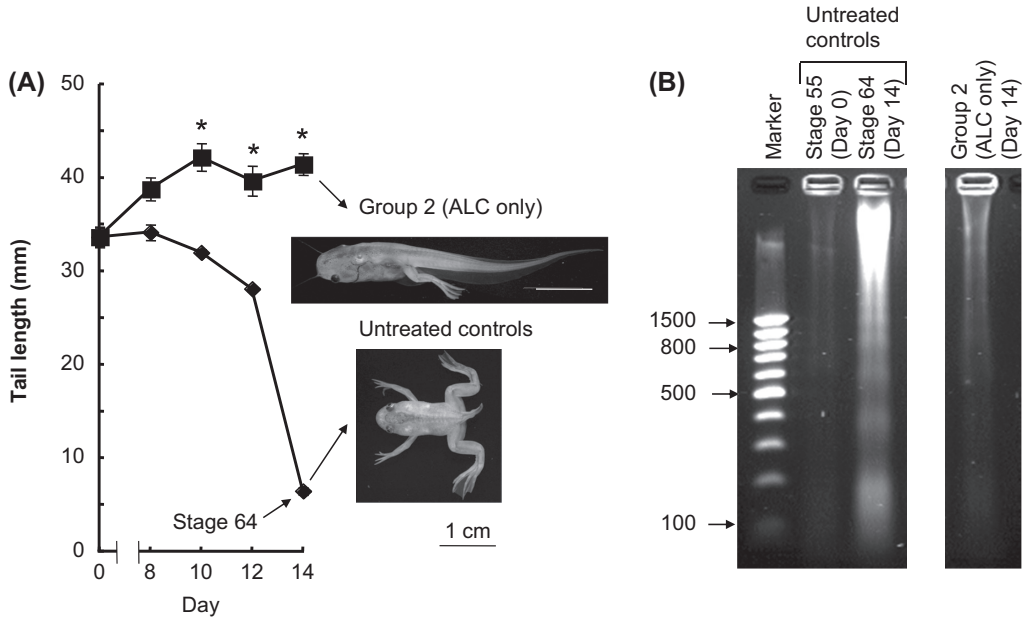

Fig. 5. Suppression of tail length shortening (**A**) and DNA ladder formation (**B**) in *X. laevis* tadpoles. Each measurement was carried out using four individuals, and values given represent the mean. *Significantly greater ($P < 0.01$) than corresponding values for untreated tadpoles.

(stage 63). A high (~1.3-fold above premetamorphosis levels) was reached at late climax stage 64 when the tail is markedly regressed. These changes were suppressed by ALC. The above findings suggest that PLA_2 activation in the tail leads to an increase in FFAs, and thus plays a crucial role in the mitochondria-dependent signaling pathway leading to apoptosis.

DISCUSSION

THs play important roles in animal metabolism, development and growth. Frog THs are structurally identical to mammalian THs (Kashiwagi et al. 2009), and frog TH receptors (TRs) are highly homologous to those of other vertebrates. T_4 generated in the thyroid gland is transported by T_4-transthyretin (TTR) to target tissues where it is converted into T_3, which is more biologically active than T_4. Nuclear TRα and TRβ form a heterodimer with a retinoid receptor (RXR), thereby binding to the TH response elements (TREs) in the target genes, followed by binding to T_3 to enhance gene transcription (Shi 2000). One of the most spectacular events during spontaneous amphibian metamorphosis is TH-dependent tadpole tail regression, which is the result of apoptosis of various types of cells, including epidermal cells, nerve cells, muscles, etc. (Rowe et al. 2005). Several investigators have shown that addition of T_3 or T_4 to the rearing solution of premetamorphic stage tadpoles results in an enhanced metamorphic process, including tail shortening (Hanada et al. 1997; Su et al. 1997a, 1997b; Kashiwagi et al. 1999). MPT is important in amphibian tadpole tail apoptosis. In a previous study we observed that T_3 induced the regression of *Rana rugosa* tadpole tails, accompanied by the activation of caspase-3 and -9, as well as an increase in DNA fragmentation and ladder formation, while CsA suppressed the effects of T_3 (Sagar and Das 1995; Hanada et al. 1997; Kashiwagi et al. 1999). Sachs et al. (1997) reported however that the expression level of *Bax* mRNA increased in *Xenopus laevis* tadpole tail muscle during spontaneous metamorphosis, and that early

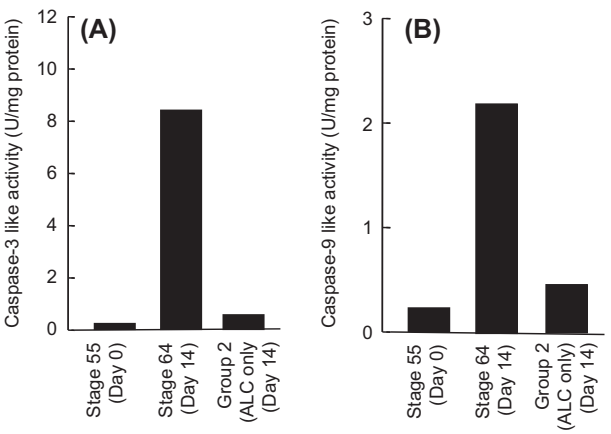

Fig. 6. Effect of acetyl-L-carnitine (ALC) on (**A**) caspase-3 and (**B**) -9 activity in tails of spontaneously developing *X. laevis* tadpoles. Each measurement was carried out using six individuals.

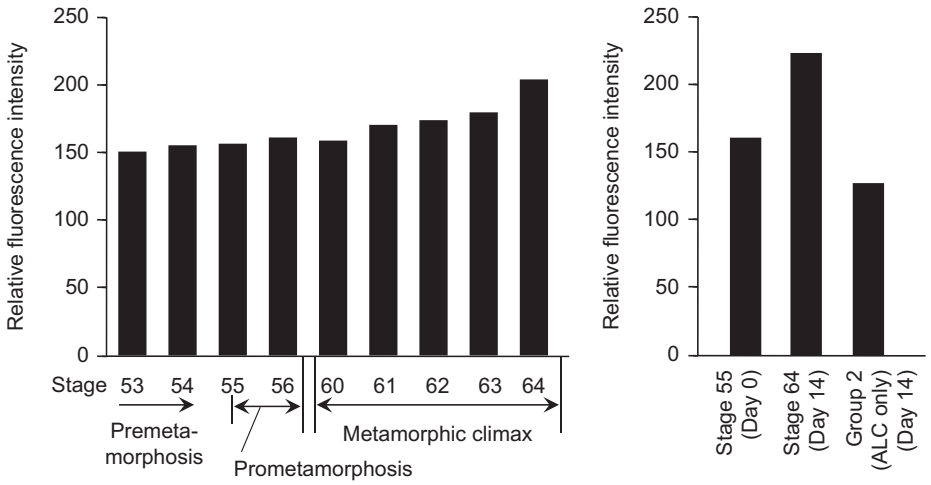

Fig. 7. Phospholipase A_2 (PLA_2) activity and acetyl-L-carnitine (ALC) suppression in *X. laevis* tadpole tails during spontaneous metamorphosis. Each measurement was carried out using six individuals.

treatment of tadpoles with T_3 also induced *Bax* expression. MPT-dependent and CsA-inhibitable apoptosis underlies the mechanism of T_3-enhanced activation of caspase-3 and -9 and tadpole tail regression (Hanada et al. 2003; Rowe et al. 2005). Moreover, we found that LC inhibited the ROS-induced MPT and T_3-induced apoptosis of the tails (Kashiwagi et al. 2001; Furuno et al. 2001; Pourahmad et al. 2001; Upadhyay et al. 2004). Frog tadpole tail thus provides perfect material for investigating a mitochondrial dependent signaling pathway leading to apoptosis.

The findings of the present investigation indicate that ALC suppresses an increase in various apoptotic indices in T_3-induced and spontaneously regressing tadpole tails.

Carnitine has been long thought to be a TH action antagonist, but its mechanism has remained obscure. Benvenga et al. (2000, 2004) reported inhibition by LC of both T_3 and T_4 entry into cell nuclei. T_3 however induced MPT and Cyt. *c* release, whereas LC and CsA inhibited this action (Kashiwagi et al. 2001), indicating that LC has a direct effect on mitochondria. Furthermore, an abrupt increase in MPT by T_3 was reported in experiments using rat liver mitochondria (Upadhyay et al. 2004).

Both free form LC and acetyl conjugates play a crucial role in mitochondrial β-oxidation of FFAs to produce ATP (Rubio-Gozalbo et al. 2004), and thus LC has a stabilizing action on mitochondrial function. Free forms of amphipathic long chain fatty acids are toxic in nature due to their detergent-like activity and hence perturb membrane/lipid bilayers of cells, leading to cell death (Six and Dennis 2000; Hardy et al. 2003).

The protective effect of LC against apoptosis seems to be responsible for the ATP synthesis and a decrease in local concentration of FFAs in and around mitochondria, resulting in stabilization of mitochondrial function. This hypothesis is supported by several studies indicating FFA-induced MPT of isolated mitochondria (Pastorino et al. 1993; Arita et al. 2001, 2003; Oyanagi et al. 2008) and apoptosis of a variety of cells including neuronal PC-12 cells by some sort of LC-inbibitable mechanism (Furuno et al. 2001; Hino et al. 2005; Zhu et al. 2008). Interestingly, the present study showed that T_3-induced tail regression was suppressed by ALC, which leads to a stabilization of mitochondrial membranes.

Cells contain a variety of PLA_2 isozymes, including extracellular, intracellular and mitochondria-bound forms, as well as calcium-dependent and -independent forms (Six and Dennis 2000; Burke and Dennis 2009). It is known that bromoenol lactone (BEL) is the selective inhibiter of Ca^{2+}-independent PLA_2 ($iPLA_2$) and p-bromophenacyl bromide (BPB) is the secretory PLA_2 ($sPLA_2$) inhibitor (Park et al. 2005; Song et al. 2005). In a preliminary study found that BEL strongly inhibits PLA_2 of *R. rugos*a tail, displaying remarkably high activity at metamorphic climax stage 64 without affecting Ca^{2+} and its chelate agent EGTA, while the suppressive effect of BPB is much weaker. These results suggest that Ca^{2+}-independent form ($iPLA_2$) of the isozymes might possible to be responsible for the degradation of membranous lipids to increase toxic free fatty acids in and around mitochondria. Furthermore, a recent investigation showed that $iPLA_2$ is important for the maintenance of mitochondrial function (Seleznev et al. 2006; Kinsey et al. 2007). This finding is inconsistent with the above-described notion. However, a possible involvement of PLA_2 activation in spontaneously regressing and T_3-induced tadpole tails requires further clarification.

It has been shown that FFAs induce MPT (Sulton and Sokolove 2001), which is thought to be a factor of tail

shortening. Hence, it seems likely that the enhancement of β-oxidation suppresses FFA accumulation, resulting in a decrease in MPT, inhibiting apoptosis, but further studies are needed to demonstrate the generation of FFAs.

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