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Prevention of paclitaxel-evoked painful peripheral neuropathy by acetyl-L-carnitine: Effects on axonal mitochondria, sensory nerve fiber terminal arbors, and cutaneous Langerhans cells

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Abstract

Prophylactic treatment with acetyl-L-carnitine (ALCAR) prevents the neuropathic pain syndrome that is evoked by the chemotherapeutic agent, paclitaxel. The paclitaxel-evoked pain syndrome is associated with degeneration of the intraepidermal terminal arbors of primary afferent neurons, with the activation of cutaneous Langerhans cells, and with an increased incidence of swollen and vacuolated axonal mitochondria in A-fibers and C-fibers. Previous work suggests that ALCAR is neuroprotective in other nerve injury models and that it improves mitochondrial dysfunction. Thus, we examined whether the prophylactic efficacy of ALCAR was associated with the prevention of intraepidermal terminal arbor degeneration, the inhibition of Langerhans cell activation, or the inhibition of swelling and vacuolation of axonal mitochondria. In animals with a confirmed ALCAR effect, we found no evidence of a neuroprotective effect on the paclitaxel-evoked degeneration of sensory terminal arbors or an inhibition of the paclitaxel-evoked activation of Langerhans cells. However, ALCAR treatment completely prevented the paclitaxel-evoked increase in the incidence of swollen and vacuolated C-fiber mitochondria, while having no effect on the paclitaxel-evoked changes in A-fiber mitochondria. Our results suggest that the efficacy of prophylactic ALCAR treatment against the paclitaxel-evoked pain may be related to a protective effect on C-fiber mitochondria.

Keywords

Allodynia; Chemotherapy; Hyperalgesia; Langerhans cell; Neuroprotection; Neurotoxicity

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Introduction

Paclitaxel (Taxol®) is one of the most effective and commonly used anti-neoplastic drugs. Its dose-limiting side effect is peripheral neuropathy, which in many patients is accompanied by chronic neuropathic pain (for reviews see Polomano and Bennett, 2001; Dougherty et al., 2004). Painful peripheral neuropathy occurs with other agents in the taxane class, as well as with chemotherapeutics in the vinca alkaloid and platinum-complex classes. The cause of the neuropathy and of the pain syndrome is unknown.

Acetyl L-carnitine (ALCAR) is a naturally occurring amino acid derivative that plays an essential role in transporting long-chain free fatty acids into mitochondria (reviewed in Fritz and Yue, 1963; Rebouche, 2004). Acute administration of ALCAR temporarily reverses the paclitaxel-evoked neuropathic pain state that is seen in experimental animals (Pisano et al., 2003; Ghirardi et al., 2005; Flatters et al., 2006; Xiao and Bennett, 2007). In addition, we have shown that a prophylactic dosing protocol using daily administration of ALCAR during the exposure to paclitaxel and for the period preceding the appearance of the pain syndrome completely prevents the development of allodynia and hyperalgesia (Flatters et al., 2006).

We have shown that rats with paclitaxel-evoked painful peripheral neuropathy have a significantly increased incidence of axonal mitochondria that are swollen and vacuolated (Flatters and Bennett, 2006). ALCAR treatment ameliorates mitochondrial dysfunction caused by a variety of neurotoxic insults (Virmani et al., 1995, 2005; Bertamini et al., 2002; Hart et al., 2004a; Sayed-Ahmed et al., 2004; Calabrese et al., 2005; Binienda et al., 2006; Mansour, 2006; Wang et al., 2007). Paclitaxel also causes degeneration of the sensory neurons' intraepidermal terminal arbors, without degeneration of the parent axons in the peripheral nerve, and it activates cutaneous Langerhans cells (Polomano et al., 2001; Flatters and Bennett, 2006; Siau et al., 2006). ALCAR has been shown to have neuroprotective and pro-regenerative effects following primary afferent neuron axotomy (Fernandez et al., 1991; Chiechio et al., 2006; Hart et al., 2002, 2004a; Wilson et al., 2003, 2007), in the peripheral neuropathy produced by antiretroviral chemotherapy (Hart et al., 2004b; Youle et al., 2007), and in diabetic peripheral neuropathy (Lowitt et al., 1995; Pacifici et al., 1992; Sima et al., 1996, 2005; DeGrandis and Minardi, 2002).

Thus, the protective effect of the prophylactic ACLAR treatment protocol might have one or more mechanisms of action: (1) prevention of the degeneration of intraepidermal nerve fibers, (2) inhibition of the activation of cutaneous Langerhans cells, or (3) prevention of paclitaxel's effect on axonal mitochondria. The present study examined each of these possibilities.

Materials and methods

These experiments conformed to the ethics guidelines of the International Association for the Study of Pain (Zimmermann, 1983), the National Institutes of Health (USA), the Canadian Institutes of Health Research, and the Canadian Council on Animal Care. All experimental protocols were approved by the Animal Care Committee of the Faculty of Medicine, McGill University.

Animals

Adult male Sprague-Dawley rats (250–300g, Harlan Inc., Indianapolis, IN; Frederick, MD breeding colony) were housed on sawdust bedding in plastic cages. Artificial lighting was provided on a fixed 12h light-dark cycle and food and water were available ad libitum.

Drug administration

Paclitaxel, 2 mg/kg per ml, was prepared by diluting Taxol®, (Bristol-Myers-Squibb: 6 mg/ml paclitaxel in Cremophor/EL vehicle) with saline. Rats were given intraperitoneal (IP) injections of paclitaxel on 4 alternate days (day 0 (D0), D2, D4 and D6) as described previously (Polomano et al., 2001). Paclitaxel-treated rats were administered either acetyl-L-carnitine hydrochloride (100 mg/kg in distilled water; Sigma-Aldrich, Oakville, ON) or vehicle (distilled water) via oral gavage (1.0 ml/kg) for 21 consecutive days (Flatters et al., 2006). ALCAR dosing began on the day of the first paclitaxel injection (D0). There is a delay of 10–14 days between the last paclitaxel injection and the onset of statistically significant pain hypersensitivity (Flatters and Bennett, 2004); the time of onset of the pain-producing pathology is thus uncertain. Therefore, we continued ALCAR dosing until D20.

Behavioral testing

Following habituation to the behavioral testing environment, baseline tests of mechanical sensitivity were conducted as previously described (Flatters and Bennett, 2004, 2006). The animals were placed on an elevated wire mesh floor and confined beneath overturned mouse cages made of clear plastic. von Frey filaments with bending forces of 4 g and 15 g were applied to the mid-plantar skin (avoiding the base of the tori) of each hind paw 5 times, with each application held for 5 seconds. Withdrawal responses from both hind paws were counted and then expressed as an overall percentage response. Normal rats rarely withdraw from the 4 g stimulus; the increased level of responding seen after paclitaxel treatment is thus indicative of mechano-allodynia. Normal animals withdraw from the 15 g stimulus 15–20% of the time (e.g., Flatters and Bennett, 2004, 2006); the increased level of responding seen after paclitaxel treatment is thus indicative of mechano-hyperalgesia. Sensitivity to heat was not assessed because the paclitaxel-evoked pain model produces no more than a minor and transient heat hyperalgesia (Polomano et al., 2001).

ALCAR-treated and vehicle-treated rats were sacrificed on D30 for immunocytochemistry and D42 for electron microscopy. The paclitaxel-evoked pain syndrome reaches maximal severity at approximately D22 and plateaus at this level for at least a month. The IENF loss is also at a maximal level by D22 and also plateaus for at least one month (Jin et al., unpublished data). Prior to sacrifice, we confirmed that significant paclitaxel-evoked mechano-allodynia and mechano-hyperalgesia were present in the vehicle-treated animals and that significant analgesia was present in the ALCAR treated animals.

Immunocytochemistry for intraepidermal nerve fibers and Langerhans cells

Rats were overdosed with sodium pentobarbital (100 mg/kg, IP) and perfused transcardially with 200 ml of perfusion buffer (0.01M phosphate buffered saline (PBS) containing 0.05% sodium bicarbonate and 0.1% sodium nitrite) followed by 250 ml of freshly prepared 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. As described previously (Siau et al., 2006), hind paw glabrous skin was excised, post-fixed overnight, and cryoprotected for 24 h in 30% sucrose in PB. The tissue was then embedded in optimum cutting temperature compound (TissueTek) and stored at −80° C. Cryostat sections (30 μm) were collected in PBS containing 0.2% Triton-X 100 (PBS + T). Following a 1 h incubation in PBS + T containing 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories; Mississauga, ON) at room temperature, sections were incubated in rabbit anti-human protein gene product 9.5 (PGP9.5) primary antibody (Research Diagnostics; Flanders, NJ) diluted 1:6400 in PBS + T containing 5% NDS for 24 h at 4° C. After rinsing in PBS + T, sections were incubated in donkey anti-rabbit IgG secondary antibody labeled with Cy3 (Jackson ImmunoReseach) diluted 1:200 for 1.5 h. Control sections incubated without primary antisera showed no specific staining. Sections from naïve rats (i.e., neither paclitaxel nor ALCAR treatment; n=7) were

stained concurrently with the sections from the paclitaxel-treated rats that had received ALCAR (n = 8) or vehicle injections (n = 6).

Quantification of intraepidermal nerve fibers and Langerhans cells

Intraepidermal nerve fibers (IENFs) and Langerhans cell (LC) counts were done by an observer blind as to the animal's group assignment using a Zeiss Axioplan 2 Imaging fluorescence microscope as described previously (Siau et al., 2006). Using a 40X objective, all ascending nerve fibers that were seen to cross into the epidermis were counted; no minimum length was required, and fibers that branched within the epidermis were counted as one. LCs were counted if their cell body and at least one of their processes were stained.

A low magnification montage of each section was made and the length of the epidermal border was measured. IENF and LC counts were expressed as the number per centimeter of epidermal border. One section of skin was analyzed for each rat in each group: naïve rats that had not received any drug (n = 7), paclitaxel-treated rats that received ALCAR treatment (n = 8), and paclitaxel-treated rats that received vehicle treatment (n = 6). The average section length was 11.9 mm (range: 8.5–15.0 mm) and thus the amount of tissue sampled per animal met or exceeded clinical guidelines for IENF quantification (Lauria et al., 2005a).

Electron microscopy of axonal mitochondria

Naive rats, ALCAR-treated rats, and vehicle-treated rats (n = 4/group) were overdosed with sodium pentobarbital and perfused transcardially with the vascular rinse described above followed by 250 ml of freshly prepared 1% glutaraldehyde and 1% paraformaldehyde in 0.1M PB, pH 7.4. As in our previous study (Flatters and Bennett, 2006) we examined mitochondria in the saphenous nerve, which contains only sensory axons. A segment (ca. 5 mm) of the nerve was dissected at the mid-thigh level and post-fixed for 3 h in the fixative described above. Nerve segments were then transferred to 10% sucrose in 0.1 M PB and kept at 4° C for a minimum of 12 h and subsequently incubated in 1% osmium tetroxide in 0.1 M PB, pH 7.4, at 4° C for 2 h, dehydrated in ascending concentrations of alcohol and propylene oxide at room temperature, and embedded in Epon. Sections (70 nm) were acquired with an ultramicrotome using a diamond knife, collected on Formvar-coated grids, and counterstained with lead citrate and uranyl acetate. Grids were observed in a Philips EM410 electron microscope operated at 80 kV. Photographs were taken and analyzed using a Megaview II CCD camera and AnalySIS 5.0 software (both from Soft Imaging System Corp., Lakewood, CO). Electron photomicrographs were taken for analysis at 3,130X for orientation and at 24,400X for counts of mitochondria. The analysis and quantification were performed by an observer who was blind as to group assignment.

Analysis of axonal mitochondria

In order to obtain random samples of unmyelinated and myelinated axons, Remak bundles that had the nuclei of their Schwann cells (nucleated Remak bundles, NRBs) in the plane of section were identified at low magnification (3,130X). The search for NRBs began at one randomly chosen hole of the grid and progressed through each hole in succession. All C-fibers within each NRB were photographed in their entirety at 24,400X. Myelinated axons that surrounded the selected NRBs were photographed at the same magnification. Each myelinated axon was imaged in its entirety; at the selected magnification this usually required the construction of a photomicrographic montage. NRBs were selected one by one until more than 60 C-fibers and 60 myelinated axons per animal had been photographed; this usually required no more than one section per animal. We made no attempt to differentiate Aδ and Aβ myelinated axons because A-fiber nociceptors in the rat can not be identified on the basis of their diameter- many have large axons conducting in the Aβ range (Djouhri and Lawson, 2004).

We counted all of the mitochondria in each axon. As in our previous study (Flatters and Bennett, 2006), normal mitochondria were identified as circular or oval profiles with double membranes and cristae that had at least one axis of at least 165 nm in length. Atypical mitochondria were identified with criteria modified from our previous report (Flatters and Bennett, 2006). As before, mitochondria were classified as atypical if they were swollen (an approximate 2- to 3-fold increase in diameter) and/or vacuolated (50% or more of the area appearing electron-lucent). Atypical mitochondria usually appeared as swollen structures with both large vacuoles and an accumulation of electron-dense material at one pole. This electron-dense material is most likely composed of collapsed cristae because particularly electron-dense linear structures are usually visible within the accumulation (Flatters and Bennett, 2006). Our prior criteria for the identification of an atypical mitochondrion required that a double membrane be present along the entire circumference of the organelle. For the current study, we decided that this criterion was too conservative because swelling may pull the inner membrane away from the outer membrane over part of the circumference (Higgins et al., 2003). Removing the criterion for a complete double membrane resulted in a clearly larger number of mitochondria being counted as atypical and it is thus not possible to equate the numbers obtained here with those of the prior work (Flatters and Bennett, 2006). However, the phenomenon of interest (the relative increase of atypical mitochondria after paclitaxel treatment) seen previously is plainly evident in the current work as well. We express the incidence of atypical mitochondria in C-fibers and A-fibers as the number of atypical mitochondria relative to the total number of mitochondria observed.

Statistics

Data were analyzed using InStat (version 3.0; GraphPad, Inc.; San Diego, CA, USA). Between-groups comparisons for the behavioral data were analyzed with unpaired t-tests. The IENF and LC counts were analyzed with one-way ANOVAs followed by the Bonferroni multiple comparisons test. Between-groups comparisons of the proportion of atypical mitochondria used the Chi-square statistic. Significance was accepted at $p < 0.05$.

Results

Behavioral assays of the analgesic effects of ALCAR treatment

As shown in Fig. 1, on D25-D27 the expected mechano-allodynia and mechano-hyperalgesia were present in the paclitaxel-treated rats that received vehicle injections. ALCAR treatment completely prevented the paclitaxel-evoked mechano-allodynia and mechano-hyperalgesia.

Intraepidermal nerve fiber counts

IENFs emerged from subepidermal cutaneous nerve fascicles and traveled vertically into the epidermis where they branched into terminal arbors consisting of multiple fine branches bearing terminal and *en passant* boutons (Fig. 2A). In the paclitaxel-treated animas, the presence of PGP9.5-positive LCs did not hinder the IENF counts because a fiber was tallied when it was seen to cross the border between the dermis and epidermis, where LC processes are sparse. Even within the epidermis, IENFs and LC processes are easily discriminated as they have different shapes and orientations (Fig. 2A,B).

In the naïve rats we counted 332 ± 23 (mean ± S.E.M.) IENFs per cm of epidermal border (Fig. 3A). Paclitaxel-treated rats that received vehicle treatment had 254 ± 24 IENFs per cm, a statistically significant reduction of 24% relative to the naïve group. Paclitaxel-treated rats that received the ALCAR treatment had 279 ± 15 IENFs per cm; this is a significant 16% reduction relative to the naïve rats. The difference between the vehicle-treated and ALCAR-treated groups was not statistically significant.

Langerhans cell counts

In naïve rats we detected a relatively small number of lightly stained LCs, while numerous intensely stained LCs were seen in paclitaxel-treated animals (Fig. 2A,B).

In the naïve rats we counted 68 ± 14 LCs per cm (Fig. 3B). Paclitaxel-treated rats that received vehicle injections had 163 ± 28 LCs per cm; a statistically significant increase of 140% compared to the naïve group. Paclitaxel-treated rats that received ALCAR treatment had 139 ± 18 LCs per cm; a significant increase of 104% relative to the naïve group. The difference between the vehicle-treated and ALCAR-treated groups is not statistically significant.

Mitochondria

We examined 659 mitochondria in 849 C-fibers. In the naïve group, 26.2% (54/206) of C-fiber mitochondria were atypical (Fig. 5A). In the paclitaxel-treated group that received vehicle treatment, 45.3% (115/254) of C-fiber mitochondria were atypical; this is a statistically significant increase relative to the naïve group. In the paclitaxel-treated group that received ALCAR, 25.1% (50/199) of C-fiber mitochondria were atypical. The difference between the vehicle-treated and the ALCAR-treated groups is statistically significant. Moreover the difference between the naïve value and the value for the ALCAR-treated group is not significantly different; indicating that ALCAR completely prevented the paclitaxel-evoked increase in atypical C-fiber mitochondria.

We examined 4,512 mitochondria in 786 A-fibers. In the naïve group, 34.5% (516/1494) of A-fiber mitochondria were atypical (Fig. 5B). In the paclitaxel-treated group that received vehicle treatment, 47.9% (775/1617) of A-fiber mitochondria were atypical; this is a statistically significant increase relative to the naïve group. In the paclitaxel-treated group that received ALCAR, 45.3% (634/1401) of A-fiber mitochondria were atypical; this is also a statistically significant increase relative to the naïve group. The difference between the vehicle-treated and the ALCAR-treated groups is not statistically significant.

Discussion

We found that an ALCAR treatment protocol that completely prevented the appearance of paclitaxel-evoked neuropathic pain also completely prevented the paclitaxel-evoked increase in the incidence of swollen and vacuolated mitochondria in C-fibers, but not in A-fibers. We found no evidence that ALCAR has a neuroprotective effect against paclitaxel-evoked degeneration of IENFs and no evidence of an effect on the paclitaxel-evoked activation of cutaneous LCs.

The effect on axonal mitochondria

Paclitaxel-treatment caused a significant increase in the incidence of atypical mitochondria. Atypical mitochondria are not a fixation artifact, but rather a consequence of the physiological state of the mitochondria at the time of its exposure to aldehydes (see Flatters and Bennett, 2006). We have hypothesized (Flatters and Bennett, 2006) that this may be related to a paclitaxel effect on the mitochondrial permeability transition pore (mPTP). The mPTP is a multi-molecular complex containing the voltage-dependent anion channel (VDAC), which spans the mitochondrial outer membrane. Isolated mitochondrial membrane fragments that contain the VDAC also contain β-tubulin (Carre et al., 2002). Paclitaxel binds to β-tubulin, suggesting a site of action for a paclitaxel effect on the mPTP. In non-neural cells, paclitaxel is known to cause mPTP opening, resulting in swollen and vacuolated mitochondria that are functionally impaired (Evtodienko et al., 1996; Andre et al., 2000; Vabiro et al., 2001; Kidd et al., 2002). There is evidence suggesting that ALCAR can prevent mPTP opening (Pastorino et al., 1993; Starkov et al., 1994). The paclitaxel-evoked opening of the mPTP has also been

shown to cause calcium release from mitochondria (Evtodienko et al., 1996; Kidd et al., 2002) and we have shown that calcium chelating agents reverse paclitaxel-evoked pain (Siau and Bennett, 2006).

ALCAR gave complete protection against the paclitaxel-evoked increase in atypical mitochondria in C-fibers but had no effect in A-fibers. We expected ALCAR to have comparable effects in A-fibers and C-fibers because we found, with the same treatment protocol used here, that ALCAR produced about the same decrease (50%) in the incidence of spontaneous discharge in both C-fibers and A-fibers (Xiao and Bennett, 2007). It may be that ALCAR inhibits spontaneous discharge in A-fibers via a mechanism different from that in C-fibers. Moreover, it may be that the myelin sheath allows less ALCAR to enter A-fibers, or that mitochondria in A-fibers and C-fibers differ in their metabolism of long-chain free fatty acids. Impulse transmission in A-fibers is far more sensitive to energy depletion (anoxia) than in C-fibers, suggesting that there may be differences in their mitochondrial metabolism. A difference in basal metabolic state is suggested by the larger percentage of atypical A-fiber mitochondria compared to C-fiber mitochondria in the naïve animals (Fig. 5).

ALCAR and the paclitaxel-evoked degeneration of IENFs

Paclitaxel doses that cause degeneration of axons at the peripheral nerve level (high doses relative to those used here) result in a very significant accumulation of drug within peripheral nerve (Cavaletti et al., 2000). It is thus probable that accumulation in nerve also occurs with the low dose used in our protocol. The accumulation of paclitaxel within nerve is due largely to its binding to the β-tubulin polymers that form axonal microtubules (Nogales et al., 1995). However, there is no convincing evidence that paclitaxel affects axonal transport, at least at drug levels relevant to the pain state produced by low doses (reviewed in Flatters and Bennett, 2006). Nevertheless, binding to microtubules would depot drug and increase the exposure of axonal mitochondria.

The consequences of exposure would be most severe in places with the highest concentration of mitochondria, and mitochondria concentrate at places with high metabolic demand (Chen and Chan, 2006). IENFs, the terminal receptor arbors of sensory neurons, are likely to be places of high metabolic demand. There is evidence that some, but not all, kinds of sensory terminals do contain very high concentrations of mitochondria (Byers and Yeh, 1984; Byers, 1985; Heppelmann et al., 1994, 2001). We thus hypothesized that the paclitaxel-evoked loss of IENFs would be inhibited by ALCAR treatment. Although we found an effect on C-fiber mitochondria at the level of the nerve, we found no effect on IENFs, many of which are likely to arise from C-fibers. It may be that paclitaxel-evoked mitochondrial dysfunction is especially severe at the level of sensory terminal arbors, and thus relatively resistant to ALCAR's beneficial effect.

We conclude that ALCAR's analgesic effect is not due to a neuroprotective action in the paclitaxel-evoked painful peripheral neuropathy model. Our results are in contrast to studies that demonstrate neuroprotective and pro-regenerative ALCAR effects in other nerve injury models (Fernandez et al., 1991; Pacifici et al., 1992; Lowitt et al., 1995; Sima et al., 1996, 2005; Hart et al., 2002, 2004a, 2004b; DeGrandis and Minardi, 2002; Wilson et al., 2003, 2007; Chiechio et al., 2006; Youle et al., 2007). Pisano et al. (2003) showed that a prophylactic ALCAR treatment protocol prevented the decrease in the sensory nerve conduction velocity (SNCV) seen after paclitaxel and cisplatin. They used the same dose of ALCAR as used here (100 mg/kg), but their cumulative dose of paclitaxel was greater than ours (25 mg/kg vs. 8 mg/kg). A high dose of paclitaxel like that used by Pisano et al. (2003) will cause degeneration of peripheral nerve axons (Authier et al., 2000; Cavaletti et al., 1995, 1997; Cliffer et al., 1998; Lauria et al., 2005b; Jimenez-Andrade et al., 2006; Peters et al., 2007) and one would thus expect a decrease in SNCV. However, the low-dose paclitaxel model that we use evokes IENF degeneration without degeneration of the parent axon in the peripheral nerve (Polomano et al.,

2001; Flatters and Bennett, 2006). The SNCV is evoked by stimulation of the peripheral nerve and would not be affected if degeneration was confined to the IENFs. These observations suggest the possibility that low-dose paclitaxel-evoked IENF degeneration and high-dose paclitaxel-evoked degeneration of peripheral nerve axons may be due to different mechanisms with different sensitivity to ALCAR treatment.

ALCAR and the paclitaxel-evoked activation of Langerhans cells

With antisera dilutions optimized for the demonstration of nerve fibers, LCs from normal animals are very lightly stained. The PGP9.5 antigen (an ubiquitin carboxyl-terminal hydrolase; Wilkinson et al., 1989) is dramatically up-regulated following paclitaxel treatment, resulting in a large increase in the number of visible cells (Siau et al., 2006). The significance of this is unknown. LCs are the skin's resident immuno-surveillance dendritic cells and their activation following paclitaxel may be due to an immunological effect of paclitaxel itself or to the appearance of self-antigens as a consequence of IENF degeneration. ALCAR treatment had no significant effect on LC activation, while paclitaxel-evoked pain was completely prevented. This result suggests that LC activation may not be a causative factor in paclitaxel-evoked pain.

The analgesic effect of the prophylactic ALCAR dosing protocol

ALCAR has a large number of actions that produce acute analgesic effects (Patacchioli et al., 1989; Piovesan et al., 1995; Taglialatela et al., 1994; Ghelardini et al., 2002; Calabrese et al., 2005; Zanelli et al., 2005; Binienda et al.,. 2006; Chiechio et al., 2006). It seems unlikely that such mechanisms could account for ALCAR's ability to completely prevent paclitaxel-evoked neuropathic pain. Our results suggest that the prevention of paclitaxel-evoked neuropathic pain produced by prophylactic treatment with ALCAR may be due to a protective effect on C-fiber mitochondria. Such an effect might prevent paclitaxel-evoked spontaneous discharge in C-nociceptors (Xiao and Bennett, 2007) and thus prevent the initiation of the central sensitization mechanisms that promote allodynia and hyperalgesia.

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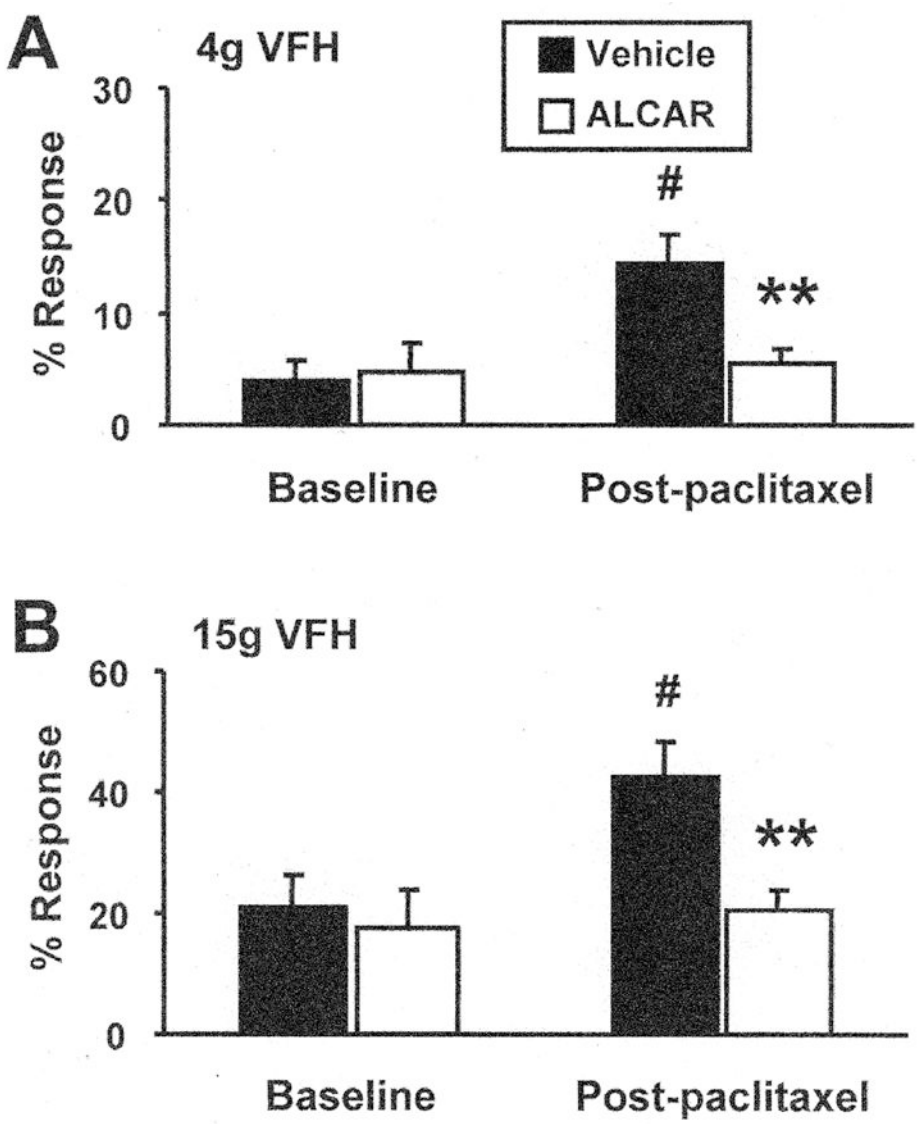

Figure 1.

The effect of ALCAR on paclitaxel-evoked pain. The expected mechano-allodynia (A) and mechano-hyperalgesia (B) developed in vehicle-treated rats. Prophylactic treatment with ALCAR completely prevented both mechano-allodynia and mechano-hyperalgesia. Baseline values are the average of tests done on three separate days prior to paclitaxel treatment. Paclitaxel effects were measured in vehicle-treated (n = 6) and ALCAR-treated (n = 8) rats on D25-D27. Means ± S.E.M. Note that the ordinates differ. # $p < 0.01$ compared to baseline; ** $p < 0.01$ compared to vehicle-treated group.

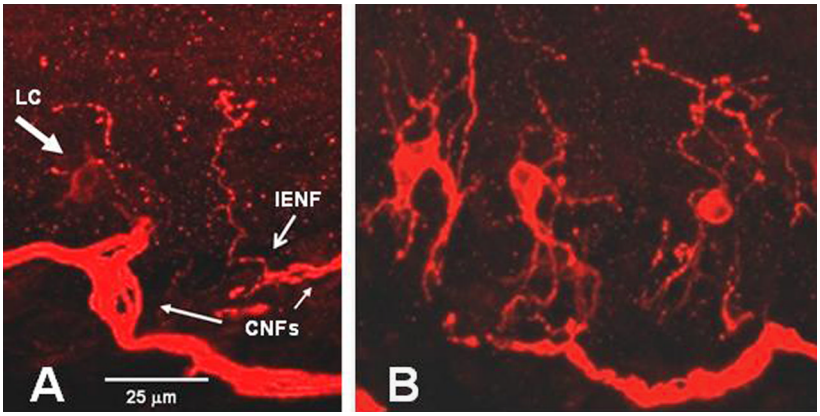

Figure 2.

Immunocytochemical demonstration of intraepidermal nerve fibers (A) and Langerhans cells (A, B) in glabrous skin stained with the anti-PGP9.5 antisera. (A) Section from a naïve animal showing a lightly stained LC and an IENF arising from a cutaneous nerve fascicle (CNF) and ascending into the epidermis. (B) Section showing the intensely stained LCs typically observed after paclitaxel-treatment.

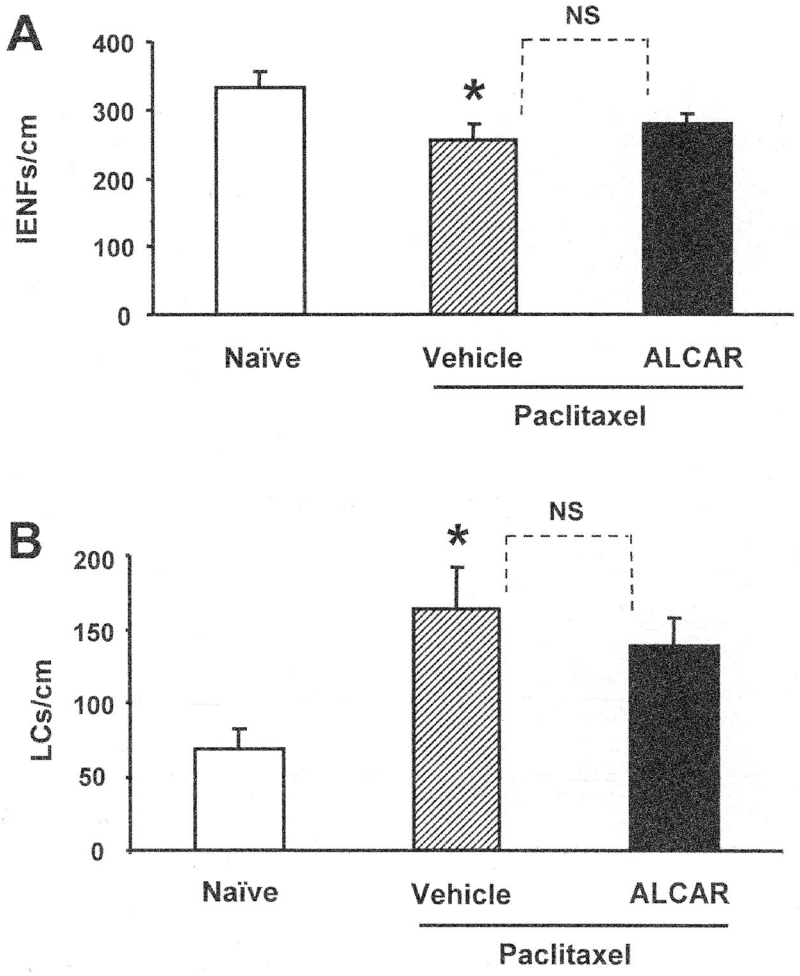

Figure 3.

ALCAR effect on the number of intraepidermal nerve fibers and Langerhans cells. Counts shown are mean ± S.E.M. per centimeter of epidermal border for naïve rats (n = 7), paclitaxel-treated rats receiving vehicle treatment (n = 6), and paclitaxel-treated rats receiving ALCAR treatment (n = 8). Paclitaxel treatment significantly reduced the number of IENFs (A) and increased the number of LCs (B) in vehicle-treated rats compared to naïve rats. ALCAR treatment had no statistically significant effect on the IENF loss or on LC activation. * $p < 0.05$ relative to the naïve group.

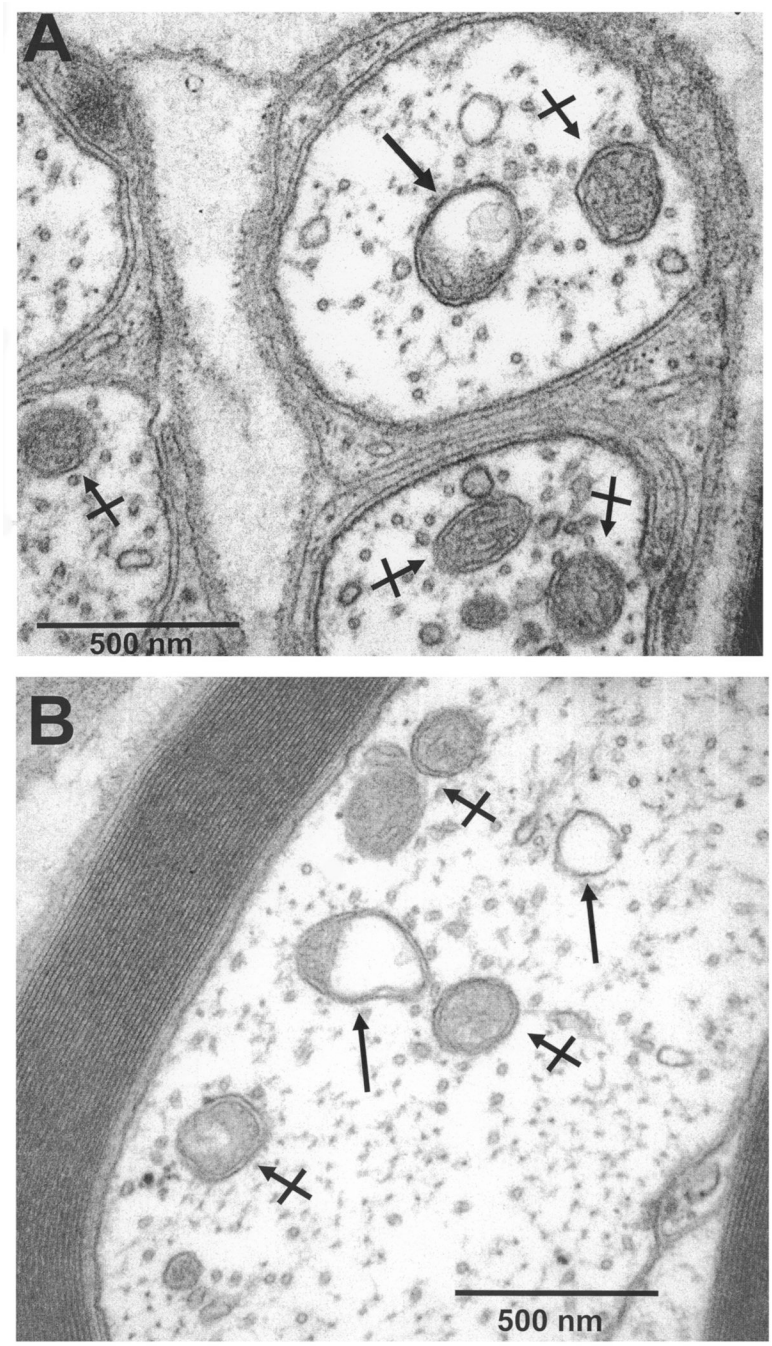

Figure 4.

Atypical and normal mitochondria in C-fibers (A) and A-fibers (B). Swollen and vacuolated mitochondria shown at arrows; barred arrows point to normal mitochondria. Note the pooling of electron dense semi-linear structures (believed to be collapsed cristae) at one pole of the swollen mitochondria. As shown here, a mixture of normal and swollen and vacuolated mitochondria were often found within the same axon. These examples are from a paclitaxel-treated rat that received ALCAR injections. 44,400X.

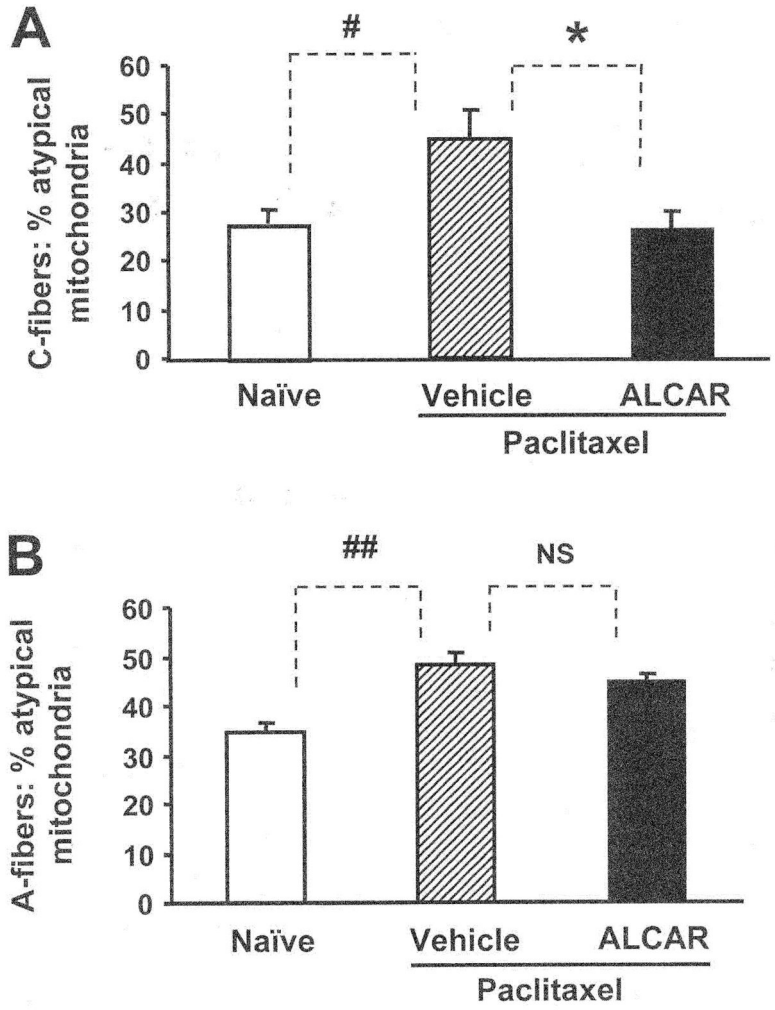

Figure 5.

Effects of ALCAR on C-fiber mitochondria (A) and A-fiber mitochondria (B). Bars show the proportion (means ± S.E.M) of atypical mitochondria relative to the total number of mitochondria in each fiber type. # $p < 0.05$, # # $p < 0.01$ compared to naïve group; * $p < 0.05$ compared to the vehicle-treated group; NS: non-significant.