Alpha-lipoic acid and docosahexaenoic acid

A positive interaction on the carrageenan inflammatory response in rats

Giuseppe Rossoni*
University of Milan, Italy
Bojidar M Stankov
Ambros Pharma, Milan, Italy

**Department of Pharmacology, Chemotherapy and Medical Toxicology*
University of Milan, Via Vanvitelli 32, 20129 Milan, Italy
tel +39 02 50317060 - fax +39 02 50317058 - email giuseppe.rossoni@unimi.it

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SUMMARY

Peripheral nerves are subject to the same types of problems as other tissues: inflammation, toxicity, swelling, permeability, leading to neuropathies. Nutrients offer an alternative for people who want to avoid the side effects of prescription drugs.
These studies were designed to assess the pharmacodynamic interaction between alpha-lipoic acid (ALA) and docosahexaenoic acid (DHA) in controlling the inflammatory response induced by carrageenan in the rat. Particularly, the activity of ALA (100 mg/kg *per os*) and DHA (200 mg/kg *per os*), given alone or in combination, was evaluated by paw oedema method.
The results of the present study clearly indicate that the new formulation containing ALA plus DHA, in a range of 2:1, had significantly greater effect than either DHA or ALA alone in antagonizing carrageenan-induced inflammatory response.
These results indicate that ALA plus DHA induce a favourable pharmacodynamic interaction which can be considered to be synergistic, at least in these experimental settings.

INTRODUCTION

Millions of people are living with some form of neuropathy (**1**). Peripheral nerves are subject to the same types of problems as other tissues, inflammation, toxicity, swelling, permeability. However, there are some differences, due to their structural uniqueness.
The nerve fibers exist as units of motor neurons with long axons that innervate muscle fibers. They are grouped by the perineural sheath, a connective tissue wrapping containing both myelinated and unmyelinated axons. Individual nerve fibers contain the nerve axon along with its Schwann cells and protective myelin sheath coating (not all axons are myelinated). Regulation of the nerve microenvironment is accomplished by three barriers, the perineural barrier, the blood-nerve barrier, and the nerve-cerebrospinal fluid barrier. When these barriers are penetrated or otherwise dysfunctional, inflammatory cells can infiltrate, causing neuropathies with demyelination, inflammation, pain and atrophy.
There is no single satisfactory treatment for most of the neuropathies, and many neuropathies have no known cause. A number of prescription drugs are used, but all have side effects, and none can actually correct the underlying nerve defect that causes the pain. Nutrients may offer an alternative for people who want to avoid the side effects of prescription drugs.

Alpha-lipoic acid

Of all the anti-oxidant nutrients, alpha lipoic acid (ALA) [(*R*)-5-(1,2-dithiolan-3-yl)pentanoic acid] seems to be the strongest. ALA was identified as a vitamin when it was isolated 50 years ago, but was reclassified upon the finding that it is synthesized in humans and animals (**2**). Dietary sources include red meat, organ meats, spinach, broccoli, potatoes, yams, carrots, beets, and yeast (**3**).
ALA is about 30% absorbed from dietary or supplemental sources, and is reduced to dihydrolipoic acid in many tissues (**4**,**5**). Exogenous ALA, and the metabolite dihydrolipoic acid, have antioxidant activity and can scavenge free radicals both intra- and extra-cellularly (**2**).
Endogenous ALA is a coenzyme that, together with pyrophosphatase, is involved in carbohydrate metabolism, and in the mitochondrial citric acid cycle which produces adenosine triphosphate. It is also a modulator of the inflammatory response and may suppress vascular inflammation (**6**). ALA is both water and fat soluble and can regenerate endogenous antioxidants, such as vitamin E, vitamin C, and glutathione, and prevent oxidative damage (**7**,**8**). Preliminary data suggests that these antioxidant effects might provide protection in cerebral ischemia, excitotoxic amino acid brain injury, mitochondrial dysfunction, muscle ischemia associated with

peripheral arterial disease, diabetes, diabetic neuropathy, and other causes of damage to brain or neural tissue (**9**,**10**). ALA does not appear to reduce levels of C-reactive protein, a marker of inflammation associated with cardiovascular disease, when used in combination with vitamins C and E (**11**).
In experimental diabetic models, ALA increases neuronal blood flow, improves neuronal glucose uptake, increases amounts of reduced glutathione in neurons, and improves neuronal conduction velocity (**12**,**13**). Preliminary evidence suggests that dihydrolipoic acid in combination with vitamin E might prevent oxidative stress in cardiac ischemia-reperfusion injury (**14**). Furthermore, the antioxidant effects of ALA might be beneficial in liver diseases in which oxidative stress is a factor (**15**).

Docosahexaenoic acid
Docosahexaenoic acid (DHA) [(4Z,7Z,10Z,13Z,16Z, 19Z)-docosa-4,7,10,13,16,19-hexaenoic acid] is an omega-3 fatty acid that falls into the larger category of polyunsaturated fatty acids (PUFAs). In humans, DHA not consumed in the diet is biosynthesized via conversion of eicosapentaenoic acid to docosapentaenoic acid, which is then converted to DHA (**16**). Many chronic conditions are associated with excessive intake of dietary saturated and trans fatty acids (including obesity, insulin resistance, coronary heart disease, and some form of cancer). Research has shown that omega-3 fatty acids, including DHA, are essential in the prevention and treatment of numerous diseases.
For example, as a predominant component of neural membranes in the brain and retina. DHA has a positive effect on membrane fluidity and permeability, receptor structure and quantity, carrier-mediated transport of nutrients in and out of the cell, enzymatic activities and cell-to-cell communication (**17**).
Furthermore, animal studies have shown that DHA inhibits amyloid plaque and tau protein formation in models of Alzheimer's disease (**18**,**19**), and clinical trials are in progress to determine whether DHA's effects are attributable to the same mechanism in humans with Alzheimer's disease (**20**).
Also, *in vitro* studies demonstrated that DHA is superior to eicosapentaenoic acid in inhibiting the expression of inflammatory markers such as pro-inflammatory cytokines, monocyte adhesion to endothelial cells, and cell-adhesion molecules, particularly vascular cell adhesion-1, intracellular adhesion molecule-1, and E-selectin (**21**). Although not clearly understood, DHA's role is thought to be attributed to its inhibition of prostaglandins F2 and E2, with subsequent delays in cervical ripening. DHA may also relax the myometrium via increased production of prostacyclin (**22**).

The present study was undertaken to evaluate the anti-inflammatory properties of ALA and DHA, given alone or in combination, in antagonizing the inflammatory response induced by carrageenan in the rat.

MATERIALS AND METHODS

Animals
Twenty-four male Wistar rats (Charles River Laboratories, Calco, Lecco, Italy), initial weight 175-200 g, were used. The animals were housed in a conditioned environment (22±1°C, 50±5% relative humidity, 12-h light/12-h dark cycle), with free access to standard laboratory rat chow (014RF21C; Mucedola, Settimo Milanese, Milan, Italy) and tap water. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animals were allowed at least five days to acclimatize before any experimental manipulations.

Carrageenan-induced paw oedema
Anti-inflammatory activity was measured by the paw oedema method (**23**,**24**). Rats were lightly anaesthetized under isofluorane and received a sub-plantar injection 0.1 mL saline containing 1% w/v carrageenan into the right hind paw. DHA (100 mg/kg) and ALA (200 mg/kg), alone (n=6/group) or in combination (n=6), were suspended in 0.5% w/v carboxymethylcellulose (vehicle) and administered orally by gavage (total volume of 2 mL/kg) 1 h before carrageenan injection. Control group (n=6) received the same volume of vehicle at the same time. The volume of the paw was measured by a plethysmometer (model 7140, Ugo Basile, Italy) immediately after the carrageenan injection. Subsequent readings of the volume of the same paw were carried out for 6 h at 60-min intervals and compared to the initial readings. The results were expressed as the paw volume variation (D mL) in relation to the basal values. The person performing these measurements was unaware of the treatments the rats had received.

Chemicals
DHA and ALA were obtained from Prochifar, Milan, Italy. λ-Carrageenan was purchased from Sigma-Aldrich, Milan, Italy. All other chemicals were of analytical or ultrapure grade.

Statistical analysis
Data are expressed as mean ± S.E.M. Differences between means were calculated using the unpaired t test and the area under the curve (AUC) was estimated according to the trapezoid method (25) All analyses were done with GraphPad Prism 4.00 software (GraphPad Software Inc., San Diego, CA, USA). Initially, one-way analysis of variance (ANOVA) was used, followed by Tukey's post-test for multiple group comparisons, as appropriate. $p<0.05$ was considered statistically significant. Subsequently, two-way ANOVA was applied for all groups and separately for the different treatment groups to scrutinize how the response was affected by the different treatments and establish the level of positive interaction between the two compounds tested (DHA and

ALA). In this case the F values for interaction, with the respective *P* values for significance, were calculated.

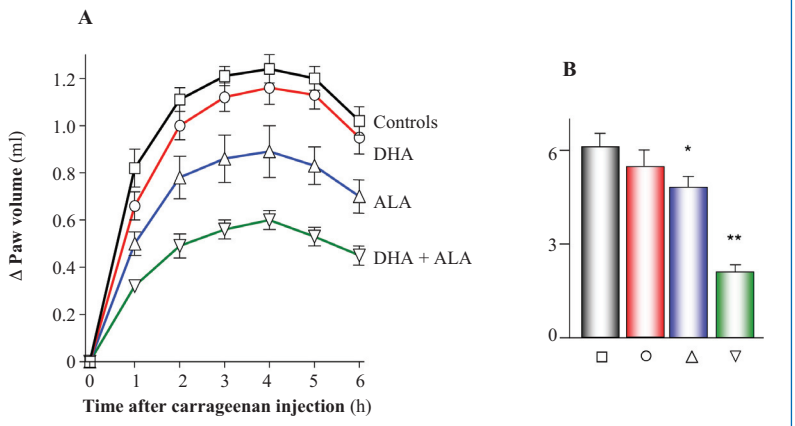
RESULTS

The change in mean paw volume for the various groups of rats is shown in *Figure 1*, *panel A*. The baseline measurements for the various groups were very similar, with mean values of 1.79-1.83 mL (data not shown). In the untreated control animals, swelling of the paw was evident by 1 h after administration of carrageenan and reached a peak at 4 h, with an increased volume of 1.24 ± 0.03 mL, decreasing slowly thereafter at 6 h.

The increase in paw volume was significantly reduced by ALA but not by DHA. In fact, the AUC value of ALA (4.81 ± 0.35 ml/6 h) and DHA (5.45 ± 0.48 ml/6 h) were 31% ($P < 0.05$) and 10% lower, respectively, than the control group (6.09 ± 0.43 ml/6 h) (***Fig 1, panel B***).

When the two drugs were given in combination, a positive synergistic effect on carrageenan-induced paw edema was observed. In fact, the AUC value of DHA plus ALA (2.72 ± 0.23 ml/6 h) showed significantly greater anti-inflammatory activity (-65%; $P < 0.01$ vs controls) (***Fig 1, panel B***). Further, when applying the two-way ANOVA analysis, the difference between all groups resulted highly significant ($F = 62.9$ and $P < 0.0001$). The separate two-way ANOVA tests for the treatment groups showed significant interaction with regard to treatment (DHA *vs* DHA+ALA: $F = 145.7$ and $P < 0.001$; ALA *vs* DHA+ALA: $F = 93.7$ and $P < 0.001$). These data are very much suggestive of a pertinent positive interaction between DHA and ALA in curtailing the inflammatory process significantly better than the respective single compounds in the model employed.

Figure 1 *Effect of DHA and ALA given alone or in combination on carrageenan-induced paw edema in rats*



DHA (100 mg/kg) and ALA (200 mg/kg) were given orally by gavage (total volume of 2 ml/kg) 1 h before carrageenan injection. A) time course of paw oedema; B) area under the curve (AUC), as estimated by the trapezoid method (**25**). Each point and bar represents the mean ± SEM of six rats per group

**p*<0.05 and ***p*<0.01 compared with the control group as determined by the unpaired t test or one-way analysis of variance (ANOVA) followed by Tukey's post-test for multiple group comparisons, as appropriate. See Results section for the two-way ANOVA analysis.

DISCUSSION

The results of the present study in rats showed that the increase in paw volume was significantly reduced by ALA but not by DHA. The results further clearly showed that the oral administration of ALA in combination with DHA brought about a greater effect than the sum of either component alone, indicating a synergic interaction in antagonizing the inflammatory effect of carrageenan, and the statistical analyses employed confirmed this notion.

The carrageenan induced paw oedema assay is a widely used model for the investigation of anti-inflammatory agents (**26**). The oedema and secondary hyperalgesia in this model involve both cyclooxygenase-dependent and -independent mechanisms, being the result of the local release of mediators of inflammation such as histamine, serotonin, prostaglandins, leukotrienes, substance P, and other (**27**,**29**).

Mechanisms

Alpha-lipoic acid

ALA is a naturally-occurring antioxidant that is unique because of its ability to act as an antioxidant in both lipid and aqueous phases of the cell. However, the potential of ALA extends beyond the strict definition of an antioxidant because its beneficial effect is achieved at low micromolar concentrations *in vivo* (**30**). Neuropathy-induced oxidative stress-associated inflammation requires the activation of NF-κB. ALA is well known for its antioxidant properties in cytokine-induced inflammation, and it is also widely known as an inhibitor of NF-κB (2). Lee *et al* (**31**) demonstrated that collagen-induced arthritis was attenuated by ALA (10 to 100 mg/kg i.p.) due to the reduced secretion of inflammatory cytokines like TNF-α and partial inhibition of NF-κB binding to DNA. Moreover, dietary supplementation of ALA reduced the severity of synovial inflammation and joint destruction in collagen-induced arthritis in mice (**32**). ALA supplementation effectively down-regulated secretion of NF-κB-dependent cytokines, such as TNF-α, IL-1β, IL-6, and sRANKL.

In addition, the use of ALA as a food supplement has increased significantly, and a number of clinical trials with ALA are underway for treatment of diabetic peripheral neuropathy (**33**,**34**).

Docosahexaenoic aid

Clinical assessment of dietary supplementation of omega-3 polyunsaturated fatty acids, including DHA, indicate their beneficial impact on human diseases, such as peripheral neuropathy, in which inflammation is suspected as a key component of the pathogenesis (**35**).

Although, the mechanism of action of DHA is still not fully defined in molecular terms. Recent studies have revealed that, during the course of acute inflammation, omega-3 polyunsaturated fatty acids-derived mediators, including DHA, with potent anti-inflammatory and pro-resolving properties are produced (**36**,**37**). More importantly, DHA represents the fatty acid that are apparently damaged in the process of development of neuropathy (**38**).

The results obtained with the new formulation ALA plus DHA (**39**) demonstrate the inventive property of this combination, since the compounds have different chemical structures and act through different modes of action but which, when acting together, interact at the cellular level to elicit a therapeutically meaningful response almost immediately. Also, the combination of DHA and ALA represent a new approach for sustaining the subjects with neuropathy, independently of its origin, such as canicolar syndromes of the hand, lumbar and cervical syndromes, phenomena of axonal degeneration or axonal oedema, and other. The administration of two components in the same pill will likely increase significantly the compliance of patients and increases the accumulation of both ingredients in the cell (**40**).

The most rationale approach for eliciting anti-inflammatory activity at the cellular level is probably in the use of a formulation allowing the lipophilic compound to reach its target at highest concentrations and in the shortest time (**40**).

CONCLUSIONS

The present findings clearly indicate that the oral administration of ALA exerted an anti-inflammatory effect in rats. In addition, we provide the first piece of evidence that when ALA was combined with DHA in a new formulation, this effect was enhanced. ALA plus DHA induced a favorable pharmacodynamic interaction which can be considered synergistic, and offer an alternative potential treatment for inflammations in the nervous system and therefore for neuropathies.

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