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4-Hydroxyhexenal and 4-hydroxynonenal are mediators of the anti-cachectic effect of n-3 and n-6 polyunsaturated fatty acids on human lung cancer cells

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ABSTRACT

Cachexia, the most severe paraneoplastic syndrome, occurs in about 80% of patients with advanced cancer; it cannot be reverted by conventional, enteral, or parenteral nutrition. For this reason, nutritional interventions must be based on the use of substances possessing, alongside nutritional and energetic properties, the ability to modulate production of the pro-inflammatory factors responsible for the metabolic changes characterising cancer cachexia. In light of their nutritional and anti-inflammatory properties, polyunsaturated fatty acids (PUFAs), and in particular n-3, have been investigated for treating cachexia; however, the results have been contradictory.

Since both n-3 and n-6 PUFAs can affect cell functions in several ways, this research investigated the possibility that the effects of both n-3 and n-6 PUFAs could be mediated by their major aldehydic products of lipid peroxidation, 4-hydroxyhexenal (HHE) and 4-hydroxynonenal (HNE), and by their anti-inflammatory properties. An "in vitro" cancer cachexia model, consisting of human lung cancer cells (A427) and murine myoblasts (C2C12), was used.

The results showed that: 1) both n-3 and n-6 PUFAs reduced the growth of lung cancer cells without causing cell death, increased lipid peroxidation and Peroxisome Proliferator-Activated Receptor (PPAR)α, and decreased TNFα; 2) culture medium conditioned by A427 cells grown in the absence of PUFAs blocked myosin production and the differentiation of C2C12 muscle cells; conversely, muscle cells grown in culture medium conditioned by the same cells in the presence of PUFAs showed myosin expression and formed myotubes; 3) adding HHE or HNE directly to C2C12 cells maintained in culture medium conditioned by A427 cells in the absence of PUFAs stimulated myosin production and myotube formation; 4) putative consensus sequences for (PPARs) have been found in genes encoding fast isoforms of myosin heavy chain, by a bioinformatics approach.

The overall results show, first, the ability of both n-3 and n-6 PUFAs and their lipid peroxidation products to prevent the blocking of myosin expression and myotube formation caused in C2C12 cells by medium conditioned by human lung tumour cells. The C2C12 cell differentiation can be due to direct effect of lipid peroxidation products, as evidenced by treating C2C12 cells with HHE and HNE, and to the decrease of pro-inflammatory TNFα in A427 cell culture medium. The presence of consensus sequences for PPARs in genes encoding the fast isoforms of myosin heavy chain suggests that the effects of PUFAs, HHE, and HNE are PPAR-mediated.

1. Introduction

Cachexia is both the most common and the most severe paraneoplastic syndrome; it was recently defined as a multifactorial condition. It is chiefly characterised by loss of skeletal muscle mass, and negative protein and energy balance, driven by a variable combination of reduced food intake and abnormal metabolism that is not fully reversed by conventional nutrition. It leads to a reduction in the response and tolerance to therapy, and in the quality and duration of life [1]. Cachexia occurs in about 80% of patients with advanced cancer; its incidence and prevalence differ in relation to the type of cancer, the highest incidence being observed in pancreatic, gastric and lung cancer [2]. Lung cancer induces sarcopenia associated to malnutrition, with a relatively high

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frequency (above 50%). Depending on the cachexia classification, about 20% of patients with non small cell lung cancer (NSCLC) are diagnosed as cachectic, and about 25% as pre-cachectic [3–5]. Malnutrition, due in part to alterations in neurohormonal mechanisms controlling food intake, contributes to metabolic alterations leading to a negative energy balance. This, in association with altered protein turnover and inflammation, drives the muscle wasting characterizing cachexia to develop [6]. With regard to the metabolic modifications occurring in cachexia, several cytokines produced by both tumour and host (TNF-α, IL-6, IL-1, INF-γ) or by tumour alone (proteolysis-inducing factor, PIF, lipid mobilizing factor, LMF) have been identified as molecular mediators of cachexia [7,8].

Due to its multifactorial pathogenesis, cachexia is unaffected by conventional dietary interventions, and limited benefits have been demonstrated by enteral and parenteral nutrition [9,10]. For these reasons, nutritional interventions focused on the use of substances with both nutritional and anti-inflammatory properties, with the aim of improving energy balance and reducing inflammatory status, have been proposed. In this perspective, and in light of their anti-inflammatory properties, fish-oil and its primary components, n-3 polyunsaturated fatty acids (PUFAs), have been proposed for treating cachexia. Moreover, in patients with cancer, a low concentration of n-3 PUFAs in the plasma phospholipids has been shown at diagnosis, the value further declining during cancer progression [11–13].

Among n-3 PUFAs, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids have been shown to down-regulate production of pro-inflammatory cytokines and acute-phase proteins in cancer patient blood [14–18], although a recent systematic review on the role of fish oil in treating cachexia concluded that there is insufficient evidence of a net benefit of n-3 PUFAs in advanced cancer. However, the same study reported that n-3 PUFA treatment can be beneficial for some selected patient populations, acting as post-operative support able to improve wound healing and reduce complications [19].

Different mechanisms have been postulated to explain the effect of n-3 PUFAs, including modulation of the activity of some transcription factors, such as NFkB and PPARs, leading to a decreased production of pro-inflammatory cytokines and acute-phase proteins. Moreover, EPA has been reported to inhibit the activation of the ubiquitin-proteasome pathway induced by proteolysis-inducing factor (PIF) and to decrease expression of the lipid mobilizing factor (LMF) [20,21]. More recently, EPA and DHA have been reported to affect the balance between anabolism and catabolism in muscles, through their effect on signalling transduction proteins and adipokines, in old and obese persons, thus suggesting another possible mechanism underlying n-3 PUFA-mediated anti-wasting effect [23,24].

The present research investigated the possibility that anti-cachectic properties of n-3 PUFAs could be mediated by the aldehydic products derived from their lipid peroxidation. 4-hydroxyhexenal (HHE) is the principal aldehyde generated by the non-enzymatic peroxidation of n-3 PUFAs. Due to its electrophilicity, HHE interacts strongly with cellular nucleophilic molecules, thus positively or negatively affecting several cell functions [22]. HHE-induced cytotoxicity has been reported in different cell types, including rat neurons and muscle cells, and human lens and renal epithelial cells [25–29]. Moreover, the HHE concentration has been shown to be significantly higher in the hippocampus/parahippocampal gyrus of patients with preclinical and late-stage Alzheimer's disease, than in that of normal subjects [30]. Conversely, it has recently been shown that, in some cases, lipoxidation of proteins can cause a gain of function/activity, suggesting that the formation of covalent protein/α,β-unsaturated aldehyde adducts, derived from lipid peroxidation, might be a step in a redox signalling pathway of physiological significance [31]. The present study was also based on a previous finding that, at a concentration found in many normal tissues and plasma, 4-hydroxynonenal (HNE), the major lipid peroxidation product of n-6 PUFA, induced the differentiation of murine erythroleukemia MEL cells and human promyelocytic HL-60 cells, by modulating the expression of several genes involved in cell cycle control [32–34]. Moreover in some tumours, arachidonic acid (AA), a fatty acid belonging to n-6 PUFAs, was shown to be present in a lower percentage in total fatty acids in comparison with corresponding normal tissues [35].

In this light, the research investigated the effect of both n-3 and n-6 PUFAs and their lipid peroxidation products (HHE and HNE) on muscle cell differentiation, using an "in vitro" cancer cachexia model, consisting of human lung cancer cells (A427) and murine myoblasts (C2C12).

Moreover, to evidence the mechanisms underlying the effect of PUFAs on C2C12 cell differentiation, the expression of peroxisome proliferator activated receptor (PPAR) α and the release of TNFα were also evaluated in A427 cells and in their culture medium, respectively. Human lung cancer cells were chosen since, in lung cancer patients, cachexia is the main cause of death despite the improvement in anticancer therapies. Moreover, little is known about the effect of PUFAs on lung-cancer-induced cachexia.

2. Materials and methods

2.1. Treatment of A427cells with PUFAs

Human lung adenocarcinoma cells A427 (ATCC, MD, USA) were seeded (20,000 cells/cm^2) in DMEM/F12 medium with 2 mM glutamine, 1% antibiotic/antimycotic solution and 10% FBS (medium A). Twenty-four hours after seeding, medium A was replaced with medium B, containing DMEM/F12 medium, 2 mM glutamine, 1% antibiotic/antimycotic solution, 2% horse serum (HS), and n-3 (EPA plus DHA) or n-6 PUFAs (AA) prepared in HS. EPA and DHA were administered simultaneously at a ratio of 1.5:1. For all treatments, the final concentrations were 10 or 50 μM. In control cells, a quantity of HS equivalent to the highest dose administered of PUFAs was added to the culture medium, in addition to the 2% HS already present in medium B.

Twenty-four hours after addition of PUFAs or HS, culture media were collected and centrifuged at 2800*g* for 10 min (centrifuge J6B Beckman, CA, USA) at room temperature. The collected media were used to determine lactate dehydrogenase (LDH) release, and as conditioned medium to culture C2C12 cells. After removing media, A427 cells were detached with trypsin/EDTA (0.25%/0.03 mM), centrifuged at 900*g* for 10 min (centrifuge J6B Beckman, CA, USA) and used for the assays listed below.

2.2. C2C12 cell culture conditions

Murine muscle C2C12 cells (ATCC, MD, USA) were seeded at 6000 cells/cm^2 in medium A for 4 days. After this time, to induce differentiation into myotubes, medium A was replaced with medium B, in which C2C12 cells were maintained for 3 further days. At the end of this period, medium B was replaced with medium conditioned by A427 cells, grown for 24 h in the presence or absence of PUFAs. Cells were analyzed after a further 4 days. C2C12 cells maintained in medium B for a further 4 days were labelled "Tdiff", and were taken as positive control.

2.3. Treatment of C2C12 cells with HHE and HNE

In these experiments, after removal of medium B (after 3 days), C2C12 cells were cultured in medium conditioned by A427 cells