Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 26 (2015) 1424–1433

Journal of Nutritional Biochemistry

Long-term omega-3 fatty acid supplementation prevents expression changes in cochlear homocysteine metabolism and ameliorates progressive hearing loss in C57BL/6J mice

Raquel Martínez-Vega[a,b], Teresa Partearroyo[c], Néstor Vallecillo[a], Gregorio Varela-Moreiras[c], María A. Pajares[a,d,*,1], Isabel Varela-Nieto[a,b,d,1]

[a]Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Arturo Duperier 4, 28029, Madrid, Spain,
[b]Unidad 761, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Instituto de Salud Carlos III, 28029, Madrid, Spain
[c]Departamento de Ciencias Farmacéuticas y de la Salud, Facultad de Farmacia, Universidad CEU San Pablo, Boadilla del Monte, Madrid, Spain
[d]Instituto de Investigación Sanitaria La Paz (IdiPAZ), Paseo de la Castellana 261, 28046, Madrid, Spain

Received 19 February 2015; received in revised form 2 July 2015; accepted 16 July 2015

Abstract

Omega-3 polyunsaturated fatty acids (PUFAs) are essential nutrients well known for their beneficial effects, among others on cognitive development and maintenance, inflammation and oxidative stress. Previous studies have shown an inverse association between high plasma levels of PUFAs and age-related hearing loss, and the relationship between low serum folate and elevated plasma homocysteine levels and hearing loss. Therefore, we used C57BL/6J mice and long-term omega-3 supplementation to evaluate the impact on hearing by analyzing their auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) thresholds. The omega-3 group showed significantly lower ABR hearing thresholds (~25 dB sound pressure level) and higher DPOAE amplitudes in mid–high frequencies when compared to the control group. These changes did not correlate with alterations between groups in plasma homocysteine or serum folate levels as measured by high-performance liquid chromatography and a microbiological method, respectively. Aging in the control group was associated with imbalanced cytokine expression toward increased proinflammatory cytokines as determined by quantitative reverse transcriptase polymerase chain reaction; these changes were prevented by omega-3 supplementation. Genes involved in homocysteine metabolism showed decreased expression during aging of control animals, and only alterations in *Bhmt* and *Cbs* were significantly prevented by omega-3 feeding. Western blotting showed that omega-3 supplementation precluded the CBS protein increase detected in 10-month-old controls but also produced an increase in BHMT protein levels. Altogether, the results obtained suggest a long-term protective role of omega-3 supplementation on cochlear metabolism and progression of hearing loss.

Keywords: Eicosapentaenoic acid; Homocysteine metabolism; Inflammation; Omega-3 fatty acids; Oxidative stress; Presbycusis

1. Introduction

The functional decline of the organism during aging is associated with the onset of a variety of chronic illnesses, including cancer, diabetes, atherosclerosis, osteoporosis and cardiovascular disease [1,2]. Aging is also associated with a progressive sensory impairment that is often concomitant with cognitive decline [1,3,4]. Most chronic diseases share common biochemical alterations leading to cellular degeneration and organ malfunction [2]. Treatment of all these disorders represents a key health challenge but also a major socioeconomic problem because, for example, the population over 65 years will presumably double by 2050 in the European Union [5]. Therefore, biomedical research is focusing in the understanding of the genetic and molecular mechanisms underlying aging and in the design of new strategies to promote active and healthy aging, including nutritional intervention.

Hearing loss is one of the fields in which nutrition intervention studies may have more preventive potential, especially age-related hearing loss (ARHL). Approximately 30% of the population over 65 years suffers ARHL, its incidence increasing exponentially with age [6,7]. Worldwide epidemiological studies have shown an association between deficiencies in several essential nutrients and hearing loss [8–12]. Moreover, other studies also provided evidences of its putative prevention by dietary supplementation with folic acid [13] or due to different levels of n-3 polyunsaturated (ω3) fatty acids (PUFAs) in the diet [14–17]. The general basis for this protection seems to rely on the relationship between vascular disease and hearing loss, which was suggested initially by the lack of presbycusis and cardiovascular disease in the Mabaan tribe and further supported by several studies in search of a correlation between cardiovascular events and hearing loss

* Corresponding author. Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM), Arturo Duperier 4, 28029, Madrid, Spain. Tel.: +34 915854414; fax: +34 915854401.
E-mail address: mapajares@iib.uam.es (M.A. Pajares).
[1] These authors share senior authorship.

http://dx.doi.org/10.1016/j.jnutbio.2015.07.011

[18–22]. These studies showed microvascular disease as the underlying cause for the atrophy of the stria vascularis [20,23], which is the structure within the cochlea that maintains its metabolic and ionic homeostasis [24]. Animal models of AHRL have provided further information on its etiology. Thus, for example, the gerbil also presents progressive blood flow reduction and microvascular alterations at the stria vascularis and spiral ligament associated with hearing loss and aging [25,26]. Altogether, these data suggest that a reduction in the blood supply of essential nutrients into the cochlea, and the subsequent metabolic alterations, could be among the main triggers of hearing loss progression.

High levels of plasma homocysteine (tHcy), a key metabolite of the folate and methionine cycles [27], are considered an independent risk factor for cardiovascular disease [28] and, more recently, also of human hearing loss [12,13]. Vascular diseases are also closely related to inflammation, a condition ameliorated by supplementation with ω3 fatty acids [29,30]. These relationships were the basis for the limited number of studies on the effects of ω3 supplementation on hearing loss and Hcy metabolism carried out to date. These studies showed an inverse correlation between the ingested levels of long-chain ω3 fatty acids and hearing loss [15,16], but opposite effects on tHcy resulted from fish oil supplementation [31,32]. Dissimilar results on tHcy and/or auditory brainstem response (ABR) were also obtained from animal studies of ω3 supplementation carried out for limited time periods in adults or during pregnancy and lactation [33–37]. Therefore, the urgent need of a better knowledge of hearing loss progression and the potential of dietary components to prevent and/or delay its onset led us to use a classical model of early hearing loss, the C57BL/6J mice, to carry out a detailed follow-up of the auditory capacity in long-term ω3 supplementation. Altogether, our results show that ω3 supplementation attenuated progression of hearing loss, this maintenance of function associating with differences in cochlear Hcy metabolism and in the balanced ratio of anti- and proinflammatory biomarkers.

2. Materials and methods

2.1. Mouse handling and experimental design

Seven-week-old C57BL/6J female mice [38,39] were purchased from Charles River Laboratories (Hollister, CA, USA) and housed under standard conditions. After an acclimatization period of 1 week, these 2-month-old mice were randomly divided into two experimental groups that were fed for 8 months either control (C-10M; $n=15$) or ω3-supplemented (ω3-10M; $n=9$) diets *ad libitum*. A group of mice within those receiving the control diet was sacrificed at 4 months of age for comparative purposes (C-4M; $n=4$). The composition of both semisynthetic diets was adjusted to mice requirements in full accordance with National Research Council directives [40], with modifications affecting only the fat content (Table 1). The AIN-93M-MX mineral mix (TD.94049) and the AIN-93-VX vitamin mix (TD.94047) used for diet preparation were purchased from Harlan Teklad (Indianapolis, IN, USA), whereas the fish oil Eupoly-3™ eicosapentaenoic acid (EPA) was gently supplied by Biosearch SA (Granada, Spain). Diet pellets were freshly made at the onset of the experiment and stored at −20°C in portions of the appropriate size until use; thawing was carried out at 4°C, and new pellets were provided every other day. Animals were weighted weekly and maintained on a 12-h:12-h dark/light cycle under controlled temperature and humidity conditions at the facilities of the Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM). All experiments were approved by the CSIC Bioethics Committee and carried out in full accordance with the European Union (2010/63/EU) and Spanish regulations (RD 53/2013) for the use of laboratory animals.

Table 1
Diet composition

	Control diet (g/kg)	ω-3 diet (g/kg)
Cornstarch	620.7	620.7
Casein (≥85%)	140.0	140.0
Cellulose	50.0	50.0
Saccharose	100.0	100.0
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
L-Cysteine	2.5	2.5
Choline	2.5	2.5
tert-Butylhydroquinone	0.008	0.008
Soybean oil	40.0	38.4
Fish oil Eupoly-3™ EPA	–	1.6

2.2. Hearing assessment

Hearing was evaluated at the beginning of the experiment (2 months of age) and monthly from 4 months of age onward (Fig. 1). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and the ABR was recorded for hearing assessment as previously described [41,42]. Briefly, broadband click and pure tone frequencies were recorded at 4, 8, 16, 20, 28 and 40 kHz at an intensity range from 90 to 10 dB sound pressure level (SPL) in 5–10-dB steps. The electrical responses were amplified and averaged to determine hearing thresholds for each stimulus. Peak and interpeak latencies were analyzed at 15–20-dB SPL above hearing threshold after click stimulation. Animals were kept thermostatized and monitored during both anesthesia and the following period of recovery. The anesthetics used in the present work are known to be metabolized in the liver and kidney within 24 h.

The recording of distortion product otoacoustic emissions (DPOAEs) was performed after stimulation with f1 and f2 primary tones, with a ratio f2/f1=1.2. An Etymotic ER-10B+™ probe was used for the generation of f1 and f2 and sound capturing. The calibration of the probe for determining the degree of attenuation of the primary frequencies was performed using a sound-attenuating cylinder with known volume (i.e., 1-ml syringe) to simulate the mouse ear canal with the aid of the TDT equipment, as described previously [43,44]. Primary tones for 8-, 10-, 14-, 18- and 22-kHz frequencies were tested. Decreasing intensity steps of 5–10 dB were performed from 80 to 30 dB SPL for each pair of frequencies (150 averages per intensity). Simultaneously, the probe was connected to a 100–50,000-μV amplifier, and the signal processing and subsequent analysis were carried out with the help of software BioSigRP™. The 2f1-f2 DPOAE component was considered positive when exceeding 5 dB SPL above background noise, this being calculated as the average amplitude of five randomly chosen points on both sides of the 2f1-f2 component. All the frequencies used were generated by SigGenRP™ (Tucker Davis Technologies TDT, Alachua, FL, USA).

2.3. Cochlea extraction and processing

Mice were sacrificed by CO_2 asphyxiation for tissue removal at 4 and 10 months of age. Tissues were immediately frozen in liquid nitrogen for protein or RNA extraction. Blood samples were collected by cardiac puncture and placed in regular or heparin-coated tubes (Laboratorios Farmacéuticos Rovi, Madrid, Spain). After centrifugation at 2500×*g* for 10 min, the corresponding serum or plasma fractions were isolated and stored at −80°C until use.

2.4. Metabolite determinations

Total plasma Hcy (tHcy) was determined after derivatization using the Reagent kit for the high-performance liquid chromatography analysis of Hcy in plasma/serum (Chromsystems Instruments & Chemicals GMBH, Munich, Germany) following manufacturer's instructions. Serum folate levels were analyzed by a microbiological method using *Lactobacillus casei* (American Type Culture Collection; ATCC 7469) [45], as modified by Tamura [46].

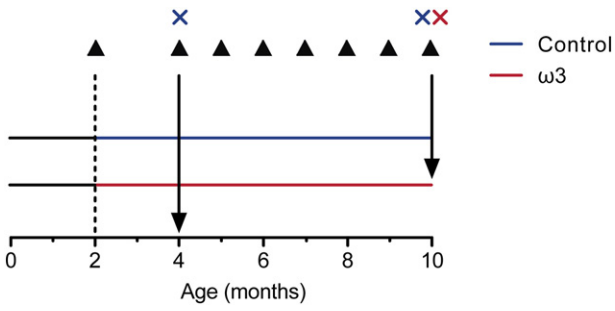

Fig. 1. Scheme of the experimental procedure. Mice were fed a standard diet until they became 2 months old ($n=24$). At this age, ABR analysis was performed, and mice were randomly divided into two experimental groups receiving the standard (control; $n=15$) or the ω3-supplemented diets (ω3; $n=9$). From 4 months of age onward, ABR was performed monthly, and sampling was carried out at 4 (only in the control group, C-4M; $n=4$) and 10 months of age (C-10M and ω3-10M). ABR analysis is represented by triangles, and sampling is indicated by a blue (control) or red "X" symbol (ω3 supplemented); points of detailed biochemical analysis are indicated by arrows.