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Eicosapentaenoic acid pre-treatment reduces biochemical changes induced in total brain and myelin of weanling Wistar rats by cuprizone feeding

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ABSTRACT

Recently, we investigated the effects of eicosapentaenoic acid (EPA), a fatty acid which modulates immune response and stimulates myelin gene expression, in an established model of multiple sclerosis (MS): the experimental autoimmune encephalomyelitis (EAE) induced in Dark Agouti rats. As scientific evidences and our previous studies have suggested that EPA could directly affect oligodendrocytes, we have now evaluated the effects of EPA in the non-immune mediate MS model characterized by selective oligodendrocytes damage induced by cuprizone (CPZ).

We found that feeding weanling rats diets containing 0.6% CPZ for 2 weeks induced variation of whole brain and myelin biochemical composition representative of a severe myelin damage. We thus administered daily and by gavage EPA or PBS to 2-day old rats up to 21 days. Afterwards, rats were fed CPZ diet for 9 days. The results show that compared to PBS/CPZ fed rats, the whole brain cerebroside content in EPA pre-treated rats was statistically increased as well as there was an overall trend of increase of all other biochemical components.

1. Introduction

Multiple sclerosis (MS) is a not yet curable immune-mediated disorder of the central nervous system (CNS). During the disease progression, myelin destruction and oligodendroglial loss are accompanied by multifocal inflammation, microglial activation, astrogliosis and axonal degeneration, all together leading to irreversible neurological disability [1,2]. The approved therapies including immunomodulatory, immunosuppressive, or antibody mediated approaches reduce disease activity, but are only partially effective [3,4]. Thus, new different strategies to slow down MS progression are under investigation on the available animal models of MS. Among the complementary approaches, there is great interest in dietary supplementation with *n*-3 polyunsaturated fatty acids (*n*-3PUFAs) which include eicosapentaenoic acid (EPA; C20:5*n*-3) and docosahexaenoic acid (DHA, C22:6 *n*-3) [5–7]. The immunomodulatory and anti-inflammatory effects of *n*-3 PUFAs have been suggested as a rationale for their potential therapeutic use in MS patients [8–10].

Recently, in a well established model of MS, the experimental autoimmune encephalomyelitis (EAE) induced in Dark Agouti (DA) rats, we obtained encouraging results with diets rich in EPA [11]. Rats fed EPA diets, administrated from the day of EAE induction or 10 days before, delayed the onset of disease and presented reduced clinical score, compared to EAE rats fed standard diet. The beneficial and protective effects of EPA were mediated through restraining immune-inflammatory responses, shown by an up-regulation of the forkhead transcription factor (Foxp3), the most specific marker for regulatory *T* (T_{reg}) cells, within the CNS. Furthermore, a stimulation of myelin repair process, shown by increased expression of myelin proteins and an improved integrity of the myelin sheath, was also observed. Our previous *in vivo* and *in vitro* studies provided evidence of the additional effect of EPA in up-regulating myelin genes [12,13] and we hypothesized that EPA could trigger oligodendrocyte maturation via AMPc pathway. More recently, Lim et al. [14] demonstrated that EPA is neuroprotective in spinal cord trauma as intravenous administration of EPA resulted in increased neuronal and oligodendrocyte survival, in the lesion epicenter. Based on evidences that EPA could have beneficial and protective effects directly on oligodendrocytes, we have now evaluated the effects of EPA in the MS model characterized by toxic demyelination induced by cuprizone (CPZ). CPZ feeding, with mechanisms not yet well established, induces oligodendrocyte

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degeneration, disruption of myelin sheath and demyelination mainly localized in the medial corpus callosus (CC) [15]. After ending CPZ exposure, remyelination occurs fast and spontaneously [16,17]. Although this is a widely used protocol in mice, the CPZ exposure does not always induce demyelination in rats [18]. However, Adamo et al. [19] found that feeding weanling (21-day old) inbred rats diets containing 0.6% CPZ for 2 weeks induced demyelinating effects similar to those induced in mice.

In the current study, the treatment proposed by Adamo was reproduced in non-inbred rats. Since in the EAE model, EPA pre-treatment ameliorated the clinical score more than EPA treatment from the induction day, EPA was daily administered by gavage to newborn rats for 21 days, and then they were fed for 9 days on the CPZ diet. The results showed that (i) the CPZ doses and intoxication time frame described by Adamo in inbred rats gave similar results in our non-inbred rats, (ii) EPA was adsorbed and metabolized, and (iii) it was able to partially protect the brain against damage induced by CPZ intoxication.

2. Materials and methods

2.1. Animals

All animal protocol were carried out according to the European Community Council Directive of 24 November 1986 (86/609/EEC) and Italian legislation (DL 116/92).

2.1.1. CPZ treatment

Pregnant Wistar rats were purchased from Harlan laboratories (Udine, Italy) and were fed a standard laboratory chow *ad libitum*. The basal diet contained 19% casein, 0.3% DL-methionine, 57% rice starch, 10% sucrose, 6% fiber, 4% salt mixture (AIN-76), 1% vitamin mixture (AIN-76), 0.2% choline chloride, and 4% fat containing 16% C16:0; 0.1% C16:1; 2.7% C18:0; 29.6% C18:1; 45.9% C18:2 *n*-6; 2.3% C18:3 *n*-3: 2.1% C20:0; 0.8% C20:1; 0.7% C22:0; 0.4% C24:0. At the parturition, litter size was adjusted to eight pups. Rats were weaned at 21 days of age and were fed pulverized regular chow pellets supplemented with 0.6% CPZ (bis-cyclohexanone oxalydihydrazone, Sigma Chemical Co., St Louis, MO) for 2 weeks. At the end of this treatment, animals were sacrificed and used to analyze the effects of CPZ treatment (CPZ group) in comparison with rats which were kept under normal diet until sacrifice (control group).

2.1.2. EPA treatment

To study the protective effect of EPA, a group of newborn pups received daily by gavage 100 μl of a solution of Ethyl-EPA (Amarin Neuroscience, Oxford, UK, purity > 98%) in PBS. EPA was administered to at the concentration 1 mg/100 μl from 2 post natal day (p.n.d.) for 5 days and then at the concentration of 2 mg/die up to 21 p.n.d. (EPA-21). Control rats received 100 μl of PBS (PBS-21) (GIBCO, Paisley, UK). At weanling, EPA and PBS rats were fed milled chow containing 0.6% (w/w) CPZ. After 9 days animals were sacrificed and used to analyze the effects of EPA/PBS treatment (EPA-21/CPZ-9; PBS-21/CPZ-9). Number of animals in each group varied between 4 and 8.

2.2. Brain tissue homogenate and myelin isolation

The animals were anesthetized with intraperitoneal injection of metedomidine:ketamine (1:1; 200 mg/kg) and then killed by CO_2 exposure. The brain (cerebral hemispheres plus brain stem) was carefully excised and the tissue homogenized at 10% (w/v) in ice-cold 0.32 M sucrose. The total homogenate was fractionated by ultracentrifugation to obtain a purified myelin fraction, following the method of Norton and Poduslo [20]. The purified myelin fractions were used immediately or stored at −20 °C for further studies.

2.3. Lipid extraction and chemical determinations

An aliquot of the purified myelin fractions, as well as an aliquot of the total brain homogenates, was used to determine total protein [21], using bovine serum albumin as standard. For the extraction of lipids, another aliquot was treated with 2:1 chloroform:methanol (v/v) according to Folch et al. [22]. Lipids were calculated as the sum of phospholipids, cerebrosides (CB) and cholesterol quantified using thin layer chromatography and densitometry as previously described [23].

2.4. Plasma, erythrocytes, and whole brain fatty acid analysis

After EPA and PBS treatment, 21-day old rats were anesthetized and blood was collected in heparinised tube from the eye. After centrifugation, lipids were extracted from 100 μl of plasma with 1 ml of isopropanol. Buffy coats obtained from heparinized blood were washed and lipids were extracted with 1.5 ml of isopropanol/chloroform 11/7. Transmethylation of fatty acids from plasma, erythrocyte, and brain lipids were obtained with methanolic HCl (Supelco, Bellefonte, PA) overnight at 70 °C. Fatty acid methyl esters (FAME) were extracted with hexane and analyzed by gas chromatography (Agilent, Palo Alto, CA) using a fused silica capillary column (Supelcowax, 30 m × 0.53 mm id, Supelco). The FAME were identified by comparison with authentic standards (Supelco) and calculated by addition of a known amount of eptadecanoic acid.

2.5. Immunohistochemistry

Rats were anesthetized with ethyl ether and perfused through the left ventricle of the heart with 30 ml of PBS followed by a 4% solution of paraformaldehyde in PBS. The brains were carefully dissected out and post-fixed in the same solution for 3 h, followed by thorough washing in PBS. Samples were cryoprotected in 5% sucrose in PBS for 30 min, followed by 30% sucrose overnight. The tissue was then frozen and cut at a cryostat to obtain 30-μm coronal sections. To detect myelin, brain sections were incubated with the primary antibody anti-MBP (1:100, Chemicon, Temecula, *CA*, USA) overnight at 4 °C and then with a fluorescent conjugated anti-mouse secondary antibody (1:10, Boehringer Ingelheim, Italy) for 2 h at room temperature. The sections were observed at an Eclipse 80i Nikon Fluorescence Microscope (Nikon Instruments, Amsterdam, Netherlands), equipped with a VideoConfocal (ViCo) system.

2.6. Splenocyte isolation

2.6.1. Ex vivo study

Splenocytes were isolated from the spleens of animals of each group. Cells were gently flushed out from the spleens using a syringe plunger with PBS and centrifuged (220 × *g*, at 4 °C). The pellet was resuspended and left 5 min at 37 °C in 5 ml of RBC lysis buffer (Sigma, 17 mM Tris, 140 mM NH_4Cl) to remove erythrocytes. Spleen cells were washed twice in PBS. Cells (5×10^5/ml) were cultured in duplicate in 24-well plates in RPMI (GIBCO) supplemented with 10% FCS (GIBCO). After 24 h of culture, the nitrite release was determined in the supernatants.

2.6.2. In vitro study

For *in vitro* study, spleen cells were isolated from adult Wistar rats and cultured in duplicate in 24-well plates in RPMI