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n-3 polyunsaturated fatty acids supplementation decreases asymmetric dimethyl arginine and arachidonate accumulation in aging spontaneously hypertensive rats

Summary *Background* Plasma accumulation of asymmetric dimethyl arginine (ADMA), is considered as a risk factor for endothelial dysfunction and a strong predictor for coronary heart diseases. Eicosapentaenoic (EPA) and docosahexaenoic (DHA) increasing plasma levels have been positively associated with reduced cardiovascular mortality with a mechanism(s) yet unclear. We hypothesised that ADMA reduction might be a part of EPA and DHA beneficial effects on the cardiovascular system. *Aim* To verify this hypothesis we measured ADMA plasma levels in aged spontaneously hypertensive rats (SHR) supplemented for 8 weeks with EPA and DHA. *Methods* 16-month-old SHR were supplemented with EPA and DHA (EPA-DHA) or with olive oil (1 g/kg/day; OLIVE). At the end of the treatments, the plasma of each animal was analysed for 1) the total fatty acid composition, by gas-cromatography, 2) ADMA levels, by high pressure liquid chromatography, 3) nitrite and homocysteine concentration by chemiluminescence and by polarisation immunoassay respectively. Moreover, the activity of dimethyl arginine dimethyl amino hydrolase, the main enzyme involved in ADMA metabolism, was measured spectrophotometrically in the kidney from each rats. *Results* Animal supplemented with EPA and DHA showed: 1) lower ADMA and arachidonate plasma levels (587.4 ± 113.7 nM and 0.49 ± 0.11 mM respectively) than the values found in OLIVE rats (1365 ± 399 nM and 1.07 ± 0.07 mM respectively) 2) higher nitrite content (0.73 ± 0.05 μM) than OLIVE (0.23 ± 0.08 μM) *Conclusions* EPA and DHA supplementation reduced ADMA accumulation in SHR in parallel with a decrease of arachidonate availability. This finding suggests that the control of the inflammatory ground of endothelium might play an important role in EPA and DHA effect on this novel and high predictive cardiovascular risk factor.

Key words hypertension – long chain fatty acids – ADMA – cardiovascular risk

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Introduction

n-3 polyunsaturated fatty acids (n-3PUFA) exert protective activities toward cardiovascular (CV) diseases [1–3]. In fact, regular consumption of a diet proportionally rich in marine n-3PUFA and low in n-6 polyunsaturated fatty acids (n-6PUFA), as well as the administration of supplements of EPA (20:5n-3) and DHA (22:6n-3) acids, is associated with a reduction in cardiovascular (CV) mortality and sudden death in subjects with or without previous myocardial infarction [4–6]. Accordingly, the American Heart Association recommends a regular consumption of n-3PUFA as a measure to reduce the risk for coronary heart disease [7]. However, despite the large body of evidences that support the beneficial effects of n-3PUFA, their mechanism(s) of action as protectors against CV disease is yet to be eluci-

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dated, although improvement of endothelial function and antiarrhythmic actions have been demonstrated [8, 9].

Recent findings suggest that – in the presence of endothelial dysfunction and impaired endothelium-dependent vasodilatation – ADMA, the most potent endogenous inhibitor of endothelial nitric oxide synthase accumulates in plasma [10]. Moreover, high ADMA plasma concentrations are currently considered as a strong predictor of coronary heart disease [11] and their reduction is included within the goals of therapeutic approaches aimed at ameliorating endothelial function. The reasons of ADMA plasma accumulation are largely unknown, even if a decrease of its renal elimination and/or dysfunctions in the DDHA, the main enzyme involved in ADMA metabolism, are thought to be important in determining plasma ADMA levels.

It is noteworthy that, while evidences on the beneficial effect of anti-hypertensive [12] or antidiabetic [13] drugs in reducing plasma ADMA accumulation have been accumulating, data on the effects of long-term treatments with n-3PUFA on plasma ADMA concentrations are lacking. Thus, the aim of our study was to investigate whether supplementation of high CV risk animals with EPA and DHA might reduce their plasma ADMA levels.

Materials and methods

Animals

Treatments were carried out in accordance with the European Communities Council Directive of 24 November 1986 for experimental animal care (86/609/EEC). All efforts were made to minimise the number of rats and their suffering. For these reasons, all measurements, including blood pressure recorded by means of a tail-cuff system (Basile, Italy), were performed at the end of treatments; in fact, previous experience in our laboratory indicates that old SHR are at high risk of sudden death when stressed by manipulation [14].

Sixteen-month old male SHR were purchased from Charles River (Calco, Italy). The animals were housed at 23 ± 1 °C with a 12-h light/dark cycle (light on at 07:00 h) and were fed a standard low calorie laboratory diet (6900 kJ/kg) with a 5.5 % fat content (2270 kJ/kg). Rats had free access to chow and tap water. At the beginning of the treatment, the animals had an average body weight of 394.30 ± 6.63 g.

The homogeneity of the animals' weight and the fact that blood pressure does not significantly change in SHR from 8 to 16 months [14] allowed us to randomise the animals and divide them into two groups. Both groups were treated daily, by gavage, with either olive oil (1 g/kg/day; OLIVE n = 8) or a mixture of DHA and EPA ethyl esters (0.9:1.5; 1 g/kg/day; EPA-DHA, n = 8), for two months. Both treatments were equicaloric: 223 kJ/100 g from diet and 405 kJ/kg from supplementation. No animal died before the end of the treatment.

Blood was collected, using Li^+ heparin as the anticoagulant, immediately placed on ice, and centrifuged within 30 minutes (600 x g x 15 min at 4 °C). Aliquots of plasma were stored at –80 °C.

Determination of dimethyl arginines in rat plasma

The determination of ADMA and of symmetric dimethyl arginine (SDMA) was performed as previously described [15], with some modifications. Briefly, ADMA and SDMA were extracted from 200 µL of deproteinised plasma using BAKERBOND SPE columns (ProKeme, Florence, Italy). The chromatographic separation of ADMA and SDMA was carried out with a multigradient program on a Waters Spherisorb ODS2 column (4.6 mm x 150 mm) using a Waters 474 Scanning fluorescence detector and the Millennium (version 3.05.01) data system supplied by Waters Corporation (Milford, MA, USA). ADMA and SDMA standards (Sigma-Aldrich, St Louis, MO, USA) were prepared in doubly distilled water and 0.1, 0.5 and 1.0 µM solutions were used to build a calibration curve.

The rat renal dimethyl arginine dimethyl amino hydrolase activity (DDHA)

The DDHA (EC 3.5.3.18) of rat kidney was assayed by incubating aliquots of kidney homogenates in 50 µl of 0.1 M sodium phosphate buffer (pH 6.5), containing ADMA (5 mM) as the substrate [16, 17]. Reactions were stopped after 1 h from the substrate addition. L-citrulline, produced by enzymatic catalysis, was evaluated spectrophotometrically and its concentration was calculated from a standard curve [18]. Heated kidney proteins were used to run assay blanks and to build L-citrulline calibration curves. In some cases, $CuSO_4$ (0.5 mM) was added to the enzyme mixture 30 min before the addition of ADMA, to verify prevention of L-citrulline formation. Results are shown as the means ± S. E. M. of four experiments performed in duplicate and are expressed as µmoles of citrulline/g of tissue/min and transformed in katals).

Plasma glucose and creatinine concentration

Creatinine and glucose plasma concentrations were assayed using commercially available diagnostic kits (Sigma-Aldrich), according to manufacturer's instructions.

Nitrite assay

Plasma nitrite concentrations were assayed with a NOA analyser (Seivers model 280, Sensor Medics, NI, Italy), which allows the measurement of nitrite levels in gaseous and liquid samples [19]. Each plasma sample was first deproteinised and then injected (25–50 µl) into an anaerobic (helium-purged) reaction vessel containing sodium iodide/glacial acetic acid sodium. The production of nitric oxide from nitrites was detected by chemiluminescence and was quantified with a photomultiplier tube/computer system.

To evaluate the eventual oxidation of nitrite to nitrate occurred in our samples, plasma were also incubated for 30 min at 37 °C with nitrate reductase (250 mU/ml; Sigma) and NADPH (100 µM; Sigma-Aldrich), before deproteinisation. The reaction mixture was then analysed for nitrites as described above. No significant differences were observed in nitric oxide content between samples treated and non-treated with NADPH-nitrate reductase.

Standard curves were built by using different concentrations of nitrites and the amount of nitrites in each sample was calculated from the standard curve. The detection limit was 3–5 pmol/25 µl.

Homocysteine levels

The plasma concentrations of total homocysteine (free and protein bound) were quantified by automated fluorescence polarisation immunoassay [20].

Kidney protein concentration

Kidney protein content was determined by using the Pierce BCA™ (Pierce, Rockford, USA) method, with bovine serum albumin as the standard.

Fatty acid composition of the total lipid fraction of OLIVE and EPA-DHA rats

Plasma from both groups of rats was processed for analysis of its total (i.e. esterified and non-esterified) fatty acid content according to Visioli et al. [21]. Briefly, total (i.e. esterified and non-esterified) fatty acids were extracted by the Folch procedure [22] and quantified by microgravimetry. Fatty acid methyl esters were prepared by the addition of 14 % BF_3 in methanol and quantified by gas liquid chromatography.

Statistical analysis of results

Data are expressed as mean ± S.E.M. Results were analysed using unpaired Student's t test with Welch's correction or P value < 0.05 was considered to be significant. Calculations were made using Graph-Pad Prism and InStat version 4.00 for Windows (GraphPad Software, San Diego, California, USA).

Results

Animal treatments did not produce differences among some metabolic parameters and systolic blood pressure levels

At the end of the treatment both groups were analysed for their body weight, systolic blood pressure, and fasting plasma glycaemia, homocysteine and creatinine levels. As shown in Table 1, no differences in the parameters observed were found between the two groups of rats.

Composition of plasma fatty acids of rat treated with olive oil OLIVE or with EPA-DHA

Analysis of total plasma fatty acids carried on in the plasma from OLIVE and EPA-DHA animals showed that both groups had the same average content of total fatty acids (981 ± 177 and 931 ± 140 µg/ml, respectively) and of the saturated palmitic and stearic acids. As expected, the OLIVE group had significantly higher plasma concentrations of oleic acid as compared with EPA-DHA rats, whereas the plasma of EPA-DHA rats was higher in total n-3PUFA, as compared with the OLIVE group (Table 2). Even though the average plasma concentration of total n-6 polyunsaturated fatty acids (n-6PUFA) did not differ between the two groups, a different profile within the n-6PUFA series was recorded at the end of the treatments. In particular, the EPA-DHA group displayed significantly lower arachidonate levels than the OLIVE group (Table 2). Consequently, the n-6PUFA/n-3PUFA

Table 1 Some metabolic parameters of fasting SHR rats at the end of the treatment

Treatment	Body weight (g)	Fasting plasma glucose (mM)	Creatinine (mg/ml)	Homocysteine (µM)
OLIVE (n = 8)	395.7 ± 24.8	9.8 ± 0.3	36.1 ± 10.3	1.6 ± 0.1
EPA-DHA (n = 8)	372.8 ± 9.2	8.6 ± 0.2	51.1 ± 11.5	2.2 ± 0.6

Metabolic parameters of our rats were measured before sacrificing the animals, at the end of the treatments as described in the Methods

Table 2 Plasma concentration of stearic, palmitic, oleic and n-6PUFA acids in OLIVE and EPA-DHA rats

	Group	
	OLIVE (n = 5)	EPA-DHA (n = 7)
Acid Concentration (mM)		
Oleic	14.9 ± 0.8*	12.2 ± 0.5
Stearic	11.5 ± 0.5	9.6 ± 0.6
Palmitic	119.1 ± 0.8	21.1 ± 1.4
n-6PUFA		
Linoleic	0.60 ± 0.11	0.98 ± 0.22
Arachidonic	1.07 ± 0.07	0.49 ± 0.11**
Docosapentaenoic	0.016 ± 0.002	0.016 ± 0.003
n-3PUFA		
alpha-Linolenic	0.009 ± 0.002	0.010 ± 0.001
Eicosahexaenoic	0.005 ± 0.001	0.063 ± 0.011**
Docosahexaenoic	0.057 ± 0.006	0.10 ± 0.02**

Fatty acid analysis of plasma from OLIVE and EPA-DHA-treated animals was performed as described in the text. Results are expressed as concentration (mM) and they represent the mean ± S. E. of the values found in each plasma sample. * Significantly different from EPA-DHA ($P < 0.05$); ** significantly different from OLIVE ($P < 0.05$)

ratio was significantly lowered in the EPA-DHA (7.8 ± 0.2 vs. 18.9 ± 1.5 in OLIVE) EPA-DHA group (7.8 ± 0.2 vs. 18.9 ± 1.5 in OLIVE).

Asymmetric and symmetric dimethyl arginines, and nitrite plasma levels

Both ADMA and its biologically inactive isomer, SDMA, were measured in the plasma of OLIVE and EPA-DHA treated rats. We found significantly higher concentrations of plasma ADMA in OLIVE than in EPA-DHA rats, whereas no significant differences in the plasma levels of SDMA were found (Fig. 1). Consequently, the ADMA/SDMA ratio, an index of ADMA metabolic activation, significantly changed only in the EPA-DHA animals (24.6 ± 6.5 in OLIVE vs 12.0 ± 2.7* in EPA-DHA rats, $p < 0.05$)

However, this selective reduction of plasma ADMA was not associated with any modification of kidney DDHA (0.011 ± 0.005 and 0.013 ± 0.003 μmol/g of tissue/min in OLIVE and EPA-DHA, respectively, for an enzyme activity, expressed in katals, of 5.7 x 10^{-8} in OLIVE and 6.5 x 10^{-8} mol/s in EPA-DHA).

Plasma nitrite concentrations were significantly higher in the plasma of EPA-DHA than in OLIVE rats (0.73* ± 0.05 vs. 0.23 ± 0.08 μM, respectively; $p < 0.05$).

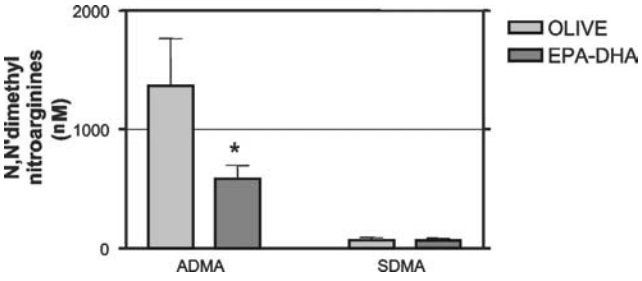

Fig. 1 ADMA plasma concentrations in OLIVE and EPA-DHA treated rats. The concentration of ADMA and SDMA were measured by HPLC analysis of denatured plasma proteins from OLIVE and EPA-DHA rats, as described in the Materials and methods section. Data are means ± S. E. M. * $p < 0.05$ as compared with OLIVE rats

Plasma asymmetric dimethyl arginine correlates with arachidonate concentrations

A positive correlation (Spearman coefficient $r = 0.923$; two tailed test $p < 0.0001$, extremely significant) between ADMA and arachidonate plasma concentrations was found. Arachidonate and ADMA plasma concentrations changed in parallel and were both reduced by ~50 % in the plasma of EPA-DHA rats as compared with OLIVE rats (Fig. 2).

Discussion

Cardiovascular diseases represent the leading causes of death and disability in developed countries and one of the most important advances in medicine has been the identification of CV risk factors as predictive of the occurrence of fatal and nonfatal CV events. The importance of monitoring risk factors relies in the ability of finding therapeutic strategies aimed at reducing their negative predictivity.

In addition to the "classical" CV risk factors, as plasma cholesterol levels, high systolic blood pressure, plasma glycaemia, increased ADMA levels, the most potent endogenous nitric oxide synthase inhibitor, is now considered a novel marker of endothelial dysfunction and an independent risk factor of CV diseases [10, 11]. Because the reduction of high ADMA plasma levels is a new target of drug activity, we were interested in studying whether an n-3PUFA supplementation could affect the plasma levels of this factor as a part of their beneficial CV effects. To verify whether a relation between n-3PUFA and ADMA existed we chose an experimental model at high CV risk and with a good predictivity for drug effectiveness for human CV diseases: the aged SHR. In this experimental animal model the simultaneous presence of several CV risks factors (long-term sustained hypertension, aging and of the onset of metabolic syndrome) concur compromising the mechanisms involved in the endothelium-dependent maintenance of

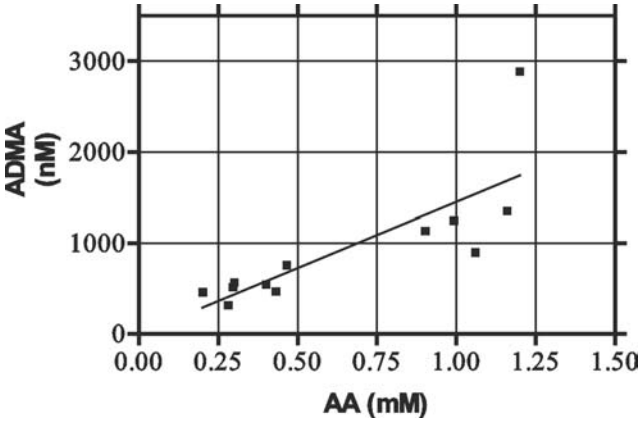

Fig. 2 Correlation between arachidonate and ADMA plasma concentrations in EPA-DHA and OLIVE oil treated rats. Arachidonate (AA) and ADMA plasma concentrations were evaluated as described in the Materials and methods section. The data were statistically analysed by using Graph-Pad Prism 4.00 and Instat 4 version for Windows

vascular tone and structure with a particular compromission of the nitric oxide pathway [23]. In addition to this, SHR are characterised by abnormal plasma and tissue fatty acid composition (high 6PUFA/n-3PUFA) when compared with age-matched normotensive rats. This difference in fatty acid profile becomes more pronounced with age thus suggesting a possible correlation between rat tissue lipid composition (high 6PUFA/n-3PUFA) and the severity and the progression of their CV disease [23].

Treating for two months the aged SHR with EPA and DHA, the pharmacologically active n-3PUFA, increasing levels of n-3PUFA and a parallel decrease of arachidonate plasma levels, with respect to that measured in placebo-olive oil treated animals, were obtained. This change in plasma fatty acid composition obtained with n-3PUFA supplementation reduced the n-6PUFA/n-3PUFA and also ADMA plasma levels over the values found in OLIVE rats, leaving unchanged the plasma level of SDMA, the biologically inactive isomer of ADMA.

The reduction of ADMA/SDMA in EPA-DHA rats was not due to ADMA metabolic activation because the activity of the kidney DDHA and the plasma concentration of homocysteine, an inhibitor of DDHA [25], were found similar in the two groups of rats. In addition, reduction of plasma ADMA levels was not the consequence of an amelioration of renal function. In fact, similar fasting creatinine was measured in both groups of rats.

Moreover, in the plasma of EPA-DHA rats increasing nitrite levels were also measured, as compared with OLIVE rats. Whether the higher nitrite concentrations found in EPA-DHA rats were causally due to the ADMA reduction or were a consequence of an effect of EPA and DHA on nitric oxide synthase is yet to be established but it is undoubted that both ADMA reduction and increased nitrite availability can concur to ameliorate endothelial function [24, 25]. n-3PUFA have already been demonstrated to have some antihypertensive effectiveness in young SHR [28]. The lack of this effect in our animals should not be a surprise considering that, at the age of 16 months, the compensatory and adaptive mechanisms, the anatomical and vascular remodelling, which derive from the sustained long-term hypertension, have been completely established [29].

Thus, the reduction of ADMA plasma levels might be linked in some ways to the reduction of arachidonate plasma levels. In fact, ADMA and arachidonate fluctuate similarly in the two groups of rats and they are both reduced by nearly 50 % in EPA-DHA with respect to OLIVE rats. The effect of n-3PUFA supplementation on arachidonate reduction is a well-assessed finding [30] which supports their use in inflammatory pathologies [26].

A common inflammatory basis for both endothelial dysfunction and atherosclerosis has been confirmed recently [27]. Plasma ADMA accumulation seems to represent an early marker for both the metabolic dysfunctions of the vasculature [10, 33] and of inflammation [34]. Arachidonate metabolism is a key step in generation of mediators crucial for controlling vasculature-platelet interaction and inflammation cascade.

A link between vascular inflammation and arachidonate metabolites was sustained further by the recent finding showing how a n-3PUFA dietary supplementation is able to blunt the arachidonate 5-lipoxygenase promoter activity and then the leukotriene-mediated inflammation that leads to atherosclerosis [35]. According to this new acquisition, the reduction of ADMA plasma accumulation observed in our EPA and DHA treated animals might mirror the decrease of the inflammatory ground of endothelial and confirms that this treatment is of some usefulness in reducing endothelial dysfunction and the sequels of atherosclerosis.

The effect of EPA-DHA on plasma ADMA accumulation is a finding that increases the knowledge on the n-3PUFA beneficial CV effects and suggests that ADMA and arachidonate plasma levels might represent the double face of common metabolic disorders. Because treatment effectiveness in SHR has usually a good predictivity for human diseases [29], the reduction of ADMA plasma levels might represent a target of n-3PUFA therapy and a tool for monitoring n-3PUFA effectiveness also in humans.

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