**Dietary unsaturated fatty acids differently affect catecholamine handling by
adrenal chromaffin cells**

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Running title: Dietary fatty acids and catecholamine handling

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Abbreviations: AA, arachidonic acid; AD, adrenaline; CA, catecholamines; COMT, catechol-*O*-methyltransferase; CDV, cardiovascular; DHA, docosahexaenoic acid; EA, elaidic acid; EP3R, prostaglandin E3 receptor; EPA, eicosapentaenoic acid; FA, fatty acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KCl, potassium chloride; LA, linoleic acid; MAO, monoamine oxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NA, noradrenaline; NET, noradrenaline transporter; OA, oleic acid; PGE2, prostaglandin E2; PNMT, phenylethanolamine N-methyltransferase; TH, tyrosine hydroxylase; VMAT, vesicular monoamine transporter; γ -LIN, γ -linolenic acid.

Abstract

Catecholamines (CA) play an important role in cardiovascular (CDV) disease risk. Namely, noradrenaline (NA) levels positively correlate whereas adrenaline (AD) levels negatively correlate with obesity and/or CDV disease. Western diets, which are typically rich in Ω -6 fatty acids (FA) and deficient in Ω -3 FA, may contribute to the development of obesity, type 2 diabetes and/or coronary artery disease. Taking this into consideration, and the fact that our group has already described that saturated FA affect catecholamine handling by adrenal chromaffin cells, this work aimed to investigate the effect of unsaturated FA upon catecholamine handling in the same model. Our results showed that chronic exposure to unsaturated FA differently modulated CA cellular content and release, regardless of both FA serie and number of carbon atoms. Namely, the Ω -6 arachidonic and linoleic acids, based on their effect on CA release and cellular content, seemed to impair NA and AD vesicular transport, whereas γ -linolenic acid selectively impaired AD synthesis and release. Within the Ω -9 FA, oleic acid was devoid of effect and elaidic acid behaved similarly to γ -linolenic acid. Eicosapentaenoic and docosahexaenoic acids (Ω -3series) impaired the synthesis and release of both NA and AD. These results **deserve attention and future development, namely in what concerns the mechanisms involved and correlative effects *in vivo*.**

Key-words: Noradrenaline, adrenaline, unsaturated lipids, tyrosine hydroxylase, phenylethanolamine N-methyltransferase

1. Introduction

Obesity prevalence is dramatically increasing worldwide, being associated with an increased risk of cardiovascular (CDV) and metabolic disorders [1]. Adrenaline (AD) and noradrenaline (NA), the main catecholamines (CA) secreted by the sympathoadrenomedullary system, play a crucial role in the regulation of metabolic and CDV homeostasis. The release of AD and NA into the circulation is controlled by a wide range of factors both in normal and stress conditions [2, 3]. Interestingly, plasma NA positively correlates with obesity and CDV risk, whereas AD levels show an inverse association with CDV mortality [4, 5]. Moreover, in humans, CA are important regulators of adipose tissue lipolysis, having a lipolytic effect by acting on β -adrenergic receptors or an anti-lipolytic effect by acting on α 2-adrenergic receptors [6].

Fatty acids (FA) act as important metabolic substrates and components of biological membranes. However, they also function as signaling molecules, regulating a wide range of physiological responses [7], such as gene transcription and metabolic and inflammatory responses [8]. Typical western diets are normally rich in Ω -6 and *trans* FA (non-essential FA used as substitutes for saturated FA in diet [9]) and deficient in Ω -3 FA being the ratio of Ω -6 to Ω -3 generally >10:1, whereas on a primitive human diet it was closer to 1:1 [10]. An elevated consumption of saturated and *trans* FA over mono and polyunsaturated FA or a dysfunction in their metabolism contributes to the development of many chronic diseases such as obesity, type 2 diabetes, cancer [7] and psychological disturbances [11]. This could be related with the fact Ω -3 FA are usually associated with cardioprotective benefits due to their multiple physiological effects on lipids, blood pressure, vascular function, cardiac rhythms, platelet function, and inflammatory responses [12]. Indeed, epidemiological, human and animal studies suggest that a Ω -3 rich diet may decrease the risk factors for metabolic syndrome including hyperglycemia, insulin resistance, hypertension, dyslipidemia and central adiposity [13]. In line with this, a recent report showed that Ω -3 FA are able to increase sympathetic activation and reduce abdominal adiposity [14].

In addition, consumption of *trans* FA raises the levels of low-density lipoproteins and triglycerides, lowers the levels of high-density lipoproteins, induces an inflammatory response, and, even at low levels of intake, significantly increases the risk of coronary events [8, 15].

Although both *in vitro* [16-18] and *in vivo* [19] studies reported that FA can have profound effects on neurosecretion, very limited information is available concerning the

effect of these molecules on CA handling by adrenal chromaffin cells [20, 21]. So, in the present work, we investigated the effect of several unsaturated fatty acids on CA synthesis and secretion in adrenal chromaffin cells. For that purpose, the effect of chronic exposure to monounsaturated, polyunsaturated and *trans* fatty acids on CA cellular content and release was investigated, using bovine adrenal chromaffin cells. Additionally, the effects of these FA on the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme of the CA synthesis, and phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts NA to AD, were also evaluated.

2. Materials and methods

2.1. Materials

Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LA), γ -linolenic acid (γ -LIN), arachidonic acid (AA), oleic acid (OA), elaidic acid (EA), MTT (Methylthiazolyldiphenyl-tetrazolium bromide), Dulbecco's Modified Eagle's medium/F12, octenylsuccinic anhydride (OSA), Tris (tris-(hydroxymethyl)-aminomethane hydrochloride), fetal calf serum, L-glutamine, HEPES, cytosine arabinoside, penicillin, streptomycin, amphotericin B, inhibitor of RNases, pargyline, perchloric acid, collagenase A, ascorbic acid (Sigma, St. Louis, MO, USA); albumin, EDTA, Titron X-100, methanol, citric acid, D (+) glucose (Merck, Darmstadt, Germany); SuperScript™ II Reverse Transcriptase, RNase H, GAPDH, SYBR Green I detection, chloroform (Invitrogen, Life Technologies, Grand Island, NY, USA); Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany).

2.2. Preparation of adrenal medullary cells

Bovine adrenal glands were obtained from a local slaughterhouse and were processed for cell culture within 2 hours of slaughter, being transported to the laboratory in ice-cold Ca^{2+} - Mg^{2+} -free buffer (containing: 154 mM NaCl, 2.6 mM KCl, 0.85 mM KH_2PO_4 , 2.15 mM K_2HPO_4 , 10 mM glucose and 12.7 mM HEPES, pH 7.4). The chromaffin cells were isolated by digestion with collagenase A as described by Livett et al. [22, 23] with some modifications. Briefly, the adrenal glands were trimmed of fat and washed with Ca^{2+} - Mg^{2+} -free buffer. The buffer was injected into the adrenal vein in order to wash the remaining blood from the gland. The adrenal glands were digested with a Ca^{2+} - Mg^{2+} -free buffer containing 0.25% collagenase A and 0.01% DNase I. Five mL of this solution was injected into the adrenal vein of each gland. This procedure was repeated every 15 min with the glands incubated at 37 °C in Ca^{2+} - Mg^{2+} -free buffer. After 45 min, the digested medullary tissue was mechanically separated from the adrenal cortex and incubated in buffer, containing collagenase A, for 30 min at 37 °C. Subsequently, cells were filtered through a 200 μm nylon mesh. Afterwards, filtered cells were centrifuged $3 \times$ at 1000 g for 3 min and washed with Ca^{2+} - Mg^{2+} -free buffer in order to remove the collagenase A/DNase. At last, they were filtered through a 100 μm nylon mesh.

2.3. Cell culture

Bovine chromaffin cells were cultured in Dulbecco's Modified Eagle's medium (nutrient mixture F-12 Ham (1:1), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 15 mM HEPES, 10 μ M cytosine arabinoside, 100 units/mL penicillin, 100 mg/mL streptomycin and 0.25 μ m/mL amphotericin B). The dispersed cells were plated in collagen-coated 24-well plastic culture dishes (2 cm²; Ø 15.6 mm) at a density of 4-5 x 10⁵-10⁶ cells/well and incubated at 37 °C in an atmosphere consisting of 5% CO₂ in air and with 99% of relative humidity. Culture medium was replaced every 24 hours and, for the experiments, cells were used after 4 days in culture. Twenty-four hours before experiment, the bovine chromaffin cells were cultured in fetal calf serum-free medium [3].

2.4. *In vitro* experiments

2.4.1. Chronic treatment with fatty acids

After 4 days in culture, and before the studies on CA handling, cells were incubated for 24 hours (chronic exposure) with ethanol or DMSO (controls), linoleic acid (18:2n-6), γ -linolenic acid (18:3n-6), docosahexaenoic acid (22:6n-3), eicosapentaenoic acid (20:5n-3) arachidonic acid (20:4n-6), oleic acid (18:1n-9, cis) or elaidic acid (18:1n-9, trans). The concentrations of the tested FA (50 and 100 μ M) are considered to be within the physiological range [24] all the fatty acids were dissolved in ethanol with the exception of oleic acid, which was dissolved in DMSO (0.1% (v/v)).

2.4.2. Studies on CA handling

The experiments were performed in buffer solution with the following composition: 125 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1.2 mM CaCl₂, 25 mM HEPES-NaOH, 5.6 mM D(+) glucose, 1 mM ascorbic acid, 0.1 mM pargyline and 0.01 mM tolcapone, pH 7.4. After the chronic treatment with the several fatty acids, cells were pre-incubated for 10 min, at 37 °C, in order to study basal release. Initially, the culture medium was removed and cells were washed with buffer at 37 °C. Afterwards, cells were incubated for 15 min in the absence (control) or presence of potassium chloride (KCl) (50 mM). Incubation was stopped by rinsing the cells with ice-cold medium (4° C). Cells were solubilized with 0.3 mL

0.1% (v/v) Triton X-100 (in 5 mM Tris.HCl, pH 7.4) or perchloric acid (0.2 M) for the determination of proteins or CA, respectively, and placed at 37°C overnight.

2.4.3. Determination of catecholamines levels

CA cellular content and release were determined by high pressure liquid chromatography with electrochemical detection (HPLC-ED) using the methodology described by Soares-da-Silva et al. [25]. The lower limits of CA detection varied from 350 to 1000 fmol.

2.4.4. Enzymatic expression by quantitative real-time PCR

Total RNA was extracted with Tripure[®] isolation reagent (Roche Applied Science, Switzerland), according to manufacturer's instructions. For cDNA synthesis, 5 µg of total RNA was incubated at 45°C for 1 hour in a total volume of 20 µL with 200 U/µL of Superscript[™] II Reverse Transcriptase, in 50 ng/µL random hexamers, 10 mmol/L per dNTP, 15 mM MgCl₂, 375 mM KCl, 250 mM Tris-HCl, pH 8.3 (at room temperature), 40 U/µL RNase inhibitor (RNaseOUT[™]). Reverse Transcriptase II was included, incubating at 25° C during 10 min, followed by 50 min at 42° C and 70° C for 15 min. Samples were incubated with 1 µL RNase H for 20 min at 37 °C. After cDNA synthesis, TH and PNMT mRNA expression levels were analyzed by relative quantification using quantitative real-time PCR (qRT-PCR) with SYBR Green I detection. The qRT-PCR was performed in a StepOne[™] qPCR machine (Applied Biosystems, Foster City, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The default thermocycler conditions have been used for all genes: 10 min of pre-incubation at 95° C followed by 40 cycles for 15 seconds at 95° C and 1 min at 60° C. Following amplification, melting curve analyses verify the authenticity of the amplified products by their specific melting temperatures (T_m) according to the instructions of the manufacturer. The quantification reactions were carried out in 20 µL volumes in a 48-well plate (Applied Biosystems[™]) containing 300 nM of forward and reverse primers, 1X FastStart universal SYBR green master (ROX) (Roche Applied Science, Switzerland) and approximately 5 µL of cDNA. The following primers for bovine TH, bovine PNMT and GAPDH were used: 5'- ATC ATG TCC CCA CGC TTC GTC-3' (forward primer bovine TH), 5'- GCC TTC CCG TCC CTC TCC AC-3' (reverse primer bovine TH), 5'- CTA CCT CCG CAA CAA CTA CGC-3' (forward primer bovine PNMT), 5'- TGT CCT CAA AGT GGG CAC

AG-3' (reverse primer bovine PNMT), 5' -CGA CAC TCA CTC TTC TAC CTT-3' (forward primer GAPDH), 5'-GTC CAC CAC CCT GTT GCT GTA-3' (reverse primer GAPDH). The data analysis was carried out using the StepOne™ Software version 2.2 (Applied Biosystems, Foster City, CA, USA) with the same baseline and threshold set for each plate, in order to generate threshold cycle (Ct) values for both genes in each sample.

2.4.5. Cellular viability

Cellular viability, after chronic treatment with the FA, was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method. MTT method is based on the activity of a mitochondrial dehydrogenase which cleaves a yellow dye (MTT), forming purpur crystals of formazan and this reaction occurs only in viable cells. The amount of product formed was determined spectrophotometrically [26].

2.4.6. Protein quantification

Protein concentration was determined as described by Bradford [27], using human serum albumin as standard.

2.5. Statistical analysis

The release of CA into the medium was calculated during periods A and B, corresponding to the first 10 min and to the following 15 min incubation periods, respectively. Release of CA from bovine adrenal chromaffin cells into the incubation medium during periods A or B was expressed as total, which corresponds to the amount of CA released into the medium in relation to the total cellular content of CA at the beginning of the release period. Basal and KCl-stimulated release correspond to release periods A and B, respectively.

Results were expressed in median (p25 – p75). Data were analyzed using IBM SPSS statistics 21 software.

Normal distribution of data was tested by Shapiro-Wilk test. The groups, of each category, were compared using one-way ANOVA (group and time) test for parametric data and Kruskal-Wallis test for non-parametric data. The homogeneity of variance was tested using Levene's test. Regarding qRT-PCR, data analysis was carried out using the computer software SPSS for Windows (version 19). The relative expression was determined using the $2^{-\Delta\Delta C_t}$ method [28], and expression values were normalized to

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251 GAPDH. The $2^{-\Delta\Delta C_t}$ method, along with Student's t-test or Mann-Whitney *U* test, were
252 used in order to evaluate statistical differences in the normalized expression of the
253 targets analyzed among the different experimental conditions tested. The *P* value
254 obtained was considered statistically significant under 0.05.
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3. Results

In order to assess whether unsaturated fatty acids alter CA handling by bovine chromaffin cells, these cells were submitted to mono-, *trans*- and polyunsaturated fatty acids exposure for 24 hours, and the following parameters were evaluated: CA total and cellular content (both basal and KCl-stimulated release) and mRNA levels of TH and PNMT.

Under control conditions, the median cellular content at the end of the experiments was found to be 604 ± 33 and 228 ± 21 nmol/mg protein for AD and NA, respectively. Basal release during 10 min of pre-incubation, was 0.015 ± 0.001 and 0.0145 ± 0.001 for AD and NA, respectively. The release of AD and NA was increased by excess KCl to about 3.31 ± 0.25 and 2.95 ± 0.36 of control, respectively.

3.1. Catecholamine total content

Regarding CA total content (which includes cellular plus released contents), we observed that LA and DHA, in both concentrations (50 and 100 μ M), were able to significantly decrease the content of both CA (tables 1 and 2). For the highest concentration, AD total content achieved after LA and DHA treatments was 220.76 (195.78 – 334.53) nmol/mg protein and 5594.61 (5252.21 – 6205.32) nmol/mg protein, respectively, and for NA the total content was 114.65 (102.74 – 164.39) nmol/mg protein and 1183.82 (533.52 – 1336.95) nmol/mg protein, respectively. We observed that EA and γ -LIN significantly decreased the content of both AD and NA, when used at the highest concentration (100 μ M). On the contrary, EPA, OA and AA did not significantly affect total content of both CA (tables 1 and 2).

None of the FA tested altered bovine chromaffin cells viability (results not shown).

3.2. Catecholamine cellular content

In agreement with what was observed for CA total content, LA diminished the cellular content of both CA in a concentration-dependent way. LA at 50 and 100 μ M was capable of reducing NA cellular content to about 73% and 12%, respectively, in comparison with controls (table 1). For AD cellular content, LA at 50 and 100 μ M reduced it approximately 79% and 11% respectively, in comparison with controls (table 2). On the other hand, both EA and γ -LIN significantly decreased the content of both

AD and NA, but only at the highest concentration tested (100 μ M). In contrast, EPA, DHA, OA and AA did not significantly modify CA cellular content (tables 1 and 2).

3.3. Catecholamine basal release

In relation to basal release, AA, LA and EA did not differentiate between AD and NA, but had opposite effects over both CA: AA significantly augmented their basal release in the same degree (to about 159% for both CA), whereas EA and LA inhibited the release of these amines. On the other hand, the chronic exposure to OA, DHA, EPA and γ -LIN did not change the basal release of both CA (tables 1 and 2).

3.4. Catecholamine KCl-stimulated release

In a concentration independent manner, EPA, DHA, EA and AA were able to significantly decrease the release of both CA induced by KCl at both concentrations (tables 1 and 2). In addition, γ -LIN was able to diminish the release of NA induced by KCl at both concentration however, this FA decreased AD release only when tested at 100 μ M (table 2). OA decreased the release of both CA to about 58% and 65% (for AD and NA, respectively) in comparison with the release induced by KCl, but only for the highest concentration tested (tables 1 and 2). On the other hand, LA was the only fatty acid capable of significantly augmenting the KCl-stimulated release of AD to about 145%, but only at 100 μ M (table 2).

3.5. TH and PNMT mRNA levels

In order to evaluate the mRNA expression of both TH and PNMT we performed qRT-PCR. As seen in Fig. 1, only EPA was capable of significantly changing the mRNA expression of TH, by decreasing its levels. As shown in Fig. 2, LA, EA, EPA, γ -LIN and DHA significantly decreased the expression of PNMT, DHA being the most effective in this reduction ($p < 0.001$, $p = 0.003$, $p = 0.031$, $p = 0.011$ and $p = 0.001$, respectively for LA, EA, EPA, γ -LIN and DHA). However, neither AA (data not shown) nor OA significantly changed the expression of these enzymes.

4. Discussion

The aim of this work was to study the effect of unsaturated FA on CA handling by bovine adrenal chromaffin cells. In general, our results showed that several unsaturated FA affected AD and NA handling through distinct ways, regardless of both the number of carbons (chain length) and double bonds. The FA tested altered four different parameters: CA synthesis and accumulation, CA basal release and CA release in response to depolarization by KCl (Fig. 3). In relation to the synthesis and accumulation of CA, fatty acids showed distinct behaviors: 1) EA, LA and γ -LIN decreased both the synthesis and accumulation of CA; 2) DHA and EPA decreased the synthesis but maintained the accumulation of CA; 3) OA and AA did not change either the synthesis or the accumulation of CA. Moreover, EA, LA and γ -LIN affected the production of AD by decreasing PNMT mRNA levels (Fig. 3 A-1 and B-1). In contrast, EPA may affect the synthesis of CA since it was able to decrease the mRNA levels of TH and PNMT, but curiously preserved their total and cellular content. Concerning DHA, it did not affect CA accumulation since it maintained the cellular content of both CA, but decreased the production of AD by interfering with PNMT levels. OA and AA did not affect either the expression of any of the enzymes or the content of CA.

Similarly to what happened with the synthesis and accumulation of CA, FA were able to differentially affect CA basal release. Among all, only AA was able to increase the basal release of both CA, whereas LA and EA decreased it for both CA. Curiously, LIN, EPA and DHA did not affect this release. At last, all fatty acids, with the exception of LA, were able to decrease the release of both CA induced by depolarization with KCl, whereas LA increased this parameter.

The consumption of *trans* FA has been associated with a higher risk of coronary heart disease, insulin resistance, sudden death and impaired early development [29-32]. Some of the mechanisms through which *trans* fatty acids contribute to this higher risk have already been described. In fact, several works showed that they increase plasmatic levels of low-density lipoproteins, triglycerides, apolipoprotein B and C reactive protein, decrease plasmatic levels of high-density lipoproteins and interfere with enzymes involved in fatty acids metabolism [30, 31, 33]. *Trans* fatty acids are present in variable quantities in a broad range of foods, and despite legislative intervention and health initiatives, dietary FA remain a concern, owing to the addition of industrially produced *trans* fat to foods and the excessive reheating of frying oils [34]. Due to this, the study of *trans* FA cellular effects on CA metabolism is of major importance. Our

work describes for first time the effect of *trans* FA upon CA handling by chromaffin cells. In our study, EA, the OA *trans* isomer, and the most representative in foods (80-90% of total *trans*), decreased all the parameters studied for NA and AD in the studied cells, except for TH expression. Given that other authors have shown that EA does not affect Ca^{2+} -induced CA secretion in these cells [35], the decrease in both CA release was probably a consequence of a lower cellular content. Regarding PNMT mRNA levels, the ability of EA to alter its gene expression was already described in other cellular models [31], whereas the lack of effect over TH is somehow in accordance with its inability to activate this enzyme [35]. In fact, the decrease of PNMT expression could explain the reduction of AD content. Although EA did not alter TH expression, it could eventually decrease its function, leading to a reduction of both NA content and release.

Chromaffin cells exposure to AA suggests that this FA affected vesicular monoamine transporter (VMAT) function, since it was able to increase the basal release of CA, decrease their release after depolarization with KCl and sustain cellular and total CA content (a reserpine-like effect) and PNMT and TH expression. In fact, the inhibition of VMAT (Fig. 3, C-1), a reserpine-like effect, could explain the increase of CA basal release associated with a lower accumulation of these amines in granules, suggested by a lower response after KCl stimulation. On the contrary, in bovine chromaffin cells, it was already described that melittin (a known activator of phospholipase A_2 (PLA_2) [36], which synthesizes AA) stimulates CA secretion in a Ca^{2+} -dependent way [37]. It was also shown that PLA_2 coupled to pertussis toxin-sensitive G-protein can be a crucial element for the late Ca^{2+} -dependent stage of exocytosis in these cells [38]. This could suggest that AA may modulate CA exocytosis, since this FA and/or its metabolites are able to affect intracellular Ca^{2+} levels through either activation of Ca^{2+} channels [39] and mobilization from intracellular stores [40].

Since it was already described that chromaffin cells express cyclooxygenase [41-43], we cannot exclude that the observed effects could also be mediated by prostaglandins originated from AA after cyclooxygenase action. In fact, chromaffin cells express prostaglandin receptors [44-46] and, after binding to prostaglandin E_3 receptor (EP_3R), prostaglandin E_2 (PGE_2) causes a decrease in intracellular Ca^{2+} levels via pertussis toxin sensitive G proteins and/or insensitive G proteins [47, 48]. The above effects are somehow in accordance with our own results since AA, probably through its

conversion to PGE2, was able to decrease CA release after depolarization by KCl (Fig. 3. C-2).

In partial agreement to what was described for AA, our results suggest that γ -LIN seems to decrease VMAT function since this FA was able to maintain CA basal release and decrease KCl-stimulated CA release. However, in contrast to AA, γ -LIN did not decrease CA basal release once there was a reduction of their content. This could be explained by the decrease of PNMT expression and impairment of TH function. Even though γ -LIN did not **change** TH expression, it could affect its function, reducing its activity and leading to diminished NA content. On the other hand, some studies showed that γ -LIN can be converted into dihomogamma-linolenic acid and AA, the precursors for PGE1 and PGE2, respectively [49]. Therefore, the conversion of γ -LIN into AA could result in a reduction of intracellular Ca^{2+} levels, through the activation of EP3R by PGE, leading to a decrease of CA KCl-stimulated release.

Another Ω -6 FA, LA, increased the KCl-stimulated release of AD, and decreased both CA basal release and both total and cellular content. The ability of LA to increase the AD release after depolarization by KCl might be related with either an increase of the expression and/or activity of VMAT (Fig. 3. B-2) or an increase of intracellular Ca^{2+} levels (Fig. 3. B-3). The rise of VMAT function can lead to a larger AD accumulation into chromaffin granules, which could be translated into a decrease of AD in cytosol, diminishing both CA content and basal release. On the other hand, several studies, in different cells types, have shown that LA has the ability to increase intracellular Ca^{2+} levels through a mechanism dependent on inositol trisphosphate [50, 51] and extracellular Ca^{2+} influx [50]. Interestingly, in mice gustatory cells positive for CD36 - a membrane protein involved in FA uptake [52] - LA was capable of inducing an increase in intracellular Ca^{2+} levels, whereas in CD36-negative gustatory cells it was unable to affect intracellular Ca^{2+} [53]. On the other hand, LA - similarly to γ -LIN and to the Ω -9 fatty acid EA- was able to lower total and cellular content of both CA, which can be a consequence of PNMT expression inhibition (Fig. 3. B-1) together with an increase in the function of enzymes involved in CA catabolism and/or a lower function of transporters involved in CA re-uptake.

Epidemiological and clinical studies have established an inverse relationship between the ingestion of Ω -3 polyunsaturated FA (eg. EPA and DHA) and mortality associated with CDV disease (reviewed in [54]). In our study, EPA was the only FA that significantly decreased the expression of enzymes involved in CA synthesis (TH

and PNMT) whereas DHA only affected the expression of PNMT. Curiously, the decrease of mRNA levels induced by these FA was not associated with either a change in the cellular content of CA or a change in their basal release. The maintenance of CA cellular content could have been achieved primarily by decreasing CA release and/or by maintaining TH and PNMT function (for instance, through an increase of their activities) (Fig. 3. D-1). In addition, other mechanisms, such as a decrease in CA catabolism (through an inhibition of monoamine oxidase and/or catechol -O-methyltransferase) (Fig. 3. D-2), and/or an increase in their cellular reuptake (Fig. 3. D-3) may have been also involved in those effects. Both EPA and DHA decreased CA release induced by KCl. This effect could be associated with an impairment of Ca^{2+} disposal, since several studies have shown that Ω -3 FA are able to affect intracellular Ca^{2+} levels through several mechanisms [55, 56].

An important point to discuss is the fact that, in order to clarify the intracellular mechanisms underlying the effects of the above FA upon CA handling further studies are needed. Since Ca^{2+} is a good candidate for the action of these molecules, its intracellular levels should be measured. In addition, the activity and expression of vesicular and membrane transporters and enzymes responsible for CA metabolism (eg. catechol-O-methyltransferase and monoamine oxidase) should also be analyzed in the future. Furthermore, in relation to TH and PNMT we only studied their mRNA levels and it would be relevant to also measure **their** protein levels and activities.

Our results showing that unsaturated FA had an impact on CA handling suggest that these amines might indeed constitute mediating factors in the well-documented relationship between unsaturated FA, metabolic syndrome and CDV disease.

The major conclusion of this work is that the unsaturated fatty acids tested were able to significantly modify CA handling by chromaffin cells, **in good agreement with previously described FA effects on neurosecretion [16-19]. Given the crucial role of CA in the regulation of metabolic CDV homeostasis, among other physiological effects, these results deserve attention and future development, namely in what concerns the mechanisms involved and correlative effects *in vivo*. Thus, after this preliminary *in vitro* study, we plan to expand our studies to determine if the fatty acids with the most relevant effects, namely elaidic and Ω -3 FA, might also have similar actions upon catecholamine handling in animal models.**

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Acknowledgements

This work was supported by U.Porto/Santander Totta (IJUP: 179/2010). A.L.T. is a recipient of a doctoral degree grant from FCT (SFRH/BD/47381/2008). The authors thank to Ricardo Escada for technical assistance with HPLC.

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Tables caption

Table 1. Evaluation of total content, cellular content, basal release and KCl-stimulated release of noradrenaline after chronic treatment with fatty acids.

Table 2. Evaluation of total content, cellular content, basal release and KCl-stimulated release of adrenaline after chronic treatment with fatty acids.

Figures caption

Figure 1. mRNA levels of tyrosine hydroxylase and phenylethanolamine N-methyltransferase after chronic treatment with fatty acids. Comparison of mRNA levels of tyrosine hydroxylase (TH) (A) and phenylethanolamine N-methyltransferase (PNMT) (B) in control conditions and after chronic treatment with elaidic acid (EA), γ -linolenic acid (γ -LIN), eicosapentaenoic acid (EPA), linoleic acid (LA) and docosahexaenoic acid (DHA) at 100 μ M by quantitative real-time PCR, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene (n=4 for each group). * $p < 0.05$, compared with control (ethanol).

Figure 2. mRNA levels of tyrosine hydroxylase and phenylethanolamine N-methyltransferase after chronic treatment with oleic acid. Comparison of mRNA levels of tyrosine hydroxylase (TH) (A) and phenylethanolamine N-methyltransferase (PNMT) (B) in control conditions and after chronic treatment with oleic acid (OA) at 100 μ M by quantitative real-time PCR, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene (n=4). * $p < 0.05$, compared with control (DMSO).

Figure 3. Putative intracellular mechanisms affected by chronic treatment with fatty acids. Intracellular mechanisms affected by chronic treatment (24 hours) with either elaidic and γ -linolenic acids (A), linoleic acid (B), arachidonic acid (C) or eicosapentaenoic and docosahexaenoic acids (D) on catecholamine handling by bovine chromaffin cells. Open circles: function increment; black cross: function impairment; TH: tyrosine hydroxylase; AAAD: Aromatic L-amino acid decarboxylase; D β H: dopamine β – Hydroxylase; PNMT: phenylethanolamine N-methyltransferase; MAO: monoamine oxidase; COMT: catechol -O- methyltransferase; VMAT: vesicular monoamine transporter; NET: noradrenaline transporter; NA: noradrenaline; AD: adrenaline.

Table 1. Evaluation of total content, cellular content, basal release and KCl-stimulated release of noradrenaline after chronic treatment with fatty acids.

Noradrenaline								
Parameters	Groups	Fatty acids						
		EPA	DHA	γ-LIN	LA	AA	OA	EA
Total Content	Control	1541.03 (1304.92 - 1733.97)	1541.03 (1304.92 - 1733.97)	522.86 (469.64 - 644.16)	522.86 (469.64 - 644.16)	209.57 (116.99 - 424.13)	209.64 (179.38 - 322.62)	405.67 (323.02 - 461.43)
	50 μM	2055.78 (1029.48 - 2766.93)	1303.39 (885.81 - 1447.75) *	488.49 (245.29 - 544.63)	406.74 (316.37 - 421.72) *	180.87 (169.41 - 192.99)	214.05 (198.06 - 235.32)	285.25 (207.37 - 325.26) *
	100 μM	929.90 (659.41 - 2386114.37)	1183.82 (533.52 - 1336.95) *	404.93 (213.37 - 451.81) *	114.65 (102.74 - 164.39) *	184.93 (148.38 - 212.64)	202.43 (200.79 - 241.54)	205.72 (129.39 - 239.19) *
Cellular Content	Control	452.93 (288.10 - 1397.12)	452.93 (288.10 - 1397.11)	279.07 (230.38 - 330.04)	279.07 (230.38 - 330.04)	179.37 (89.83 - 275.42)	182.91 (136.38 - 221.94)	137.95 (127.39 - 148.39)
	50 μM	1313.76 (409.53 - 2588.07)	828.03 (366.27 - 1296.59)	237.59 (91.62 - 296.65)	205.25 (165.83 - 217.74) *	144.76 (115.18 - 172.01)	165.813 (148.99 - 202.44)	101.11 (49.23 - 137.32)
	100 μM	359.45 (215.79 - 2233.76)	1054.66 (156.56 - 1153.47)	186.54 (66.68 - 233.73) *	32.90 (28.07 - 62.14) *	158.68 (90.12 - 173.59)	170.66 (140.05 - 205.39)	70.58 (32.31 - 86.90) *
Basal Release	Control	0.97 (0.57 - 1.31)	0.97 (0.57 - 1.31)	0.96 (0.68 - 1.21)	0.96 (0.68 - 1.21)	0.90 (0.80 - 1.29)	1.00 (0.73 - 1.17)	1.05 (0.68 - 1.27)
	50 μM	0.89 (0.43 - 1.54)	0.68 (0.51 - 1.05)	0.66 (0.56 - 0.87)	0.79 (0.75 - 0.85)	1.06 (0.97 - 1.15)	0.93 (0.77 - 1.24)	0.52 (0.35 - 0.59) *
	100 μM	0.82 (0.29 - 1.03)	0.56 (0.44 - 0.76)	0.8545 (0.72 - 1.22)	0.51 (0.45 - 0.54) *	1.43 (1.02 - 1.89) *	0.88 (0.71 - 1.37)	0.44 (0.42 - 0.49) *
KCl-stimulated release	Control	2.57 (2.22 - 3.33)	2.58 (2.22 - 3.33)	1.67 (1.47 - 1.79)	1.67 (1.47 - 1.80)	1.02 (0.94 - 1.34)	1.17 (0.88 - 1.59)	1.58 (1.50 - 1.78)
	50 μM	0.38 (0.26 - 0.64) *	0.70 (0.63 - 0.92) *	1.22 (1.10 - 1.44) *	1.09 (0.85 - 1.44)	0.74 (0.54 - 0.84) *	1.00 (0.69 - 1.08)	1.08 (0.97 - 1.18) *
	100 μM	0.71 (0.63 - 0.91) *	0.64 (0.46 - 0.78) *	0.86 (0.78 - 0.98) *	1.79 (1.67 - 2.17)	0.59 (0.30 - 0.85) *	0.76 (0.60 - 0.89) *	1.05 (0.83 - 1.14) *

Results are presented as nmol /mg protein median (p25 – p75).

* $p < 0.05$, compared with control group. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; OA: oleic acid; EA: elaidic acid; γ-LIN: γ-linolenic acid; LA: linoleic acid; AA:arachidonic acid.

Table 2. Evaluation of total content, cellular content, basal release and KCl-stimulated release of adrenaline after chronic treatment with fatty acids.

Adrenaline								
Parameters	Groups	Fatty acids						
		EPA	DHA	γ-LIN	LA	AA	OA	EA
Total Content	Control	8985.59 (6834.22 - 10642.50)	8985.59 (6834.22 - 10642.50)	1033.42 (950.86 - 1304.29)	1033.42 (950.86 - 1304.29)	420.33 (267.32 - 673.32)	457.94 (371.53 - 526.28)	1073.92 (1019.43 - 1144.13)
	50 μM	9986.71 (9247.85 - 11002.50)	8094.28 (5712.06 - 8665.94) *	1003.29 (515.97 - 1135.75)	776.39 (585.87 - 819.48) *	346.27 (297.18 - 385.52)	407.81 (368.53 - 485.09)	999.57 (795.50 - 1163.65)
	100 μM	8499.87 (7127.78 - 9929.24)	5594.61 (5252.21 - 6205.32) *	824.81 (420.75 - 947.15) *	220.76 (195.78 - 334.53) *	377.57 (273.34 - 422.33)	392.23 (356.65 - 493.63)	819.99 (513.35 - 889.27) *
Cellular Content	Control	3839.46 (2460.60 - 5804.53)	3839.46 (2460.60 - 5804.53)	618.98 (549.81 - 775.71)	618.98 (549.81 - 775.71)	355.13 (197.80 - 493.88)	397.17 (291.75 - 426.20)	499.60 (473.30 - 544.06)
	50 μM	6686.84 (3342.07 - 10455.66)	4577.85 (3305.44 - 5309.11)	594.22 (220.19 - 728.03)	488.95 (385.95 - 516.25) *	285.90 (224.74 - 344.17)	329.70 (299.25 - 207.10)	387.28 (210.36 - 518.56)
	100 μM	2869.04 (1752.05 - 9511.40)	4517.29 (1370.19 - 4908.64)	450.70 (158.90 - 580.28) *	69.89 (61.66 - 149.72) *	318.74 (189.93 - 349.51)	335.96 (288.79 - 423.92)	301.75 (132.07 - 375.07) *
Basal Release	Control	0.87 (0.79 - 1.06)	0.87 (0.79 - 1.06)	0.87 (0.69 - 1.36)	0.87 (0.69 - 1.36)	0.90 (0.75 - 1.21)	0.99 (0.73 - 1.25)	0.99 (0.87 - 1.18)
	50 μM	0.90 (0.84 - 1.37)	0.87 (0.78 - 1.06)	0.65 (0.56 - 0.88)	0.78 (0.70 - 0.86)	1.15 (1.06 - 1.33)	1.01 (0.80 - 1.23)	0.68 (0.60 - 0.89) *
	100 μM	0.86 (0.72 - 1.11)	0.83 (0.68 - 0.91)	1.02 (0.88 - 1.61)	0.53 (0.40 - 0.59) *	1.43 (1.06 - 1.91) *	0.90 (0.73 - 1.30)	0.83 (0.74 - 0.89)
KCl-stimulated release	Control	3.24 (2.73 - 4.11)	3.24 (2.73 - 4.11)	1.37 (1.24 - 1.56)	1.37 (1.24 - 1.56)	1.08 (0.91 - 1.56)	1.12 (0.86 - 1.69)	1.24 (1.21 - 1.37)
	50 μM	0.36 (0.32 - 0.72) *	0.42 (0.33 - 0.84) *	1.27 (1.01 - 1.42)	1.12 (0.93 - 1.45)	0.72 (0.62 - 0.83) *	0.84 (0.72 - 1.02)	0.79 (0.69 - 0.84) *
	100 μM	0.58 (0.47 - 0.82) *	0.46 (0.34 - 0.52) *	0.73 (0.60 - 0.95) *	1.99 (1.78 - 2.47) *	0.58 (0.23 - 0.71) *	0.65 (0.55 - 1.12) *	0.87 (0.80 - 1.00) *

Results are presented as nmol/mg protein median (p25 – p75).

* $p < 0.05$, compared with control group. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; OA: oleic acid; EA: elaidic acid; γ-LIN: γ-linolenic acid; LA: linoleic acid; AA:arachidonic acid.

Figure 2

Figure 3