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Increased plasma concentrations of Palmitoylethanolamide, an endogenous fatty acid amide, affect oxidative damage of human low-density lipoproteins: An in vitro study

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Abstract

Fatty acid ethanolamides (NAEs) are naturally occurring hydrophobic molecules usually present in a very small amount in many mammalian tissues and cells. Moreover, these compounds have been isolated in mammalian biological fluids, such as blood. Palmitoylethanolamide (C16:0) (PEA) is a fully saturated NAE, which presents some possible pharmaceutical activities, such as anti-inflammatory and antinociceptive effects.

PEA is physiologically present in the mammalian blood at concentrations ranging from 9.4 to 16.7 pmol/ml.

Since increasing evidence indicates that oxidative modification of low-density lipoproteins (LDL) is an important determinant in atherogenesis, the aim of this study was to evaluate the effect of physiologically relevant concentrations of PEA on Cu^{2+}-induced LDL oxidation (measured as conjugated dienes formation). Our experiments indicate both anti-oxidative and slightly pro-oxidative effects of PEA. The anti-oxidative effect is obtained at low PEA concentrations (0.01 and 0.1 μM), while the pro-oxidative effect is obtained at a higher PEA concentration (1 μM). Fluorescence and circular dichroism data indicate that the effect of PEA occurs mainly by affecting the conformational features of ApoB-100.

Keywords: Palmitoylethanolamide; LDL; Copper; Lipid peroxidation; Tryptophan oxidation; Fluorescence

1. Introduction

N-Acylethanolamines (NAEs) (fatty acid ethanolamides) are naturally occurring hydrophobic molecules usually present in a very small amount in many mammalian tissues and cells [1,2]. Moreover, NAEs are normally present in biological fluids, such as blood [2], in very low concentrations. The physiological levels of important NAEs in mammalian blood plasma are in the range 2.8–5.2 pmol/ml for anandamide (AEA); 9.4–16.7 pmol/ml for PEA; 8.1–10.3 pmol/ml for oleylethanolamide (OEA) [2–4]. However, the NAEs levels in blood plasma could be modified in pathological conditions, e.g., the physiological concentrations of AEA in human plasma are 4 pmol/ml, but these concentrations are increased up to 18–30 pmol/ml in sera of patients with endotoxic shocks [5]. In vivo studies demonstrated that NAEs could accumulate in injured tissues, such as, e.g., in myocardium infarcted areas [6], and in post decapitative brain ischemia [7].

The discovery that polyunsaturated NAEs (called "endocannabinoids") are endogenous ligands of cannabinoid-receptors CB1 and CB2 [6] raised the interest on these compounds, for the possible development of new drugs with applications similar to those suggested for cannabis [8].

NAEs with saturated or monounsaturated acyl chains are generally thought to be cannabinoid-receptor inactive [9], although this point is debated. They are suggested to pro-

Abbreviations: NAE, fatty acid ethanolamides; PEA, palmitoylethanolamide; LDL, low-density lipoproteins; Laurdan, 2-dimethylamino-(6-lauroyl)-naphtalene; GP, generalised polarization; CD, circular dichroism; AEA, anandamide

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long and enhance anandamide biological activity, likely by preventing its degradation ("entourage effect") [10,11]. This raises the possibility that these compounds could be used therapeutically to affect anandamide actions.

Palmitoylethanolamide (C16:0) (PEA), a shorter and fully saturated analogue of anandamide, exhibits a number of biochemical, physiological and pharmacological effects [12,13]. However, its mechanism of action remains unclear [12,13] and its effects are not always reproducible. Among the others, it was identified as the anti-inflammatory principle present in many natural products, and its anti-inflammatory properties were confirmed by recent research [12–14], although they seem less marked in human systems [13]. In vitro studies demonstrated that PEA inhibits the nitric oxide production in macrophages [15], affects the time course of capacitation of human spermatozoa [16], and increases the PLA_2 hydrolytic activity [17]. In those studies, PEA concentrations inducing significant effects ranged from 5 [16] to 30 μM [17]. Physiologically relevant concentrations of PEA (3 nM–3 μM) [18] may also have important physiological and/or pharmacological effects. For example, 300 nM PEA was shown to protect rat isolated heart against ischemia [19].

The aim of this study was to evaluate the possible effect of physiologically relevant concentrations of PEA on the resistance of plasma lipoproteins to oxidation. Interest in the study is supported by previous studies which have demonstrated that PEA [20] and its analogues stearoylethanolamide (SEA)[20] and oleylethanolamide (OEA)[21] have an antioxidant effect by inhibiting the radical-induced in vitro oxidation of lipids in liver [20], and cardiac mitochondria [21], although those results were obtained with large NAE concentrations. In the present work, the effect of PEA on lipid peroxidation of plasma lipoprotein was studied using low-density lipoprotein (LDL) as a lipoprotein model. Low-density lipoproteins are the main carriers of cholesterol in the human circulation. Several studies have shown structural and functional alterations of oxidised LDL and their likely involvement in some pathologies, such as in atherosclerosis, diabetes, and in ageing [22].

PEA was incubated with plasma, at concentrations similar to those observed in human diseases. After incubation, LDL were subsequently isolated, and their resistance to copper-induced oxidation was evaluated. Copper ions were used to induce lipoprotein lipid peroxidation, because the compositional changes and the structural alterations induced by metal ions-triggered oxidative damage on LDL have been characterised in previous studies.

2. Materials and methods

2.1. Materials

The fluorescent probe 2-dimethylamino-(6-lauroyl)-naphthalene (Laurdan) was purchased from Molecular Probes (Eugene, OR). Palmitoylethanolamide (PEA) was synthesised as previously described [23]. PEA stock solutions were prepared in ethanol immediately before use.

2.2. Isolation of plasma and incubation of plasma with Palmitoylethanolamide

Human plasma has been obtained from 20 healthy normolipemic blood donors (25–50 years) from the local blood bank. Informed consent was obtained from each participating donors. The study was approved by the Institutional Review Board of the Polytechnic University of Ancona and was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. Blood was collected in vacutainer tubes containing citrate phosphate dextrose (CDP) solution as anticoagulant (anticoagulant:whole blood ratio is 1.4:10). Plasma was prepared by centrifugation at 4 °C for 20 min at 3000 rpm. In order to minimize the individual subject variability of plasma endogenous anti-oxidant concentrations and the LDL particle size in the samples, the same pool of plasma was used to carry out the experiments. Plasma was pooled, divided in portions and stored at −80 °C no more than 1 month [24]. The pooled plasma was then used for in vitro modifications and separation of LDL. Before each experiments, levels of biochemical markers of lipid peroxidation (levels of lipid hydroperoxides and TBARS: thiobarbituric acid reactive substances) were carried out to evaluate oxidative damage in stored samples [25,26]. In our experimental conditions no significant changes of lipid hydroperoxides and TBARS were observed (data not shown).

Pooled plasma was incubated in the absence or in the presence of different concentrations (0.01–1 μM) of PEA dissolved in ethanol, at 37 °C, in the dark and under argon, for 1 h. In these samples, ethanol was always 1% of total plasma volume; control samples were prepared by incubating plasma with the same ethanol concentration. Plasma was thereafter used for the preparation of lipoproteins.

2.3. Isolation of low-density lipoproteins (LDL)

LDL (d = 1.019–1.063 g/ml) were isolated from control or PEA-treated plasma by single vertical spin density gradient ultracentrifugation for 1.30 h at 65,000 rpm [27].

At the end of the ultracentrifugation, LDL were dialysed at 4 °C for 24 h against 10 mM phosphate-buffer saline (PBS), pH 7.4. To maintain anti-oxidant conditions, all salt solutions used to adjust solvent densities and all dialysis buffer solutions contained 270 μM (0.01% w/v) EDTA, and dialysis buffer solutions were bubbled with nitrogen before use. According to Abuja et al. [28], practically no ethanol should be present in the final LDL preparation used for the experiments, as it is removed during LDL isolation.

2.4. LDL oxidation

LDL isolated from control or PEA-treated plasma were resuspended in 10 mM PBS pH 7.4 (final protein concen-