Food Chemistry 153 (2014) 1–8

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Inhibition of human neutrophils NEP activity, CD11b/CD18 expression and elastase release by 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde, oleacein

Monika E. Czerwińska, Anna K. Kiss, Marek Naruszewicz *

Department of Pharmacognosy and Molecular Basis of Phytotherapy, Faculty of Pharmacy, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland

ARTICLE INFO

Article history:
Received 17 August 2013
Received in revised form 7 November 2013
Accepted 4 December 2013
Available online 11 December 2013

Keywords:
DHPEA-EDA
Oleacein
Olive oil
Human neutrophils
Cardiovascular diseases

ABSTRACT

Polyphenols, such as oleacein (3,4-DHPEA-EDA; 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde), are believed to play a role in the prevention of cardiovascular diseases. Due to an increase of neutrophil mediators in acute myocardial infarction the aim of this study was to establish the effect of oleacein on neutral endopeptidase (NEP) activity and other functions of human neutrophils, such as elastase, MMP-9 and IL-8 production. The effect on CD62L and CD11b/CD18 expression on neutrophils was also determined.

Oleacein with a concentration of 100 μM inhibited NEP activity, elastase, MMP-9 and IL-8 release from neutrophils by 77.7 ± 2.7%, 21.3 ± 7.8%, 22.7 ± 4.2% and 25.2 ± 5.6%, respectively. Oleacein with a concentration of 50 μM suppressed CD11b/CD18 expression by 63.6 ± 3.1% and to a lesser extent, increased CD62L expression by 27.3 ± 8.3% on the surface of neutrophils, in comparison with stimulated cells.

Oleacein by inhibiting NEP activity, adhesion molecules expression and elastase release might play a role in the protective effects of olive oil against endothelial injuries.

1. Introduction

Extra virgin olive oil is a major source of fatty acids in the traditional Mediterranean diet (Buckland et al., 2012). Olive oil consumption is associated with a reduced risk of major cardiovascular events among high-risk people, as well as mortality in the healthy Mediterranean population (Buckland et al., 2012; Estruch et al., 2013). It has been suggested that the high concentration of phenolic compounds, in olive oil, may contribute to the health benefits of the Mediterranean diet (Paiva-Martins et al., 2009; Visioli & Galli, 1998). Among phenolic compounds present in olive oil, such as hydroxytyrosol (3,4-DHPEA; 3,4-dihydroxyphenylethanol), tyrosol (*p*-HPEA; *p*-hydroxyphenylethanol) and the isomer of oleuropein aglycon (3,4-DHPEA-EA; 3,4-dihydroxyphenylethanol-elenolic acid), the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA; 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde named oleacein, Fig. 1) is one of the most abundant components in olive oil (Fabiani et al., 2008; Selvaggini et al., 2006; Tovar, Motilva, & Romero, 2001). It has been previously demonstrated that phenolic compounds of olive oil inhibit copper sulphate-induced LDL oxidation (Visioli, Bellomo, Montedoro, & Galli, 1995), reduce oxidative-induced hemolysis of erythrocytes (Paiva-Martins et al., 2010), reduce reactive oxygen species (ROS) generation and myeloperoxidase release from human neutrophils (Czerwińska, Kiss, & Naruszewicz, 2012). Recently, we have also observed that oleacein might play a role in preventing the endothelial progenitor cells senescence, induced by angiotensin II (Parzonko, Czerwińska, Kiss, & Naruszewicz, 2013).

Neutrophils, also known as polymorphonuclear leukocytes (PMN), stand as the first line of defence of the innate immune system. Early inflammatory events attract neutrophils to the injured tissues, where damage is extended by proinflammatory mediators, released from neutrophils. However, during the chronic inflammatory process, neutrophils are still activated by a great variety of stimuli and an increase in neutrophil mediators can be observed. Neutrophils constitute the reservoir of membrane-bound receptors for endothelial adhesion molecules, extracellular matrix proteins, soluble mediators of inflammation and adhesion molecules, which are involved in the pathogenesis of atherosclerosis (Baetta & Corsini, 2010; Freitas, Lima, & Fernandes, 2009). The activation and recruitment of dysregulated neutrophils severely damages normal tissues (Witko-Sarsat, Rieu, Descamps-Latscha, Lesavre, &

Abbreviations: PMNs, polymorphonuclear cells, neutrophils; NEP, neutral endopeptidase; f-MLP, formyl-Met-Leu-Phenylalanine; LPS, lipopolysaccharide; MMP-9, metalloproteinase 9; IL-8, interleukin 8; SAAVNA, *N*-Methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide; SAAP-AMC, *N*-Succinyl-Ala-Ala-Pro-Phe-7-amino-4-methyl-coumarin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

* Corresponding author. Tel./fax: +48 22 572 09 85.
E-mail address: marek.naruszewicz@wum.edu.pl (M. Naruszewicz).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.12.019

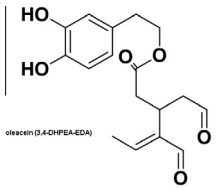

Fig. 1. Chemical structure of oleacein (3,4-DHPEA-EDA).

Halbwachs-Mecarelli, 2000). Neutrophils are also the source of neutral endopeptidase (NEP), which is a membrane ectoenzyme degrading and inactivating a variety of peptides, including atrial (ANP) and brain natriuretic peptide (BNP). Natriuretic peptides play a role in diuresis, vasodilation and the inhibition of the renin–angiotensin–aldosterone system. NEP enzymatic activity and natriuretic peptides plasma levels markedly increased in patients with acute myocardial infarction (Matsumura et al., 1996).

Therefore, the aim of this study was to determine the effect of oleacein on neutrophil NEP activity and other functions of human neutrophils that are involved in the pathophysiology of vascular, endothelial and myocardial injury. As a reference compound, a synthetic NEP inhibitor phosphoramidon was used. We evaluated the effect of oleacein on elastase, metalloproteinase 9 (MMP-9) and interleukin 8 (IL-8) release from neutrophils, as well as CD62L (L-selectin) and CD11b/CD18 (β_2 integrin) expression. Additionally, we established the simultaneous effect of oleacein with natriuretic peptides on these mentioned proteases and cytokines production, as well as the expression of adhesion molecules.

2. Materials and methods

2.1. Materials

Aminopeptidase (leucine aminopeptidase, microsomal from porcine kidney, type IV-S), ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide), f-MLP (formyl-Met-Leu-Phenylalanine), LPS (from *Escherichia coli* 0111:B4), cytochalasin B, HEPES, L-glutamine and *N*-Methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (SAAVNA), *N*-Succinyl-Ala-Ala-Pro-Phe-7-amino-4-methylcoumarin (SAAP-AMC), phosphoramidon disodium salt (PMD) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island NY, USA). Penicillin–streptomycin was obtained from PAA (Pasching, Austria). Human IL-8 and MMP-9 Quantikine ELISA Kits were purchased from R&D System (Minneapolis, USA). Anti-human CD62L (conjugated with fluorescein isothiocyanate, FITC) and anti-human CD11b (conjugated with phycoerythrin, PE) were purchased from eBioscience (San Diego, CA).

Phosphate buffered saline (PBS) was purchased from Biomed (Lublin, Poland). Hanks' balanced salt solution (HBSS) and RPMI 1640 medium were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Source of oleacein

Oleacein was isolated from leaves of *Ligustrum vulgare* L. (Oleaceae) as previously described (Kiss, Mańk, & Melzig, 2008). The structure of oleacein was confirmed by ultraviolet (UV) spectra, nuclear magnetic resonance (NMR) and mass spectrometry (MS). The compound purity was confirmed by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) methods. Oleacein was >95% pure.

Oleacein was dissolved in DMSO and then (Ca^{2+})-free HBSS, (Ca^{2+})-free PBS buffers at pH 7.4 or RPMI 1640 medium was added to obtain the proper concentration for each sample. The concentration of DMSO used (<0.1%) did not influence the performed assays.

2.3. Blood collection and neutrophil isolation

Peripheral venous blood was obtained from healthy adult volunteers (<35 years old) from the Warsaw Blood Donation Centre. Donors were recognised as healthy, according to medical history and routine laboratory test. Donors declared that they were non-smokers and were not taking any medication. They were clinically confirmed to be healthy, and a routine laboratory test showed values within a normal range. Neutrophils were isolated by dextran sedimentation and centrifugation in a Ficoll Hypaque gradient. Erythrocytes were removed by hypotonic lysis. The purity of neutrophils preparation was >97%. Following isolation, the cells were suspended in an appropriate medium, such as (Ca^{2+})-free HBSS, (Ca^{2+})-free PBS buffers at pH 7.4 or RPMI 1640 medium and were maintained at 4 °C before use (Böyum, 1968).

2.4. NEP activity

A two-step assay was used to determine the NEP activity. In the first enzymatic reaction, the cell suspension (5×10^5 cells) was preincubated with 50 μl of oleacein in the concentrations of 20, 50 and 100 μM and 100 μl of substrate Suc-L-Ala-L-Ala-Phe-7-amino-3-methyl-coumarin (100 μM) for 15 min at 37 °C. The second reaction was initiated by the addition of 20 μl of leucine aminopeptidase (APN) solution (1:235). Neutrophils were stimulated with 10 μl of f-MLP (10 μg/ml). The fluorescence of released 7-amino-3-methyl-coumarin (AMC) was measured for 1 h at intervals of 15 min at λ_{exi} = 367 nm and λ_{em} = 440 nm using a microplate reader (BioTek). The NEP activity was determined as the amount of released product (nmol) per hour (nmol AMC $\times 10^6$ cells/h). The changes of activity were presented as a percentage in comparison with the control (100%). Phosporamidon, a synthetic NEP inhibitor, was used as the reference compound.

The inhibitory effect of oleacein was calculated according to the formula: inhibition [%] = $100 - F_{sample}/F_{control} \times 100$.

2.5. Elastase release

Neutrophil elastase release was determined using *N*-Methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (SAAVNA) as a substrate, and *p*-nitrophenol was measured spectrophotometrically according to a modified assay of Liou et al. (2013). The cell suspension (5×10^5 cells) was preincubated with 50 μl of oleacein in the concentrations of 20, 50 and 100 μM for 15 min, at 37 °C and then stimulated with cytochalasin B (0.6 μg/ml) and f-MLP (1 μg/ml), for 15 min. After the addition of SAAVNA (100 μM), the extent of *p*-nitrophenol was measured spectrophotometrically, for 1 h at intervals of 20 min at 405 nm, using a microplate reader (BioTek). The inhibitory effect on elastase release was calculated according to the formula: inhibition [%] = $(A_{control} - A_{sample})/A_{control} \times 100$.

2.6. MMP-9 and IL-8 production

Neutrophils (2×10^6 cells) were cultured in a 24-well plate in RPMI 1640 medium with 10% FBS, 10 mM HEPES and 2 mM L-glutamine in the absence or presence of LPS (10 ng/ml) for 24 h at 37 °C with 5% CO_2. Oleacein was added at 20, 50 and 100 μM, 1 h before the stimuli. The MMP-9 (Shimizu et al., 2006) and IL-8 (Wang et al., 1994) which were released into cell supernatants were measured by enzyme-linked immunosorbent assay (ELISA), following the indications of the manufacturer (R&D System). The