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Original Article

Oleacein may inhibit destabilization of carotid plaques from hypertensive patients. Impact on high mobility group protein-1

Agnieszka Filipek[a], Monika E. Czerwińska[a], Anna K. Kiss[a], Jerzy A. Polański[b], Marek Naruszewicz[a,*]

[a] Department of Pharmacognosy and Molecular Basis of Phytotherapy, Faculty of Pharmacy, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland
[b] Chair and Department of General, Vascular and Oncologic Surgery, Medical University of Warsaw, Żwirki i Wigury 61, 02-091 Warsaw, Poland

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ABSTRACT

Background: In patients with hypertension the haemorrhage into carotid atherosclerotic plaque increases risk of plaque destabilization and rupture. Our previous study showed that oleacein, a secoiridoid present in extra virgin olive oil, enhanced uptake of haemoglobin-haptoglobin complex and change macrophage phenotype from pro-inflammatory M1 to anti-inflammatory M2.

Purpose: The aim this study was to investigate a potential role of oleacein in attenuation of carotid plaque destabilisation *ex vivo*.

Methods: Samples of atherosclerotic plaque were harvested from 20 patients with hypertension /11 women and 9 men/, who underwent carotid endarterectomy after transient ischemic attacks. Matching pieces of each plaque were incubated with increased concentration of pure oleacein /range 0–20 μM/ for 24 h. HMGB1, MMP-9, MMP-9/NGAL, TF and IL-10, as well as HO-1 secretion from plaque was measured by enzyme–linked immunosorbent assay /ELISA/. Statistical significance was set at $P < 0.05$ and $P < 0.001$.

Results: Oleacein at the concentrations of 10 and 20 μM significantly ($P < 0.001$) decreased secretion of HMGB1 (up 90%), MMP-9 (up to 80%), MMP-9/NGAL complex (up to 80%) and TF (more than 90%) from the treated plaque, as compared to control. At the same time IL-10 and HO-1 release increased by more than 80% ($P < 0.001$).

Conclusion: Our results indicate that oleacein possess ability to attenuate the destabilization of carotid plaque and could be potentially useful in the reduction of ischemic stroke risk.

Introduction

In recent years, the incidences of ischemic stroke represent the leading cause of mortality in older population, worldwide (Soler and Ruiz, 2010). Clinical research has proven that independent risk factors of cerebral ischemia include high pulse pressure and hypertension, primarily associated with tobacco smoking (Selwaness et al., 2013). These factors lead to the progression of carotid atherosclerotic plaque, as well as to its destabilization and rupture, which together with thrombotic occlusion may cause a transient ischemic attack or a stroke (Takaya et al., 2005). Many studies indicate that intraplaque haemorrhage play a significant role in pathogenesis of atherosclerotic lesion instability (Gao et al., 2007). This is related to intraplaque accumulation of erythrocyte constantly leaking from a network of immature neovessels developed mainly from the adventitia (Kolodgie et al., 2003; Virmani et al., 2005). As a result, erythrocytes release free cholesterol as well as haemoglobin, which induce oxidative stress and chronic inflammation (Ishihara et al., 1987; Kockx and Herman, 1988). These are associated with increased apoptosis and necrosis of macrophages, neutrophils, as well as plaque-stabilizing cells, *i.e.* smooth muscle cells and endothelial cells (Tabas, 2005). In fact, the destabilized plaque expresses and releases high-mobility group protein-1 (HMGB1) a DNA-binding cytokine, which is a specific biomarker of cell lethality (Porto et al., 2006).

It appears that carotid atherosclerotic plaque may be stabilized by oleacein – a secoiridoid found mainly in olives fruit paste, extra virgin olive oil (*Olea europaea* L., Oleaceae), but also in aqueous extracts from common

Abbreviations: OC, oleacein; OC5, oleacein at concentration of 5 μmol/l; OC10, oleacein at concentration of 10 μmol/l; OC20, oleacein at concentration of 20 μmol/l; OL20, oleuropein at concentration of 20 μmol/l; Simv40, simvastatin at concentration of 40 mg/ml; HMGB1, high mobility group protein-1 or high mobility group box 1; TF, tissue factor; MMP-9, matrix metalloproteinase 9; MMP-9/NGAL, matrix metalloproteinase 9 and neutrophil gelatinase-associated lipocalin complex; IL-10, interleukin-10; HO-1, heme oxygenase 1; TIA, transient ischemic attacks

* Corresponding author.
E-mail address: marek.naruszewicz@wum.edu.pl (M. Naruszewicz).

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Table 1
Clinical features of carotid plaques donors.

		OC5	OC10	OC20	OL20	Simv40
Number of subjects (male sex)	20 (9)	7 (3)	6 (3)	7 (3)	1 (1)	1 (1)
Age (years)	65 ± 12	59 ± 4	54 ± 9	60 ± 11	59	62
Body mass index (kg/m^2)	26.1 ± 1.5	26 ± 2	29 ± 4	26 ± 2	26.8	25.7
Current smokers (n)	18	6	6	6	1	1
Systolic BP	156 ± 1	149 ± 9	154 ± 3	157 ± 11	145	151
Diastolic BP	87 ± 13	81 ± 3	87 ± 6	85 ± 4	92	97
TC (mg/dl)	175 ± 22	168 ± 9	170 ± 1	179 ± 8	161	170
TG (mg/dl)	112 ± 43	119 ± 14	106 ± 9	109 ± 7	123	105
LDL-C (mg/dl)	103 ± 26	107 ± 11	98 ± 6	94 ± 9	120	119
HDL-C (mg/dl)	43.1 ± 15.2	37 ± 9	40 ± 8	42 ± 5	39	41
Creatinine (mg/dl)	0.86 ± 0.35	0.81 ± 0.15	0.78 ± 0.23	0.89 ± 0.24	0.72	0.91
Glucose (mg/dl)	94 ± 16	95 ± 4	98 ± 11	96 ± 7	103	99
Medication (n)						
Aspirin	15	4	6	5	1	1
Statin	17	4	7	6	1	1
ACE-inhibitors	20	7	6	3	1	1
Diuretics	7	1	2	4	1	1
Beta-blocking agents	9	–	4	5	–	–

privet leaves (*Ligustrum vulgare* L., Oleaceae) (Czerwińska et al., 2013). The potential contribution of oleacein in the prevention and treatment of cardiovascular diseases is based on its ability to inhibit the LDL oxidation, myeloperoxidase release, reduction of the expression of adhesion molecules in immune system and endothelial cells, as well as decrease angiotensin II production (Naruszewicz et al., 2015) . Additionally, the interaction between oleacein and red blood cells membrane has been previously suggested (Paiva-Martins et al., 2010).

Our recent studies have also confirmed that oleacein, bound to haemoglobin and haptoglobin, has the ability to increase the absorption of this complex by macrophages with the CD163 receptor (Filipek et al., 2015). This alters macrophage phenotype from pro-inflammatory M-1 to anti-inflammatory M-2, which is manifested by an increase of interleukin-10 (IL-10) production by these cells (Martinez et al. 2014).

In fact, it has been shown that CD163 expression in atherosclerotic tissue plays a significant role in attenuation of plaque destabilization induced by haemorrhages (Finn et al., 2012).

Taking into consideration anti-inflammatory and anti-oxidative effects of oleacein, we have investigated its potential influence on the stabilisation process of carotid plaques isolated from patients during planned endarterectomy procedures in an *ex vivo* experiment. For this purpose, the plaques were incubated with both oleacein and a factor that intensifies its destabilisation, *i.e.* with a lipopolysaccharide (LPS) that stimulates neutrophils and monocytes/macrophages to increase secretion of biomarkers having prognostic value in atherothrombotic plaque rupture. The analysis included HMGB1, matrix metalloproteinase 9 (MMP-9), matrix metalloproteinase 9 / neutrophil gelatinase-associated lipocalin complex (MMP-9/NGAL), tissue factor (TF). Additionally, we have investigated the secretion of IL – 10 and heme oxygenase 1 (HO-1), which could play a protective role in prevention of the plaque destabilisation.

Materials and methods

Chemicals

Quantitine ELISA Human MMP-9 Immunoassay, Quantitine ELISA Human MMP-9/NGAL Complex Immunoassay, Quantitine ELISA Human Coagulation Factor III/ Tissue Factor Immunoassay, Quantitine HS Human IL-10 Immunoassay and DuoSet IC Human Total HO-1/HMOX1 were purchased from R&D Systems a Bio-techne Brand, USA. Enzyme–linked Immunosorbent Assay Kit for High Mobility Group Protein 1 (HMGB1) was purchased from Uscnk Life Science Inc. Aprotinin, Leupeptin, Peptasin, Phenylmetylsulfonylfluoride (PMSF), Sodium azide, Triton® X-100, Bovine Serum Albumin (BSA), Dimethyl sulfoxide (DMSO), Liposaccharide (LPS) and Simvastatin (Simv) were purchased from Sigma, USA. Color Reagent A, Color Reagent B and Stop Solution were purchased from R&D Systems, USA. Dubelcco's Phosphate Buffered Saline without Ca^{2+}/Mg^{2+} (DPBS) was purchased from Gibco by Life Technologies, UK.

Oleacein (OC) and oleuropein (OL) were isolated from *Ligustrum vulgare* L. (Oleaceae) leaves in the Department of Pharmacognosy and Molecular Basis of Phytotherapy as previously described (Kiss et al., 2008). The structures and purity of compounds were confirmed by performing UV, NMR and MS spectra. The purity of compounds were confirmed by TLC and HPLC methods. All substances were used of > 95% purity (Filipek et al., 2015).

Oleacein, oleuropein and simvastatin

Oleacein, oleuropein and simvastatin were dissolved in DMSO and then in (Ca^{2+}/Mg^{2+})-free DPBS buffer at pH 7.4 to final concentration of 5 μmol/l, 10 μmol/l and 20 μmol/l (oleacein) and 20 μmol/l (oleuropein), as well as 40 mg/ml (simvastatin). The final concentration of DMSO did not exceed 0.01% and did not influence the performed assays.

Patients

Carotid plaques were obtained during the planned endarterectomy of twenty patients (9 men and 11 women) in age 50–79 (mean 65 ± 12 years) with transient ischemic attacks (TIA) lasting less than 24 h (clinical characteristics in Table 1). The study was conformed to the principles of the Declaration of Helsinki.

Study designed

The experiments were conducted no later than 3 h after endarterectomy. Matching pieces of the largest plaque (mass > 100 mg) burden from each patient were incubated in sterile condition in PBS (Abela et al., 2011) or in PBS with oleacein in the concentration range from 5 to 20 μmol/l (OC5, OC10, OC20) by 24 h in the presence of LPS (1 μg/ml) at 37 °C.

Due to the lack of sufficient material from endarterectomy, experiments including a positive and negative control have been limited to two patients from whom we received two plaques from the same vessel. As a positive control, we used a dose of 40 mg/ml simvastatin (Abela et al., 2011). In contrast, as a negative control we used an oleacein precursor, *i.e.* oleuropein, which in our research (Filipek et al., 2015) did not show any activity on the function of macrophages.

After incubation, all supernatants were collected, centrifuged (4000