International Journal of Biological Macromolecules 51 (2012) 183–187

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

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

Protective effects of grape seed extract against oxidative and nitrative damage of plasma proteins

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ARTICLE INFO

Article history:
Received 25 March 2012
Received in revised form 24 April 2012
Accepted 5 May 2012
Available online 11 May 2012

Keywords:
Grape seeds
Antioxidant
Catechins
Carbonyl groups
3-Nitrotyrosine

ABSTRACT

Oxidative stress, vascular inflammation, endothelial dysfunction plays a crucial role in the pathogenesis of cardiovascular diseases.

The aim of our *in vitro* study was to examine the antioxidative properties of grape seed extract, and its potential protective effect on the haemostatic function of human fibrinogen under oxidative stress conditions, induced by peroxynitrite (100 μM).

The preincubation of plasma with the tested extract (0.5–50 μg/ml or 0.5–300 μg/ml) reduced the formation of 3-nitrotyrosine and diminished oxidation of thiol groups in plasma proteins. The low concentrations (0.5–50 μg/ml) of grape seed extract also decreased the level of carbonyl groups, however at higher concentrations (100–300 μg/ml) this effect was not observed. Furthermore, grape seed extract counteracted the inhibitory effect of peroxynitrite on human plasma clotting.

The results obtained in this study indicate that components of the grape seed extract posses antioxidative properties and may be promising substances for the creation of new dietary supplements.

1. Introduction

Grapes, the berries of *Vitis vinifera* L. ssp *sativa*, have been well known and consumed since the ancient times. The nutritional and medicinal values of these fruits have been confirmed by both traditional medicine and the growing number of scientific reports [1,2]. Grape seeds, a by-product of the winery and grape juice industry, are also a source of numerous substances with the positive effect on human health. These seeds contain about 5–8% polyphenols (depending on the variety), including the flavan-3-ol monomers: catechin, epicatechin, gallocatechin, epigallocatechin and epicatechin 3-*O*-gallate, as well as procyanidin dimers, trimers, and highly polymerised procyanidins [3,4]. Since the seeds of grapes are rich in monomeric and oligomeric flavan-3-ols (consisting of up to 5 flavanol units), they are widely studied mainly to elucidate their mechanisms of action to prevent cardiovascular diseases (CVDs). Polyphenolic compounds from grape seed have been demonstrated to display favourable health effects, such as antioxidative [5], cardioprotective [6], immunomodulatory and antitumour activity [7].

The research the natural substances that exert cardioprotective effects, including the reduction of oxidative stress, modulation of blood coagulation cascade and anti-platelet properties, as well as anti-inflammatory effects, are intensively developed. Oxidative stress, vascular inflammation, endothelial dysfunction and the loss of its anti-thrombotic properties plays a crucial role in the pathogenesis of CVDs [8]. A variety of modifications of biologically active compounds such as lipids, nucleic acids and chiefly proteins occur under the influence of oxidative and nitrative agents. High activity of reactive oxygen and nitrogen species and the impaired effectiveness of antioxidant and proteolytic systems lead to the accumulation of oxidized protein products and may cause various pathological changes, including the aging of organisms. Oxidative and nitrative modifications of protein structure lead to the loss of their biological properties, affect the degradation and cause accumulation of modified protein products, that has been observed under various conditions, such as aging, cell differentiation and apoptosis [9]. Thus, the biologically active substances with antioxidative properties which are present in the extract from grape seed might be the therapeutic potential of this extract in the treatment of inflammation, oxidative stress, and other pathological alterations in the cardiovascular system.

The aim of our *in vitro* study was to examine the antioxidative properties of grape seed extract and its possible protective effect on the haemostatic function of human fibrinogen under peroxynitrite-induced oxidative stress conditions, assessed by measurements of polymerization properties of human plasma and by the estimation of oxidative stress markers.

All amino acid residues, occurring in proteins are susceptible to oxidation. Oxidation of amino acid residues leads to the

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http://dx.doi.org/10.1016/j.ijbiomac.2012.05.009

formation of relatively stable carbonyl groups that can be qualitative and quantitative markers enabling to assess the oxidative damage to proteins [10,11]. Aldehydes and ketones react with ammonia derivatives, including hydroxylamine, hydrazine or phenylhydrazine to form stable compounds, which can be used to identify oxidation of proteins. 2,4-Dinitrophenylhydrazine (DNPH) is usually used followed by spectrophotometric or immunoenzymatic methods. Specific anti-DNP antibodies enable determination of carbonyl groups in proteins using ELISA method [12,13]. The other markers of reactive oxygen/nitrogen agents action *in vivo* are 3-nitrotyrosine and thiol groups. Both may be estimated spectrophotometrically and 3-nitrotyrosine can also be determined by competitive ELISA method [14].

2. Materials and methods

2.1. Chemicals

Bicinchoninic Acid Protein Assay Kit and o-phenylenediamine dihydrochloride (OPD peroxidase substrate) were obtained from Sigma (St. Louis, MO, USA). Anti-Dinitrophenyl (DNP) antibodies developed in rabbit were purchased from Sigma–Aldrich (Saint Louis, USA). Anti-3-Nitrotyrosine goat polyclonal antibodies were obtained from Abcam (Cambridge, UK). StreptABComplex/HRP Polyclonal Swine Anti-Goat, Mouse, Rabbit Immunoglobulins, Multi-Link were from DAKO (Glostrup, Denmark). Bovine thrombin was purchased from BIOMED, (Lublin, Poland). All other commercial reagents were of analytical grade. Peroxynitrite was synthesized according to the method of Pryor and Squadrito [15], and stored not longer than 2–3 months at −70 °C.

2.2. Plants material

The commercial extract from grape (*Vitis vinifera*) seed – OMNIVIR® was purchased from C.E. Roeper GmbH (Hamburg, Germany). Extract was dissolved in 50% DMSO to the stock concentration of 10 mg/ml. Composition of the examined extract and the identification of its components was described previously [16].

2.3. Samples preparation

Blood was obtained from young (20–25 years) healthy, non-smoking volunteers and collected into citrate solution (0.32% final concentration), and immediately centrifuged (3000 × *g*, 15 min) to obtain plasma. Subjects did not take addictive substances or antioxidant supplements and their diet was balanced (meat and vegetables). Plasma samples were preincubated for 5 min at 37 °C with the grape seed extract, added to the final concentration range of 0.5–50 μg/ml or 0.5–300 μg/ml (as the determination of protein carbonyl groups), and then exposed to 100 μM peroxynitrite ($ONOO^-$). For the determination of the polymerization process, before the exposure to 100 μM peroxynitrite, plasma was diluted 3 times. To assess the antioxidative effect of the tested extract, the samples of plasma treated with peroxynitrite in the absence of grape seed extract were performed. The control samples (plasma untreated with the extract and/or peroxynitrite), were also prepared. To exclude the possibility of direct interaction of the tested extract with plasma components, the experiments with blood plasma and the extracts only (without adding of $ONOO^-$) were performed; no pro-oxidative effect was found. Concentration of plasma proteins was estimated with the use of bicinchoninic acid, according to the method described by Smith et al. [17]. The study was performed under the guidelines of the Helsinki Declaration for Human Research and approved by the committee on the Ethics of Research in Human Experimentation at the Medical University of Lodz (No. RNN/13/07/KB).

2.4. The measurement of fibrin polymerization in human plasma

Thrombin-catalysed polymerization was monitored every 6 s for 20 min as the change of turbidity at 360 nm in spectrophotometer T60. Plasma samples were clotted with thrombin (at the final concentration of 0.5 U/ml). The maximally velocity of polymerization (V_{max}, ΔmOD/min) was recorded for each absorbance curve [18].

2.5. Determination of protein carbonyl groups in human blood plasma by ELISA method

The detection of protein carbonyl groups was performed in human blood plasma using ELISA method according to Alamdari et al. [13]. Human plasma proteins were non-specifically adsorbed onto ELISA plates, reacted with dinitrophenylhydrazine (DNPH), washed with ethanol: PBS, and finally carbonyl groups were detected by anti-DNP antibodies (I step) and peroxidase-linked antibodies (II step).

2.6. Determination of 3-nitrotyrosine in the proteins of human plasma the competitive ELISA test

The detection of 3-nitrotyrosine in blood plasma was performed according to the method described by Khan et al. [19], and modified by Olas et al. [20]. The concentrations of nitrated plasma proteins were estimated from the standard curve, constructed with the use of 3-nitrotyrosine-containing fibrinogen (3-NT-Fg). 3-NT-Fg was prepared by the exposure of human fibrinogen to 1000 μM peroxynitrite action. The presence of 3-nitrotyrosine in fibrinogen was confirmed spectrophotometrically at 302 nm, at pH 11.5 (molar absorption coefficient for nitrotyrosine, $\varepsilon = 4400\ M^{-1}\ cm^{-1}$) [21], and then the obtained nitro-fibrinogen was used for the preparation of the standard curve, ranging from 10 to 1000 nmol/l of 3-nitrotyrosine-fibrinogen equivalent.

2.7. Determination of thiols

Thiol groups in blood plasma proteins were determined using Ellman's reagent, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The thiol-disulfide interchange reaction between DTNB and thiol is the basis of this spectrophotometric assay [22]. Plasma samples were mixed with DTNB, and at the end of the incubation period (1 h, 37 °C) the values of absorbance were recorded at 412 nm. The concentrations of thiol groups were calculated by using $\varepsilon_{max} = 13{,}600\ M^{-1}\ cm^{-1}$, and the results were expressed as μmol/l.

2.8. Statistics

The statistical analysis was performed using StatSoft Inc. "Statistica" v. 6.0. All the values in this study were expressed as mean ± SD. Results were analysed under the account of normality with Shapiro–Wilk test and Equality of Variance with Levene test. The significance of differences between the values was analysed depending on the Levene test by ANOVA, followed by Tukey multiple comparisons test or Kruskal–Wallis test; $p < 0.05$ was accepted as statistically significant.

3. Results

The addition of peroxynitrite (100 μM) to blood plasma induced oxidative and nitrative alterations in plasma proteins; this effect