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Research paper

Punicalagin, an active pomegranate component, is a new inhibitor of PDIA3 reductase activity

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ABSTRACT

Background: Polyphenolic compounds isolated from pomegranate fruit possess several pharmacological activities including anti-inflammatory, hepatoprotective, antigenotoxic and anticoagulant activities. The present work focuses the attention on PDIA3 interaction with punicalagin and ellagic acid, the most predominant components of pomegranate extracts. PDIA3, a member of the protein disulfide isomerase family involved in several cellular functions, is associated with different human diseases and it has the potential to be a pharmacological target.
Methods: The interaction of polyphenols with PDIA3 purified protein was explored by fluorescence quenching and calorimetric techniques and their effect on PDIA3 activity was investigated.
Results: A higher affinity was observed for punicalagin which also strongly affects PDIA3 reductase activity *in vitro* as a non-competitive inhibitor. Isothermal titration calorimetry confirmed the high affinity of punicalagin for PDIA3. Considering the PDIA3 involvement in oxidative cellular stress response observed in neuroblastoma cells after treatment with hydrogen peroxide, a comparative study was conducted to evaluate the effect of punicalagin on wild type and PDIA3-silenced cells. Punicalagin increases the cell sensitivity to hydrogen peroxide in neuroblastoma cells, but this effect is drastically reduced in PDIA3-silenced cells treated in the same experimental conditions.
Conclusions: Punicalagin binds PDIA3 and inhibits its redox activity. Comparative experiments conducted on unsilenced and PDIA3-silenced neuroblastoma cells suggest the potential of punicalagin to modulate PDIA3 reductase activity also in a biological model.
General significance: Punicalagin can be used as a new PDIA3 inhibitor and this can provide information on the molecular mechanisms underlying the biological activities of PDIA3 and punicalagin.

1. Introduction

Protein disulfide isomerase isoform A3 (PDIA3), also known as ERp57, GRP58 and 1,25-D3-MARRS, is a member of the protein disulfide isomerase family; it is a protein of 505 aminoacids and molecular weight of 57 kDa with a structure characterized by four tioredoxin-like domains: *a*, *b*, *b*' and *a*'. The *a* and *a*' domains contain the catalytic active sites constituted by the tetrapeptide CGHC, which provides PDIA3 with redox activity, while *b* and *b*' domains are redox inactive but are required for the PDIA3 complete activity [1–3]. PDIA3 is localized predominantly in the endoplasmic reticulum, where it is involved in the correct folding of newly synthetized glycoproteins and in the assembly of the MHC class I complex [4–6]. It is also present in the cytosol where it can interact with other proteins such as the Redox Effector Factor 1 (Ref-1) [7]

Abbreviations: DiE-GSSG, dieosin glutathione disulfide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSSG, oxidized glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PDIA3, protein disulfide isomerase isoform A3; PBS, phosphate buffered saline; TCEP, tris(2-carboxyethyl) phosphine; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; XTT, sodium 3′- [1-(phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy- 6-nitro) benzene sulfonic acid hydrate.

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and the Signal Transducer and Activator of Transcription 3 (STAT3) [8], and in the nucleus where it can directly bind to DNA regions rich in A/T [9]. Furthermore, many studies showed that PDIA3 can be found on the cell surface where it binds 1α, 25-dihydroxycholecalciferol, followed by the activation of a fast response pathway [10]. PDIA3 is thought to play a role in cell protection against oxidative stress through its redox and chaperone activities, it can prevent the development of diseases related to unfolded/misfolded proteins accumulation and it has been involved in gene regulation [11,12]. An increasing interest in PDIA3 role in pathological processes has been demonstrated by several authors since PDIA3 has been associated with human neurodegenerative diseases such Alzheimer and Parkinson [13–15]. Besides, PDIA3 activity and expression are altered in several types of cancers, resulting in increased tumor aggressiveness and reduced overall survival. PDIA3 may play a role in the oncogenic transformation because of its ability to control intracellular and extracellular redox state via thiol-dependent reductase activity [16,17].

Given that PDIA3 is involved in many biological processes and can interact with several macromolecules and small ligands, such as green tea catechins and flavonoids [18,19], the investigation of modulators of PDIA3 activities could be interesting. Data from literature indicate that several phytochemicals can be found in pomegranate fruits and can be a valuable aid in counteracting oxidative stress and preventing some major diseases. Pomegranate extracts possess important biological properties, including anti-atherosclerotic, antioxidant, anti-inflammatory and anti-genotoxics, helpful in preventing the development of chronic and debilitating diseases such as cardiovascular illnesses, type 2 diabetes and cancer [20,21]. These activities have been attributed to the high content of phenolic compounds [22,23]. The nutraceutical properties of pomegranate are not limited to the edible part of the fruit; in fact, non-edible fractions of fruit and tree (peel, flower, etc.) contain even higher amounts of biologically active components. The peel of the pomegranate is rich in ellagitannins, such punicalagin, punicalin, gallagic acid, ellagic acid and glycosides [24,25]. Punicalagin, a unique pomegranate compound of high molecular weight soluble in water, is the predominant ellagitannin [26]. Punicalagin owns important biological activities, including anti-inflammatory, hepatoprotective and anti-genotoxic activities. It seems to be the polyphenol responsible for more than 50% of antioxidant and antigenotoxic activities of pomegranate juice [20,27,28] and together with ellagic acid are the major bioactive constituents of the fruit with promising therapeutic properties. However, there are currently few studies on punicalagin biological efficacy and for this reason we characterized its capability to bind and modulate PDIA3 activity.

2. Materials and methods

2.1. Chemicals

Punicalagin, ellagic acid, phosphate buffered saline (PBS), tris(2-carboxyethyl) phosphine (TCEP), dimethyl sulfoxide (DMSO), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dithiothreitol (DTT), oxidized glutathione (GSSG), eosin isothiocyanate, glutamine, sodium pyruvate, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Sigma-Aldrich. EDTA (ethylenediaminetetraacetic acid) 0.5 M solution pH 8.0 was from IBI Scientific and sodium 3′- [1- (phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy- 6-nitro) benzene sulfonic acid hydrate (XTT) from Biotium.

2.2. Protein expression and purification

Human recombinant PDIA3 was cloned into pET21 vector (Novagen) and expressed in *E. coli* strain BL21 as previously described [9]. Protein was purified by ammonium sulphate fractionation, ion exchange and heparin chromatography [7,29]. Protein purification was evaluated by SDS-PAGE and concentration was spectrophotometrically determined (ε_{280} reduced form = 44,810 $M^{-1}cm^{-1}$).

2.3. Fluorescence quenching measurements

The PDIA3-polyphenol interaction was evaluated by fluorimetric titration. Fluorescence spectra were recorded using a SPEX-FluoroMax spectrofluorimeter (Horiba Scientific) from 300 to 500 nm with excitation at 290 nm using a 10 mm path length quartz fluorescence cuvette and under continuous stirring. The excitation and emission slits were both set to 5 nm and scan speed was 120 nm min^{-1}. The reduction of PDIA3 was obtained by adding 2 mM TCEP to 50 μM PDIA3 stock solution. Aliquots of freshly reduced PDIA3 (0.1 μM final concentration) were diluted in PBS containing EDTA 0.2 mM and DTT 0.1 mM, and titrated in quartz cuvette by stepwise additions, at 5 min time intervals, of polyphenols solution (punicalagin 0.2 mM in PBS freshly prepared from a 20 mM stock solution in water, ellagic acid 0.2 mM in PBS/ethanol 50:50 v/v freshly prepared from a 20 mM stock solution in DMSO). Quenching analysis on PDIA3 in non-reduced form was conducted as above described using unreduced PDIA3 and in the absence of DTT. All experiments were carried out at 25 °C. The blank spectra (polyphenol addition without protein) were recorded under the same experimental conditions and subtracted from the corresponding polyphenol-protein system to correct the fluorescence background. Fluorescence intensities recorded at 338 nm were used for quenching analysis and obtained data, as the average of at least three independent titration experiments, were analyzed as previously described [19].

2.4. Determination of protein disulfide reductase activity

Disulfide reductase activity of PDIA3 was monitored by sensitive fluorescent assay using dieosin glutathione disulfide (DiE-GSSG) as fluorogenic probe. DiE-GSSG is synthesized by the reaction of eosin isothiocyanate with oxidized glutathione (GSSG) according to the method of Raturi and Mutus [30] with some modifications [[18]]. DiE-GSSG purification was determined in HPLC and it concentration was calculated spectrophotometrically (ε_{525} = 88,000 $M^{-1}cm^{-1}$). Punicalagin or ellagic acid effects on PDIA3 disulfide reductase activity were evaluated using DiE-GSSG 200 nM, PDIA3 50 nM, DTT 5 μM in reaction buffer (PBS and EDTA 0.2 mM). DiE-GSSG reduction was monitored at 545 nm with excitation at 520 nm, at 25 °C under continuous stirring. Polyphenols were tested at 0.2, 0.5, 2, 5 and 20 μM with 2 min incubation before the analysis. A better investigation on PDIA3 reductase activity was performed by DiE-GSSG titration (31 nM–1000 nM) in reaction buffer added with 20 nM final concentration of PDIA3 and DTT 5 μM. GSSG reduction was monitored for 3 min at 545 nm with excitation at 520 nm, at 25 °C under continuous stirring. To assess the effect on the PDIA3 activity, before DiE-GSSG titration, PDIA3 20 nM was incubated with different concentrations of punicalagin (0.2, 0.5, 1, 2 and 5 μM) for 2 min. Values were fitted using the enzymatic kinetic equation on Graph Pad Prism 5.0 software (GraphPad Software, Inc.) to calculate Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) for PDIA3 and their modifications after punicalagin treatment. Punicalagin inhibition constant (K_i) was determined using a