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Rescue of ER oxidoreductase function through polyphenolic phytochemical intervention: Implications for subcellular traffic and neurodegenerative disorders

Rituraj Pal [a], Elaine A. Cristan [b], Karina Schnittker [b], Mahesh Narayan [a,*]

[a] Department of Chemistry, University of Texas at El Paso, El Paso, TX 79968, USA
[b] Department of Biological Sciences, University of Texas at El Paso, El Paso, TX 79968, USA

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ABSTRACT

Protein disulfide isomerase (PDI), the chief endoplasmic reticulum (ER) resident oxidoreductase chaperone that catalyzes maturation of disulfide-bond-containing proteins is involved in the pathogenesis of both Parkinson's (PD) and Alzheimer's (AD) diseases. *S*-nitrosylation of PDI cysteines due to nitrosative stress is associated with cytosolic debris accumulation and Lewy-body aggregates in PD and AD brains. We demonstrate that the polyphenolic phytochemicals curcumin and masoprocol can rescue PDI from becoming *S*-nitrosylated and maintain its catalytic function under conditions mimicking nitrosative stress by forming stable NO_x adducts. Furthermore, both polyphenols intervene to prevent the formation of PDI-resistant polymeric misfolded protein forms that accumulate upon exposure to oxidative stress. Our study suggests that curcumin and masoprocol can serve as lead-candidate prophylactics for reactive oxygen species induced chaperone damage, protein misfolding and neurodegenerative disease; importantly, they can play a vital role in sustaining traffic along the ER's secretory pathway by preserving functional integrity of PDI.

Introduction

Flow of traffic in the endoplasmic reticulum (ER) consists of (a) incoming newly-synthesized (unstructured proteins), (b) exiting biologically functional folded forms to be secreted outside the cell or into the cell membrane (via the secretory pathway), and (c) terminally misfolded folded forms that must transit to the cytosol via the retrotranslocatory pathway for proteosomal degradation [1–3]. A central event that modulates protein flux through secretory and retrotranslocatory pathways is the ability of the nascent polypeptide to rapidly acquire native disulfides and native structure through oxidative protein folding [4–6]. A host of subcellular factors and machinery is charged with assisting this process. Among them is the chief ER resident oxidoreductase chaperone, protein disulfide isomerase (PDI), which catalyzes the oxidative folding reactions required for maturation of disulfide-bond-containing proteins [1,7]. PDI function is thus pivotal in regulating traffic along the secretory pathway, for prevention of terminally misfolded proteins that would otherwise constitute traffic along the retrotranslocatory pathway and for mitigation of neurodegenerative onset [2,3].

PDI functions primarily through redox-active CGHC sequences in two of its five domains (Fig. 1A); surface-exposed cysteines in these sequences are capable of catalyzing thiol oxidation and thiol-disulfide isomerization reactions in substrate proteins without structural consequence for the 56-kDa oxidoreductase [8–11]. However, recent work has demonstrated that nitrosative stress, originating from elevated levels of nitric oxide (NO), results in the *S*-nitrosylation of PDI cysteines (i.e., the covalent modification of PDI active site cysteines by NO) [12–14]. NO-induced chemical modification of PDI abolishes its ability to participate in thiol-disulfide exchange reactions (both isomerase and oxidoreductase) and its ability to attenuate neuronal cell death triggered by protein misfolding and/or proteosome inhibition [12]. Coupled with *S*-nitrosylation of PDI is the accumulation of Lewy-body aggregates in the cytosol human neuronal cells and their abrogation by introduction of unmodified PDI both of which make PDI a valuable target in preventing neurodegeneration [13].

In this study, we have examined the ability of the polyphenols, masoprocol (from *Larrea Tridentata*) and curcumin [a turmeric (*Curcuma Longa*) spice] to protect PDI catalytic function under conditions of nitrosative and oxidative stress. Curcumin has a wide spectrum of biological and pharmacological functions including anti-inflammatory, antimicrobial, and anticarcinogenic (Fig. 1B) [15–21]. It has already been used clinically and is approved by the FDA as a safe food additive [15]; recent efforts to improve its

* Corresponding author. Address: Department of Chemistry, UT El Paso, 500W. Univ. Ave., El Paso, TX 79968, USA. Fax: +1 915 747 5748.
E-mail address: mnarayan@utep.edu (M. Narayan).

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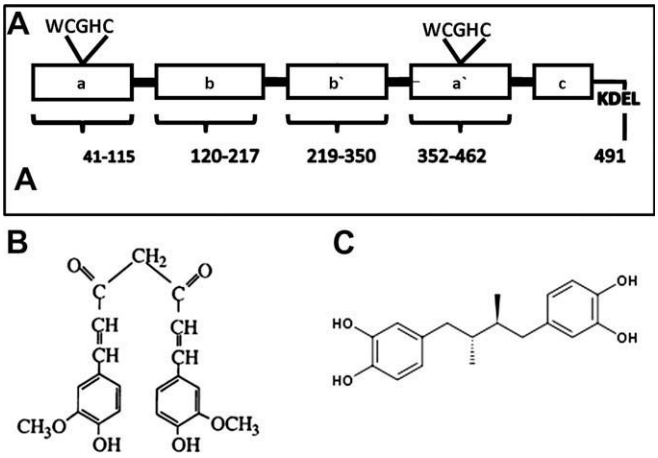

Fig. 1. (A) Schematic of protein disulfide isomerase (PDI)m (B) curcumin and (C) masoprocol.

bioavailability (co-administration with other agents and structural modifications) have met with success [21]. Masoprocol (nordihydroguaiaretic acid (NDGA)) is a potent antioxidant whose derivatives and analogues are potentially useful in treating diseases related to cancers, diabetes, viral, bacterial infections, and inflammation [22,23] (Fig. 1c). Terameprocol, a tetra-*O*-methyl derivative of nordihydroguaiaretic acid, is currently in Phase I/II clinical trials as an anticancer agent [23].

In this study, we chose to assay the PDI-catalyzed folding of bovine pancreatic ribonuclease A (RNase A), a model ER-processed four disulfide-bond-containing protein [4,24,25] under control and nitrosative stress conditions. The select polyphenols were then introduced to determine whether they can rescue PDI function and furthermore, prevent the formation of misfolded forms that are likely destined constitute the retrotranslocatory pathway. Our results indicate that curcumin and masoprocol can scavenge NO_x and hydroxyl radicals, prevent PDI *S*-nitrosylation and promote traffic along the secretory pathway by preventing the formation of PDI-resistant polymeric aggregates. The implications for prevention of reactive oxygen species induced misfolding and associated neurodegenerative disorders are discussed.

Materials and methods

RNase A was purchased from Sigma and purified as previously described [24]. PDI was expressed and purified as previously described [25]. Masoprocol was a gift from Wayne Stevens (Western Engineering Research Co., El Paso). Curcumin was obtained from the Sigma Chemical Co. Both polyphenols were found to be >99% pure using reversed-phase HPLC analysis, and used without further purification. Hydrogen peroxide (30%) and tetranitromethane were obtained from Sigma Chemical Co. Oxidized and reduced dithiothreitol (DTT^{ox} and DTT^{red}, respectively), were purchased from Sigma and used without further purification. All other chemicals were of the highest grade commercially available.

Preparation of fully-reduced RNase A. Fully-reduced RNase A (R) was prepared by incubating native protein (10 mg/ml) in 6 M Gdn HCl and 100 mM DTT^{red} (pH 8, 100 mM Tris–HCl, 1 mM EDTA) for a period of 2 h [24]. The mixture was then repeatedly dialyzed against 50 mM acetic acid at 4 °C prior to lyophilisation. The fully-reduced protein was dissolved into 10 mM acetic acid to obtain a stock solution (5 mg/ml) that was kept frozen (−20 °C) until further use.

PDI-catalyzed oxidative folding of RNase A under nitrosative stress. Fully-reduced RNase A (36 μM final concentration) was incubated into a solution (pH 8, 100 mM DTT^{ox}, 100 mM Tris–HCl, 25 °C) containing 4 μM of WT PDI and oxidative regeneration followed as previously described [25,26]. A control experiment was run in parallel and did not contain any PDI. Aliquots from the regeneration mixture were periodically withdrawn after initiation of oxidative folding and subjected to a reduction pulse (application of 2 mM DTT^{red} for a period of 2 min) [27], before addition of glacial acetic acid (which reduced the pH to 3). Samples were desalted on a G-25 column prior to application on a C-18 column (Supelco Discovery® BIO Wide Pore C18, 5 μm, 15 cm × 100.0 mm) for reversed-phase chromatographic analysis using an acetonitrile gradient (1%/min) [25].

In other experiments, varying concentrations of masoprocol or curcumin (0–50 μM final concentration from stock acetonitrile solutions prepared by weight) were incubated with the regeneration mixture prior to the addition of tetranitromethane (0–5 μM obtained by dilution of the neat compound). The regeneration and sample analysis was carried out as above.

Some samples were further analysis described in the next Section.

The rate of regeneration of the native protein (N) was determined by integrating the areas of the peaks corresponding to the native protein (N), structured intermediates and the fully-reduced protein (R) at each time point. The fractional increase in N was plotted as a function of time; the data were fitted to a single-exponential function to obtain the rate constant for the formation of N from R [28].

NO_x scavenge assays and post-regeneration polyphenol analysis. Samples from the regeneration mixture (*PDI-catalyzed oxidative folding of RNase A under nitrosative stress*) were fractionated on a C-18 reversed-phase column (Supelco Discovery® BIO Wide Pore C18, 5 μm, 15 cm × 100.0 mm) and lyophilized. Peaks corresponding to the polyphenols (identified using wavelengths of 360 and 418 nm) were collected and subjected to ESI-FTMS (LTQXL, Thermo Fisher Scientific, San Jose, CA) and UV–Vis analysis (Perkin–Elmer). Solutions of curcumin (5–200 μM) and masoprocol (5–200) μM in acetonitrile were obtained by dilution from stock solutions that were prepared by weight. Freshly prepared stock solutions of tetranitromethane (TNM) in acetonitrile were separately added to curcumin and masoprocol. Added tetranitromethane concentrations varied between 0–10 μM. Samples were immediately analyzed using UV–Vis spectroscopy and by reversed-phase HPLC (Supelco Discovery® BIO Wide Pore C18, 5 μm, 15 cm × 100.0 mm) using an acetonitrile gradient (1%/min). Fractions were collected and analyzed using mass spectrometry.

Formation of PDI-resistant aggregates. Fully-reduced RNase A (2 mg/mL, pH 8, 100 mM Tris–HCl) was incubated with $FeSO_4$ (1 mM) and H_2O_2 (0–5 μM) for a period of 5 min prior to fractionation using gel filtration chromatography or by SDS gel electrophoresis. Quantification of high molecular weight protein fractions was determined by densitometric analysis of SDS gels.

PDI (4 μM) was added to the high molecular weight RNase A and regeneration was initiated as in *2.2* prior to analysis as previously described [24,25]. The rates of PDI-catalyzed regeneration of high molecular weight RNase A and monomeric RNase A were determined a previously described [25].

Finally, fully-reduced RNase A (2 mg/mL) was subjected to oxidative stress as described above in the presence of curcumin (0–50 μM). Fractionation of RNase A, regeneration, and kinetic analysis was performed as described above.

Results

Regeneration of studies of RNase A by PDI

Fig. 2 shows the regeneration profile of fully-reduced RNase A. The addition of PDI (4 μM) to the regeneration mixture enhanced