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Dietary curcumin counteracts extracellular transthyretin deposition: Insights on the mechanism of amyloid inhibition

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ABSTRACT

The transthyretin amyloidoses (ATTR) are devastating diseases characterized by progressive neuropathy and/or cardiomyopathy for which novel therapeutic strategies are needed. We have recently shown that curcumin (diferuloylmethane), the major bioactive polyphenol of turmeric, strongly suppresses TTR fibril formation *in vitro*, either by stabilization of TTR tetramer or by generating nonfibrillar small intermediates that are innocuous to cultured neuronal cells.

In the present study, we aim to assess the effect of curcumin on TTR amyloidogenesis *in vivo*, using a well characterized mouse model for familial amyloidotic polyneuropathy (FAP). Mice were given 2% (w/w) dietary curcumin or control diet for a six week period. Curcumin supplementation resulted in micromolar steady-state levels in plasma as determined by LC/MS/MS. We show that curcumin binds selectively to the TTR thyroxine-binding sites of the tetramer over all the other plasma proteins.

The effect on plasma TTR stability was determined by isoelectric focusing (IEF) and curcumin was found to significantly increase TTR tetramer resistance to dissociation. Most importantly, immunohistochemistry (IHC) analysis of mice tissues demonstrated that curcumin reduced TTR load in as much as 70% and lowered cytotoxicity associated with TTR aggregation by decreasing activation of death receptor Fas/CD95, endoplasmic reticulum (ER) chaperone BiP and 3-nitrotyrosine in tissues. Taken together, our results highlight the potential use of curcumin as a lead molecule for the prevention and treatment of TTR amyloidosis.

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1. Introduction

Amyloidosis constitute a large group of acquired or hereditary disorders caused by extracellular deposition of abnormal insoluble fibrils composed of misfolded proteins or fragments thereof, which can damage tissue architecture and function, thus causing disease [1,2]. Nearly 30 different unrelated proteins, which share high content of lamellar cross β-sheet structure, are reported to be capable of forming amyloid fibrils *in vivo*, though they are associated with clinically distinct conditions [1,2].

Human transthyretin (TTR), carrier of virtually all of the retinol binding protein (RBP) in blood and about 15% of total thyroxine (T_4) in plasma, is associated with different forms of amyloidosis. That is the case of senile systemic amyloidosis (SSA), a late onset disease in which non-mutated TTR forms amyloid that deposits preferentially in the heart, affecting about 25% of individuals over 80 years, and the case of familial amyloidotic polyneuropathies (FAP) and cardiomyopathies (FAC) in which single amino acid substitutions resulting from single point mutations in the TTR gene result in deposition of amyloid aggregates in peripheral and autonomic nervous systems and heart, respectively [3,4].

Regarding hereditary TTR-related amyloidosis, since the first report describing the TTR V30M mutant, the most common among FAP patients [5], more than one hundred TTR variants have been found to cause TTR amyloidosis (amyloidosismutations.com). The amyloidogenic potential of the TTR variants has been related to the decrease of protein tetramer conformational stability [6], which leads to its dissociation into partially unfolded non-native monomeric species, the rate-limiting step for the process of amyloid fibril formation associated to neurodegeneration and cell death [7]. Moreover, studies using a combination of polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) under semi-dissociating conditions have shown that amyloidogenic TTR tetramers present a greater tendency to dissociate compared with wild-type (WT) TTR [8]. It has been shown that binding of T_4 to TTR efficiently inhibits TTR fibrillogenesis *in vitro* and does so by stabilizing the tetramer against dissociation, thus preventing the

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subsequent conformational alterations required for amyloid fibril formation [9]. Accordingly, it has been proposed that small aromatic molecules might act analogously by binding preferentially to the central hydrophobic channel and stabilizing the native state of TTR over its dissociative transition state [7]. Very recently, our group reported that curcumin (diferuloylmethane), a naturally occurring polyphenol, competes with T_4 for the binding to TTR and inhibits different steps of the process of TTR amyloid fibril formation *in vitro* [10]. In the current work, we present the *in vivo* effects of curcumin using a well characterized FAP mice model.

2. Materials and methods

2.1. Ethics statement

All the experiments described herein were approved by the Portuguese General Veterinarian Board (authorization number 024976 from DGV-Portugal) and are in compliance with national rules and the European Communities Council Directive (86/609/EEC), for the care and handling of laboratory animals.

2.2. Transgenic mice

Seven month-old transgenic mice for human TTR V30M in a TTR null background [11], labeled as hTTR V30M mice, were fed either standard mouse chow (controls, $n=10$) or standard mouse chow containing 2% (w/w) curcumin (Sigma-Aldrich, St. Louis, MO, USA) (treated, $n=10$) over 6 weeks. After the treatment period, animals were sacrificed following anesthesia with ketamine/xylazine and blood samples were collected. Plasma was separated by centrifugation and stored at −20 °C prior to analysis. Mice tissues, in particular whole gastrointestinal tract (GI), including esophagus, stomach, colon and duodenum, were immediately excised and frozen at −80 °C or fixed in 4% neutral buffered formalin and embedded in paraffin for light microscopy techniques.

2.3. Analytical procedure and sample preparation for the determination of curcumin in mice plasma

The frozen plasma samples were thawed at room temperature. Then, 0.3 ml of 134 U/ml β-glucuronidase solution in sodium acetate buffer (pH 5.0) was added to 100 μl of each plasma. The mixtures were vortexed and incubated at 37 °C for 1 h. The buffered plasmas were extracted with 2 ml of ethyl acetate by vortex mixing for 2 min. After centrifugation at 2000 rpm for 2 min, the upper organic layer was removed into a clean microcentrifuge tube and evaporated to dryness under nitrogen stream. The extracts were re-suspended in 200 μl of methanol.

2.3.1. Chromatographic procedure

The HPLC system consisted of a variable loop Accela autosampler, an Accela 600 LC pump and an Accela 80 Hz PDA detector (Thermo Fisher Scientific, San Jose, CA, USA). Analyses were carried out in using a Supelco Discovery® C-18 (15 cm × 2.1 mm × 5 μm) column (Agilent Technologies, Waldbronn, Germany). The compounds were separated using a gradient elution program at a flow rate of 0.2 ml min^{-1}, at 25 °C. The mobile phases consisted in water:acetonitrile (90:10, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following linear gradient was applied: 0–20 min: 0–100% B; 20–23 min: 100% B; 23–30 min: 100–0% B; followed by re-equilibration of the column for 10 minutes before the next run. Single online detection was carried out in DAD detector, at 280 nm, and UV spectra in a range of 210–600 nm were also recorded.

2.3.2. ESI–MS^n analysis

The HPLC system was coupled to a LCQ Fleet ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) with an ESI source and operating in negative mode. The spray voltage was 5 kV and capillary temperature 300 °C. The capillary and tune lens voltages were set at −28 V and −115 V, respectively. CID-MS^n experiments were performed on mass-selected precursor ions in the range of m/z 100–1000. The scan time was equal to 100 ms and the collision energy was optimized between 15 and 40 (arbitrary units), using helium as collision gas. Data were acquired using Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA).

2.3.3. Calibration curve

The curcumin calibration curve was obtained taking into account the possibility of a non-complete efficient extraction. Thus, six different concentrations of curcumin standards were added to plasma control samples in the range 0.7–14 μM. The samples were then extracted using the methodology above described. The injection in HPLC, under the same chromatographic conditions, gave a linear regression between the peak area and concentration (expressed as μg ml^{-1}) with $R^2=0.982$, intercept of 948224 and slope of 2.87×10^6. The detection range was established from 0.36 to 1.20 μg ml^{-1}.

2.4. Determination of TTR levels in mice plasma by sandwich enzyme-linked immunosorbent assay (ELISA)

The concentration of plasma TTR was determined by ELISA. Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C, with rabbit anti-human TTR polyclonal antibody (Abcam, Cambridge, UK). After blocking and washes, TTR standards (2–25 ng/ml) and the diluted mice plasma were applied to different wells in triplicate and incubated. Following sheep anti-human TTR was added and incubated for one hour (Abcam, Cambridge, UK). After washing, anti-sheep conjugated alkaline phosphatase was added. p-Nitrophenyl phosphatase was employed in color development. The absorbance was measured at 405 nm in a Multiskan® Ascent microplate spectrophotometer (Thermo Electron Instruments). Data were fitted to 2nd-order polynomial (quadratic equation).

2.5. Thyroxine (T_4) binding gel electrophoresis

Five microliters of plasma from curcumin treated mice and from controls (non-treated mice) were incubated with $[^{125}I]$-T_4 (specific radioactivity 1250 μCi/μg; Perkin–Elmer, MA, USA). The plasma proteins were then separated by native PAGE [12]. The gel was dried, subjected to phosphor imaging (Typhoon 8600; Molecular Diagnostics, Amersham Biosciences), and analyzed using the ImageQuant program version 5.1.

2.6. Isoelectric focusing (IEF) in semi-dissociating conditions

Thirty microliters of mice plasma from treated and non-treated animals were subjected to native electrophoresis (PAGE). The TTR gel band was excised and applied to a semi-dissociating (4 M urea) pH 4–6.5 IEF gel run for 6 hours at 1200 V [12]. Proteins were stained with Coomassie Blue. The gels were scanned and subjected to densitometry analysis using the ImageQuant program version 5.1.

2.7. Immunohistochemistry

Tissue sections (5 mm thick) were deparaffinated in histoclear and dehydrated in a descent alcohol series. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide/ 100% methanol, and sections were blocked in 4% fetal bovine serum and 1% bovine serum albumin in PBS. The primary antibodies and the respective dilutions used were: rabbit polyclonal anti-TTR (1:1000) (Carpinteria, CA, USA), goat polyclonal anti-BiP (1:50) and rabbit polyclonal anti-Fas (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-3-nitrotyrosine (1:500) (Chemicon, Temecula,

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