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Proteomics screening of molecular targets of curcumin in mouse brain

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ABSTRACT

Aims: Curcumin is one of the most important constituent of *Curcuma longa* L. with antioxidant, anti-inflammatory and anticancer effects. In this study, we investigated potential intracellular targets of curcumin by affinity chromatography based on target deconvolution. Identification of curcumin interacting proteins may help in evaluating biological and side effects of this natural compound.
Main methods: Curcumin was immobilized through a linker to sepharose beads as solid matrix. Pull down assay was performed by passing tissue lysate of mouse brain through the column to enrich and purify curcumin interacting proteins. Then proteins were separated using two-dimensional gel electrophoresis and identified using MALDI/TOF/TOF mass spectrometry.
Key findings: Our results show that curcumin physically binds to a wide range of cellular proteins including structural proteins, metabolic enzymes and proteins involved in apoptosis pathway.
Significance: Finding curcumin interacting proteins may help in understanding a part of curcumin pharmacological effects.

Introduction

Curcumin, a polyphenol compound, is an active biological constituent of the perennial herb *Curcuma longa* L. Turmeric is an Indian spice derived from the rhizomes of the plant from Zingiberaceae family. Turmeric has a long history of use in Ayurvedic medicine as a treatment for inflammatory conditions (Goel et al., 2008). Recently several studies have been performed on curcumin to reveal its cellular targets. For example, targets of curcumin in neuroblastoma cell line have been already identified (D'Agnano et al., 2012).

Numerous studies revealed that curcumin has antioxidant and radical scavenger, anti-inflammatory, anti-infectious, cardioprotective and anticancer properties (Calabrese et al., 2008). Moreover, curcumin, as an antioxidant, could potentially inhibit lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Chan et al., 2005; Ak and Gülçin, 2008). Various studies have shown that cardioprotective, neuroprotective, memory enhancing, and anti-aging effects of curcumin are related to its high antioxidant capacity (Thiyagarajan and Sharma, 2004; Miriyala et al., 2007).

In addition curcumin has the ability to inhibit the proliferation of an extremely wide array of cancer cell types. Other study showed that curcumin inhibited tumor necrosis factor (TNF-α) and induced apoptosis in PC-12 cells through modulating mRNA expression of Bcl-2 family proteins (Shishodia et al., 2007). Previous studies have shown that curcumin has a neuroprotective effect in some central nervous system such as cerebral ischemia, traumatic brain injury and memory impairment (Miriyala et al., 2007; Shishodia et al., 2007).

Curcumin may show its neuroprotective effects through some mechanisms including anti-oxidation, anti-apoptosis, anti-inflammation, anti-amyloidogenic, metal chelating properties, preventing BBB damage, and reducing edema. Some clinical trials, such as application of curcumin Alzheimer's disease, are currently in progress (Sun et al., 2011). Mechanistic study of curcumin may provide a deeper understanding of the therapeutic potential of curcumin and other curcuminoids in neurological disorders (Baum et al., 2008).

In this study affinity chromatography based target deconvolution was chosen to find potential targets of curcumin in the brain. Target deconvolution is a high-throughput technique to discover drug targets and predict potential therapeutic and side effects as well as toxicity of chemicals in early study of a new drug development (Terstappen et al., 2007; Raida, 2011).

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix. In our study,

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curcumin was covalently coupled to epoxy activated sepharose as chromatographic matrix through phenolic group (Lee and Bogyo, 2013).

Following the affinity chromatography-based purification of curcumin targets, proteins were separated using two-dimensional gel electrophoresis and were identified using, matrix-assisted laser desorption/ionization/time of flight (MALDI/TOF/TOF) and MASCOT database.

Material and methods

Materials and reagents

Reagents were obtained from Sigma (USA). Deionized water was used for all experiments and all other chemical substances were purchased from Merck (Germany).

Animals

Animal study was approved by the Mashhad University of Medical Sciences Ethics Committee (#87639). Male BALB/c mice weighing 20–25 g were obtained from the animal house of Mashhad University of Medical Sciences. Mice were kept in a room at 25 ± 2 °C on a 12-h light/dark period and let to eat and drink ad libitum. 6 mice were sacrificed by decapitation and brain tissues were rinsed using 0.9% normal saline solution. Then tissues were quickly frozen in liquid nitrogen and stored at −80 °C until further processing.

Curcumin preparation

Curcumin 65–70% was purchased from Sigma (Cat. # C 1386). The curcumin 65–70% further purification was performed according to the previous work using silica column chromatography and dichloromethane as solvent (Péret-Almeida et al., 2005). The isolated curcumin was also crystallized with methanol–water. TLC experiments showed that the curcumin was completely free of demethoxycurcumin and bisdemethoxycurcumin (Moghaddam et al., 2009).

Preparation of curcumin-activated sepharose 6-B complex

Curcumin was immobilized on epoxy activated sepharose 6-B as chromatographic matrix, according to previously described method (Conboy et al., 2009). 0.5 g of epoxy activated sepharose 6B (sigma, USA) was suspended in 50 mL deionized water to swell and washed five times with 50 mL coupling buffer containing 50% dimethylformamide, 0.1 M Na_2CO_3, 10 mM NaOH at 4 °C. Swallowed beads were immediately coupled to curcumin. Briefly, curcumin solution (20 mM solution in 50% dimethylformamide, 0.1 M Na_2CO_3, 10 mM NaOH) was added to beads with the ratio of 2:1 (v: v) and incubated overnight at 30 °C. Unreacted epoxy groups were blocked by incubation and shaking beads with 1 M ethanolamine at 30 °C overnight.

Control beads were prepared by incubation of the swallowed epoxy activated sepharose 6-B with 1 M ethanolamine (pH 11). The control and curcumin coupled beads were finally washed in three cycles of alternating low pH (0.1 M acetate buffer, pH = 4) and high pH (0.1 M Tris–HCl buffer, pH 8, containing 0.1 M NaCl) buffers and stored at 4 °C until use (Conboy et al., 2009).

Separation of target proteins

200 mg of brain tissues was homogenized in 1 mL lysis buffer containing 50 mM Tris pH 7.4, 2 mM ethylene glycol tetraacetic acid, 2 mM ethylenediaminetetraacetic acid, 2 mM Na_3VO_4, 1% Triton X-100 and 10 mM 2-mercaptoethanol, 2 μL complete protease inhibitor cocktail (Sigma P8340, USA), 0.2 (w/v) 1 mM phenylmethylsulfonyl fluoride using a Polytron Homogenizer (Kinematica, Switzerland). After sonication (UP100H, Hielscher) for 40 s, homogenates were centrifuged (Hettich Universal 320R, Germany) at 25,000 *g* for 10 min at 4 °C and total protein contents in supernatants were determined using a Bradford protein assay kit (BioRad, #500-0002). The same amount of proteins was applied for each test (Osawa et al., 1995).

Affinity chromatography

Tissue extracts were incubated with control beads for 30 min at 4 °C. During this contiguous the proteins that capable to link to the epoxy activated sepharose were out, while proteins that can be band to curcumin were free in supernatant. After centrifugation at 1000 *g* for 1 min, supernatants were collected and transferred to curcumin coupled beads and incubated for 30 min at 4 °C. Beads were washed four times, each time with 2 mL of binding buffer, and supernatants were discarded. Then curcumin interacting proteins were eluted with 2 mL of 2 M NaCl. The later step was repeated 3 times and fractions were pooled and dialyzed for against ddwater for 3 days at 4 °C using a membrane with a 2000 Da cutoff (Spectra, USA). Desalted samples were freeze dried and stored at −80 °C until use (Kunnumakkara et al., 2008).

2D gel electrophoresis

Freeze-dried samples were dissolved in rehydration buffer containing 6 M urea, 2 M thiourea, 2% 3-[(3 cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), 20% Bio-Lyte (BioRad) and concentration was adjusted to 1 μg protein/1 μL. For isoelectric focusing (IEF) experiment, 125 μL of lysate was loaded to 7 cm non linear immobilized pH gradient (IPG) strips (pH range: 3–10, BioRad). To separate curcumin target proteins based on their isoelectric point (pI), IPG strips were actively rehydrated for 12 h at 50 V and subsequently isoelectric focusing was carried out using PROTEAN IEF CELL (BioRad) at 4000 V for 11 h. The IPG strips were equilibrated in buffer containing 50 mM Tris–HCl, 6 M urea, 30% glycerol and 2.5% sodium dodecyl sulfate (SDS), 1.5% DTT for 20 min. For the second dimension, strips were placed on 12% SDS-PAGE and electrophoresis was carried out to separate proteins according to molecular weight. Gels were stained by MS-compatible silver staining method and protein spots were excised and transferred to Center of Genomic Sciences at University of Hong Kong for in gel digestion and curcumin targets identification (Rashedinia et al., 2013).

In gel digestion

Gel slices were destained and dehydrated in acetonitrile for 30 min and dried in vacufuge. Protein reduction was performed by incubation of gel slices in 10 mM DTT and alkylated by incubation in 55 mM iodoacetamide in dark at room temperature. Gel pieces were washed and diluted with 10 mM ammonium bicarbonate before digestion. Gel pieces were covered with 12.5 ng/μL of trypsin and incubated 30 min at 4 °C. Gel pieces were incubated by 20 μL of 10 mM ammonium bicarbonate overnight at 37 °C. 100 μL stop solution containing 50% acetonitrile and 5% formic acid was added. Peptide extraction was performed in 3 steps. 100 mL of 100 mM ammonium bicarbonate in step 1, 100 mL extraction solutions (50% acetonitrile and 5% formic acid) in step 2 and 150 mL extraction solution in step 3 were added. To extract peptides, all fractions were pooled and dried down in vacufuge and then resuspended in 0.1% formic acid. Samples were desalted using ZipTip® μC-18 (Millipore). Eluted samples were stored at −20 °C until use. Samples were prepared in MALDI matrix containing 10 mg/mL α-Cyano-4-hydroxycinnamic acid in 50% water/acetonitrile and 0.1% formic acid and dried before applying matrix.