Curcumin induces paraoxonase 1 in cultured hepatocytes in vitro but not in mouse liver in vivo

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Abstract
Paraoxonase 1 (PON1) is an enzyme that is mainly synthesised in the liver and protects LDL from oxidation, thereby exhibiting antiatherogenic properties. Using a luciferase reporter gene assay, we tested curcumin for its ability to induce PON1 in Huh7 hepatocytes in culture. Curcumin (≥ 10 μmol/l) dose-dependently induced PON1 transactivation in Huh7 cells. However, dietary supplementation of female B6C3F1 mice with curcumin (500 mg/kg diet) for 2 weeks did not increase the hepatic PON1 mRNA and protein levels. No curcumin was detectable in the plasma of the 12 h fasted mice. In conclusion, curcumin may be a potent PON1 inducer in cultured cells in vitro, but not in the liver of curcumin-fed mice because of its low concentrations in vivo.

Key words: Curcumin; Paraoxonase; Hepatocytes; Mice; Atherosclerosis

Paraoxonase 1 (PON1) is a serum esterase which is primarily synthesised in the liver, binds to HDL and circulates in the blood(1). PON1 protects LDL from oxidation, thereby mediating potential antiatherogenic activity(2–4). PON1-deficient mice are highly susceptible towards atherosclerosis(5). The overexpression of PON1 in transgenic mice, on the other hand, counteracts atherogenesis and enhances plaque stability(6). Dietary secondary plant products, such as polyphenols, may increase PON1 expression(7–9). However, little is known regarding the effect of curcumin on PON1 activity.

In the present study, we investigated the impact of curcumin on PON1 transactivation in cultured hepatocytes. We then compared our in vitro data with the effect of a dietary curcumin supplementation on PON1 mRNA and protein levels in mouse liver in vivo.

Materials and methods

Chemicals

Curcumin (CAS no. 458-37-7) was purchased from Alexis Biochemicals (Lausen, Switzerland) for cell culture experiments. Curcumin used in the feeding experiment was obtained from LKT Laboratories, Inc. (St Paul, MN, USA). Resveratrol and 7-ketocholesterol were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Cell culture conditions

Cell culture experiments were carried out in cultured hepatocytes. Huh7 liver hepatoma cells of human origin, stably transfected with a 1000 bp fragment of the human PON1 promoter (PON1-Huh7; originating from X. Coumoul and R. Barouki; INSERM, Paris, France), were cultivated in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, streptomycin (55 mg/ml) and penicillin (100 mg/ml) (all from PAA Laboratories, Coelbe, Germany).

Cytotoxicity measurement

Cytotoxicity was determined via the neutral red assay(10).

Reporter gene assay

PON1-Huh7 cells were seeded at an initial density of 150 000 cells/well in twenty-four-well plates and incubated
with a medium supplemented with 1–20 μmol/l of curcumin for 48 h as recently described(7). Afterwards, the cells were washed with PBS, lysed and subjected to luciferase activity measurement (Luciferase assay system; Promega, Madison, WI, USA) by luminescence reading (Infinite 200 reader; Tecan, Crailsheim, Germany). Results were normalised to the total cell protein content (bicinchoninic acid protein assay; Pierce, Rockford, IL, USA).

**Animals and diets**

The animal experiment was performed in accordance with the guidelines for the care and use of animals for experimental procedures with approval of the Ministry of Agriculture, Environment and Rural Areas of the state of Schleswig-Holstein (Germany). Sixteen female B6C3F1 mice with an initial body weight of 18–24 g were purchased from Harlan Winkelmann GmbH (Borchen, Germany). Four animals per cage (type III polypropylene cages) were housed in a controlled environment (22 ± 2°C, 55 (SEM 5) % relative humidity, 12 h light–12 h dark cycle). The mice were randomly divided into two groups of eight animals each and fed *ad libitum* for 14 d a semi-synthetic diet (38% maize starch, 20% maize oil, 20% casein, 10% sucrose, 6% mineral premix, 4% cellulose powder and 2% vitamin premix; Altromin Spezialfutter GmbH & Company KG, Lage, Germany) or the same diet enriched with curcumin at a dose of 500 mg/kg. The experimental diets were stored at 4°C. At the end of the experiment, the mice were deprived of food for 12 h, anaesthetised with CO2 and decapitated. Blood was collected into heparinised tubes, and the plasma obtained by centrifugation (3000 g, 10 min) was stored at −80°C until analyses. Liver tissue was quickly excised, rinsed with 0·9 % NaCl solution, frozen in liquid N2 and stored until analyses. Liver tissue was quickly excised, rinsed with 0·9 % NaCl solution, frozen in liquid N2 and stored until further analysis.

**RNA isolation and real-time quantitative RT-PCR**

RNA was isolated from liver samples (20–30 mg) using TRIzol lysis reagent (Bioline, Luckenwalde, Germany) and quantified photometrically (Spectrophotometer DU800; Beckman Coulter, Krefeld, Germany). Real-time quantitative PCR was performed as a one-step procedure (SensiMix™ One-step Kit; Quantace, Berlin, Germany) with SybrGreen detection, using the Rotorgene 6000 cycler (Corbett Life Science, Sydney, NSW, Australia)(11). Quantification was done using a standard curve. Transcription levels of target genes were related to transcription of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. Primers were designed by standard tools (Spidey, Primer3 and NCBI Blast) and purchased from MWG (Ebersberg, Germany). A 178 bp fragment of the murine PON1 gene (gene ID 18979), specific for this isoform (no homology to PON2 and PON3 mRNA), was amplified using a forward primer (5'-CACCGTTCATCCTGTCTCA-3') and a reverse primer (5'-CACCGGTTCATCCTGTCTTA-3') and normalised to the mRNA levels of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (gene ID 14433; 131 bp fragment; forward primer 5'-CGCATCTTCTTGAGTCT-3' and reverse primer 5'-GGCAAAATCCTCAGCTTGC-3').

**Western blotting**

Liver cell protein was extracted from tissue samples (30 mg) using radio immunoprecipitation assay buffer (50 mm-Tris–HCl, 150 mm-NaCl, 0·5 % deoxycholate, 0·1 % SDS and 1 % NP-40; pH 7–4 with protease-inhibitor cocktail, 1:100; Sigma, St Louis, MO, USA) by incubation on ice for 30 min and subsequent centrifugation at 12 000 g (4°C, 30 min). Protein concentrations were determined in the supernatants by the bicinchoninic acid assay (Pierce, IL, USA). Protein (60 μg) was separated on a 12 % SDS-polyacrylamide gel and transferred onto an immunoblot polyvinylidenefluoride membrane(12). The membrane was blocked with 3 % non-fat dried milk in Tris-buffered saline, pH 7–4, with 0·05 % Tween-20 (TBS/T) for 2 h and probed with a polyclonal rabbit anti-PON1 antibody (1:1000; Abcam, Cambridge, UK) at 4°C overnight. The membranes were incubated with a goat anti-rabbit IgG secondary antibody (1:4000) conjugated with horseradish peroxidase (BioRad, Munich, Germany) for 45 min. Specific bands were visualised by an enhanced chemiluminescence reagent on a ChemiDoc system and quantified densitometrically with the program Quantity One® (all from Bio-Rad Laboratories, München, Germany). The membranes were stripped with 10 ml strip buffer (8 g glycine, 2·5 ml HCl and 1 litre water) and subsequently incubated with a rabbit polyclonal antibody against β-actin, which was used as a loading control (1:800; Santa Cruz Biotechnology, Heidelberg, Germany), and processed as described earlier. The predicted sizes for PON1 and β-actin were 40 and 42 kDa, respectively, which were checked against molecular-weight markers.

**Curcumin analysis**

Curcumin was extracted from the mouse plasma with ethyl acetate–methanol (95:5, v/v) and quantified (against authentic standards) by HPLC (Jasco, Gross Umstadt, Germany) with fluorescence detection (excitation/emission, 420/470 nm), as recently described(13). The mobile phase (acetonitrile–methyl alcohol–water–1 % acetic acid, 49:20:30:1, v/v) was pumped through a Jasco Reprosil-Pur Basic C18 column (75 × 2 mm, 1.8 μm) protected by a guard column (1 × 4 mm, 5 μm), both maintained at 39°C, at a flow rate of 0·4 ml/min.

**Statistical analysis**

Statistical analysis was performed using PASW Statistics 18 (IBM, Chicago, IL, USA). Data were analysed for normality...
of distribution (Kolmogorov–Smirnov and Shapiro–Wilk tests) and equality of variance (Levene’s test) before the $t$ test for independent samples, ANOVA and Mann–Whitney $U$ test. The data are expressed as the means with their standard errors and significance was accepted at $P<0.05$.

Results and discussion

Although mainly affected by genetic, lifestyle and environmental factors, improvement of PON1 status by dietary factors, including plant bioactives, is considered as a promising approach in maintaining vascular health. There is increasing interest in the potential antiatherogenic activity of curcumin; however, its exact cellular and molecular modes of action in this context are not completely understood\textsuperscript{(14)}.

To study the potential induction of PON1 transactivation by curcumin, we tested the compound in our PON1-Huh7 reporter gene assay. Under the conditions investigated, curcumin was not cytotoxic to PON1-Huh7 cells at concentrations of up to 20 $\mu$mol/l. Curcumin dose-dependently induced PON1 transactivation \textit{in vitro} ($P<0.001$; Fig. 1). At 20 $\mu$mol/l, curcumin induced PON1 transactivation 4.2-fold and was even more potent than 25 $\mu$M-resveratrol, which was used as a positive control\textsuperscript{(15)}. The concentrations required to induce a significant PON1 transactivation in our cellular model ($\approx$ 10 $\mu$mol/l) were comparably high.

Interestingly, PON1 induction by curcumin was significantly counteracted (approximately 50% inhibition at 20 $\mu$M-curcumin) due to 20 $\mu$M-7-ketocholesterol (data not shown). It has been previously shown that 7-ketocholesterol is an inhibitor of the aryl hydrocarbon receptor\textsuperscript{(16)}. Thus, PON1 induction by curcumin seems to be, at least partly, mediated by an aryl hydrocarbon receptor-dependent signal transduction pathway.

To investigate whether dietary curcumin (500 mg/kg diet) might also induce PON1 \textit{in vivo}, a 2-week feeding experiment with female B6C3F1 mice was conducted. Curcumin consumption did neither affect feed intake and body weight (data not shown) nor relative PON1 mRNA (Fig. 2(a)) or protein expression (Fig. 2(b)) in the livers of the mice.

To understand the dose–response relationship for curcumin-induced PON1 transactivation \textit{in vivo}, we aimed to determine curcumin concentrations in the plasma. However, the curcumin concentrations in the plasma of our fasted animals were below the limit of detection of our HPLC method of 0.2 ng (amount injected)\textsuperscript{(13)}. Hence, 12 h after their last feed intake, curcumin plasma concentrations in mice were below 10 nmol/l. This is in agreement

![Fig. 1. Dose-dependent induction of paraoxonase 1 transactivation in stably transfected Huh7 liver cells by incubation with curcumin. Values are means, with their standard errors of three independent experiments performed in triplicate represented by vertical bars. Resveratrol (25 $\mu$mol/l) was used as a positive control. ***Mean values were significantly different ($P<0.001$).](https://www.cambridge.org/core/terms).
with our previously published data, which shows that no free curcumin was detectable in the plasma and liver 30, 60 or 90 min after oral administration of curcumin (50 mg/kg body weight) to mice\textsuperscript{(13)}. On the other hand, intraperitoneal injection of mice with curcumin (100 mg/kg body weight) resulted in detectable amounts of curcumin in the brain (about 5 mg/g tissue)\textsuperscript{(13)}. In human subjects, intake of gram doses of curcumin was required to detect curcumin in the blood at concentrations <0.2 \mu mol/L\textsuperscript{(17)}.

Thus, the lack of induction of PON1 by dietary curcumin observed in mice is probably caused by the low in vivo concentrations of the phytochemical. Data from our cell model suggest that concentrations of 10 \mu mol/l or more are required for significant PON1 induction (Fig. 1), a concentration that is unlikely to be ever achieved in vivo. However, it has recently been shown that the formulation of curcumin-loaded nanoparticles significantly enhanced cellular curcumin uptake and increased its bioactivity. Furthermore, nano-formulated curcumin exhibited a superior bioavailability as compared with curcumin in mice\textsuperscript{(18)}. Thus, future studies are warranted to test whether nano-formulated curcumin may induce PON1 in vivo.

In summary, the present results indicate that curcumin induces PON1 in cultured hepatocytes in vitro, but not in the livers of mice in vivo, and emphasises the importance of verifying cell culture data using an appropriate in vivo model.

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References
