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Original Contribution

Iron chelation in the biological activity of curcumin☆

Yan Jiao [a], John Wilkinson IV [a,c], E. Christine Pietsch [b], Joan L. Buss [a], Wei Wang [a,c], Roy Planalp [d], Frank M. Torti [a,c], Suzy V. Torti [b,c,*]

[a] *Department of Cancer Biology, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA*
[b] *Biochemistry, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA*
[c] *Comprehensive Cancer Center, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA*
[d] *Department of Chemistry, University of New Hampshire, Durham, NH, USA*

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Abstract

Curcumin is among the more successful chemopreventive compounds investigated in recent years, and is currently in human trials to prevent cancer. The mechanism of action of curcumin is complex and likely multifactorial. We have made the unexpected observation that curcumin strikingly modulates proteins of iron metabolism in cells and in tissues, suggesting that curcumin has properties of an iron chelator. Curcumin increased mRNA levels of ferritin and GSTα in cultured liver cells. Unexpectedly, however, although levels of GSTα protein increased in parallel with mRNA levels in response to curcumin, levels of ferritin protein declined. Since iron chelators repress ferritin translation, we considered that curcumin may act as an iron chelator. To test this hypothesis, we measured the effect of curcumin on transferrin receptor 1, a protein stabilized under conditions of iron limitation, as well as the ability of curcumin to activate iron regulatory proteins (IRPs). Both transferrin receptor 1 and activated IRP, indicators of iron depletion, increased in response to curcumin. Consistent with the hypothesis that curcumin acts as an iron chelator, mice that were fed diets supplemented with curcumin exhibited a decline in levels of ferritin protein in the liver. These results suggest that iron chelation may be an additional mode of action of curcumin.

Keywords: Chelator; Chemoprevention; Curcumin; Iron; Ferritin; Glutathione *S*-transferase; Free radicals

Introduction

Curcumin (diferuloylmethane, or (*E, E*)-1, 7-*bis* (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione) is a natural yellow colored product extracted from the Indian herb, turmeric. This low molecular weight polyphenol is the major curcuminoid in *Curcuma longa*, a rhizome used in India for centuries as a spice, for coloring, and as a medicinal agent (reviewed in [1,2]). Recent studies have demonstrated that curcumin has a wide range of beneficial pharmacological effects, including anti-inflammatory [3], antioxidant [4], antiviral [5], antiangiogenic and antitumorigenic [1] effects.

Particular interest has focused on curcumin's antitumor effects and its potential utility as a cancer chemopreventive agent. Curcumin has been shown to inhibit tumor formation in the skin, forestomach, duodenum, and colon in mice [6], and the mammary glands, colon, tongue, and sebaceous glands of rats (reviewed in [7]). These and other results have led to clinical trials exploring the utility of curcumin as a cancer chemopreventive agent in humans. Recently completed Phase I clinical trials have demonstrated that curcumin is exceptionally well tolerated, and curcumin has been recommended for further evaluation in Phase II trials [8,9].

The mechanism of curcumin's cancer chemopreventive activity has not been completely defined, and is likely

Abbreviations: GST, glutathione *S*-transferase; NQO1, NAD(P)H:quinone oxidoreductase 1; IRPs, iron regulatory proteins; IRE, iron-responsive element; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium; TfR, transferrin receptor; BSA-PBS, bovine serum albumin-phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; UTR, untranslated region.

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* Corresponding author. Department of Biochemistry, Wake Forest University Health Sciences, Winston Salem, NC 27157. Fax: +1 336 716 0255.
E-mail address: storti@wfubmc.edu (S.V. Torti).

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multifactorial. Curcumin inhibits tumor growth and induces tumor cell apoptosis in animal and cell culture models [10]. Curcumin also inhibits activation of NFκB through blockade of IκB kinase, and inhibits activation of COX2 [11–14]. Curcumin also alters AP1 complexes [15] and inhibits Akt [16]. Another contributor to curcumin's chemopreventive activity is the induction of cytoprotective phase II enzymes. These include proteins such as glutamate-cysteine ligase [15], isoforms of glutathione *S*-transferase (GSTs) [17], as well as heme oxygenase [18], and NAD(P)H:quinone oxidoreductase 1 (NQO1) [19]. In addition to these effects on transcription and cell signaling, curcumin possesses chemical features that may further modulate its chemopreventive activity. For example, curcumin is an antioxidant and free radical scavenger [20]. Curcumin has also been shown to conjugate directly to thioredoxin reductase and modulate its activity [21]. Direct chemical measurements using cyclic voltammetry have recently shown that curcumin has a high affinity for iron, particulary Fe(III), with a formation constant of 10^{22} M^{-1} [22]. However, the extent to which these chemical properties contribute to curcumin's activity in vivo is uncertain.

Ferritin is a key protein in the maintenance of intracellular iron homeostasis. Our previous results have shown that ferritin transcription is induced concomitantly with other Phase II enzymes in response to chemical inducers of the Phase II response [23]. The ferritin apoprotein is a 480 kDa multimer of 24 subunits. The subunits of mammalian ferritin are of two types, termed ferritin H and ferritin L. The ratio of these subunits within the ferritin protein varies in a tissue-specific fashion, and is further modulated by inflammatory and environmental stimuli (reviewed in [24]). In addition to its regulation by Phase II inducers, ferritin is regulated by iron. This occurs via a well-studied regulatory circuit mediated by iron regulatory proteins (IRPs). IRPs interact with the iron-responsive element (IRE) in the 5′ UTR (untranslated region) of ferritin H and L mRNAs, as well with IREs in the 3′ UTR of transferrin receptor 1 [25,26], a protein with a critical role in iron transport [26,27]. The activity of the IRP proteins is in turn modulated by intracellular levels of "free" or "labile" iron. Under conditions of iron deprivation, the IRP proteins are activated to repress ferritin and stabilize transferrin receptor 1 mRNA; conversely, when iron is abundant, ferritin translation is induced and transferrin receptor 1 mRNA is degraded (reviewed in [24]). Translational repression of ferritin, activation of IRP, and induction of transferrin receptor 1 thus all serve as indicators of reduced intracellular iron, and are observed in cells treated with iron chelators [28].

The ability of curcumin to induce some proteins of the Phase II response led us to query whether curcumin also induced ferritin. Unexpectedly, we found that although curcumin induced GSTα and ferritin mRNA, it reduced ferritin protein. Curcumin also induced transferrin receptor 1 and activated IRP. These properties suggest that curcumin may exhibit iron chelator activity, and imply that iron chelation may be a novel mechanism that contributes to the potent cancer chemopreventive activity of curcumin.

Materials and methods

Animals and treatment

Mice were treated according to the guidelines established by our institutional animal care and use committee. All experimentation followed approved protocols. Female FVB mice, 5 weeks of age, weighing an average of 13.5 g were purchased from Charles River (Wilmington, MA). Mice were maintained in a room controlled at 25°C with a relative humidity of 60% and 12 h light/dark cycle. After arrival, mice were allowed to acclimate for 1 week using standard pellet rodent diet, followed by acclimatization to a basal AIN76A diet for 1 week. Mice were then randomly divided into 12 cages. Six cages were designated for the 2-week endpoint, and six cages for the 12-week endpoint. For each endpoint, two cages continued to receive AIN76A basal diet, two cages were changed to basal diet with 0.5% curcumin, and two cages were changed to basal diet with 2.0% curcumin. Curcumin for use in animal diet preparation was purchased as Turmeric type 97 (stock number 12-970-05) from Kalsec (Kalamazoo, MI) and added to a dietary base of AIN76A diet. The diet was prepared by Dyets Inc. (Bethlehem, PA). The analysis of type 97 curcumin is 77% curcumin, 17% dexethoxycurcumin, and 3% bisdemethoxycurcumin. Thus, the final concentration of curcumin in the 0.5% (w/w) curcumin diet was 3.85 g/1000 g of diet and 15.4 g/1000 g diet in the 2% (w/w) curcumin diet. There were 0.21g of iron per 1000 g diet. At the 2-week and 12-week endpoints, mice were sacrificed and tissues snap-frozen in liquid nitrogen. Tissues were stored at −80°C for subsequent biochemical assays. A total of nine mice from each group was analyzed.

Tissue homogenate preparation

For Western blot analysis, livers were ground using a liquid nitrogen-chilled mortar and pestle after the addition of tissue lysis buffer (TLB) (25 mM Tris, pH 7.4; 1% Triton X100, 1% SDS, 1% Na deoxycholate, 150 mM NaCl, 2 μg/ml aprotinin, 1 mM PMSF). Tissues were further homogenized on ice using a polytron style homogenizer and subjected to freeze/thawing, and the homogenate was clarified by centrifugation at 18,000*g* for 15 min at 4°C. Supernatants were removed and protein concentration was determined by Bio-Rad protein assay (Hercules, CA.).

Cell culture and treatment

The mouse normal liver cell line BNL CL.2 was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Cells were plated at a density of 1×10^6 in 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation Co., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products Co., Woodland, CA), 100 units/ml penicillin, and 100μg/ml streptomycin (Life Technologies, Inc.). Cells were cultured at 37°C in 5% CO_2 in a humidified incubator. Control cells received basal media, while treated cells received basal media

containing curcumin (Sigma-Aldrich, St. Louis, MO) at final concentrations of 12.5, 25, and 50 μM. In selected experiments, cells were treated with 200 μM ferric ammonium citrate (Sigma-Aldrich) in growth medium. Viability was measured by fixing cells with 10% formaldehyde and staining with 0.1% crystal violet. Staining was quantified by measuring absorbance at 595 nm.

Cytosolic extract preparation

Extracts for Western blot analysis were prepared as described [29]. Cell debris was removed by centrifugation at 18,000*g* for 2 min at 4°C and the supernates were subjected to Western blot analysis.

Antibodies

Rabbit anti-rat GSTα polyclonal antibody was purchased from Biotrin International (Dublin, Ireland). This antibody cross-reacts with mouse GSTya and GSTyc subunits [30]. Polyclonal rat anti-human GSTπ antibody, which cross-reacts with mouse GSTπ, was purchased from BD Biosciences (San Jose, CA). Rabbit anti-ferritin H antibody and rabbit anti-ferritin L antibody were prepared by Biosource International. Rat anti-mouse transferrin receptor (TfR) antibody was purchased from US Biological (Swampscott, MA). Rabbit anti-mouse β-actin antibody was purchased from Sigma Chemical Co. Secondary antibodies were goat anti-rabbit IgG purchased from Bio-Rad, and goat anti-rat IgG, and goat anti-mouse IgG HRP purchased from Calbiochem (San Diego, USA).

Western blot assay

Western blots were performed essentially as described [29]. Protein (10-20 μg) was resolved on 12% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, USA). Equivalent protein loading was demonstrated by staining membranes in 1% Ponceau S. Membranes were blocked for 1 h in PBS containing 5% nonfat dry milk, followed by 30 min in PBS containing 2% BSA. The blots were cut to isolate particular molecular weight ranges, rinsed briefly in PBS, incubated with primary antibodies in 1% BSA-PBS, washed, and then incubated with appropriately matched HRP-labeled secondary antibodies followed by visualization using the Enhanced Chemiluminescence (ECL) System (Amersham Biosciences). Films were quantified using UN-SCANIT Automatic digitizing system Version 5.1. Fold changes were calculated after normalization to β-actin.

RNA extract preparation and Northern blot analysis

BNL CL.2 cells were treated with curcumin as described above, and total RNA was extracted using the TRIzol reagent (Invitrogen, Inc.). Northern blots were performed as described previously using 10 μg of total RNA [27]. RNA was transferred to Immobilon Ny+ nylon membranes (Nytran NY^+; Millipore, Bedford MA) and hybridized with ^{32}P-labeled cDNA fragments prepared by random priming. Ferritin H RNA was detected using a 500 bp *Eco*R1 fragment of pUC-mFH 500 [28]. Ferritin L was detected using a 650 bp Pst1 fragment of pLF108 [29]. To detect GSTα RNA, the plasmid pGTB was digested with Pst1 to release a 542 bp fragment [30]. The plasmid pHFBA-1 was digested with *Bam*H1 to release a 1.9 kb fragment which recognizes β-actin RNA [28,31]. Fold changes were calculated after normalizing to β-actin.

RNA-binding protein gel-shift assay

Cytosolic extracts were prepared and band shift assays were conducted as described [32]. Cytosolic extracts (10 μg) were incubated with a [α-^{32}P]UTP-radiolabeled human ferritin H IRE probe prepared from in vitro transcription of *Bam*HI linearized pST18 plasmid (a generous gift of Dr. P. Ponka). RNA-protein complexes were analyzed on a 6% nondenaturing polyacrylamide gel. Gels were dried and subjected to autoradiography and Phosphorimaging. Quantification of IRE/IRP complexes was based on Phosphorimaging analysis (ImageQuant version 5.2, Molecular Dynamics).

Results

Curcumin induces GST and ferritin mRNA in cultured liver cells

One mechanism of action of synthetic chemopreventive agents is the induction of cytoprotective proteins [31]. These include ferritin, a protein that functions as a cytoprotective protein by virtue of its ability to bind iron and reduce oxidative stress. We have previously observed that oltipraz [23] as well as novel synthetic chemopreventive agents [29] induce ferritin as well as GSTα and NQO1. To test whether a natural chemopreventive agent such as curcumin might also function to induce ferritin, we treated cultured liver cells with curcumin and measured its effect on levels of ferritin and GSTα mRNA. As shown in Fig. 1, BNL CL.2 cells treated with curcumin exhibited a dose-dependent induction of ferritin H, ferritin L, and GSTα mRNA. GSTα demonstrated the greatest induction, rising an average of 9-fold over control levels in cells treated with 50 μM curcumin for 24 h. At 8 h, the average increase in ferritin H mRNA (normalized to β-actin) relative to controls was 2.3-fold in cells treated with 12.5 or 25 μM curcumin, and 3.1-fold in cells treated with 50 uM curcumin. At 24 h, average increase in ferritin H mRNA was 1.9-, 2.1-, and 2.1-fold in cells treated with 12.5, 25, and 50 μM curcumin, respectively. By 48 h, ferritin H mRNA levels had declined to near baseline in all cases (not shown).

Curcumin has divergent effects on GST and ferritin protein in cultured liver cells

Western blotting was then used to assess the effect of curcumin on cognate proteins. As seen in Fig. 2, levels of

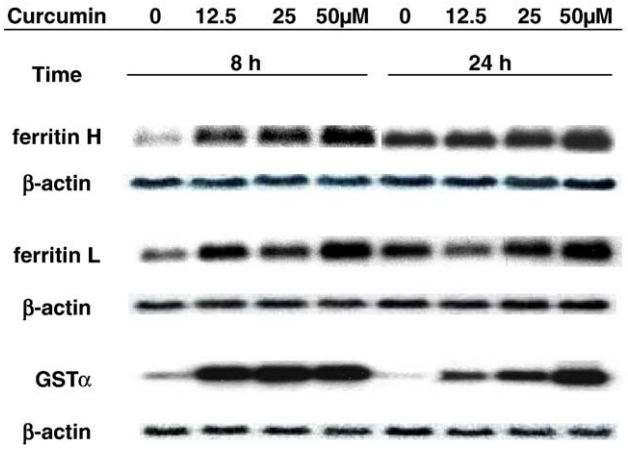

Fig. 1. Curcumin increases levels of mRNA of ferritin H, ferritin L, and GSTα in cultured normal mouse liver cells. BNL CL.2 cells were treated with 0, 12.5, 25, or 50 μM curcumin for 8 or 24 h. RNA was isolated and analyzed by Northern blot analysis using cDNA probes specific to ferritin H, ferritin L, and GSTα. β-actin was used as a loading control. The experiment was repeated 3 times with similar results.

GSTα increased in response to curcumin. Consistent with the rise in GSTα mRNA, the increase in GSTα protein was dramatic, rising an average of 5.3-fold over controls in cells treated for 24–48 h with 50 μM curcumin (Fig. 2). Levels of β-actin protein, used as a control, were unchanged with curcumin treatment. Surprisingly, however, levels of ferritin H and L protein did not increase in response to curcumin, despite the increase in ferritin mRNA. In fact, levels of ferritin subunits declined to approximately 65% of control in cells treated with curcumin. Average decline in ferritin H protein at 24 h was 20 ± 8% in cells treated with 12.5 μM curcumin, and 40 ± 6% in cells treated with 25 or 50 μM curcumin (normalized to β-actin). Sustained incubation with curcumin did not result in a further decline in ferritin H. In two experiments, average decline in ferritin H at 48 h was 18% with 12.5 μM curcumin, and 53% in cells treated with either 25 or 50 μM curcumin. Effects of curcumin on cell survival were discernable but modest, and increased in a dose- and time-dependent manner. Survival was 99–82% of control in cells treated with 12.5 μM curcumin, 87–75% of control in cells treated with 25 μM curcumin, and 78–57% of control in cells treated with 50 μM curcumin (Table 1).

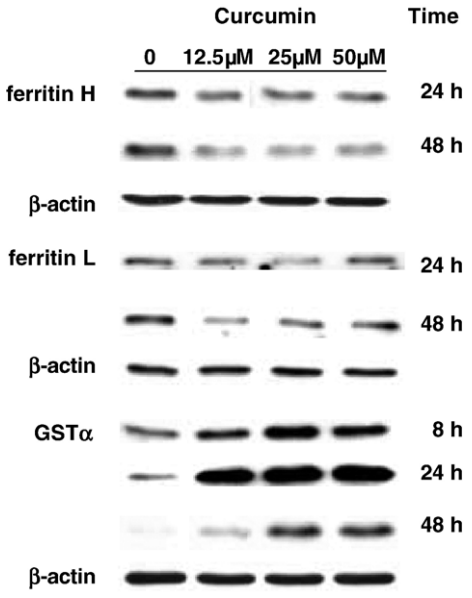

Fig. 2. Curcumin increases GSTα protein but decreases ferritin H and ferritin L in cultured mouse liver cells. Cytosolic extracts were obtained from BNL CL.2 cells treated with curcumin at 0, 12.5, 25, and 50 μM for 24 and 48 h, subjected to SDS PAGE, and analyzed by Western blotting using antibodies against ferritin H, ferritin L, and anti-GSTα. Western blotting with β-actin demonstrated equivalent protein loading in all lanes (only one β-actin blot is shown). A typical result of 3 replicate experiments is shown.

Dietary curcumin increases GSTα but decreases ferritin protein in mouse liver

To determine whether similar effects of curcumin on GST and ferritin proteins were seen in vivo, mice were fed diets containing 0, 0.5, and 2% curcumin for either 2 or 12 weeks. Mice were weighed 3 times per week. As depicted in Fig. 3, dietary curcumin at either dose had no effect on weight gain. Livers were obtained from animals at sacrifice, and analyzed by Western blotting for expression of ferritin H, ferritin L, and GSTα as well as MnSOD and GSTπ, classic phase II enzymes. Two weeks of dietary curcumin had little effect on levels of any of these proteins, and resulted in a slight but not statistically significant reduction in ferritin H (not shown). Following a more prolonged 12-week exposure to dietary curcumin, GSTα protein levels were induced (Fig. 4). Levels of GSTα increased an average of 2-fold in mice receiving either 0.5 or 2.0% dietary curcumin. The overall effect of curcumin on expression of GSTα was highly significant (P = 0.0014, ANOVA), with the group receiving 0.5% curcumin and the group receiving 2.0% curcumin, both expressing higher levels of GSTα than controls (P = 0.0009 and 0.002, respectively). There was also a trend to induction of GSTπ and MnSOD, although the fold induction was more modest (1.4- to 1.5-fold), and did not attain statistical significance. In contrast, as shown in Fig. 4, ferritin H and L proteins were both reduced to approximately 50% of control in mice receiving 2.0% dietary curcumin. For ferritin H and ferritin L, the overall effect was very significant (P = 0.004 and 0.002, respectively), with the 2.0% curcumin dose signifi-

Table 1
Effect of curcumin on cell viability

Time	Viability		
	12.5 μM curcumin	25 μM curcumin	50 μM curcumin
0	100%	100%	100%
8 h	99 ± 2	87 ± 1	78 ± 2
24 h	89 ± 3	78 ± 2	66 ± 1
48 h	82 ± 1	75 ± 1	57 ± 3

Viability was measured using a crystal violet assay. Percentage viability was calculated by dividing staining intensity at the indicated times and concentrations by staining intensity at time zero. Measurements were made in duplicate. Means and standard deviations of 3 independent experiments are shown.

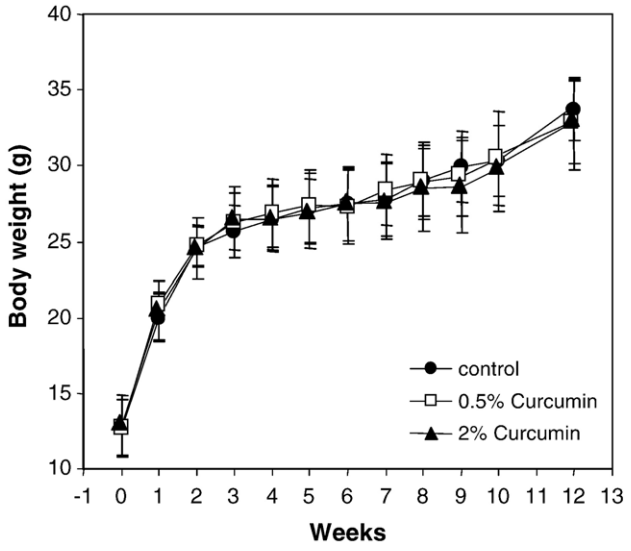

Fig. 3. Dietary curcumin does not affect mouse growth. Animals were placed on diets with or without dietary curcumin at 6 weeks of age (week 0) and maintained on diets for 12 weeks. Mice were weighed every week and weights of mice in each group were averaged. ● Control; □ 0.5% curcumin; ▲ 2% curcumin (Means ± SD).

cantly lower than control (P = 0.001). The 0.5% curcumin dose was not significantly different from control. Thus, dietary curcumin has divergent affects on iron-related and non-iron-related Phase II proteins in vivo as well as in vitro: whereas GSTα was induced by curcumin, ferritin protein was reduced. These results suggest that curcumin may have a selective effect on proteins of iron metabolism that is distinct from its effects on other cytoprotective proteins.

Curcumin increases transferrin receptor 1 and IRP activity

Iron chelators are known to translationally repress ferritin through the activation of IRP proteins [24]. The discordance between the effects of curcumin on ferritin mRNA and protein as well as curcumin's apparently selective ability to reduce ferritin protein levels suggested that curcumin may act as an iron chelator in vivo. To test this hypothesis, we first measured levels of transferrin receptor 1 in curcumin-treated cells. This iron transporter is induced by iron chelators through IRP-mediated mRNA stabilization [32]; thus, if curcumin acts as an iron chelator, levels of transferrin receptor 1 should be increased in response to curcumin treatment. As shown in Fig. 5, levels of transferrin receptor 1 were increased in curcumin-treated cells, consistent with IRP activation. We then directly tested the effect of curcumin on IRP activation using an RNA band-shift assay. As shown in Fig. 6, curcumin enhanced IRP activity in BNL CL2 cells. IRP activity rose until 24 h and then declined back to baseline by 48 h. At 24 h, the effect of curcumin equaled that of desferrioxamine, a known iron chelator. The increase in IRP activity was not due to an unanticipated effect of curcumin on IRP mRNA, as IRP mRNA did not increase in cells treated with curcumin (not shown).

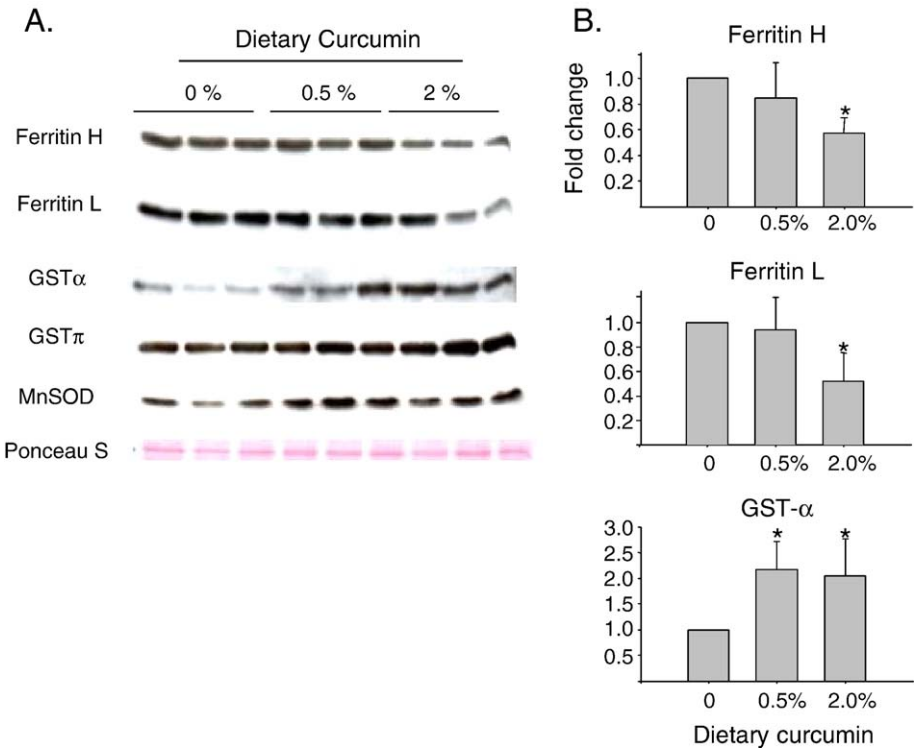

Fig. 4. Dietary curcumin increases GSTα protein but reduces ferritin protein in vivo. (A) Livers of mice fed diets containing 0, 0.5, and 2% curcumin for 12 weeks were analyzed by Western blotting as described in Fig. 2. Equivalent loading and transfer were demonstrated by staining the membrane with Ponceau S. A typical blot is shown. (B) Western blots for all mice (9 for each group, total of 27) were quantified by Unscanit automatic digitizing system and normalized to control. Averages ± SD are shown. Data were analyzed for statistical significance using SAS software. *Indicates difference from control ($P \leq 0.002$, ANOVA).

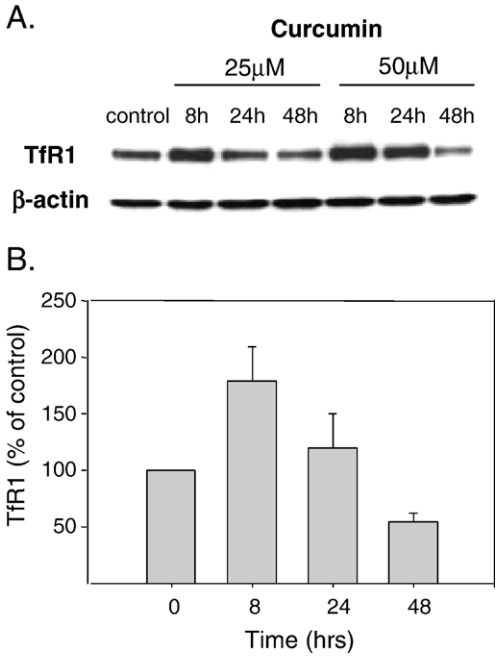

Fig. 5. Curcumin increases transferrin receptor 1. (A) BNL CL.2 cells were treated with curcumin at the doses described for the indicated lengths of time and levels of transferrin receptor 1 analyzed by Western blotting. β−Actin was used as a loading control. (B) The relative increase in transferrin receptor in cells treated with 50 μM curcumin was quantified in 4 independent experiments. Means and standard errors are shown.

Effects of curcumin on ferritin are blocked by iron

If iron depletion underlies the curcumin-dependent decrease in levels of ferritin protein, then it should be possible to reverse the effect of curcumin on ferritin by repleting cells with iron. To test this prediction, BNLCL2 cells were pretreated with ferric ammonium citrate for 4 h before the addition of curcumin. Cells were then exposed to curcumin alone or to curcumin plus iron for an additional 48 h, and analyzed for ferritin H by Western blotting (Fig. 7). Treatment with iron increased basal levels of ferritin H, as expected from iron's known ability to derepress ferritin translation [24]. In contrast to the effect of curcumin on cells grown in normal medium, under conditions of iron repletion, curcumin was unable to reduce levels of ferritin H protein. Thus, iron blocks curcumin-dependent ferritin repression.

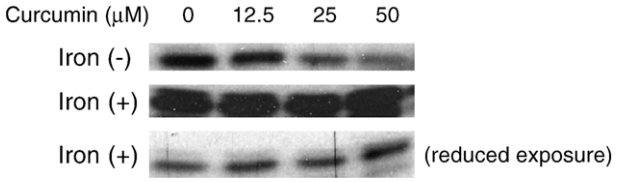

Fig. 7. Effects of curcumin on ferritin are blocked by iron. Cells were treated for 48 h with the indicated concentrations of curcumin in the presence and absence of 200 μM ferric ammonium citrate and analyzed for ferritin H protein by Western blotting. The upper and middle images were exposed for the same length of time (ca 1 s). The lower image is identical to the middle image; however, it was exposed for shorter length of time (ca 1/5 s) so that band intensities can be more easily compared.

Discussion

There is a growing interest in the use of dietary agents in the prevention of cancer. Curcumin represents a particularly promising candidate chemopreventive agent. Curcumin prevents tumor formation in a number of animal models, including models of skin, colon, liver, esophageal, stomach, and breast cancer [33–36]. Curcumin has also demonstrated the ability to improve patient outcomes in Phase I clinical trials [8]. Equally important to its potential application as a chemopreventive agent, curcumin exhibits exceptionally low toxicity in both animal and human studies. For example, no toxicity was seen in a Phase I trial of 25 subjects consuming up to 8000 mg of curcumin per day for 3 months [8]. Five other human trials using 1125–2500 mg of curcumin per day also reported no toxicity [37].

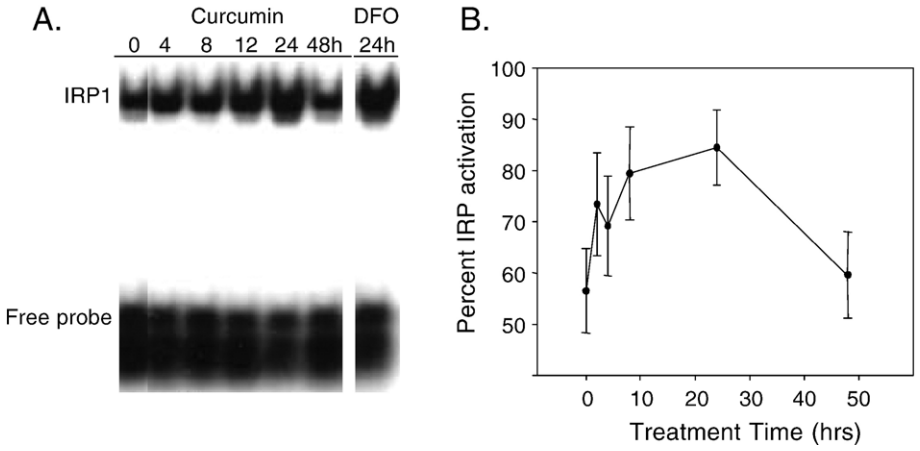

Fig. 6. Curcumin increases IRE-binding activity. (A) BNL CL.2 cells were incubated with 50 μM curcumin for 8, 24, and 48 h or with 250 μM DFO (an iron chelator) for 24 h. Cytosolic extracts were isolated and incubated with ^{32}P-labeled human ferritin-IRE probe. The RNA-protein complexes were separated by 6% nondenaturing polyacrylamide gel electrophoresis. (B) Means and standard errors of 6 independent experiments are shown. 100% refers to IRE binding activity in cells treated with 250 μM DFO for 24 h.

The mechanism of action of curcumin is complex and multifaceted. In part, curcumin acts by activating various cytoprotective proteins that are components of the phase II response. We have recently observed that ferritin is a constituent of this response. Thus, ferritin is induced by chemopreventive agents such as oltipraz [23] and D3T [38].

To explore whether ferritin was induced as part of the cellular response to curcumin, we treated cultured liver cells with curcumin and measured mRNA levels for ferritin and other Phase II enzymes. Although curcumin can inhibit proliferation and induce apoptosis of cultured cancer cells [16,39], curcumin exerted relatively modest antiproliferative effects on these noncancer cells under these conditions (Table 1). Curcumin induced a rapid and robust induction of both ferritin H and ferritin L mRNA, as well as GSTα. Despite the induction of GSTα, cellular levels of glutathione were not depleted, and in fact increased modestly, (data not shown), perhaps due to the ability of curcumin to induce enzymes involved in the synthesis of glutathione, such as glutamate-cysteine ligase [15]. Modest induction of MnSOD was also observed (not shown). These results are consistent with our previous observations demonstrating the ability of synthetic chemopreventive agents to induce ferritin [23,29], and suggest that like these agents, curcumin induces ferritin H and L mRNA via a transcriptional mechanism.

To demonstrate that curcumin-mediated induction of ferritin and GSTα mRNA was reflected in a similar increase in protein levels, we performed Western blot analysis. As expected, the induction of GSTα mRNA led to a corresponding increase in GSTα protein (Fig. 1). The increase in GSTα protein was demonstrable at all time points tested, although at 48 h GSTα protein levels had begun to decline. We did not study the mechanism of this decline; however, a decline in curcumin-mediated effects at later time points was consistently observed in our studies (Figs. 2, 5, and 6), and likely relates to the instability of curcumin itself, which was not replenished during the course of these in vitro experiments. Curcumin is rapidly metabolized in vivo and in isolated hepatocytes [40–42]. We observed that the pronounced yellow color observed in curcumin-treated samples (indicative of the intact diferuloylmethane structure [40]) exhibited a similar marked decay with increasing time of incubation (unpublished observations).

Unexpectedly, during the course of these experiments, we observed that the curcumin-mediated increase in ferritin H and ferritin L mRNA was not accompanied by a corresponding increase in the levels of their cognate proteins. In fact, in contrast to several synthetic chemopreventive agents that we have shown to induce both ferritin mRNA and protein [23,29,38], levels of ferritin protein declined in cells treated with curcumin (Fig. 2).

To test the relevance of these observations to the response induced by curcumin in vivo, we fed mice diets containing curcumin for 2 or 12 weeks and then measured levels of GSTα and ferritin protein in the liver. Consistent with published results demonstrating the safety of curcumin, no toxicity or adverse effects on growth or weight gain were observed (Fig. 3). No significant difference in GSTα or ferritin levels between controls and curcumin-supplemented mice was seen after 2 weeks (data not shown). However, pronounced differences were observed after 12 weeks (Fig. 4). As we had observed in cultured liver cells in vitro, curcumin caused a significant increase in GSTα and a more modest increase in GST π and MnSOD, while decreasing ferritin H and L protein (Fig. 4).

The striking discord between the effects of curcumin on GSTα and ferritin at the protein level despite a curcumin-mediated increase in both ferritin and GSTα mRNA suggested that curcumin may selectively inhibit the translation of ferritin mRNA. Since iron chelators act as inhibitors of ferritin translation, we considered that curcumin may act as an iron chelator. Iron chelators repress ferritin translation by activating IRP proteins. IRP proteins bind to a specific IRE element in the 5′ UTR of ferritin H and L and repress their translation (reviewed in [28]). IRP proteins also bind to IRE elements in the 3′ UTR of TfR1, stabilizing TfR1 mRNA. The binding of IRPs to TfR mRNA therefore results in an increase in levels of TfR1 protein in cells treated with iron chelators. Thus if curcumin acts as an iron chelator, we would expect both an increase in levels of TfR1 and activation of IRP proteins. As shown in Figs. 5 and 6, cells treated with curcumin exhibited both of these properties.

The demonstration that curcumin has properties consistent with in vivo activity as an iron chelator is congruent with direct chemical measurements of its iron-binding activity. Cyclic voltammetry studies have shown that curcumin binds iron, particularly Fe(III) [22]. Iron binding may occur via the β-diketonate group, a known bidentate chelator of Fe(III), as shown in the simple analogous complex of Fe(III) and acetylacetonate [43]. Based on the formation constant of [Fe(III)(curcumin)] of 10^{22} M^{-1} [22], we calculate the pFe(III) (“pM”) of curcumin as 16.6 (calculated at pH 7.4 for 10 μM curcumin and 1 μM Fe(III)). Although this is not as high as iron chelators used in the treatment of iron overload (e.g., deferiprone, pM 20; desferrioxamine, pM 26 [44]), it compares favorably to the pM of the iron chelator nitrilotriacetic acid (NTA) and many other iron chelators [43], and is consistent with iron chelator activity in vivo. Supporting this interpretation, iron was recently shown to attenuate the cytotoxic effects of curcumin in cultured squamous cell carcinoma [39].

Iron chelators could be important as chemopreventive agents, and have mechanisms of action consistent with that role. Iron chelators prevent the participation of iron in the Fenton reaction, which reductively cleaves hydrogen peroxide to produce the hydroxyl radical. By inhibiting this and other iron-catalyzed pathways of oxidative stress [45], iron chelators substantially reduce oxidative injury to critical cellular targets, including DNA, lipids, and protein [46,47]. Such an ability to protect against oxidative stress is a hallmark of a chemopreventive agent [48].

Curcumin has many effects, and dissecting the contribution of iron chelation to the overall cancer chemopreventive activity of curcumin will require considerable additional experimentation. Nevertheless, this finding has potentially important ramifications in the clinical use of curcumin. For

example, it is possible that iron, a dietary supplement in widespread use, may bind to and interfere with the beneficial activity of curcumin. We are currently exploring this possibility.

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