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Inhibition of Proteasomal Function by Curcumin Induces Apoptosis through Mitochondrial Pathway*

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Curcumin is a natural polyphenolic compound having an antiproliferative property, which recent evidence suggests is due to its ability to induce apoptosis. However, the molecular mechanisms through which curcumin induces apoptosis are not fully understood. Here, we report that the curcumin-induced apoptosis is mediated through the impairment of the ubiquitin-proteasome system. Exposure of curcumin to the mouse neuro 2a cells causes a dose-dependent decrease in proteasome activity and an increase in ubiquitinated proteins. Curcumin exposure also decreases the turnover of the destabilized enhanced green fluorescence protein, a model substrate for proteasome and cellular p53 protein. Like other proteasome inhibitors, curcumin targets proliferative cells more efficiently than differentiated cells and induces apoptosis via mitochondrial pathways. Addition of curcumin to neuro 2a cells induces a rapid decrease in mitochondrial membrane potential and the release of cytochrome *c* into cytosol, followed by activation of caspase-9 and caspase-3.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the major yellow pigment extracted from turmeric, a commonly used spice, derived from the rhizome of the plant *Curcuma longa*. It has been demonstrated to have anti-inflammatory, antioxidant, and anti-proliferative properties (1, 2). Ample evidence exists to support its use in cancer prevention for its anti-proliferative and anti-carcinogenic properties. *In vivo*, curcumin suppresses carcinogenesis of the skin (3), the stomach (4, 5), the colon (4, 6), the breast (7), and the liver (8) in mice, and *in vitro*, it has been shown to inhibit the growth of a wide variety of tumor cells (9–14). Although its precise mode of action remains elusive, studies have shown that chemo-preventive action of curcumin might be due to its ability to induce apoptosis (9–14). How curcumin induces apoptosis remains unclear. Curcumin has been shown to inhibit the activation of transcription factors NF-κB and AP-1 (15, 16) and inhibit the activity of c-Jun N-terminal kinase (17) and protein tyrosine kinase (2). Modulation of the activities of these factors might be linked with the initiation of the apoptotic signal. Curcumin also has been reported to induce mitochondrial abnormalities and promote p53-dependent apoptosis and the activation of caspase-8 and caspase-3 (9, 10, 18–20).

The ubiquitin-proteasome pathway (UPP)[1] is the principal mechanism in the cell for controlled protein degradation. The pathway has been shown to be involved in the regulation of critical cellular processes such as transcription, cell cycle progression, oncogenesis, growth, and development, selective elimination of abnormal proteins, and antigen processing (21). Degradation of a protein by UPP involves two distinct and successive steps: (*i*) covalent attachment of multiple ubiquitin molecules to the target protein, and (*ii*) degradation of the targeted protein by 26S proteasome. 26S proteasome is a 2.1-MDa complex of which ~65 subunits are divided into three sub-complexes: 20S, 19S, and 11S. The 20S core catalytic complex is a cylindrical stack of four seven-membered rings and is flanked on both sides by 19S regulatory complexes. Three distinct types of proteolytic activities have been defined for 20S proteasome: chymotrypsin-like (Tyr or Phe at P1), trypsin-like (Arg or Lys at P1), and post-glutamyl peptidyl hydrolytic-like (Glu at P1).

Because UPP plays a crucial role in the degradation of many regulatory proteins that are necessary for cell growth, it is not surprising that the altered function of this pathway will affect cell survival. In fact, it has long been known that the inhibition of proteasome function induces apoptosis, depending on the cell types and conditions (22–28). In this investigation, we provide evidence that the curcumin-induced apoptosis is mediated through the inhibition of UPP.

EXPERIMENTAL PROCEDURES

Materials—Curcumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N^6,2′-O-dibutyryl-adenosine-3′,5′-cyclic monophosphate (dbcAMP), lactacystin, MG 312, and all cell culture reagents were obtained from Sigma. LipofectAMINE 2000 and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) were purchased from Invitrogen and Molecular Probes, respectively. Proteasome substrates were obtained from the Peptide Institute, Osaka, Japan. Expression plasmid for p53 (with V5 tag) was purchased from Invitrogen, and ubiquitin (with HA tag) was a kind gift from Dr. R. Takahashi, RIKEN Brain Science Institute, Japan. The plasmid pd1EGFP-N1 was purchased from Clontech. Mouse monoclonal anti-GFP and anti-HA were obtained from Roche, rabbit polyclonal anti-ubiquitin was obtained from Dako, rabbit polyclonal anti-caspase-3 (recognizes only active form) was from NEB (Beverly, MA), goat polyclonal anti-lamin B (6217) was from Santa Cruz Biotechnology, and mouse monoclonal anti-caspase-9 was from MBL, Japan, mouse mono-

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[1] The abbreviations used are: UPP, ubiquitin-proteasome pathway; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; dbcAMP, N^6,2′-O-dibutyryl-adenosine-3′,5′-cyclic monophosphate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBST, Tris-buffered saline Tween-20; d1EGFP, destabilized enhanced green fluorescent protein.

clonal anti-cytochrome *c* was from PharMingen, mouse monoclonal anti-V5 was from Invitrogen, and mouse monoclonal anti-β-tubulin was from Sigma. Goat anti-rabbit IgG-FITC was purchased from Molecular Probes, HRP-conjugated anti-mouse and anti-rabbit IgG were from Amersham Life Science and HRP-conjugated anti-goat IgG was from Santa Cruz Biotechnology. Neuro 2a and HeLa cells were obtained from National Centre for Cell Science, India.

Cell Culture, Transfection, Treatments, and Viability Assay—Neuro2a and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. For transfection experiments, neuro 2a cells were plated into six-well tissue cultured plates at a sub-confluent density. Twenty-four hours later, cells were transiently transfected with expression vectors using LipofectAMINE 2000 reagent according to the manufacturer's instruction. Transfection efficiency was about 80–90%. After 24 h of transfection, cells were treated with curcumin and then processed for co-immunoprecipitation and immunoblotting experiments. For cell viability assay, cells (5×10^3 cells/well) were seeded into 96-well plates; 24 h after seeding, media were replaced, and cells were treated with different doses of curcumin. Cell viability was measured by MTT assay as described previously (29). Statistical analysis was performed using a Student's *t* test; $p < 0.05$ was considered to indicate statistical significance.

Co-immunoprecipitation and Immunoblotting Experiment—Twenty-four hours after transfection of ubiquitin or pd1EGFP plasmids, cells were treated with curcumin for 8 h. Cells were then washed with cold PBS, scraped, pelleted by centrifugation, and lysed on ice for 30 min with radioimmune precipitation assay buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10 mM NaF, 5 mM $Na_4P_2O_7$, 0.1 mM Na_2VO_5, complete protease inhibitor mixture). Cell lysates were briefly sonicated, centrifuged for 10 min at 15,000 × *g* at 4 °C, and the supernatants (total soluble extract) were used for immunoprecipitation. Protein concentration was measured according to the method of Bradford (43) using bovine serum albumin as a standard. For each immunoprecipitation experiment, 200 μg of protein in 0.2 ml radioimmune precipitation assay buffer was incubated either with 5 μl (2 μg) of GFP antibody or 4 μl (2 μg) of HA antibody. After 5–6 h of incubation at 4 °C with rotation, 20 μl of protein G agarose beads were added, and incubation was continued at 4 °C overnight. The beads were washed 6× with radioimmune precipitation assay buffer. Bound proteins were eluted from the beads with SDS (1×) sample buffer, vortexed, boiled for 5 min, and analyzed by immunoblotting. The total cell lysate or the immunoprecipitated proteins were separated through SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were successively incubated in blocking buffer (5% skim milk in Tris-buffered saline Tween-20 (TBST, 50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween)), with primary antibody in TBST and then with secondary antibody conjugated with horseradish peroxidase in TBST. Detection was carried out with enhanced chemiluminescence reagent. All primary antibodies were used in 1:1000 dilutions for immunoblotting.

Immunofluorescence Staining—Cells grown on 60-mm tissue cultured plates were treated with curcumin. Eight hours after treatment, cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed extensively, then blocked with 5% nonfat dried milk in TBST for 1 h. Primary antibody (anti-cytochrome *c*) incubation was carried out overnight at 4 °C. After several washings with TBST, cells were incubated with FITC-conjugated secondary antibody for 1 h, washed several times, and visualized by using a water lance in the confocal microscope. The digital images were assembled using Adobe Photoshop.

Measurement of Mitochondrial Membrane Potential and Cytochrome c Release—Neuro 2a or HeLa cells were plated in 60-mm tissue cultured plates at sub-confluent density. Twenty-four hours later, cells were exposed to curcumin for 8 h and then incubated with 5 μM JC-1 fluorescence dye for 30 min in the CO_2 incubator and washed several times with PBS. Mitochondrial membrane potential was evaluated qualitatively under a confocal microscope using 568 nm argon-krypton laser sources.

To study the release of cytochrome *c* from mitochondria, neuro 2a or HeLa cells grown on dishes were treated with different doses of curcumin for different time periods. The cells were then scraped, washed in the PBS, washed in the sucrose buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride containing 250 mM sucrose) and resuspended in the same buffer. After 1 h of incubation on ice, cells were lysed by a B-type pestle Dounce homogenizer with 30 strokes. Homogenates were centrifuged at 750 × *g* for 10 min at 4 °C, and the supernatants were re-centrifuged at 10,000 × *g* for 15 min at 4 °C. The final supernatants were used for immunoblotting experiments of cytochrome *c*.

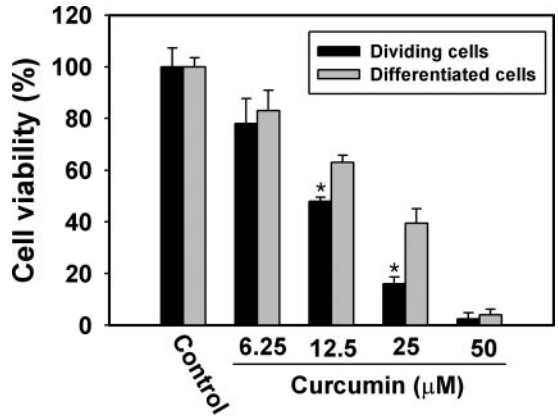

FIG. 1. **Induction of cell death by curcumin.** Neuro 2a cells (5×10^3 cells/well) were plated onto 96-well tissue cultured plates. Cells were left untreated or treated with 5 mM of dbcAMP for 24 h to differentiate the cells; media was replaced and then exposed to varying doses of curcumin. Cells were then subjected to MTT assay after 8 h of curcumin exposure. *, $p < 0.01$, as compared with differentiated cells.

Assay of Proteasome Activity—Neuro 2a cells were plated in a six-well tissue cultured plate, and on the following day, cells were treated with varying doses of curcumin for 8 h. Cells were then isolated and suspended in 100 μl of proteasome assay buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 5 mM ATP, 5 mM dithiothreitol, and 20% (v/v) glycerol), lysed by sonication, and then centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant (25 μg) was incubated in the proteasome activity assay buffer (50 mM Tris, pH 7.4, 0.5 mM EDTA, and 50 μM of each proteasome substrate) for different time periods to obtain linearity of the reaction. The substrates Boc-Leu-Arg-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Z-Leu-Leu-Glu-MCA were used to determine trypsin, chymotrypsin, and post-glutamyl peptidyl hydrolytic-like activity, respectively. Protease activities at a particular time point (30 min) within the linear range were used to calculate the data. The fluorescence intensity was measured at 380-nm excitation and 460-nm emissions by using a Wallac multi-label counter. Statistical analysis was performed by using the Student's *t* test, and $p < 0.05$ was considered to indicate statistical significance.

Cycloheximide-chase Experiment—Neuro 2a cells were plated in a six-well tissue cultured plate, and on the following day, cells were transiently transfected with p53 expression plasmid. Twenty-four hours of post-transfection, cells were chased with 15 μg/ml of cycloheximide for different time periods in the presence or absence of curcumin. Cells collected at each time point were then processed for immunoblotting by anti-V5.

RESULTS

Curcumin Kills Growing Neuro 2a Cells More Rapidly than Differentiated Neuro 2a Cells—Curcumin has been reported to inhibit the proliferation of various tumor cells, but it was not known whether the sensitivity of curcumin differs between dividing and differentiated cell. Here, we first studied the effect of curcumin on cell death between the dividing and the differentiated neuro 2a cells. Cells were plated onto 96-well tissue cultured plates, left untreated or treated with 5 mM of dbcAMP for 24 h for differentiation, and then exposed to various doses of curcumin for 8 h. Curcumin exposure dose-dependently induced the death of both dividing and differentiated cells (Fig. 1). However, the dividing neuro 2a cells were more sensitive to curcumin in comparison with the differentiated form. Curcumin also dose-dependently inhibited the proliferation of HeLa cells (data not shown). Dibutyryl cyclic AMP modulates the apoptotic program in a wide variety of cells, accelerating apoptosis in some and delaying the rate of apoptosis in others (30, 31). We do not know what kinds of role dbcAMP are playing in the neuro 2a cell. Although we completely removed

the dbcAMP from the media before adding curcumin, we still could not rule out the possibility that the partial resistance of differentiated cells toward curcumin could be due to the dbcAMP. In other words, the curcumin-induced cell death pattern seems to be very similar to the proteasome inhibitor-induced cell death, because proteasome inhibitors also target growing cells more rapidly than the differentiated cells (23, 26). Curcumin exposure also induces neurite outgrowth in a manner similar to proteasome inhibitors (see Fig. 4*A*).

Inhibition of Proteasome Function by Curcumin—Because the curcumin-induced cell death pattern is very similar to the proteasome inhibitors, we thought that curcumin might be affecting the cellular proteasomal function, and thereby induce cell death. Therefore, we tested the effect of curcumin on cellular proteasome activity. Neuro 2a cells were treated with varying doses of curcumin for 8 h, cell lysates were made and processed for proteasome activity assays. As shown in Fig. 2, proteasome activity was dose-dependently inhibited by curcumin exposure. We measured chymotrypsin (Fig. 2*A*), trypsin, and post-glutamyl peptidyl-like (Fig. 2*B*) protease activity of the proteasome and observed that the activity of all three enzymes was dramatically inhibited by curcumin. We then compared the inhibitory effect of curcumin with lactacystin and MG132, the known proteasome inhibitors, and found that the curcumin was about 5-fold less potent than lactacystin and about 3.5-fold less potent than MG132 at a dose of 10 μM (Fig. 2*C*). No difference in proteasome activity was observed between dividing and differentiated neuro 2a cells. A similar inhibitory effect of curcumin on proteasome activity was also observed in HeLa cells.

Because ubiquitinated proteins are degraded by the proteasome, inhibition of proteasome activity will result in increased accumulation of ubiquitinated proteins. Therefore, we next checked the ubiquitination profile. The neuro 2a cells were exposed to different doses of curcumin and cells were collected and processed for immunoblotting using ubiquitin antibody. As expected, curcumin treatment caused a massive accumulation of ubiquitinated proteins, depending on the dose (Fig. 3, *A* and *B*). Curcumin treatments also dose-dependently decreased the free ubiquitin levels (Fig. 3, *A* and *B*). We do not know how exactly curcumin decreases the free ubiquitin levels. Curcumin exposure might lead to a massive build-up of misfolded and/or oxidized proteins, which ubiquitinates and ultimately decreases the free ubiquitin pool. Alternately, it might inhibit the expression of ubiquitin gene directly. We re-confirmed the accumulation of ubiquitinated proteins by transient transfection of the ubiquitin construct into neuro 2a cells followed by co-immunoprecipitation with HA antibody and detection of the blot by anti-ubiquitin (Fig. 3*C*).

Inhibition of proteasomal function by curcumin was further confirmed by using destabilized enhanced green fluorescent protein (d1EGFP), a model substrate for proteasome with a 1-h half-life. This d1EGFP vector contains a PEST (proline (P), glutamate (E), serine (S), and threonine (T)) signal sequence in its C-terminal, which can be targeted for degradation by the proteasome. Impaired proteasomal activity will increase the half-life of this protein. Neuro 2a cells were transiently transfected with the pd1EGFP expression plasmids. Twenty-four hours later, cells were exposed to various doses of curcumin or lactacystin for 8 h. Cells were then fixed, washed several times with PBS, and observed under the microscope. Cells were also processed for immunoprecipitation experiments by using anti-GFP followed by immunoblotting using ubiquitin antibody. Results showed that curcumin exposure increased the half-life and the accumulation of d1EGFP protein (Fig. 4, *A* and *B*). Curcumin treatment also increased the accumulation of ubiquitinated derivatives of d1EGFP (Fig. 4*C*). Similar results were also obtained upon exposure of lactacystin.

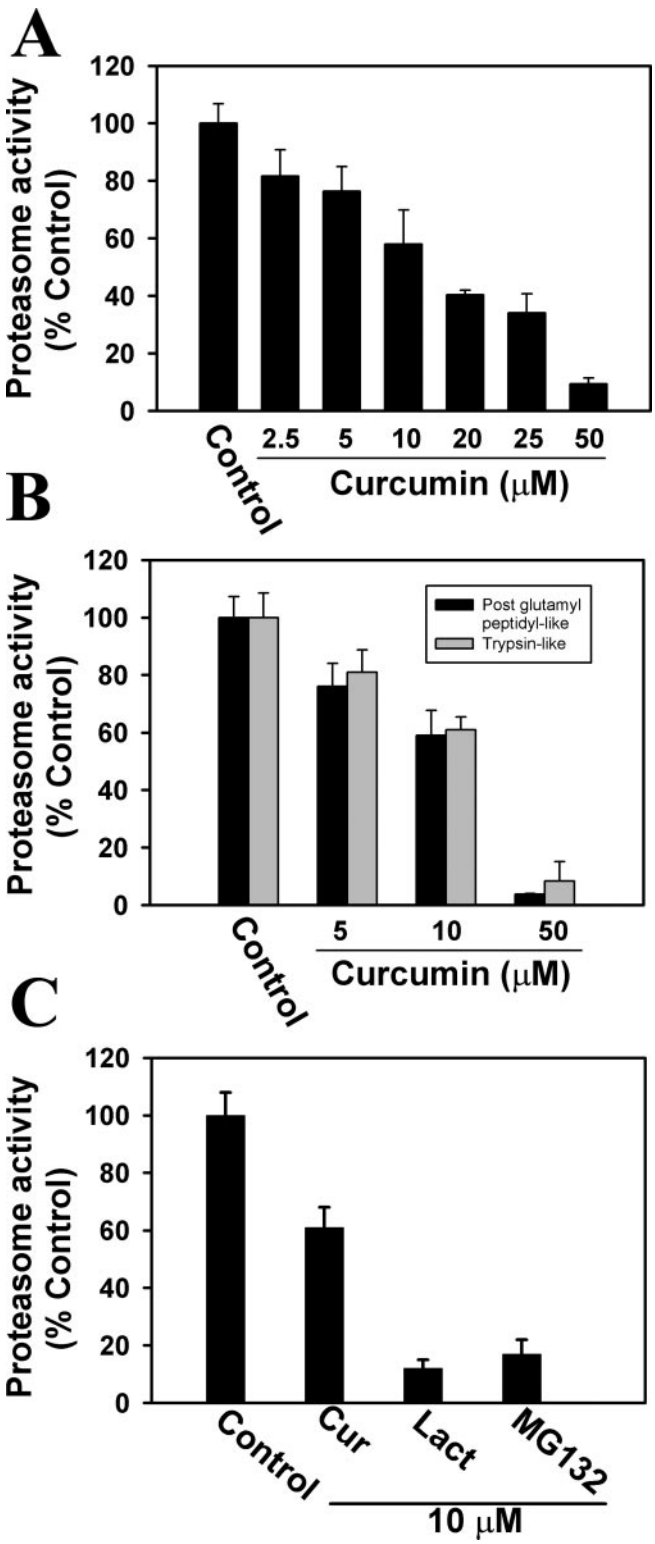

FIG. 2. **Curcumin inhibits proteasome activity.** Neuro 2a cells were treated with curcumin (different doses), lactacystin, and MG 132 for 8 h. Cells were then collected and processed for proteasome activity assays as described under "Materials and Methods." *A*, chymotrypsin-like protease activity. *B*, trypsin and post-glutamyl peptidyl hydrolytic-like protease activity. *C*, comparison of the proteasome inhibitory effect (chymotrypsin-like protease activity) of curcumin with the known proteasome inhibitors lactacystin and MG 132. *Cur*, curcumin; *Lact*, lactacystin.

Curcumin Induces Apoptosis Involving Mitochondria—Pro-

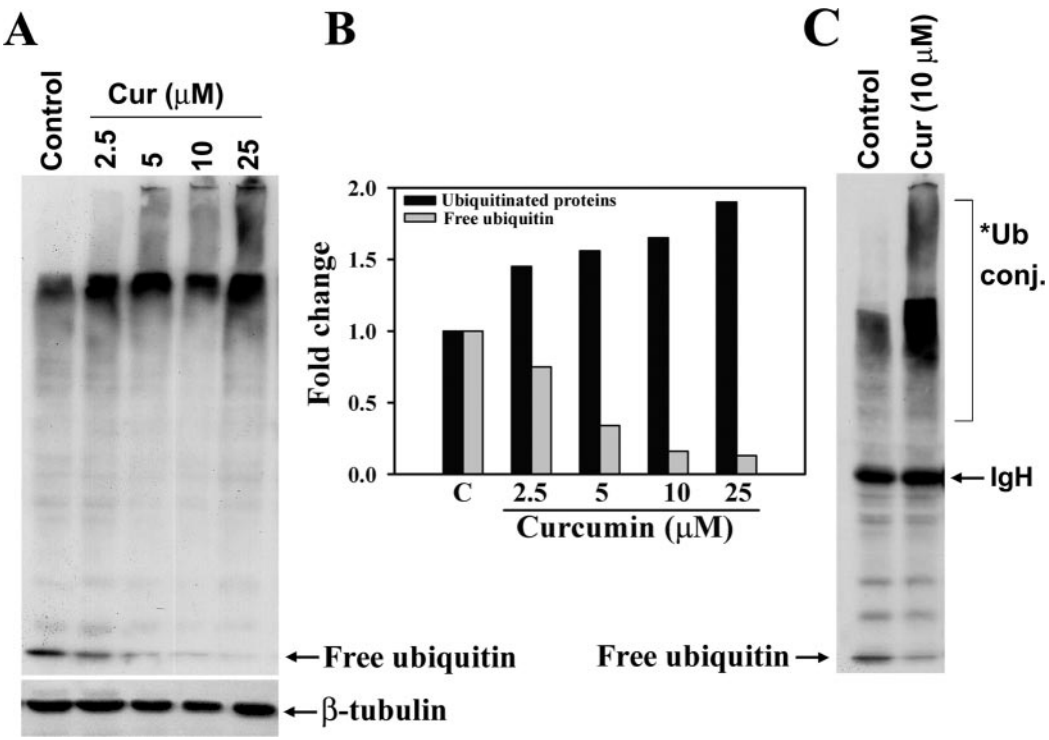

FIG. 3. **Curcumin exposure results in increased accumulation of ubiquitinated proteins.** *A*, neuro 2a cells were treated with curcumin, and the cell lysates were made in a manner similar to that described in Fig. 2. Cell lysates were processed for immunoblotting using anti-ubiquitin. *B*, quantitation of the free ubiquitin and ubiquitinated protein levels from the blot represented in *A*. Quantitation was performed using NIH Image analysis software. Data were normalized against β-tubulin. *C*, cells were transiently transfected with ubiquitin plasmid. Twenty-four hours later, cells were exposed to curcumin for 8 h; the cells were then processed for co-immunoprecipitation by using HA antibody, as described under "Materials and Methods." Blot was detected with ubiquitin antibody. *Cur*, curcumin; *IgH*, immunoglobulin heavy chain.

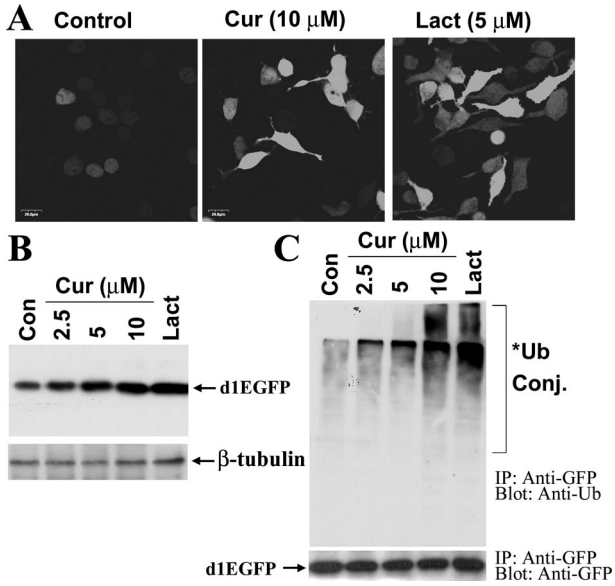

FIG. 4. **Decreased turnover of the destabilized EGFP upon curcumin exposure.** Neuro 2a cells were transiently transfected with pd1EGFP plasmids in a manner similar to that described in Fig. 3. Twenty-four hours later, cells were exposed to either curcumin or lactacystin for 8 h. Cells were then processed for microscopic observation (*A*), anti-GFP immunoblotting (*B*), and co-immunoprecipitation using anti-GFP, followed by blot detection with ubiquitin antibody (*C*). In *B*, β-tubulin was used as a loading control; in *C*, immunoprecipitated d1EGFP (detected by anti-GFP) was used as the reference. Lactacystin (*Lact*) was used at a dose of 5 μM. *Cur*, curcumin; *Con*, control.

teasome inhibition is known to induce apoptosis through mitochondrial cytochrome *c* release followed by sequential activation of caspase-9 and caspase-3. Because curcumin inhibits the proteasomal function, we tested the possible involvement of curcumin in mitochondrial dysfunction and subsequent events. HeLa cells were treated with different doses of curcumin for different time periods and then were either subjected to JC-1 staining to study the changes of mitochondrial membrane potential or processed for immunofluorescence staining and immunoblot analysis of cytochrome *c* to study its release into cytosol from mitochondria. JC-1 is a potentially sensitive fluorescent dye that detects specifically polarized mitochondria as a red color. Treatment of curcumin dramatically changed the mitochondrial membrane potential, depending upon the dose, as can be evaluated from the disappearance of the red-dotted color in mitochondria (Fig. 5, *top panel*). Curcumin exposure also dose-dependently induced cytochrome *c* release from the mitochondria (Fig. 5, *bottom panel*). The changes in the mitochondrial membrane potential (Fig. 6*A*) and the release of cytochrome *c* (Fig. 6*B*) were noticed from two hours onwards, when curcumin was added at a dose of 10 μM. Similar results were also observed in neuro 2a cells (data not shown).

Because the caspase-9 activation is initiated with the release of cytochrome *c* from mitochondria followed by binding to apoptosis protease activating factor-1, we further examined the activation of caspase-9 and subsequent events after curcumin exposure. The neuro 2a cells were treated with different doses of curcumin for different time periods, cells lysates were made and then processed for immunoblotting by using antibody against caspase-9, caspase-3, and lamin B. As shown in Fig. 7, treatment of curcumin dose- and time-dependently activated caspase-9 and caspase-3, followed by the cleavage of lamin B.

Curcumin Exposure Increases the Half-life of p53—Several key cellular proteins, including p53, are known to be degraded by the ubiquitin-proteasome pathway. Therefore, inhibition of proteasomal function by curcumin is expected to alter the stability of various proteasome substrates. To test this possibility, we transiently transfected p53 expression plasmid to neuro 2a

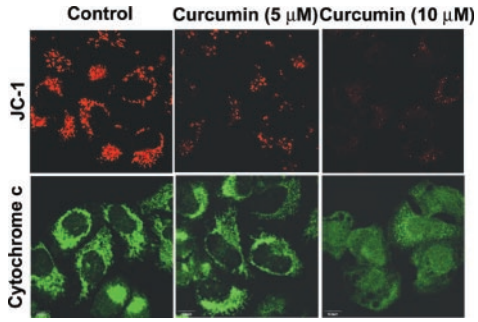

FIG. 5. **Dose-dependent effect of curcumin upon changes of mitochondrial membrane potential and cytochrome *c* release.** HeLa cells were treated with different doses of curcumin for 8 h and then subjected to JC-1 staining to study the changes in the mitochondrial membrane potential (*upper panel*) and immunofluorescence staining of cytochrome *c* to observe its release from mitochondria (*lower panel*).

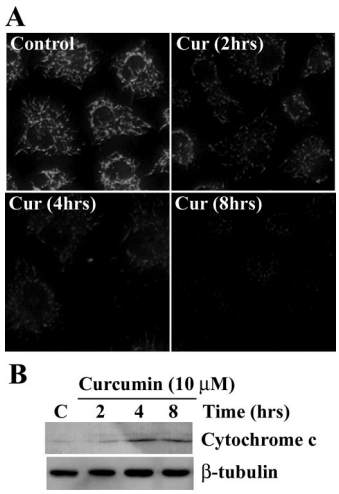

FIG. 6. **Time response of curcumin upon changes of mitochondrial membrane potential and cytochrome *c* release.** HeLa cells were treated with 10 μM of curcumin for different time periods as indicated. The cells were then processed for JC-1 staining (*A*) and cytochrome *c* immunoblotting (*B*). In *B*, β-tubulin was used as a loading control. *Cur*, curcumin.

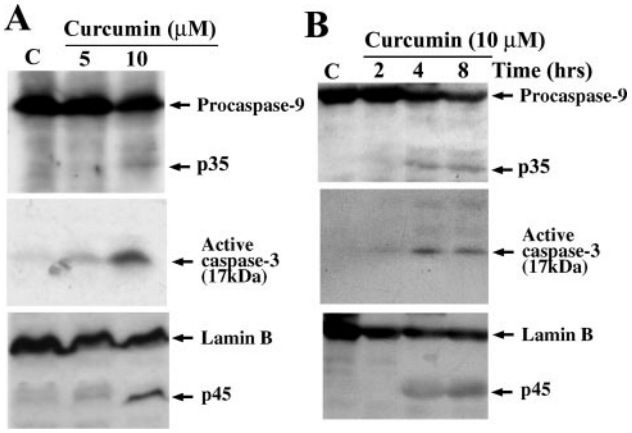

FIG. 7. **Activation of caspase-9 and caspase-3 by curcumin.** Neuro 2a cells were treated with different doses (*A*) of curcumin for different time periods (*B*) in a manner similar to that described in Fig. 6. Cell lysates were used for immunoblotting using antibody against caspase-9, caspase-3, and lamin B. *C* indicates control.

cells. Twenty-four hours later, cells were exposed to various doses of curcumin for 8 h and then processed for immunoblotting using V5 antibody. As expected, curcumin exposure increased the accumulation of p53 protein, depending upon the

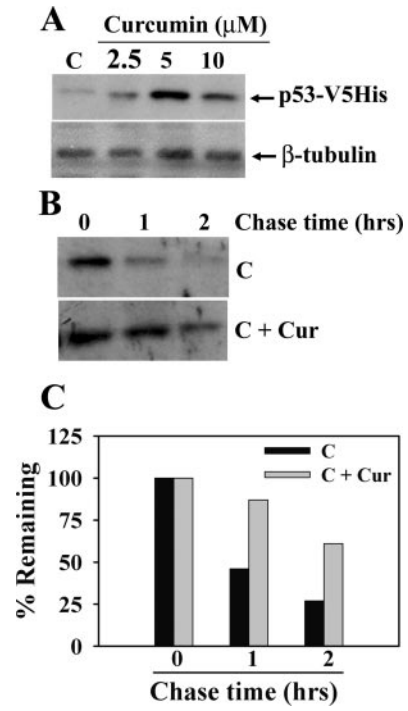

FIG. 8. **Exposure of curcumin increases the stability of p53.** *A*, neuro 2a cells were transiently transfected with p53 expression plasmid. Twenty-four hours later, cells were treated with different doses of curcumin for 8 h and then processed for immunoblot analysis using V5 and β-tubulin antibody. *B*, twenty-four hours after transfection of p53 plasmid, cells were treated with cycloheximide (15 μg/ml) and chased in the presence or absence of curcumin for different time periods. Blots were detected with V5 antibody. *C*, quantitation of the band intensities of the blot shown in *B* using NIH Image analysis software. *C* indicates control; *C + Cur* indicates control + curcumin.

dose (Fig. 8*A*). Next, we performed cycloheximide-chase experiment to reconfirm the increased half-life of p53. Twenty-four hours after transfection, cells were treated with cycloheximide (15 μg/ml) in the presence or absence of curcumin (10 μM) for different time periods. As shown in Fig. 8*B*, treatment of curcumin increased the half-life of p53.

DISCUSSION

Curcumin has long been used as a popular dietary spice and herbal medicine in several southeastern countries. Recent evidence suggests that the curcumin has chemopreventive and anti-tumor activities because of its ability to induce apoptosis. Here, we report that the curcumin-induced apoptosis is mediated through the impairment of UPP function.

We have shown that exposure of curcumin dose-dependently inhibits the cellular proteasome activity over time and increases the accumulations of ubiquitinated proteins. Curcumin also inhibits the degradation of destabilized enhanced green fluorescent protein (a model substrate for proteasome, with a half-life of about 1 h) and causes increased accumulations of its ubiquitinated derivatives. Second, we have observed that the curcumin induces apoptosis through mitochondrial pathways, an apoptotic pathway reported to be activated during pharmacological inhibition of proteasome function. Finally, like other proteasome inhibitors, curcumin also kills growing cells more rapidly than differentiated cells.

How curcumin disturbs the cellular proteasome function is not clear. It could inhibit the proteasome function directly by blocking the 20S proteasome core cavity, or indirectly either by inhibiting the ubiquitin isopeptidase activity or by the generation of oxidative stress. Recently, curcumin has been shown to inhibit the ubiquitin isopeptidase activity (32). Inhibition of

isopeptidase activity probably leads to the accumulation of ubiquitin-protein conjugates and polyubiquitin because of the lack of ubiquitin recycling. Excessive accumulation of ubiquitin-protein conjugates could conceivably create proteasome dysfunction. Curcumin could also induce proteasomal malfunction through the generation of oxidative stress. Oxidative stress is known to inhibit the proteasome function (33, 34), and curcumin has been reported recently to induce the oxidative stress (14, 35). Our result favors this hypothesis, because severe mitochondrial abnormalities (observed by us and others) will ultimately lead to the generation of oxidative stress inside the cell. However, this hypothesis contrasts with several earlier reports that demonstrate the antioxidant role of curcumin (36, 37).

Several key proteins involved in pathways regulating transcription, growth, and apoptosis are the substrate for proteasomal degradation, like IκB, p53, c-myc, and JNK (21, 38, 39). Proteasome inhibition also down-regulates various transcripts involved in cell growth/survival pathways and up-regulation of molecules implicated in proapoptotic pathway (38). Though the precise link between proteasome inhibition and induction of apoptosis is unknown, it is expected that the altered degradation or altered expression of those proteins will affect cell survival and promote apoptosis. In fact, we have shown that curcumin exposure led to the up-regulation of p53 by increasing its stability.

Our observations of curcumin-induced proteasomal malfunction can now explain several earlier reported effects of curcumin-like down-regulation of NF-κB activity, generation of stress response, and apoptosis. Curcumin is very well known to inhibit the NF-κB activation (15). Impaired proteasome function will inhibit the degradation of IκB and, thereby, block the nuclear translocation and transcriptional activity of NF-κB. Because NF-κB activity is very essential for cell survival, its down-regulation would definitely promote cell death. Curcumin also has been reported to inhibit cell survival signal protein kinase B/Akt (40), induce the expression of Hsp-70 (41), and activate p53 (19), which can also be observed during proteasome inhibition (24, 27, 38). However, contrasting results were reported by others (42), where they have shown down-regulation of cyclin D1 (a substrate of proteasome) levels by curcumin and its reversal by lactacystin. One of the explanations could be the direct inhibitory effect of curcumin on cyclin D1 transcription (42) that will ultimately decrease the protein levels. Treatment of lactacystin along with curcumin can increase the low levels of cyclin D1 protein by increasing the stability. Lactacystin could also affect the transcription of cyclin D1 levels. Further investigation is required to confirm the fact.

Proteasome inhibition has been shown to induce dual (caspase-8 and caspase-9) apoptotic signaling pathways, consisting of early release of mitochondrial cytochrome *c* into the cytosol and caspase-9 activation, followed by independent activation of the Fas/caspase-8 pathway (38). Cytochrome *c* binds to apoptosis protease-activating factor-1 and forms the apoptosome complex that activates caspase-9 and, in turn, cleaves and activates downstream executioner caspases. Here, we have observed curcumin-induced apoptosis through mitochondrial pathway involving caspase-9 activation, whereas others have reported the involvement of Fas/caspase-8 pathways (10, 20). Both apoptotic pathways are most likely involved in the curcumin-induced apoptosis.

Altogether, our results indicate that curcumin-induced apoptosis is mediated through the impairment of UPP. Proteasome inhibitors are considered to be one of the promising groups of anticancer agent. Because curcumin inhibits proteasome function and has been found to be safe pharmacologically, it has enormous potential in the prevention and therapy of cancer.

REFERENCES

1. Ammon, H. P., and Wahl, M. A. (1991) *Planta Med.* **57,** 1–7
2. Aggarwal, B. B., Kumar, A., and Bharti, A. C. (2003) *Anticancer Res.* **23,** 363–398
3. Huang, M. T., Ma, W., Yen, P., Xie, J. G., Han, J., Frenkel, K., Grunberger, D., and Conney, A. H. (1997) *Carcinogenesis* **18,** 83–88
4. Huang, M. T., Lou, Y. R., Ma, W., Newmark, H. L., Reuhl, K. R., and Conney, A. H. (1994) *Cancer Res.* **54,** 5841–5847
5. Li, N., Chen, X., Liao, J., Yang, G., Wang, S., Josephson, Y., Han, C., Chen, J., Huang, M. T., and Yang, C. S. (2002) *Carcinogenesis* **23,** 1307–1313
6. Kim, J. M., Araki, S., Kim, D. J., Park, C. B., Takasuka, N., Baba-Toriyama, H., Ota, T., Nir, Z., Khachik, F., Shimidzu, N., Tanaka, Y., Osawa, T., Uraji, T., Murakoshi, M., Nishino, H., and Tsuda, H. (1998) *Carcinogenesis* **19,** 81–85
7. Mehta, K., Pantazis, P., McQueen, T., and Aggarwal, B. B. (1997) *Anticancer Drugs* **8,** 470–481
8. Chuang, S. E., Kuo, M. L., Hsu, C. H., Chen, C. R., Lin, J. K., Lai, G. M., Hsieh, C. Y., and Cheng, A. L. (2000) *Carcinogenesis* **21,** 331–335
9. Mukhopadhyay, A., Bueso-Ramos, C., Chatterjee, D., Pantazis, P., and Aggarwal, B. B. (2001) *Oncogene* **20,** 7597–7609
10. Anto, R. J., Mukhopadhyay, A., Denning, K., Aggarwal, B. B. (2002) *Carcinogenesis* **23,** 143–150
11. Bharti, A. C., Donato, N., Singh, S., and Aggarwal, B. B. (2003) *Blood* **101,** 1053–1062
12. Pal, S., Chaudhury, T., Chattopadhyay, S., Bhattacharya, A., Datta, G., Das, T., and Sa, G. (2001) *Biochem. Biophys. Res. Commun.* **288,** 658–665
13. Khar, A., Ali, A. M., Pardhasaradhi, B. V. V., Begum, Z., and Angum, R. (1999) *FEBS Lett.* **445,** 165–168
14. Woo, J. H., Kim, Y. H., Choi, Y. J., Kim, D. G., Lee, K. S., Bae, J. H., Mindo, S., Ch, J. S., Jeong, Y. J., Jee, Y. H., Park, J. W., and Kwon, T. K. (2003) *Carcinogenesis* **24,** 1199–1208
15. Singh, S., and Aggarwal, B. B. (1995) *J. Biol. Chem.* **270,** 24995–25000
16. Huang, T. S., Lee, S. C., and Lin, J. K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5292–5296
17. Chen, Y. R., and Tan, T. H. (1998) *Oncogene* **17,** 173–178
18. Didier, M., Sophie, B., Ronald, Z., Serge, L., and Jean-Paul, T. (2001) *FEBS Lett.* **495,** 131–136
19. Jee, S. H., Shen, S. C., Tseng, C. R., Chiu, H. C., and Kuo, M. L. (1998) *J. Investig. Dermatol.* **111,** 656–661
20. Bush, J. A., Cheung, K. J., Jr., and Li, G. (2001) *Exp. Cell Res.* **271,** 305–314
21. Glickman, M. H., and Ciechanover, A. (2001) *Physiol. Rev.* **82,** 373–428
22. Sadoul, R., Fernandez, P. A., Quiquerez, A. L., Martinou, I., Maki, M., Schroter, M., Becherer, J. D., Irmler, M., Tschopp, J., and Martinou, J. C. (1996) *EMBO J.* **15,** 3845–3852
23. Drexler, H. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 855–860
24. Lopes, U. G., Erhardt, P., Yao, R., and Cooper, M. (1997) *J. Biol. Chem.* **272,** 12893–12896
25. Qiu, J. H., Asai, A., Chi, S., Saito, N., Hamada, H., and Kirini, T. (2000) *J. Neurosci.* **20,** 259–265
26. Drexler, H. C., Risau, W., and Konerding, M. A. (2000) *FASEB J.* **14,** 65–77
27. Meriin, A. B., Gabai, V. L., Aglom, J., Shifrin, V. I., and Sherman, M. Y. (1998) *J. Biol. Chem.* **273,** 6373–6379
28. Jana, N. R., Zemskov, E. A., Wang, G., and Nukina, N. (2001) *Hum. Mol. Genet.* **10,** 1049–1059
29. Wang, G. H., Mitsui, K., Kotliarova, S., Yamashita, A., Nagao, Y., Tokuhiro, S., Iwatsubo, T., Kanazawa, I., and Nukina, N. (1999) *Neuroreport* **10,** 2435–2438
30. Martin, M. C., Dransfield, I., Haslett, C., and Rossi, A. G. (2001) *J. Biol. Chem.* **276,** 45041–45050
31. Kiefer, J., Okret, S., Jondal, M., and McConkey, D. J. (1995) *J. Immunol.* **145,** 1227–1230
32. Mullally, J. E., and Fitzpatrick, F. A. (2002) *Mol. Pharmacol.* **62,** 351–358
33. Ding, Q., and Keller, J. N. (2001) *J. Neurochem.* **77,** 1010–1017
34. Okada, K., Wangpoengtrakul, C., Osawa, T., Toyokuni, S., Tanaka, S., and Uchida, K. (1999) *J. Biol. Chem.* **274,** 23787–23793
35. Bhaumik, S., Anjum, R., Rangaraj, N., Pardhasarradhi, B. V. V., and Khar, A. (1999) *FEBS Lett.* **456,** 311–314
36. Ruby, A. J., Kuttan, G., Babu, K. D., Rajasekharan, K. N., and Kuttan, R. (1995) *Cancer Lett.* **94,** 79–83
37. Reddy, A. C., and Lokesh, B. R. (1994) *Mol. Cell. Biochem.* **137,** 1–8
38. Mitsiades, N., Mitsiades, C. S., Poulaki, V., Chauhan, D., Fanourakis, G., Gu, X., Bailey, C., Joseph, M., Libermann, T. A., Treon, S. P., Munshi, N. C., Richardson, P. G., Hideshima, T., and Anderson, K. C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 14374–14379
39. Mitsiades, N., Mitsiades, C. S., Poulaki, V., Chauhan, D., Richardson, P. G., Hideshima, T., Munshi, N., Treon, S. P., and Anderson, K. C. (2002) *Blood* **99,** 4079–4086
40. Chaudhary, L. R., and Hruska, K. A. (2003) *J. Cell. Biochem.* **89,** 1–5
41. Kato, K., Ito, H., Kamei, K., and Iwamoto, I. (1998) *Cell Stress Chaperones* **3,** 152–160
42. Mukhopadhyay, A., Banerjee, S., Stafford, L. J., Xia, C., Liu, M., and Aggarwal, B. B. (2002) *Oncogene* **21,** 8852–8861
43. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254

Inhibition of Proteasomal Function by Curcumin Induces Apoptosis through Mitochondrial Pathway

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