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Curcumin Inhibits UV Irradiation-Induced Oxidative Stress and Apoptotic Biochemical Changes in Human Epidermoid Carcinoma A431 Cells

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Abstract Ultraviolet (UV) light is a strong apoptotic trigger that induces caspase-dependent biochemical changes in cells. Previously we showed that UV irradiation can activate caspase-3, and the subsequent cleavage and activation of $p21^{Cdc42/Rac}$-activated kinase 2 (PAK2) in human epidermoid carcinoma A431 cells. In this study we demonstrate that curcumin (Cur), the yellow pigment of *Curcuma longa* with known anti-oxidant and anti-inflammatory properties, can prevent UV irradiation-induced apoptotic changes, including c-Jun N-terminal kinase (JNK) activation, loss of mitochondrial membrane potential (MMP), mitochondrial release of cytochrome C, caspase-3 activation, and cleavage/activation of PAK2 in A431 cells. Flow cytometric analysis using the cell permeable dye 2′,7′-dichlorofluorescin diacetate (DCF-DA) as an indicator of reactive oxygen species (ROS) generation revealed that the increase in intracellular oxidative stress caused by UV irradiation could be abolished by Cur. In addition, we found that SP600125, a JNK-specific inhibitor, reduced UV irradiation-induced JNK activation as well as caspase-3 activation, indicating that JNK activity is required for UV irradiation-induced caspase activation. Collectively, our results demonstrate that Cur significantly attenuates UV irradiation-induced ROS formation, and suggest that ROS triggers JNK activation, which in turn causes MMP change, cytochrome C release, caspase activation, and subsequent apoptotic biochemical changes. J. Cell. Biochem. 90: 327–338, 2003.

Key words: curcumin; UV irradiation; apoptosis; oxidative stress; caspase-3; PAK2

In mammalian cells, exposure to ultraviolet (UV) light leads to the damage of cellular constituents, resulting in a complex cell response that includes induction of genes and perturbation of a variety of signaling pathways [for reviews see Bender et al., 1997; Schwarz, 1998]. If damaged constituents in UV-irradiated cells cannot be repaired, cells are likely to die via the process of apoptosis. Apoptosis, which is widely observed in different cells of various organisms, has a unique morphological cell death pattern characterized by chromatin condensation, membrane blebbing, and DNA fragmentation [Kerr et al., 1972; Ellis et al., 1991]. A variety of physical and chemical signals can trigger apoptosis, indicating the existence of a core death program in cells. Considerable evidence has shown that caspases, a cysteine protease family identified in recent years, play crucial roles in this core apoptotic program [for reviews see Martins and Earnshaw, 1997; Nicholson and Thornberry, 1997]. Caspases exist as proenzymes and can be activated at the onset of apoptosis via proteolytic processing into two smaller subunits, which then form functionally active proteases that can act on many proteins and enzymes involved in

Abbreviations used: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PAK2, $p21^{Cdc42/Rac}$-activated kinase 2; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; MMP, mitochondrial membrane potential.

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diverse cell functions. Caspase-3 (also known as CPP32/Yama/apopain) has been implicated as a downstream effector protease that can be activated by other upstream caspases [Fernandes-Alnemri et al., 1994; Enari et al., 1996].

Curcumin (Cur), a common dietary pigment and spice, is used as a traditional Indian medicine [Nadkarni, 1976]. The medicinal roles of Cur have been demonstrated in wound healing, liver ailments, hepatitis, urinary tract disease, and as a cosmetic compound [Kuttan et al., 1985]. Cur exhibits a wide range of pharmacological effects, including anti-inflammatory, anti-carcinogenic, hypocholesterolemic, and anti-infection activities [Barthelemy et al., 1998; Ramirez-Tortosa et al., 1999; Ramsewak et al., 2000]. As a potent anti-oxidant, Cur has also been shown to display anti-proliferative and anti-carcinogenic properties in a wide variety of cell lines and animals [Huang et al., 1994; Jiang et al., 1996; Jee et al., 1998; Mahmoud et al., 2000]. Recent studies have shown that the anti-tumor activity of Cur is attributed to its ability to induce apoptosis via caspase-3 activation [Khar et al., 1999]. Although multiple biological functions of Cur have been identified, the precise molecular mechanisms underlying its actions appear unknown.

Reactive oxygen species (ROS) are oxygen-containing molecules having either unpaired electrons or the ability to abstract electrons from other molecules. UV irradiation, like many chemical and physical treatments capable of inducing apoptosis, is known to provoke oxidative stress via generating ROS in cells [Halliwell and Gutteridge, 1990], suggesting a close relationship between oxidative stress and apoptosis. Direct evidence establishing oxidative stress as a mediator of apoptosis came from observations that addition of oxidants such as H_2O_2 can trigger apoptosis, whereas addition of anti-oxidants block this effect [for review see Buttke and Sandstrom, 1994].

Recently, protein phosphorylation has been shown to be involved in regulating apoptosis. Alteration in activities of several protein kinases can be observed during apoptosis in a variety of cell types [reviewed in Anderson, 1997]. It appears that c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) can serve as a key component in regulating entry into apoptosis in several cell types under certain circumstances [Xia et al., 1995; Verheij et al., 1996; Seimiya et al., 1997]. JNK/SAPK, an emerging member of the mitogen-activated protein kinase (MAPK) family, can be rapidly activated by environmental stress and inflammatory cytokines [Derijard et al., 1994; Kyriakis et al., 1994], which can also induce ROS generation in target cells [Kyriakis and Avruch, 1996; Anderson, 1997]. Activation of JNK by UV irradiation can be prevented by the anti-oxidant N-acetylcysteine [Alder et al., 1995; Tao et al., 1996], suggesting that JNK is activated by oxidative stress. The components in the JNK/SAPK regulation pathway are identified as being MAPK kinase kinase 1 (MEKK1), SAPK/extracellular-signal-regulated kinase kinase 1 (SEK1), JNK/SAPK, and c-Jun [Sanchez et al., 1994; Yan et al., 1994; Derijard et al., 1995; Lin et al., 1995]. Using dominant, interfering mutants of c-Jun or SEK1 as tools, activation of the JNK/SAPK pathway has been demonstrated to be required for stress-induced apoptosis in U937 leukemia cells and for growth factor withdrawal-triggered apoptosis in PC-12 pheochromocytoma cells [Xia et al., 1995; Verheij et al., 1996]. However, in contrast, two recent reports show that the activation of JNK/SAPK was not essential for Fas-mediated apoptosis in Jurkat T cells and for dexamethasone-induced apoptosis in multiple myeloma cells [Lee et al., 1997; Rudel and Bokoch, 1997]. Hence, the role of JNK/SAPK in apoptosis signaling remains to be better understood.

Previously, we showed that UV irradiation activated caspase-3, and subsequent $p21^{Cdc42/Rac}$-activated kinase 2 (PAK2) cleavage/activation and DNA fragmentation [Tang et al., 1998]. PAK2 is a member of the PAK family of serine/threonine kinases that can bind to and be activated by the small (21 kDa) guanosine triphosphatases (GTPases) Cdc42 and Rac that regulate actin polymerization [for review see Sells and Chernoff, 1997]. After removal of the PAK2 N-terminal regulatory region by caspase-mediated cleavage, the activity of the C-terminal catalytic fragment (36 kDa) was easily detected by in-gel kinase assays [Tang et al., 1998].

In this article, we investigated the effects of Cur on UV irradiation-induced apoptosis. We found that Cur inhibited apoptotic biochemical changes, such as JNK activation, mitochondrial membrane potential (MMP) change, cytochrome C release, caspase-3 activation, and PAK2 cleavage/activation in A431 cells. We also

found that the inhibition of JNK activation by SP600125 (a specific JNK inhibitor) prevented UV irradiation-induced caspase-3 activation. The formation of ROS during UV irradiation was significantly attenuated by Cur, which may account for the inhibitory effects of Cur on the apoptotic signal cascade elicited by UV irradiation.

EXPERIMENTAL PROCEDURES

Materials

[γ-^{32}P]ATP was purchased from Amersham (Buckinghamshire, UK). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). Dulbecco's modified Eagle's medium (DMEM), Cur, 2′,7′-dichlorofluorescin diacetate (DCF-DA), goat anti-rabbit and anti-mouse Immunoglobulin G (IgG) antibodies conjugated with alkaline phosphatase were from Sigma (St. Louis, MO). Anti-JNK1 and anti-α-PAK (C19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-CPP32/caspase-3 antibody was from Transduction Laboratories (Lexington, KY). Monoclonal anti-cytochrome c antibody (6H2.B4) was from Imgenex (San Diego, CA). CDP-Star^{TM} chemiluminescent substrate for alkaline phosphatase was from Boehringer Mannheim (Mannheim, Germany). Myelin basic protein (MBP) was purified from porcine brain, as previously described [Yu and Yang, 1994a].

Cell Culture and UV Irradiation

Human epidermoid carcinoma A431 cells were cultured as previously described [Yu and Yang, 1994b]. Cells ($\sim 5-6 \times 10^6$) were plated on 100 mm culture dishes, and approximately 24 h later medium was removed, and with lids off, cells were exposed to 200 J/m^2 UV light (254 nm, UV-C) using a UV gene linker apparatus (UV Stratalinker, model 1800, Stratagene, La Jolla, CA) equipped with an energy output control. Medium was added immediately after irradiation and the dish incubated at 37°C in a CO_2 incubator for 2 h. Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in 600 μl solution A (20 mM Tris-HCl at pH 7.4, 1 mM ethylene-diaminetetraacetic acid (EDTA), 1 mM ethylene-glycol-bis(b-aminoethylether) N, N, N′, N′-tetraacetic acid (EGTA), 1% Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, and 1 mM sodium orthovanadate) on ice for 10 min. Cell lysates were collected and sonicated on ice (Sonicator model W-380, Heat Systems-Ultrasonics, Farmingdale, NY) for 3×10 s at 50% power output, and then centrifuged at 15,000*g* for 20 min at 4°C. The resulting supernatants were used as cell extracts.

Immunoblots

Immunoblot analysis was carried out essentially as previously described [Yu and Yang, 1994c]. Briefly, proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to PVDF membranes, probed with anti-α-PAK (C19) (0.2 μg/ml) or anti-CPP32/caspase-3 (0.25 μg/ml) monoclonal antibodies, and visualized using goat anti-rabbit or anti-mouse IgG antibody conjugated with alkaline phosphatase and CDP-Star^{TM} (chemiluminescent substrate for alkaline phosphatase), according to the manufacturer's protocol.

In-Gel Kinase Assay

In-gel kinase assays were performed as previously described [Tang et al., 1998]. Briefly, cell extracts (40 μg) were resolved in 10% SDS–polyacrylamide gels that were polymerized in the presence of 0.5 mg/ml MBP. After removing SDS from gels (allowing protein renaturation), in-gel kinase assays were performed by incubating the gel in phosphorylation buffer containing [γ-^{32}P]ATP. Gels were stained, destained and dried, and ^{32}P-labelled MBP was detected by autoradiography.

Caspase-3 Activity Assay

Caspase-3 activity was measured by the fluorogenic substrate Z-DEVD-AFC. Cell lysates (100 μg) were incubated in a 250-μl caspase assay buffer (25 mM HEPES at pH 7.5, 0.1% CHAPS, 10 mM dithiothreitol, 100 U/ml aprotinin) containing 0.1 mM Z-DEVD-AFC for 3 h at 37°C. Ice-cold caspase assay buffer (1.25 ml) was then added to the mixture and the relative caspase-3 activity was determined by a fluorescence spectrophotometer (Hitachi, F-2000; excitation 400 nm, emission 505 nm).

Fluorescent Measurement of Intracellular Oxidants

DCF-DA was used to detect ROS production in cells. The fluorescence of this cell-permeable agent significantly increases after oxidation

[Kehrer and Paraidathathu, 1992; LeBel et al., 1992]. DCF-DA was dissolved in Dimethyl sulphoxide (DMSO) and stored as 50 mM stock at −20°C. A431 cells were incubated with Cur or other compounds for 1–3 h at 37°C and then with 10 μM DCF-DA for another 1 h. Cells were harvested at the indicated time points after UV irradiation, washed three times with PBS, and then immediately analyzed (3×10^5 cells/sample) by flow cytometry using a FACScan (Becton-Dickinson, Mountain View, CA) with a 488 nm excitation beam.

Flow Cytometric Detection of MMP

To assess changes in membrane potential, UV-treated A431 cells were incubated with 40 nM DiOC6(3) for 1 h at 37°C [Vermes et al., 2000], then washed twice with PBS, suspended in PBS, and analyzed immediately using a FACScan flow cytometer with a 488 nm excitation beam.

Cytochrome C Release Assay

Mitochondrial cytochrome c release assay were performed according to the method as described by Yang et al. [1997]. A431 cells (1×10^7) were either left untreated or UV-irradiated, then harvested by centrifugation at 800*g* at 4°C for 15 min. After washing three times with ice-cold PBS, cell pellets were resuspended in HEPES-buffer (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1mM PMSF, pH 7.5) containing 250 mM sucrose, homogenized with a homogenizer, and centrifuged at 800*g* at 4°C for 15 min. The supernatants were centrifuged at 10,000*g* for 15 min at 4°C, and the mitochondrial pellets were dissolved in SDS sample

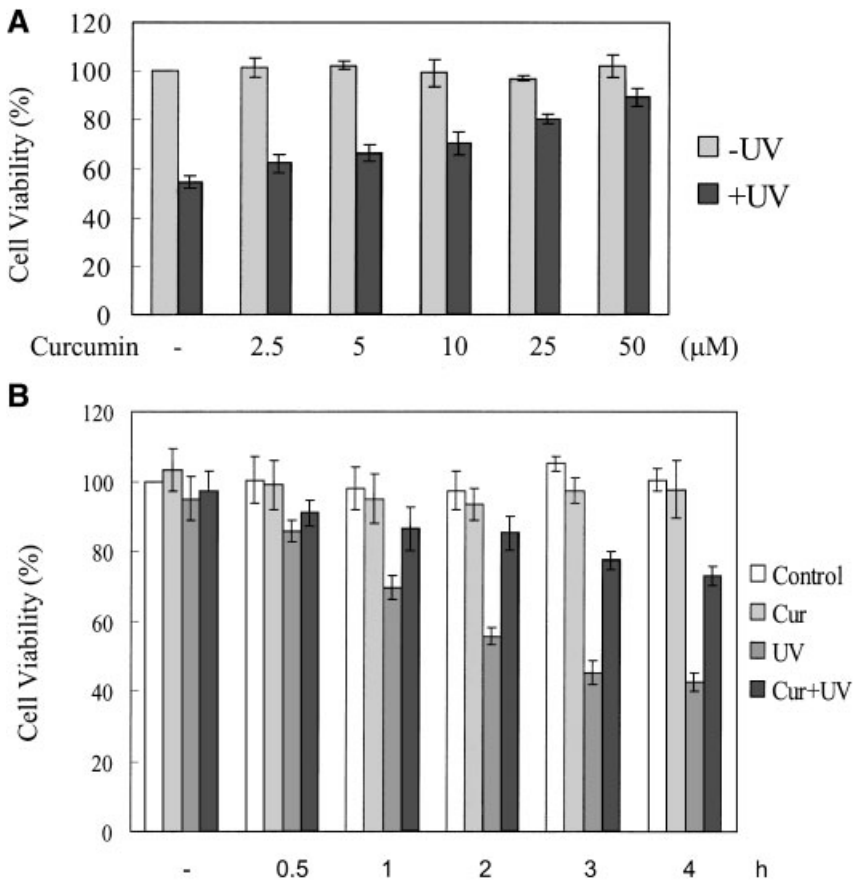
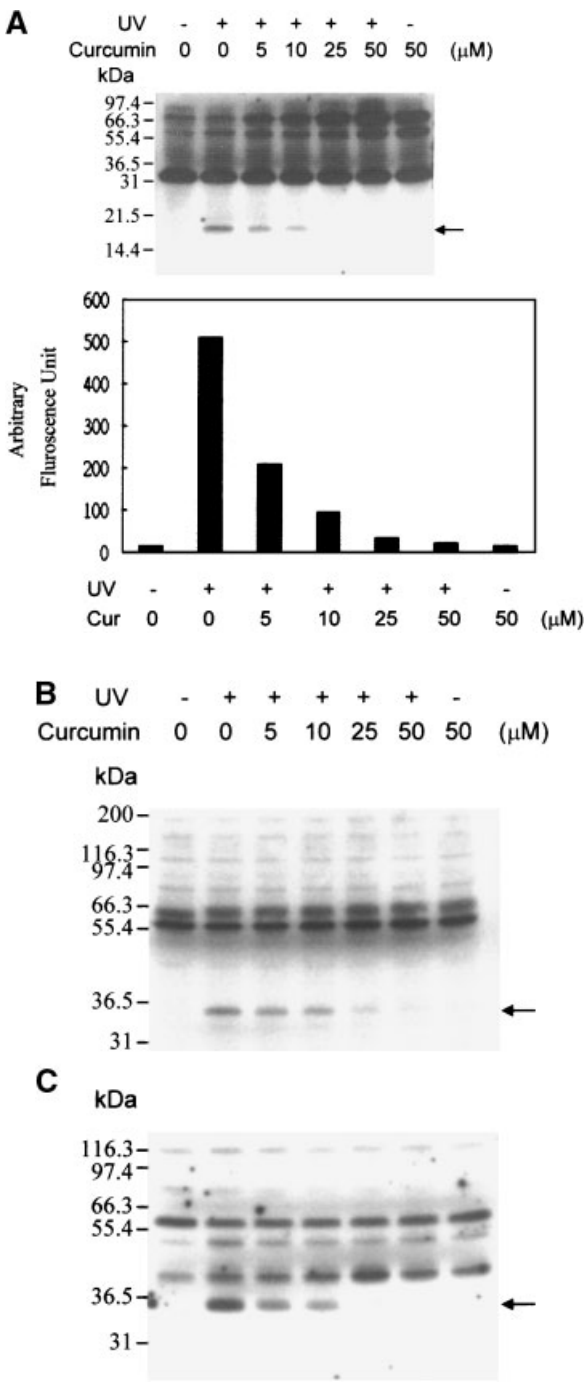


Fig. 1. Curcumin (Cur) prevents ultraviolet (UV) irradiation-induced cell death. **A**: A431 cells were either incubated with various concentrations of Cur at 37°C for 1 h, or were irradiated with UV light (200 J/m^2), or were preincubated with Cur and then irradiated. After 2 h of UV irradiation, cells were detached by trypsin digestion and viable cells counted using microscopy after trypan blue staining. **B**: A431 cells preincubated with or without 50 μM Cur for 1 h were either left untreated or irradiated with UV light (200 J/m^2). Viable cells were counted as in (A) at various time points after UV irradiation. The results shown are averages of three independent experiments, and standard deviations are all <5%. The number of viable cells in control samples was given the value of 100%.

buffer, subjected to 15% SDS–PAGE, and analyzed by immunoblotting with a monoclonal antibody against cytochrome C.

Immunoprecipitation and JNK Activity Assay

Cell extract protein concentration was adjusted to 1.0 mg/ml with solution A, then 0.5 ml cell extract was incubated with 10 μl JNK1(C17) antibody (200 μg/ml) at 4°C for 1.5 h, and then with 40 μl protein A-Sepharose CL-4B (30% v/v) for another 1.5 h with shaking. Immunoprecipitates were collected by centrifugation, washed three times with 1 ml buffer B (20 mM Tris-HCl pH 7.0, and 0.5 mM dithiothreitol) containing 0.5 M NaCl, and resuspended in 40 μl buffer B. To measure JNK activity, immunoprecipitates were incubated in a 50 μl mixture containing 20 mM Tris-HCl at pH 7.0, 0.5 mM dithiothreitol, 0.2 mM [γ-^{32}P]ATP, 20 mM $MgCl_2$, and 0.1 mg/ml GST-cJun (1–79) at room temperature for 10 min with shaking. The reaction was stopped by addition of 2× Laemmli sample buffer and samples analyzed by 12.5% SDS–PAGE. Phosphorylated GST-c-Jun (1–79) (~37 kDa) was identified by autoradiography.



Analytical Methods

Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). SDS–PAGE was performed according to the method of Laemmli [1970] using 10 or 15% gels.

RESULTS

Inhibition of UV irradiation-induced cell death and apoptotic biochemical changes in A431 cells by Cur. We examined the effect of Cur on UV irradiation-induced cell death. A431 cells were incubated with various doses of Cur, or were exposed to UV light irradiation, or were exposed to UV light irradiation after pre-incubation with Cur, and cell viability was determined. Cur alone up to 50 μM had no effect on viability. Approximately 50% of cells died after 200 J/m^2 UV irradiation, and this cell death was reduced by Cur in a

Fig. 2. Effect of Cur on UV irradiation-induced activation of caspase-3 and cleavage/activation of p21$^{Cdc42/Rac}$-activated kinase 2 (PAK2). A431 cells were preincubated with various concentrations of Cur at 37°C for 1 h, exposed to UV light (200 J/m^2), and cell extracts prepared 3 h later. Cell extracts (60 μg) were separated by 15% SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted using anti-CPP32/caspase-3 antibody (**A**, **upper panel**). The same cell extracts (60 μg) were analyzed for caspase-3 activity using Z-DEVD-AFC as the substrate (A, **lower panel**). Cell extracts (40 μg) were separated by 10% SDS–PAGE and immunoblotted using anti-α-PAK (C19) antibody (**B**), or separated by 10% SDS–PAGE containing myelin basic protein (MBP) and analyzed by in-gel kinase assay (**C**). Arrows indicate the position of the proteolytically processed p17 subunit of caspase-3 (in A), or the 36 kDa catalytic fragment of PAK2 (in B and C). The data are representative of three independent experiments.

dose-dependent manner (Fig. 1A). Further experiments showed an UV irradiation time-dependent decrease in viable cells, and that Cur pretreatment significantly increased the number of viable cells (Fig. 1B). We investigated whether prevention of UV-induced cell death by Cur was due to the inhibition of apoptosis by analyzing the effect of Cur on UV-induced cleavage/activation of caspase-3 and PAK2, two biochemical events that occur during UV-induced apoptosis [Tang et al., 1998]. Although low dose Cur had little effect, higher doses (>25 μM) completely blocked cleavage/activation (Fig. 2A,C). Taken together, the results demonstrate that Cur is a potent inhibitor of UV irradiation-induced apoptosis.

Cur attenuates formation of ROS in A431 cells during UV irradiation. UV irradiation-induced apoptosis in A431 cells generates ROS [Chan and Yu, 2000]. We examined the effect of Cur on ROS formation in UV-irradiated cells using DCF-DA as the detection reagent. Pretreatment of cells with Cur significantly attenuated the increase in intracellular ROS content triggered by UV irradiation (Fig. 3), with 50 μM Cur reducing intracellular ROS levels to ~35% of control.

Cur inhibits UV irradiation-induced loss of MMP and cytochrome C release. MMP change and cytochrome C release are directly associated with apoptosis [Li et al., 1997; Zou et al., 1997; Weber et al., 2003], and both events are observed in UV irradiated cells [Tournier et al., 2000]. We investigated the effect of Cur on MMP change and cytochrome C release. We found that UV irradiation decreased DiOC6(3) uptake into mitochondria in A431 cells (Fig. 4A–C). Significant loss of MMP was detected 1 and 2 h after UV light exposure, and this loss was completely blocked by Cur (Fig. 4D,E). For analysis of cytochrome C release, cytosolic fractions from A431 cells were isolated, and cytochrome C levels were determined by immunoblotting. We found that UV irradiation caused a significant release of cytochrome C into the cytosol of A431 cells when compared with non-irradiated controls (Fig. 5). Cur pretreatment inhibited this cytochrome C release in a dose-dependent manner (Fig. 5).

Cur inhibits JNK activation required for caspase-3 activation during UV irradiation-induced apoptosis. To determine the relationship between JNK and caspase-3 activities in UV irradiation-induced apoptosis, we examined the effect of the specific JNK inhibitor SP600125 [Bennett et al., 2001] on UV-irradiated A431 cells. We found that SP600125 reduced UV irradiation-stimulated JNK activity to 20–40% of that in control cells,

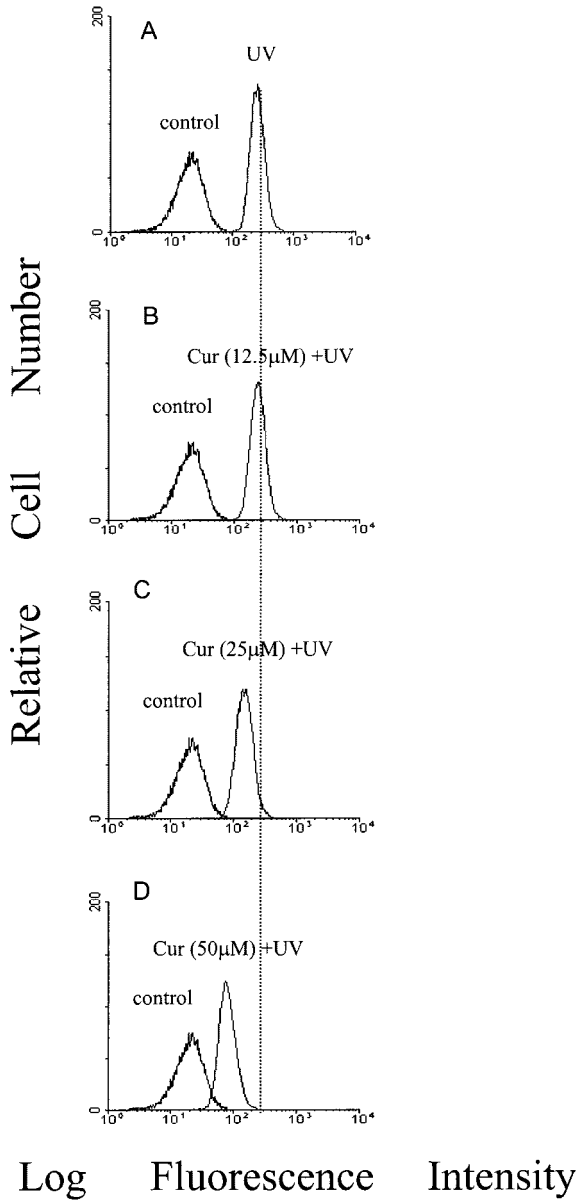


Fig. 3. Cur attenuates UV irradiation-induced oxidative stress. **A:** A431 cells preloaded with 10 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA) for 1 h were left untreated or exposed to UV light (200 J/m^2). **B–D**: A431 cells were left untreated or treated with various concentrations of Cur for 1 h, loaded with 10 μM DCF-DA for another 1 h, then exposed to UV light (200 J/m^2). Cells were collected 2 h after irradiation and analyzed by flow cytometry. The data are representative of three independent experiments.

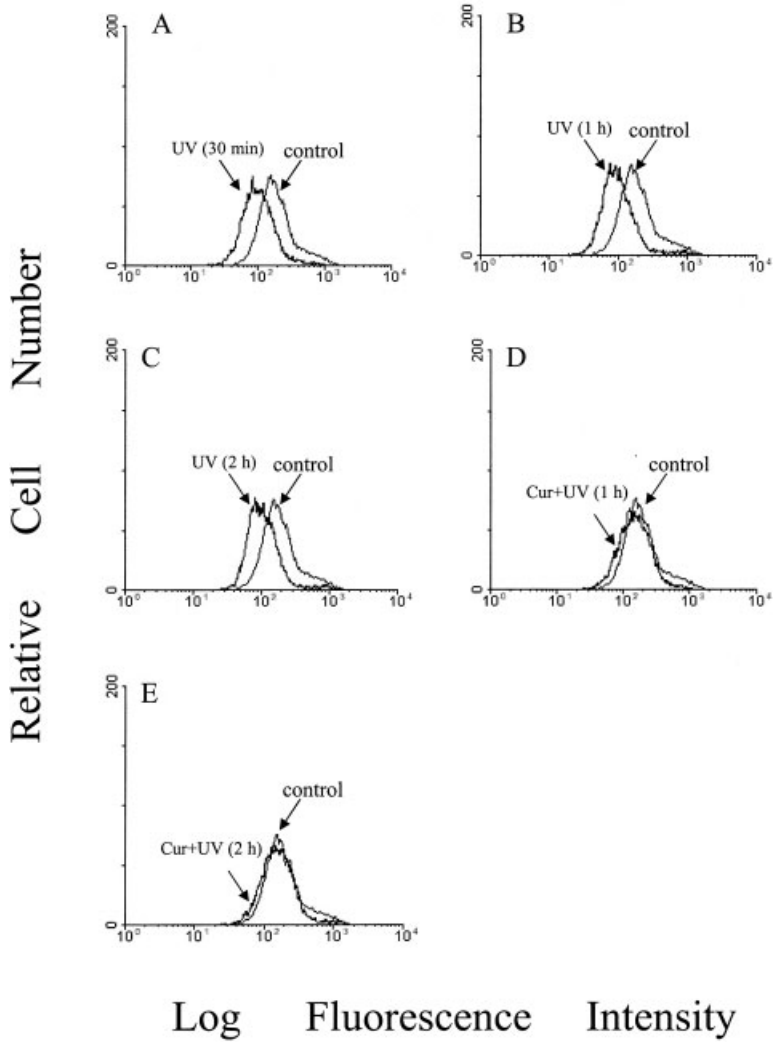


Fig. 4. Flow cytometry analysis of mitochondrial membrane potential (MMP) in UV irradiated cells. **A–C**: A431 cells were treated with or without UV irradiation (200 J/m^2) for 30, 60, or 120 min and then loaded with 40 nM DiOC6(3) at 37°C for 1 h. **D**, **E**: A431 cells were pretreated with 50 μM Cur at 37°C for 1 h and then exposed to UV light (200 J/m^2). After 1 or 2 h cells were incubated with 40 nM DiOC6(3) at 37°C for 1 h, and then collected and analyzed by flow cytometry. The data are representative of three independent experiments.

but had no effect on JNK protein levels (Fig. 6A). We also found that SP600125 inhibition of JNK activity reduced caspase-3 cleavage/activation (Fig. 6B). These findings indicate that JNK activity is required for caspase-3 activation during UV irradiation-induced apoptosis. We examined the effect of Cur on UV irradiation-induced JNK activity. We found that Cur inhibited JNK activity in a dose-dependent manner, with 50 μM Cur pretreatment causing ~70% reduction in activity, but having little effect on JNK protein levels (Fig. 7).

DISCUSSION

UV irradiation triggers multiple intracellular signaling pathways causing a diverse array of functional changes in cells [Bender et al., 1997]. The effects of this simple physical perturbation appear due to the ability of UV irradiation to interact directly with multiple cell membrane and cytoplasmic targets besides DNA [Schwarz, 1998]. Considerable evidence indicates that UV irradiation-induced ROS generation plays an important role in the effect of UV on cells

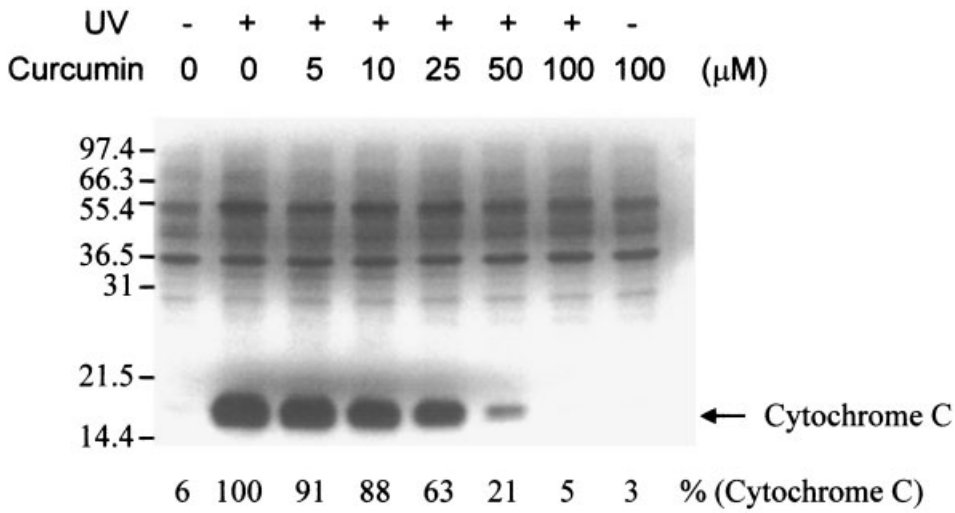


Fig. 5. Effect of Cur on UV irradiation-induced cytochrome C release from mitochondria. A431 cells were preincubated with various concentrations of Cur at 37°C for 1 h and then exposed to UV light (200 J/m^2). Cytosolic and mitochondrial fractions were separated, and cytosol aliquots (40 μg) were resolved by 15% SDS–PAGE then immunoblotted using anti-cytochrome C antibody. The data are representative of three independent experiments. The position of cytochrome C is denoted by an arrow.

[Buttke and Sandstrom, 1994; Scharffetter-Kochanek et al., 1997].

We have previously shown that UV irradiation directly elicits ROS formation in A431 cells, and that anti-oxidants or ROS scavengers prevent UV irradiation-induced caspase-3 cleavage/activation and subsequent PAK2 activation [Chan and Yu, 2000]. In the present study, we show that Cur at doses higher than 25 μM inhibits UV irradiation-induced, caspase-dependent apoptotic biochemical changes in A431 cells (Fig. 1). Others have shown that Cur can both stimulate and inhibit apoptotic signaling. For instance, Cur induced apoptosis in human melanoma cells (30–60 μM for 24 h) [Bush et al., 2001], human leukemia (HL) 60 cells (10–40 μM for 16–24 h) [Kuo et al., 1996; Anto et al., 2002], AK-5 tumor cells (10 μM for 18 h) [Bhaumik et al., 1999; Khar et al., 1999], and MCF-7 breast cancer cells (25 μM for 24 h) [Choudhuri et al., 2002]. In contrast, both dexamethane-induced apoptosis in rat thymocytes and chemotherapy-induced apoptosis in breast cancer cells were inhibited by Cur (10 μM for 12 h) [Jaruga et al., 1998; Somasundaram et al., 2002]. These observations suggest that the treatment protocol and the type of cell may determine the effect of Cur.

The inhibitory effect of Cur on apoptotic biochemical changes triggered by several stimuli has been attributed to its anti-oxidant properties [Ruby et al., 1995]. Oxidative stress is now recognized as a stimulator of cell responses such as apoptosis. Not only can direct exposure of cells to oxidative stress induce apoptosis, but anti-oxidants also protect cells against apoptosis induced by various stimuli that do not exert direct oxidant effects [Buttke and Sandstrom, 1994; Slater et al., 1995]. Mechanisms underlying the anti-oxidant and anti-inflammatory properties of Cur may involve glutathione (GSH)-linked detoxification. Cur treatment can increase non-protein sulfhydryls (NPSH), a surrogate for GSH, in a dose-dependent fashion. This increase may be attributed to increased γ-glutamyl cysteine synthetase (γ-GCS) activity, which catalyzes GSH biosynthesis [Piper et al., 1998]. Recently, it was found that Cur inhibited the singlet oxygen-dependent formation of 2,2,6,6-tetramethypiperidine N-oxyl (TEMPO) formation at very low concentrations, showing that Cur is able to effectively quench singlet oxygen [Das and Das, 2002]. Our finding that Cur attenuated UV irradiation-induced intracellular ROS formation supports the notion that Cur suppresses apoptosis by quenching ROS that form after UV irradiation (Figs. 2 and 3).

It appears that mitochondria act as important conduits for signals during programmed cell death, and that loss of mitochondrial integrity can be promoted or inhibited by many key

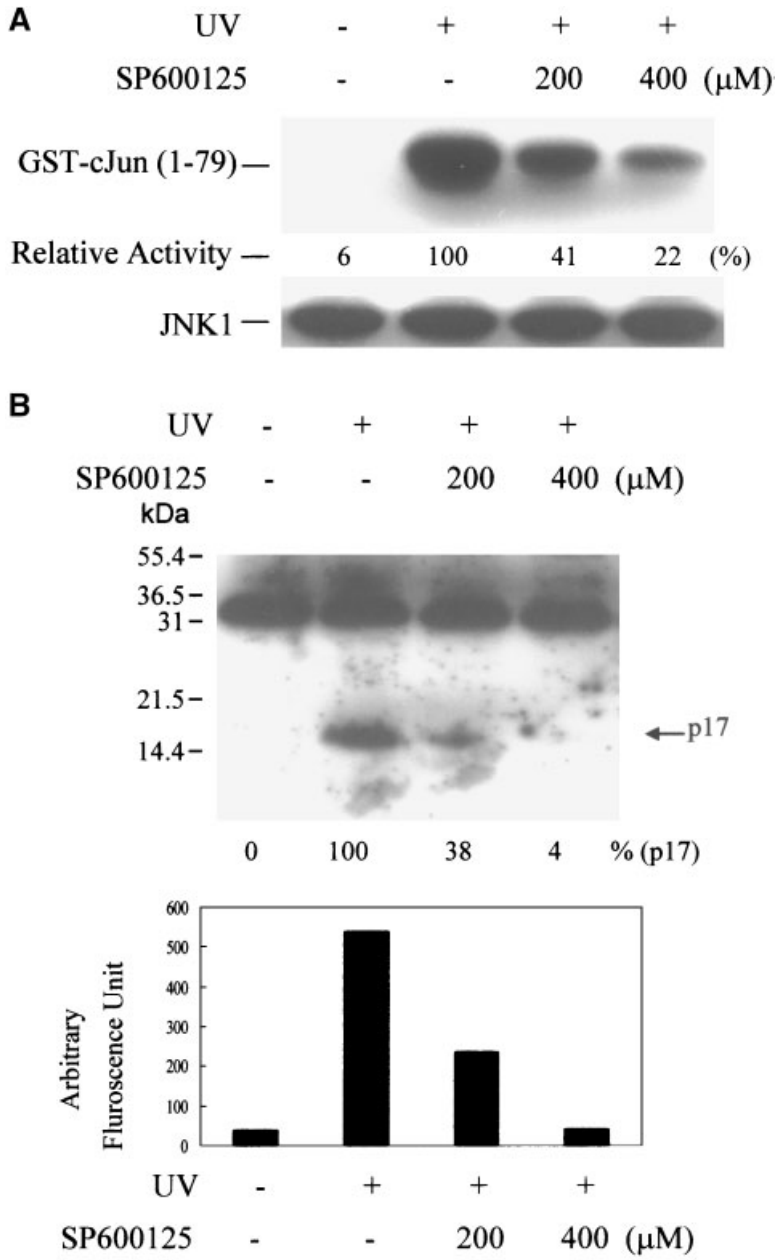


Fig. 6. Effect of the c-Jun N-terminal kinase (JNK) specific inhibitor SP600125 on UV irradiation-induced caspase-3 activation. A431 cells were preincubated with various concentrations of SP600125 at 37°C for 1 h and then exposed to UV light (200 J/m^2). **A**: After 15 min of UV exposure, cell extracts were prepared, from which JNK1 was immunoprecipitated and kinase activity assayed. The **lower panel** represents the immunoblot of JNK1 protein from 60 μg of cell extract. **B**: After 2 h of UV exposure, cell extracts were prepared and 60 μg separated by 15% SDS–PAGE and immunoblotted with anti-CPP32/caspase-3 antibody (B, **upper panel**). In addition, the same cell extracts (60 μg) were analyzed for caspase-3 activity using Z-DEVD-AFC as the substrate (lower panel). Arrow indicates the position of the p17 subunit of caspase-3.

regulators of apoptosis [Kroemer et al., 1997; Green and Reed, 1998]. For instance, divergent cellular stresses, including heat shock, DNA damage, and oxidative stresses, result in caspase activation through cytochrome C release from the mitochondrial intermembrane space into the cytoplasm [Liu et al., 1996; Green and Reed, 1998]. To further elucidate mechanisms underlying Cur inhibition of UV irradiation-induced apoptosis, we examined MMP and mitochondrial release of cytochrome C. We found that Cur concentration-dependently

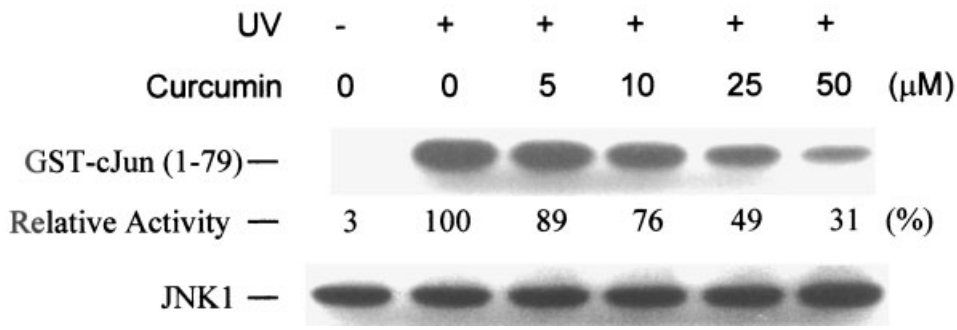


Fig. 7. Effect of Cur on UV irradiation-induced JNK activation. A431 cells were preincubated with various concentrations of Cur at 37°C for 1 h, and then exposed to UV light (200 J/m^2). Cell extracts were prepared 15 min later, from which JNK1 was immunoprecipitated and kinase activity assayed using GST-cJun (1–79) as the substrate. Kinase reaction products were analyzed by 12.5% SDS–PAGE and phosphorylated GST-cJun (1–79) was identified by autoradiography. The signal intensity from autoradiographs was determined using a densitometer, and the maximum signal was given the value of 100%. **Lower panel** shows immunoblot analysis of JNK1 protein from 60 μg cell extract at each dose point.

inhibited UV irradiation-induced loss of MMP (Fig. 4), and that mitochondrial release of cytochrome C was also inhibited in a dose-dependent manner by Cur (Fig. 5).

JNK plays roles in many cell responses, including entry into apoptosis. While it has been reported that Cur can block JNK activation by various agents or treatments, including UV-C radiation [Ruby et al., 1995; Singh and Aggarwal, 1995; Chen and Tan, 1998], the mechanisms by which it operates remain unclear. Using SP600125, an inhibitor specific for JNK, we demonstrated that UV irradiation-induced caspase-3 activation in A431 cells was mediated by JNK activity (Fig. 7). These observations taken together with the findings that ROS generation and JNK activation triggered by UV irradiation can be blocked by Cur, support the hypothesis that Cur inhibits UV irradiation-induced apoptotic biochemical changes by blocking ROS formation and JNK activation, both of which are important triggers for cytochrome C release from mitochondria into the cytoplasm and subsequent activation of caspases. A model illustrating the inhibitory effects of Cur on UV irradiation-induced apoptosis in A431 cells is shown in Figure 8.

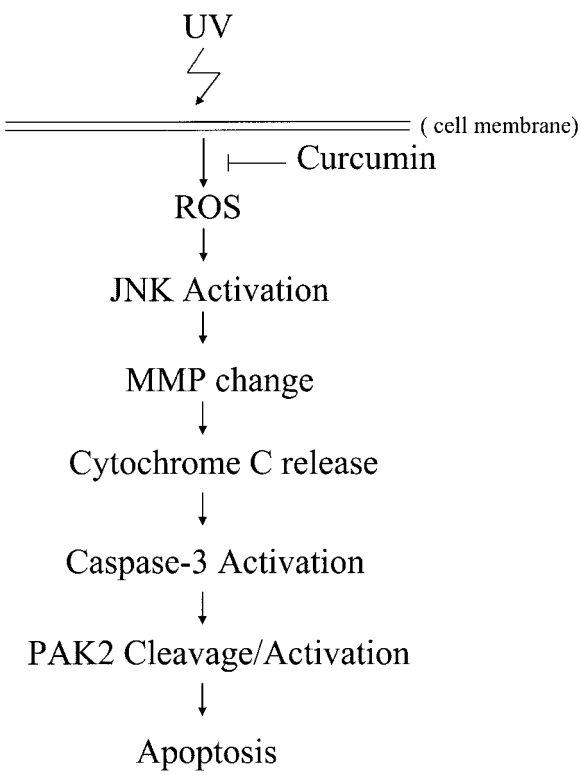


Fig. 8. Model illustrating Cur inhibition of the UV irradiation-induced apoptotic signaling cascade in A431 cells.

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