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Journal of Cellular Biochemistry 109:933–942 (2010)

Journal of Cellular Biochemistry

Capsaicin Induces Apoptosis Through Ubiquitin–Proteasome System Dysfunction

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ABSTRACT

Capsaicin is an active component of red pepper having an antiproliferative effect in a variety of cancer cells, which recent evidence suggests due to its ability to induce apoptosis. However, the molecular mechanisms through which capsaicin induces apoptosis are not well understood. Here we demonstrate that capsaicin-induced apoptosis is mediated via the inhibition cellular proteasome function. Treatment of capsaicin to mouse neuro 2a cells results in the inhibition of proteasome activity in a dose- and time-dependent manner that seems to correlate with its effect on cell death. The effect of capsaicin on cellular proteasome function is indirect and probably mediated via the generation of oxidative stress. Exposure of capsaicin also causes increased accumulation of ubiquitinated proteins as wells as various target substrates of proteasome like p53 and Bax and p27. Like many other classical proteasome inhibitors, capsaicin also triggers the intrinsic pathway of apoptosis involving mitochondria and induces neurite outgrowth. Our results strongly support for the use of capsaicin as an anticancer drug. J. Cell. Biochem. 109: 933–942, 2010.

KEY WORDS: CAPSAICIN; APOPTOSIS; PROTEASOME; CELL CYCLE; BAX; p27

Capsaicin (*N*-vanillyl-8-methyl-1-noneamide) is the active principle ingredient and spicy component of hot chilli pepper, genus *Capsicum*. It is widely used as food additives in many South Asian and Latin American countries [Holzer, 1991; Cordell and Araujo, 1993]. Topical application of capsaicin has been used to treat a variety of neuropathic pain conditions including rheumatoid arthritis, diabetic neuropathy, cluster headaches, herpes zoster, etc. [Watson et al., 1988; Sicuteri et al., 1989; Holzer, 1991; Matucci Cerinic et al., 1995]. The pain relieving effect of capsaicin is mediated through transient potential vanilloid receptor (TRPV-1) predominantly present in the sensory neurons [Holzer, 1991; Cordell and Araujo, 1993; Raisinghani et al., 2005]. However, several experimental studies have demonstrated that capsaicin has a profound antiproliferative effect in several cancer cell lines because of its ability to induce apoptosis [Kim et al., 2005, 2006; Aggarwal and Shishodia, 2006; Sanchez et al., 2006; Amantini et al., 2007; Baek et al., 2008; Czaja et al., 2008; Gil and Kang, 2008; Huang et al., 2009]. The role of TRPV-1 receptor in the apoptosis induced by capsaicin is controversial [Shin et al., 2003; Ito et al., 2004; Kim et al., 2006; Mori et al., 2006; Amantini et al., 2007]. Interestingly, capsaicin-induced apoptosis is observed in cancer cells devoid of TRPV-1 receptor, indicating the involvement of other cellular target in the initiation of apoptosis [Ito et al., 2004; Mori et al., 2006]. But the molecular mechanisms through which capsaicin induce apoptosis it not well understood. So far, several mechanisms have been postulated for capsaicin-induced apoptosis, including generation of oxidative stress, down-regulation of NF-κB activity, up-regulation of several proapoptotic proteins, and activation of intrinsic (the mitochondrial) pathway of caspase activation [Kang et al., 2003; Macho et al., 2003; Shin et al., 2003; Ito et al., 2004; Jin et al., 2005; Mori et al., 2006; Tsou et al., 2006; Wu et al., 2006; Jun et al., 2007; Zhang et al., 2008]. But how capsaicin increases the levels of various proapoptotic proteins and alters the ratio of proapoptotic to antiapoptotic molecules is not well known.

The ubiquitin–proteasome system (UPS) is the major non-lysosomal intracellular protein degradation pathway in eukaryotes [Glickman and Ciechanover, 2002]. This pathway plays a central role in the targeted degradation of cellular proteins that are important for cell cycle progression and apoptosis in normal and malignant cells [Orlowski, 1999]. Proteins to be degraded through this pathway are first covalently attached with multiple ubiquitin molecules and then the multi-ubiquitinated proteins are degraded by 26S proteasome. The 26S proteasome is a 2,000 kDa multi-subunit protenase complex comprised of a 20S core catalytic component capped at either end by

Grant sponsor: Department of Biotechnology, Government of India.
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Received 24 July 2009; Accepted 24 November 2009 • DOI 10.1002/jcb.22469 • © 2010 Wiley-Liss, Inc.
Published online 12 January 2010 in Wiley InterScience (www.interscience.wiley.com).

19S regulatory component. The 20S proteasome consist three different enzymatic sites known as trypsin-like, chymotrypsin-like, and post-glutamyl peptide hydrolase-like. The 19S subunit recognizes and binds to polyubiquitinated proteins [Glickman and Ciechanover, 2002].

It has long been known that the inhibition of proteasome function induces apoptosis depending on cell types and conditions [Sadoul et al., 1996; Drexler, 1997; Lopes et al., 1997; Orlowski, 1999; Drexler et al., 2000; Jana et al., 2004]. Cancer cells are also shown to be more sensitive to proteasome inhibition than normal cells, indicating the potential role of proteasome inhibitors as anticancer drug [Adams, 2004a,b]. Indeed, proteasome inhibitor, bortezomib (PS-341, Velcade) recently approved by the Food and Drug Administration (FDA) for the treatment of multiple myeloma [Adams, 2004a]. In the present report, we demonstrate that capsaicin inhibits cellular proteasome function indirectly and induces apoptosis through mitochondrial pathway.

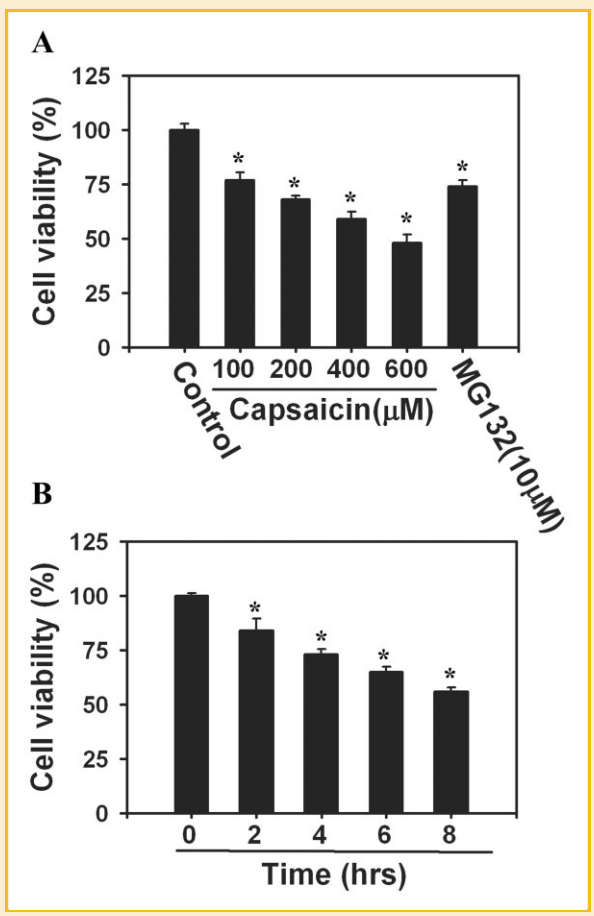

Fig. 1. Capsaicin induces cell death in a dose- and time-dependent manner. A,B: Mouse neuro 2a cells were plated onto 96-well tissue culture plates at subconfluent density. On the following day, cells were left untreated or treated with different doses of capsaicin for 6 h (A). In another experiment, cells were treated with 300 μM of capsaicin for different time periods as indicated in the figure (B). Cell viability was determined by MTT assay. Values are mean ± SD of two independent experiments each performed triplicates. $^{*}P < 0.05$ as compared to control.

MATERIALS AND METHODS

MATERIALS

Capsaicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactacystin, *N*-acetyl cysteine (NAC), MG132, proteasome, caspase-9 and caspase-3 substrates, rabbit polyclonal anti-ubiquitin, and all cell culture reagents were obtained from Sigma. Lipofectamine 2000 and JC-1 (5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide) were purchased from Invitrogen and Molecular Probes respectively. Mouse monoclonal anti-HA was from Roche, mouse monoclonal anti-cytochrome *c* and anti-p27 were from Pharmingen, rabbit polyclonal anti-IκB-α, anti-Bax, anti-Bcl2, and anti-p53 were from Santa Cruz Biotechnology. Alkaline phosphatase and fluorophore conjugated secondary antibodies were purchased from Vector Laboratories. The NF-κB-luciferase construct (containing multiple copies of NF-κB response element) was purchased from Clontech.

CELL CULTURE AND TREATMENTS

Neuro 2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and

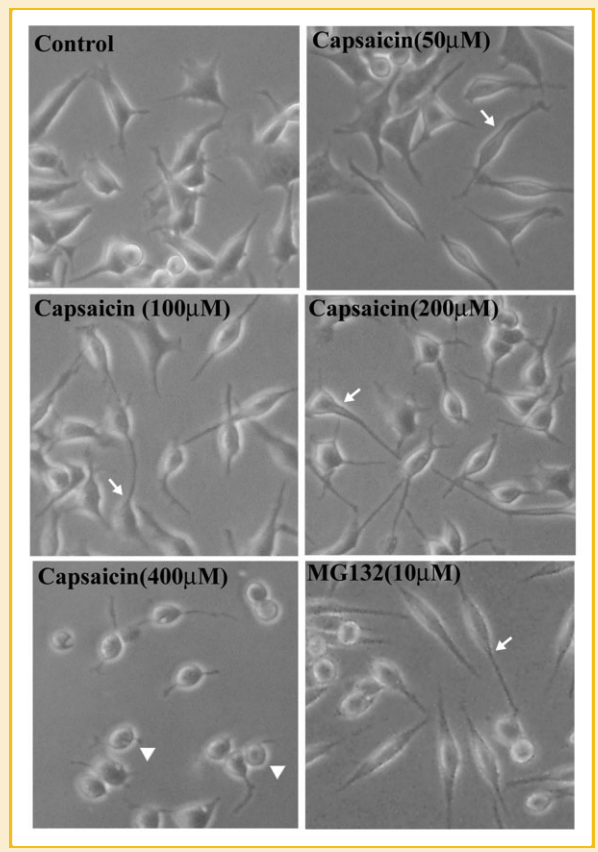

Fig. 2. Exposure of capsaicin stimulates neurite outgrowth. Neuro 2a cells were plated onto 60 mm tissue culture plates and on the following day, cells were left untreated or treated with different doses of capsaicin or MG132 for 4 h. The morphology of cells was then observed under light microscope. Arrow indicates the cell that is having long neurites and arrowhead shows the cell undergoing apoptosis.

antibiotics penicillin/streptomycin. For routine experiments, cells were plated into 6-well tissue culture plate at sub confluent density. After 24 h of plating, cells were treated with different doses of capsaicin, for different time periods and then processed for immunoblotting experiments. In some experiments, cells were transiently transfected with either HA-tagged ubiquitin or NF-κB-luciferase plasmid and 24 h later cells were treated with different doses of capsaicin for different time points. The collected cells were then subjected to immunoblot analysis using HA antibody.

CELL VIABILITY ASSAY

For cell viability assay, cells (approximately 5×10^3 cells/well) were seeded into 96-well tissue culture plates and 24 h after seeding, media was changed and cells were exposed to different doses of capsaicin for different time periods. Cell viability was measured by MTT assay as described earlier [Jana et al., 2004].

MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL

Neuro 2a cells were plated into 60 mm tissue cultured plate at subconfluent density. On the following day, cells were treated with different doses of capsaicin and MG132 for 6 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 pre-warmed at 37°C. Mitochondrial membrane potential was evaluated qualitatively under a fluorescence microscope using 568 nm filter.

IMMUNOFLUORESCENCE STAINING OF CYTOCHROME *c*

To study the release of cytochrome *c* from mitochondria, neuro 2a cells grown on 2-well chamber slides were treated with different doses of capsaicin and MG132 (as positive control). Six 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

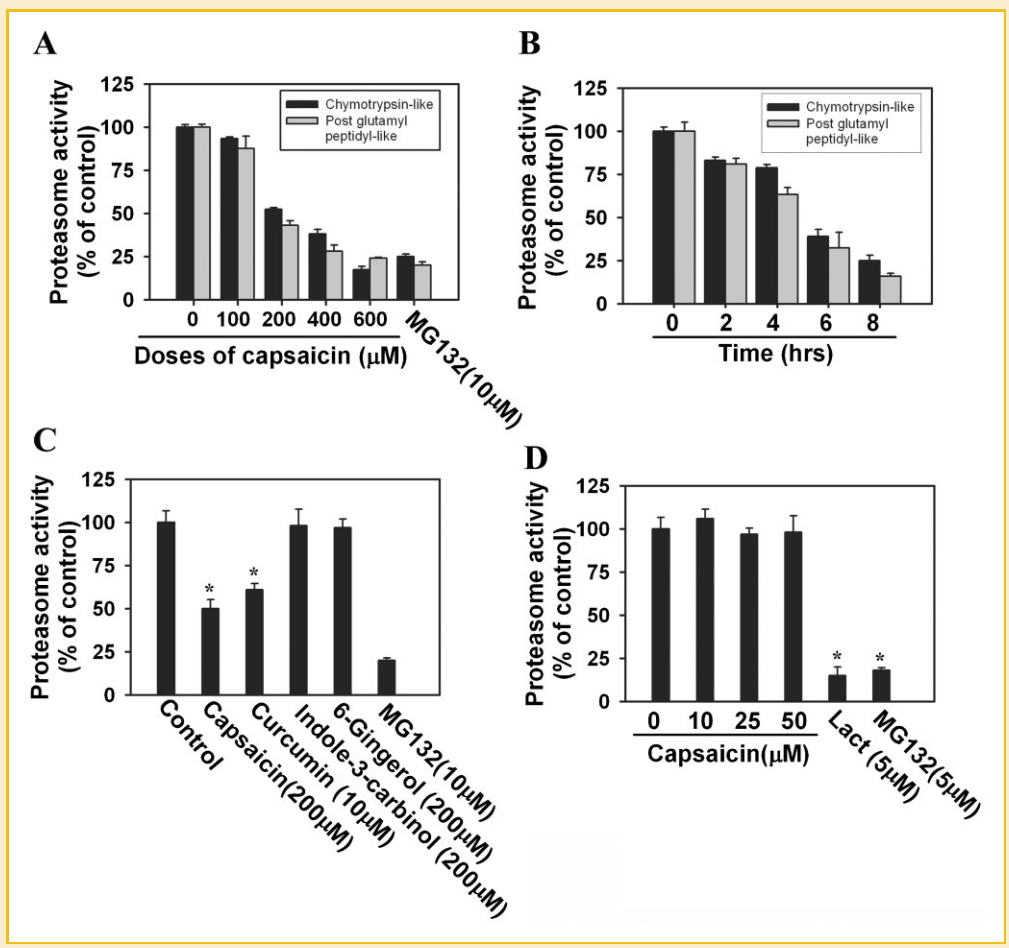

Fig. 3. Capsaicin inhibits proteasome activity. A,B: Neuro 2a cells were plated onto 6-well tissue culture plates at about 60% confluency. Twenty-four hours later, cells were exposed to different doses of capsaicin for 6 h (A) or treated with 300 μM of capsaicin for different time periods (B). Cells were collected and subjected to proteasome activity assays (chymotrypsin and post-glutamyl peptidyl hydrolytic-like protease activity) as described in the Materials and Methods Section. C: Effect of various natural compounds on the chymotrypsin-like activity of proteasome. Cells were exposed different compound for 6 h. MG132 and curcumin were used as positive control. D: Partially purified 20S proteasome was incubated with different concentrations of capsaicin and proteasome inhibitors lactacystin (Lact) and MG132 in the presence of chymotrypsin-like substrate of proteasome. Values are mean ± SD of two independent experiments each performed triplicate. In experiment A, significant ($P < 0.01$) decrease in proteasome activity was observed from 200 μM onwards and in experiment B, all the time points showed significant decrease in proteasome activity. $^*P < 0.001$ as compared with control in experiments C and D.

with 5% non-fat dried milk in TBST for 1 h. Primary antibody (anticytochrome *c*) incubation was carried out overnight at 4°C. After several washings with TBST, cells were incubated with FITC-conjugated secondary antibody for 5 h, washed several times, and visualized using a fluorescence microscope. The digital images were assembled using Adobe Photoshop.

ASSAY OF PROTEASOME, CASPASE-9 AND CASPASE-3-LIKE PROTEASE ACTIVITY

Neuro 2a cells were plated in a 6-well tissue cultured plate and on the following day, cells were treated with varying doses of capsaicin for different time periods. Cells were then isolated and processed for proteasome activity assay as described earlier [Jana et al., 2004]. The substrates Suc-Leu-Leu-Val-Tyr-MCA and Z-Leu-Leu-Glu-MCA were used to determine chymotrypsin and post-glutamyl peptidyl hydrolytic-like activity respectively. To evaluate the direct effect of capsaicin on proteasome's protease activity, pure 20S proteasome (250 ng/reaction) was used instead of cell supernatant in the protease activity assay buffer. 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 using a fluorescence plate reader. The caspase-9 and caspase-3-like protease activities were determined as described earlier [Dikshit et al., 2006a]. The substrates Ac-Asp-Glu-Val-Asp-MCA and Ac-Leu-Glu-His-Asp-MCA were used to determine the caspase-3 and caspase-9-like protease activity respectively.

MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS) AND NF-κB ACTIVITY

The generation of ROS was measured using 2′7′-dichlorodihydro-fluorescein diacetate (CM-H_2DCFDA; Molecular Probes) as described earlier [Sharma et al., 2007]. Briefly, cells were treated with either

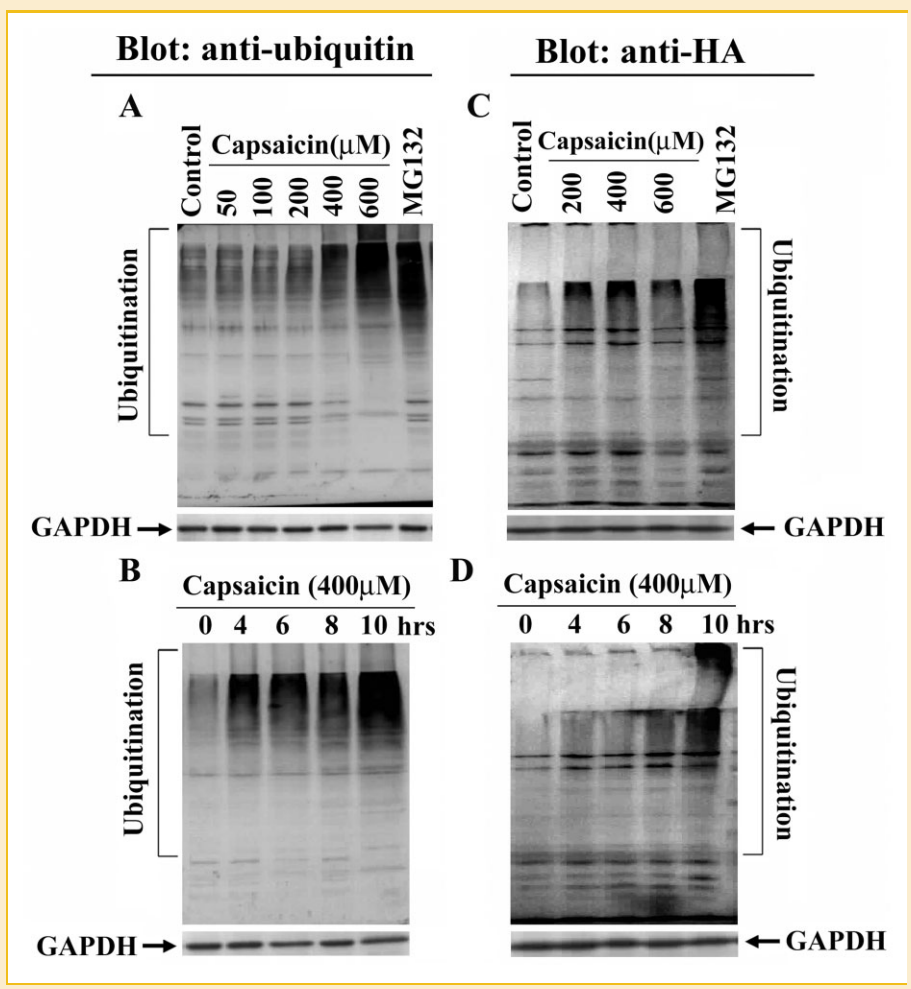

Fig. 4. Treatment of capsaicin results in increased accumulation ubiquitinated protein. A,B: Neuro 2a cells were treated with different doses of capsaicin for different time periods in the similar way as described in Figure 3. Cells were then collected and processed for immunoblot analysis using antibodies against ubiquitin and GAPDH. C,D: Cells were transiently transfected with HA-tagged ubiquitin expression plasmid and 24 h later cells were exposed varying doses of capsaicin for different time periods as indicated in the figure. Cells were collected and subjected to immunoblotting using HA and GAPDH antibodies.

different doses of capsaicin alone or along with NAC for 6 h. Cells were then incubated with H_2DCFDA (10 μM) for 30 min at 37°C, washed with PBS, lysed and the fluorescence intensity was measured using a spectofluorimeter (excitation, 500 nm; emission, 530 nm). The data were normalized as per μg of protein and expressed as fold change in comparison with control. For measuring the NF-κB activity, cells were transiently transfected with NF-κB-luciferase reporter plasmids and 24 h later cells were treated with varying doses of capsaicin for 6 h. Cell lysate were then processed for luciferase activity assay using the assay kit (Roche). The data were normalized as per μg of protein and expressed as percent change in comparison with control.

CYCLOHEXIMIDE-CHASE EXPERIMENT

Neuro 2a cells were plated onto a 6-well tissue culture plate and 24 h later; cells were chased with 15 μg/ml of cycloheximide for different time periods in the presence and absence of capsaicin. Cells were then collected and processed for immunoblot analysis using Bax and p27 antibodies.

IMMUNOBLOTTING EXPERIMENT

After each experiment, cells were washed with cold PBS, scraped, pelleted by centrifugation, suspended in NP-40 lysis buffer (50 mM Tris; pH 8.0, 150 mM NaCl, 1% NP-40, complete protease inhibitor cocktail) and sonicated. Total cell lysates were then centrifuged at 10,000*g* for 10 min and the protein amounts were quantified using Bradford reagent. The samples were then separated through SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were successively incubated in blocking buffer (5% skim milk in 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 AP in TBST. Detection was carried out using NBT and BCIP. Most primary antibodies were used as 1:1,000 dilutions.

STATISTICAL ANALYSIS

Statistical analysis was carried out using Microsoft Excel software. Values were presented as mean ± SD. Inter group comparisons were performed by two-tailed Student's *t*-test and $P < 0.05$ was considered statistically significant.

RESULTS

CAPSAICIN INDUCES NEURITE OUTGROWTH AND APOPTOSIS IN MOUSE NEURO 2a CELLS

In order to investigate the effect of capsaicin on the viability of neuro 2a cells, we plated cells onto 96-well tissue culture plate and on the following day, cells were treated with different concentrations of capsaicin for 6 h. The cell viability was measured by MTT assay. Figure 1A demonstrates that exposure of capsaicin resulted a dose-dependent increase in the death of neuro 2a cells. Capsaicin at a dose of 600 μM caused about 50% reduction of cell viability. Time course study further revealed that capsaicin at a dose of 300 μM significantly inhibited cell viability from 2 h onwards and 50% cell death was observed at 8 h (Fig. 1B). We have also noticed DNA fragmentation as well as TUNEL-positive cells in capsaicin-treated neuro 2a cells (data not shown). Interestingly, capsaicin treatment also induced neurite outgrowth in neuro 2a cells, depending upon the dose (Fig. 2). Similar kind of neurite outgrowth was also observed when neuro 2a cells were treated with proteasome inhibitor MG132.

CAPSAICIN INHIBITS CELLULAR PROTEASOME FUNCTION

The generation of oxidative stress is considered to be one of the important mechanisms of capsaicin-induced apoptosis [Macho et al., 2003; Ito et al., 2004; Wu et al., 2006; Zhang et al., 2008]. Since oxidative stress can be generated because of the proteasomal dysfunction or vice versa [Okada et al., 1999; Ding and Keller, 2001], we further explored the possible involvement of proteasome system in capsaicin-induced cell death. Moreover, capsaicin-induced neurite outgrowth, similar like classical proteasome inhibitor MG132 also prompted us to study proteasomal dysfunction. Neuro 2a cells were treated with varying doses of capsaicin for different time periods; cells were collected and then subjected to assays of various enzymatic

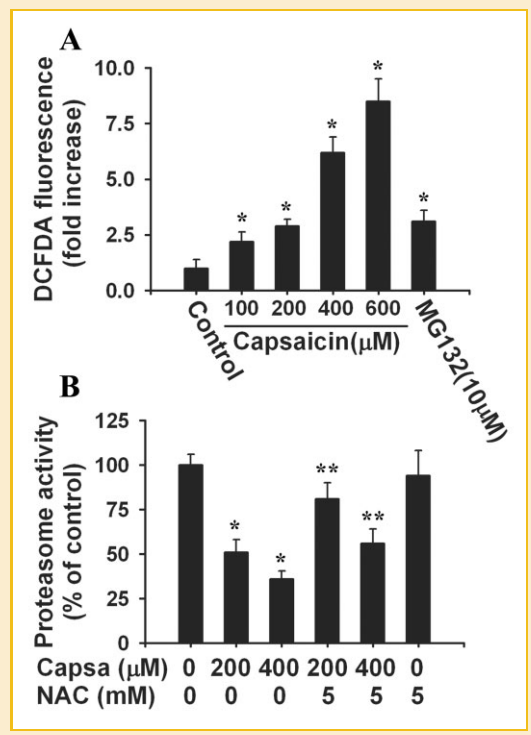

Fig. 5. Capsaicin-induced proteasome inhibition is mediated by ROS formation. A: Cells were treated with different doses of capsaicin or MG132 as described in Figure 3. Cells were then subjected to analysis of ROS using fluorescent dye DCFDA as described in the Materials and Methods Section. B: Cells were treated with different doses of capsaicin (Capsa) along with NAC for 6 h and then processed for proteasome activity assay (chymotrypsin-like). Values are mean ± SD of two independent experiments each performed triplicate. $^{*}P < 0.01$ as compared to control. $^{**}P < 0.01$ in comparison with capsaicin-treated group.

activity of proteasome. As shown in Figure 3A,B, exposure of capsaicin resulted in a dose- and time-dependent decrease in chymotrypsin and post-glutamyl peptidyl-like protease activity of the proteasome. More than 50% of inhibition of the cellular proteasome activity was observed when capsaicin was exposed at a dose of 300 μM for 6 h. In the similar dosing condition, capsaicin inhibited cell viability to about 30%, which indicates proteasome inhibition might be associated with the cell death. The capsaicin-induced proteasome inhibition seems to be very specific, because several natural compounds like indole 3-carbinol or 6-gingerol had no effect on the cellular proteasome activity (Fig. 3C). MG132 and curcumin were used as positive controls. We and others have reported earlier that curcumin inhibits the cellular proteasome function [Jana et al., 2004; Dikshit et al., 2006b; Milacic et al., 2008]. Capsaicin was less potent than curcumin not only on proteasome inhibition but also on induction of cell death. Next, we checked the direct effect of capsaicin on the 20S proteasome activity. In a standardized assay condition, partially purified 20S proteasome was incubated with different concentrations of capsaicin. Figure 3D shows that capsaicin in fact had no direct effect on the chymotrypsin-like protease activity of 20S proteasome. In the similar assay condition, lactacystin and MG132 showed dramatic inhibition of 20S proteasome activity.

To further confirm the effect of capsaicin on cellular proteasome dysfunction, we checked cellular total ubiquitination profile upon capsaicin exposure. The inhibition of proteasome function is

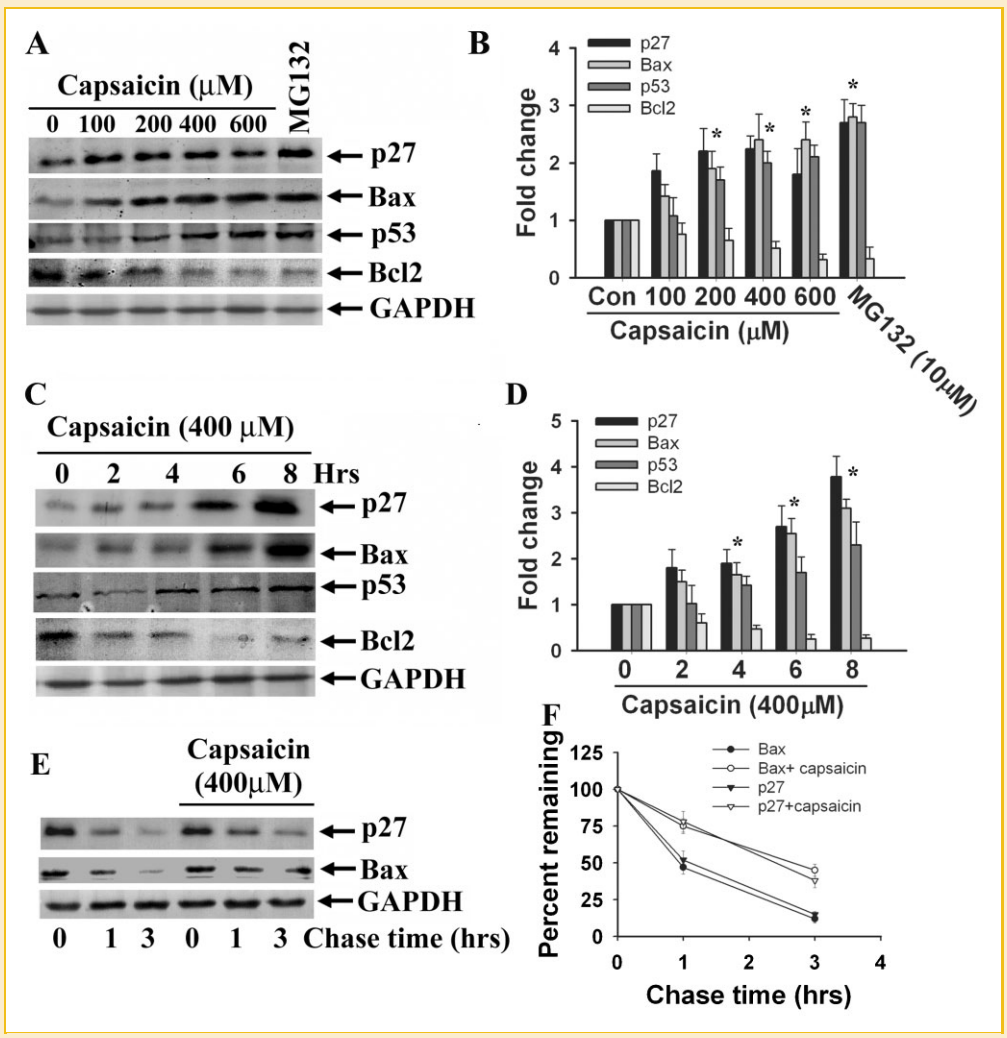

Fig. 6. Capsaicin exposure alters the levels of various target substrates of proteasome. Neuro 2a cells were plated and treated with different doses of capsaicin for different time periods as described in Figure 3. The collected cells were processed for immunoblot analysis using antibody against p27, Bax, p53, Bcl2, and GAPDH. A,B: Dose-dependent effect. C,D: Time-dependent effect. B,D: The band intensities of the different proteins were measured using NIH Image analysis software. Data were normalized against GAPDH. Values are mean ± SD of three independent experiments. $^{*}P < 0.05$ as compared to control. E,F: Neuro 2a cell were treated with cycloheximide and chased in the presence or absence of 400 μM of capsaicin for different time periods as described in the Materials and Methods Section. Collected cells were then subjected to immunoblot analysis using antibodies against Bax, p27 and GAPDH. B: Quantitation of the Bax and p27 protein levels in the chase experiment described in E from three independent experiments. Data were normalized against GAPDH. Capsaicin treatment significantly increased ($P < 0.01$) the accumulations of p27 and Bax in comparison with control at all the time point tested.

expected to increase the accumulation of ubiquitinated proteins. As shown in Figure 4A,B, exposure of capsaicin into neuro 2a cells caused a dose- and time-dependent increase in the accumulation of ubiquitinated derivatives of various cellular proteins. This finding was re-confirmed upon transient transfection of HA-ubiquitin plasmid into neuro 2a cells followed by the treatment of different doses of capsaicin. The blot was detected with HA antibody. As expected, capsaicin treatment also showed increased accumulation of HA-tagged ubiquitinated proteins (Fig. 4C,D).

To study the involvement of ROS in the inhibition of proteasome activity, we first tested the effect of capsaicin on ROS production. As shown in Figure 5A, capsaicin was found to dose-dependently increase ROS generation. Treatment of NAC along with capsaicin significantly prevented ROS production (data not shown). Interestingly, NAC treatment also prevented capsaicin-induced proteasome inhibition (Fig. 5B). This finding suggests that capsaicin-induced proteasome dysfunction is mediated via ROS production.

TREATMENT OF CAPSAICIN INCREASES THE LEVELS OF VARIOUS TARGET SUBSTRATES OF PROTEASOME

Since capsaicin inhibits the cellular proteasome function, we further tested the effect of capsaicin on the various cellular substrates of proteasome required for cell survival or cell death. Neuro 2a cells were treated with varying doses of capsaicin for different time periods. Cells were collected and subjected to immunoblot analysis using antibodies of various proapoptotic, antiapoptotic, and cell cycle regulatory proteins. Figure 6 demonstrates that the levels of p27, p53, and Bax were increased in capsaicin-treated cells in a dose- and time-dependent manner. The cycloheximide chase experiment further confirmed that the stability of some those proteins were increased in capsaicin-treated cells (Fig. 6E,F). The levels of Bcl2 were decreased in capsaicin-treated cell. Similar results were also observed in MG132-treated cell, which has been reported earlier. Surprisingly, we have also noticed decreased levels of IκB-α in capsaicin-treated cells, although there was significant inhibition of NF-κB activity (Fig. 7).

CAPSAICIN INDUCES MITOCHONDRIAL MEMBRANE DEPOLARIZATION, CYTOCHROME *c* RELEASE AND ACTIVATION OF CASPASES

Proteasome inhibitors are well known to induce the intrinsic pathway of apoptosis involving mitochondria. Therefore, we first tested the effect of capsaicin on the mitochondrial membrane potential and cytochrome *c* release. Cells were treated with different doses of capsaicin for 6 h and then either processed for JC-1 staining to study the mitochondrial membrane potential or immunofluorescence staining of cytochrome *c* to check its release from mitochondria. JC-1 is a voltage sensitive fluorescent dye that detects normal polarized mitochondria as red color, and depolarized mitochondrial membranes changes to green color. The exposure of capsaicin to neuro 2a cells caused loss of mitochondrial membrane potential (Fig. 8, top panel). MG132 was used as positive control, which showed similar results. The immunofluorescence staining of cytochrome *c* also confirmed it release from mitochondria in response to capsaicin treatment (Fig. 8, bottom panel). Interestingly, capsaicin treatment also resulted in accumulation of mitochondria in a pericentriolar region as evident from both JC-1 and cytochrome *c* staining. Since the release of cytochrome *c* from mitochondria activates caspase-9 upon binding to cytoplasmic apaf-1, we further tested the effect of capsaicin on the activation of caspase-9 and the downstream caspase-3. As expected, exposure of capsaicin leading to the activation of caspase-9 and caspase-3 in a dose- and time-dependent manner (Fig. 9).

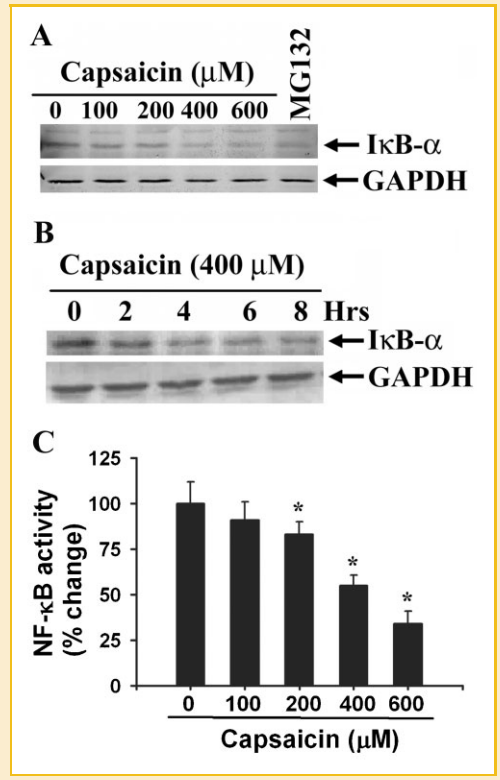

Fig. 7. Effect of capsaicin on IκB-α levels and NF-κB activity. A,B: Neuro 2a cells were treated with different doses of capsaicin for different time periods as described in Figure 3. Cell lysates were then subjected to immunoblot analysis using IκB-α and GAPDH antibodies. C: Cells were transiently transfected with NF-κB-luciferase plasmid, treated with varying doses of capsaicin and processed for luciferase activity assay as described in the Materials and Methods Section. Values are mean ± SD of two independent experiments each done triplicate. $^{*}P < 0.05$ as compared to control.

DISCUSSION

Capsaicin has been reported to induce apoptosis in several cancer cells. However, the molecular mechanism that triggers capsaicin-induced apoptosis is poorly understood. In the present investigation we demonstrate that apoptosis induced by capsaicin is mediated at least in part through the impairment of cellular proteasome function.

First, we have found that capsaicin inhibits cellular proteasome function indirectly in a dose- and time-dependent manner and

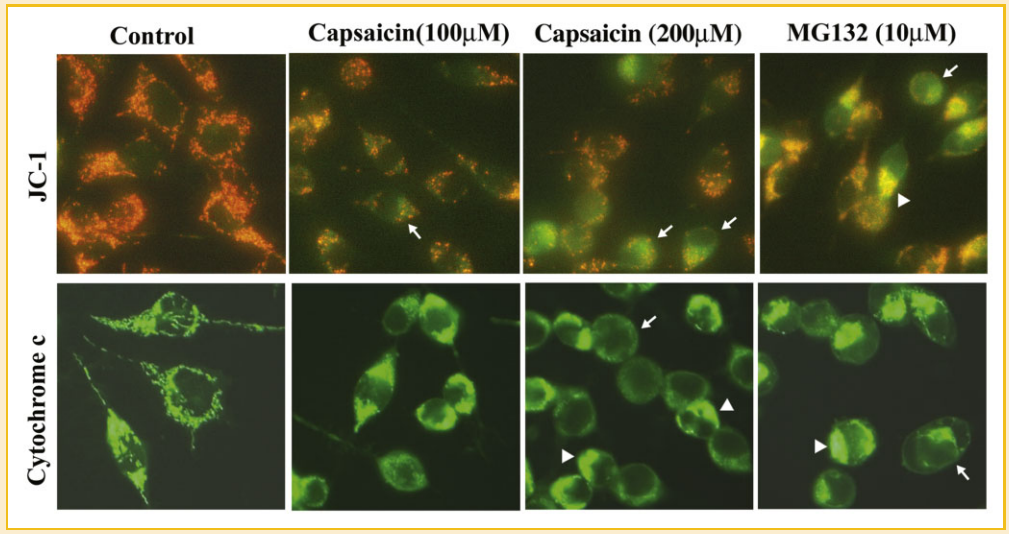

Fig. 8. Exposure of capsaicin leads to dose-dependent changes in the mitochondrial membrane potential and release of cytochrome c. Neuro 2a cells were treated with different doses of capsaicin or proteasome inhibitor MG132 for 6 h and then subjected to JC-1 staining to evaluate the changes in mitochondrial membrane potential (top panel) and to immunofluorescence staining using cytochrome c antibody (bottom panel). Arrow indicates the cell showing changes in mitochondrial membrane potential (color changes from red to diffuse green) and cytochrome c release in cytosol. Arrowhead shows the accumulation of mitochondria in a perinuclear region. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

causes increased accumulation of ubiquitinated proteins. The neurite outgrowth and cell death induced by capsaicin also correlate with its inhibitory effect on proteasome. Secondly, capsaicin treatment increased the levels of many cellular target substrates of proteasome, including various proapoptotic and cell cycle regulatory proteins. Finally, like any classical proteasome inhibitors, capsaicin exposure also leads to severe mitochondrial abnormalities and induction of caspase-9 and caspase-3. All of these findings clearly indicate that capsaicin-induced proteasome dysfunction might be one of the initial events leading to apoptosis. Since capsaicin does not have any direct inhibitory effect on 20S proteasome activity, we suspected possible involvement of oxidative stress. Oxidative stress is well known to inhibit cellular proteasome function [Okada et al., 1999; Ding and Keller, 2001] and there are several reports that demonstrate the generation of oxidative stress in capsaicin-treated cells [Macho et al., 2003; Ito et al., 2004; Wu et al., 2006; Zhang et al., 2008]. In the neuro 2a cell, we have also observed production of ROS upon capsaicin exposure and antioxidant NAC partially recovered capsaicin-induced proteasome inhibition. This finding suggest that capsaicin-induced proteasome dysfunction is most likely mediated through the generation of oxidative stress. Oxidative stress and proteasomal dysfunction could potentially damage mitochondria, which can again generate oxidative stress and creates a vicious cycle. Therefore, both oxidative stress and proteasome malfunction might be linked with each other in capsaicin-induced apoptosis.

The capsaicin-induced proteasome dysfunction also might increase the levels of Bax and p53 and decrease the expression of Bcl2 and the altered ratio of these proteins could further affect the integrity of mitochondria. Once cytochrome *c* is released into cytosol, it will trigger the sequential activation of caspase-9 and caspase-3. In fact, several earlier reports and our findings clearly demonstrated the release of mitochondrial cytochrome *c* and activation of caspases-9 and caspase-3 in capsaicin-treated various cancer cells [Shin et al., 2003; Ito et al., 2004; Jin et al., 2005].

Capsaicin treatment is also known to down-regulate the NF-κB activity, cell cycle arrest, generation of ER stress, and induction of autophagy [Mori et al., 2006; Wu et al., 2006; Oh et al., 2008; Oh and Lim, 2009]. All of these phenomena can be explained based on its inhibitory effects on proteasome, because proteasome inhibitors are known to produce a similar effect. The NF-κB pathway is very much essential for cell survival and tightly regulated by UPS [Orlowski, 1999]. Altered proteasomal function will inhibit the turn over of IκB-α and thereby block the nuclear translocation of NF-κB subunits leading to inhibition of transcription. Capsaicin also has been shown to down-regulate the NF-κB activity through altered degradation of IκB-α [Mori et al., 2006]. Surprisingly, we have observed decreased levels of IκB-α in capsaicin-treated neuro 2a cells, although there was a significant decrease in NF-κB activity. Currently, we do not know how capsaicin decreases NF-κB activity when IκB-α level also decreased. Probably, capsaicin-induced down-regulation of NF-κB activity is mediated via multiple pathways. Like many classical proteasome inhibitors, we have also found that capsaicin increases the levels of p27. The p27 is a cyclin-dependent kinase inhibitor, tightly regulated by proteasome during cell cycle and therefore, its increased levels could potentially arrest cell cycle at G1/S phase [Wu et al., 2006]. Our findings of neurite outgrowth in response to capsaicin could be due to the increased levels of p27. However, the selective effect of capsaicin on either proteasomal dysfunction or apoptosis in normal or cancer cells are not known and require further investigation.

Taken together, our findings suggest that capsaicin-induced proteasomal dysfunction might be involved at least in part in the induction of apoptosis. Proteasome inhibitors are being considered

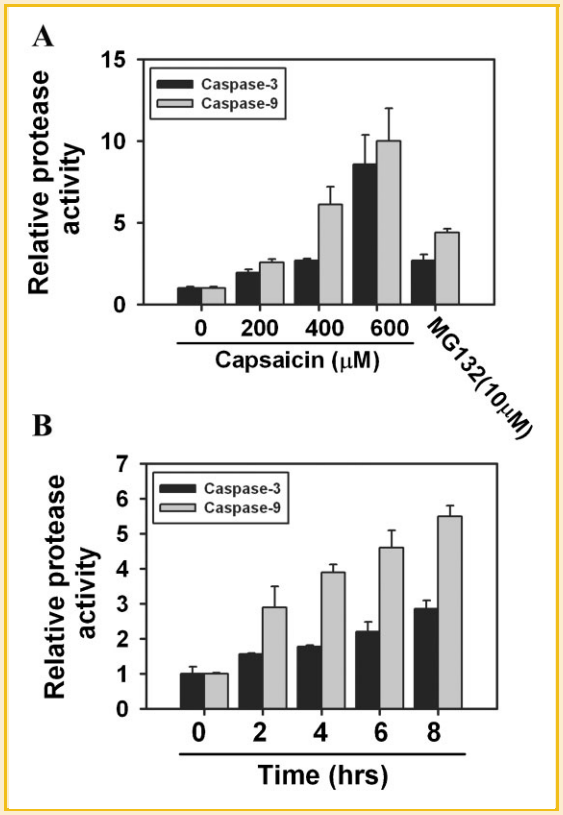

Fig. 9. Activation of caspase-9 and caspase-3 upon capsaicin treatment. Neuro 2a cells were plated and treated with either different doses of capsaicin for 6 h (A) or 400 μM of capsaicin for different time periods (B) as indicated in the figure. Cells were collected; lysates were made and then subjected to caspase-9 and caspase-3 activity assays. MG132 was used as positive control. Values are mean ± SD of two independent experiments each performed triplicates. All the doses of capsaicin used in experiment A and all the time points in experiment B showed significant ($P < 0.05$) increase in caspase activity.

as a new class of potential anticancer agents because of their preferential effect on cancer cells. Since capsaicin inhibits proteasome function and induces apoptosis in cancer cells, it has enormous potential as an anticancer drug.

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