Title: Endoplasmic Reticulum Stress-Mediated Autophagy/Apoptosis Induced by Capsaicin and Dihydrocapsaicin (DHC) is Regulated by the Extent of JNK/ERK Activation in WI38 Lung Epithelial Fibroblast Cells

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Running title: Molecular mechanisms of DHC-induced ER-stress

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Abbreviations: DHC, dihydrocapsaicin; LC3, microtubule-associated protein 1 (MAP1) light chain-3; 3MA, 3-methyladenine; MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide]; zVAD, benzyl-Oxcarbonyl-Val-Ala-Asp-fluoromethyl ketone; TG, thapsigargin; TM, tunicamycin; BaF1, bafilomycin A1; PI3K, phosphatidylinositol-3-kinase.

Sections: Cellular and Molecular
Abstract

Endoplasmic reticulum (ER)-stress causes cell survival or death which is dependent on the type of cell and stimulus. Capsaicin and its analogue dihydrocapsin (DHC) induced caspase-3 independent/dependent signaling pathways in WI38 lung epithelial fibroblast cells. Here we describe the molecular mechanisms induced by both chemicals. Exposure to capsaicin or DHC caused induction of p53, p21, and G0/G1 arrest. DHC induced massive cellular vacuolization by dilation of the ER and mitochondria. Classic ER-stress inducers elicited the unfolded protein response (UPR) and upregulation of LC3II. DHC induced ER-stress by the action of Bip, IRE1, Chop, eIF2a and caspase-4, and to a lesser level by capsaicin treatment. DHC treatment induced autophagy that was blocked by 3-methyladenine (3MA) and accumulated by bafilomycin A1 (BaF1). Blocking of DHC-induced autophagy by 3MA enhanced apoptotic cell death that was completely inhibited by treatment of cells with zVAD. Knockdown of Ire1 downregulated the DHC-induced Chop and LC3II and enhanced caspase-3 activation. DHC induced rapid and high-sustained JNK/ERK activation, but capsaicin induced transient activation of JNK/ERK.
The JNK inhibitor SP600125 downregulated the expression of IRE1, Chop, and LC3II induced by DHC, thapsigargin and MG132. Pharmacological blockade or knockdown of ERK downregulated LC3II. Capsaicin and DHC induced Akt phosphorylation, and the phosphatidylinositol 3-kinase (PI3K) inhibitors, wortmannin and LY294002, induced autophagy via ERK activation. Our results indicate that the differential responses of capsaicin and DHC for cell protection are caused by the extent of the UPR and autophagy that are both regulated by the level of JNK and ERK activation.
Introduction

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), a representative pungent ingredient found in the red pepper of the genus Capsicum, has been known to induce selectively apoptosis in malignant cells but not in normal cells, which is ascribed to the generation of reactive oxygen species (ROS) by blocking of the plasma membrane electron transport system (Morre et al., 1995; Macho et al., 1999). In a previous study, capsaicin-induced apoptosis in ras-transformed human breast epithelial cells (H-ras MCF10A), but not in the parental MCF10A cells, was regulated by the mitogen-activated protein kinase (MAPK) JNK/p38, suggesting that MAPK may have a critical role in the selective effect of capsaicin in malignant cells (Kang et al., 2003). However, the molecular mechanisms of the selective effect on malignant cells induced by capsaicin exposure are still unclear. Furthermore, previous studies have underscored the prosurvival effect of capsaicin on normal cells. Therefore, it is of interest to explore the effect of capsaicin acting on normal cells for use as a chemopreventive agent. In a preliminary study, we found that capsaicin induced caspase-3-dependent/independent signaling pathways in WI38 normal lung.
epithelial fibroblast cells and we investigated the molecular mechanisms underlying the effects of both capsaicin and its analogue dihydrocapsaicin (DHC) in WI38 cells.

The endoplasmic reticulum (ER) performs several functions including protein folding and trafficking, and the regulation of the intracellular calcium concentration. Upon disruption of the ER functions by accumulation of unfolded/misfolded proteins in the ER, cells trigger the unfolded protein response (UPR) as a self-protective mechanism (Schröder and Kaufman, 2005).

Under normal conditions, the ER-stress sensors IRE1, PERK and ATF6 reside with binding to Bip/GRP78, but release from Bip/GRP78 by ER-stress and transfer downstream signals to the cytoplasm. IRE1 activates the downstream target XBP1 and activates several UPR target genes. PERK phosphorylates eIF2a to block translation. Activation of ATF6 increases transcription of ER chaperones, including BiP/GRP78 that is involved in stress mitigation. In addition, the UPR activates the ER-associated degradation (ERAD) system. In this pathway, misfolded/unfolded proteins are translocated from the ER lumen to the cytosol and are then degraded by the ubiquitin proteosome pathway.
Consequently, the UPR contributes to the reduction of ER-overload and functions to protect cells against ER-stress. However, if the ER functions are severely affected, cells are removed by apoptosis. ER-stress-mediated apoptosis is triggered by the activation of ER-membrane resident caspase-12 (mice) and caspase-4 (humans), as well as induction of Chop (Rao et al., 2001; Morishima et al., 2002; Oyadomari and Mori, 2004).

Autophagy is a cellular defense mechanism that occurs through degradation and recycling of cytoplasmic constituents. Starvation-induced autophagy is known to have an important role in cell survival, while excessive autophagy triggers cell death (Baehrecke, 2005; Codogno and Meijer, 2005). During autophagy, cytoplasmic constituents are sequestered into double-membrane vesicles (autophagosomes) that subsequently fuse with lysosomes to form autolysosomes; eventually, degraded by lysosomal hydrolases. Autophagic cell death is thus characterized by the accumulation of vacuoles (Klionsky and Emr, 2000). Accumulating evidence has suggested that ER-stress is linked to autophagy (Ogata et al., 2006; Yorimitsu et al., 2006). However, the cellular consequences of ER-stress-mediated autophagy appear to vary depending on
the cell type and stimulus. As relevant in pathogenesis, disruption of autophagy may be a cause of several neurodegenerative disorders such as Parkinson’s disease, Huntington disease and Alzheimer’s disease (Kaufman, 2002). Under autophagy-deficient conditions, toxic proteins that accumulate in the ER can be effectively removed by autophagy (Teckman and Perlmutter, 2000; Fujita et al., 2007), suggesting that ER-stress-induced autophagy may play an important role in cell protection. A recent study showed that autophagy induced by ER-stress inducers mitigates ER-stress and protects cells. By contrast, autophagy that was induced by the same chemicals contributed to cell death in non-transformed cells (Ding et al., 2007). However, it is still unclear whether ER-stress-mediated autophagy is involved in cell survival or cell death.

In the present study, we investigated whether the distinct response to normal cells of capsaicin is associated with ER-stress response or autophagy. Furthermore, we compared the molecular mechanisms induced by capsaicin with those of induced by its structural analogue DHC.
Methods

Cell cultures and chemicals. WI38 normal lung epithelial fibroblast cells were maintained in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum, 50 μg/ml penicillin and 50 μg/ml streptomycin at 37 °C in a 5% CO₂ - 95% air-humidified incubator. The compounds capsaicin, dihydrocapsaicin, 3-metyladenine and MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma (St. Louis, MO USA). Benzyl-Oxcarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD) and caspase-3 substrate (Ac-DEVD-pNA) were purchased from Biomol (Plymouth Meeting, PA USA) and Calbiochem (La Jolla, CA USA), respectively. Other chemicals used were of the purest grade available from Sigma.

Cytotoxicity assay. The viability of the cultured cells was determined by use of the MTT assay. In brief, cells were suspended at a concentration of 0.5–1 × 10⁵ cells/ml. A 200 μl sample of the cell suspension was seeded onto a 48-well plate. After culturing overnight, cells were exposed to chemicals for 24 h. After 4 h of incubation with MTT (0.5 mg/ml), the medium was removed and the formazan crystals were dissolved with DMSO. Absorbance was then measured.
Flow cytometric analysis. Cells were harvested and washed twice with cold PBS buffer. After fixing 70% ethanol for 30 min at 4 °C, cells were washed with cold PBS buffer, and resuspended in 1 ml PBS buffer containing 500µg/ml propidium iodide (PI). At least 10,000 events were analyzed by a FACScan (BD FacsCalibur and CellQuest software (Macintosh, Facstation; Becton Dickinson). The percentage of nuclei with hypodiploid content was evaluated as apoptotic cells, and also calculated percentage of cells in each cell cycle phase (G0/G1, S, or G2/M).

Transmission electron microscopy. Cells were fixed with 4% paraformaldehyde and 0.1%glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and post fixed with 1% OsO4 for 2 h. The cells were then dehydrated with increasing concentrations of alcohol (30, 50, 70, 90 and 100 %), infiltrated with LR White resin two times for 1 h each, and subsequently embedded in LR White resin. The solidified blocks were cut into 60 nm thicknesses and were then stained with uranyl acetate and lead citrate. Samples were observed under a transmission electron microscope (Hitachi H-7600, Hitachi, Tokyo,
Transfection. Dr. Xiao-Ming Yin (University of Pittsburgh School of Medicine, Pittsburgh, PA USA) kindly provided adenoviral GFP-LC3B. After cells were washed with OPTI-MEM medium (Invitrogen, Carlsbad, CA), DNA was transfected into cells using Lipofectamine 2000 according to the supplier’s protocol (Invitrogen). After 4 h of incubation, the medium was exchanged to a complete medium containing 10% serum and antibiotics. The cells were incubated for an additional 24 h, treated as indicated in the figure legends and the cells were then observed under a fluorescence microscope (Nikon TE2000U; Nikon, Tokyo, Japan). Alternatively, cells were transfected with siRNA corresponding to human Ire1 (5'-CUGCCCGGCUCGGGAUUU-3' and 5'-AAUCCCGAGGCCGGGCAG-3'), Atg5 (5'-GGACGAAUCCAACUUGU-3' AND 5'-AACAAGUUGGAAUUCGUCC-3'), ERK (5'-GCAUGACCAUUCUGCUA-3' AND 5'-UAGCAGAUUGGUCAUUGC-3') or control siRNA for EGFP (Ambion, Austin, TX) using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol. Cells were then cultured in complete medium for 48 h before further analysis.
Immunoblot analysis. Cells were washed with PBS and lysed in 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 50 mM glycerophosphate, 20 mM NaF, 1 mM Na3VO4, 2 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin. The cell lysates were then centrifuged, and the protein content was quantified. Equal amounts of protein were separated by the use of SDS-polyacrylamide gel electrophoresis (on 12–15 % gels), and the separated proteins were transferred to a PVDF membrane and then immunoblotted with the corresponding antibodies. Anti-Atg8/LC3 antibody was obtained from Absent (San Diego, CA USA). Anti-IRE 1 and antibodies against Akt, phospho-Akt, ERk, phospho-ERK, p38, phospho-p38, p21, cleaved caspase-3, phosphor-p53, mTor, and phosphor-mTor were purchased from Cell Signaling Technology (Danvers, MA USA). Antibodies against Chop, ATF4, ATF6, PERK, Atg5, β-actin, caspase-3 and α-tubulin were purchased from SantaCruz Biotechnology (Santa Cruz, CA USA). Anti-caspase-4 was obtained from Abcam (Cambridge, UK). Immobilized proteins were incubated with goat anti-mouse IgG and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA USA), and signals were detected using a chemiluminescence
kit (Amersham Biosciences, Amersham, UK).

**Statistical analysis.** All experiments were repeated at least three times and the significance of the differences between treatments and respective controls was analyzed by use of the Student's *t* test. Values are expressed as the mean ± SD.
Results

Antiproliferative effect of capsaicin and DHC on WI38 cells. Capsaicin and DHC constitute over 90 percent of capsaicinoids extracted from red pepper, and the only difference between both chemicals is the presence of a carbon-carbon double bond (Nelson, 1919). To examine the cytotoxicity of DHC and capsaicin, WI38 cells were treated for 24 h with various concentrations of DHC or capsaicin as indicated in Figure 1A, and cell viability was measured using the MTT assay. At a concentration of 200 µM, the effect of capsaicin on WI38 cells was minimal, but treatment with DHC significantly decreased viability, and viability was further decreased with a concentration of 400 µM.

To examine if the cytotoxic effect by capsaicin or DHC is associated with cell cycle arrest, the expression of proteins that regulate cell cycle progression were analyzed on immunoblots. Cells were treated with 200 µM capsaicin or DHC up to 24 h. As p53 is involved in both cell cycle arrest and apoptosis, expression and phosphorylation of p53 and p21 (a downstream target of p53) were analyzed. Capsaicin treatment induced a small but significant elevation in the phosphorylation of p53 at serine 15, as well as induced p53 accumulation that
remained elevated up to 24 h. The level of p53 phosphorylation following DHC treatment was markedly increased in a time-dependent manner. Despite the accumulation of p53, treatment with DHC did not affect the level of p21 accumulation up to 6 h, but the level of p21 greatly increased after the initial 6-hour period (Fig. 1B). To determine further if DHC and capsaicin induce cell cycle arrest, cells were treated with 200 μM DHC or capsaicin for 18 h and flow cytometric analysis was performed after PI staining. In capsaicin-treated cells, the percentage of the M1 portion representing apoptotic cells did not change and cell cycle arrest showed in G0/G1 phase that was more potent than DHC. DHC-treated cells also showed a slight increase in the M1 DNA content (Fig. 1C). Therefore, these results suggest that the cytotoxicity induced by capsaicin or DHC, in part, was caused by cell cycle arrest.

**DHC induces massive cytoplasmic vacuolization.** When WI38 cells were treated with 200 μM DHC, massive cytoplasmic vacuoles were observed on phase-contrast microscopy. After 6 h of DHC treatment, the vacuoles were first observed (arrows) and nuclear detachment from the nuclear membrane in many cells was observed (arrowheads). The number of vacuoles increased
with time and peaked at 12 h. After 12 h, most of cells shrank as seen by the presence of distinctive large vacuoles and the cells ultimately died (Fig. 2A). Very few cytoplasmic vacuoles were seen in the capsaicin-treated cells (data not shown).

Ultrastructural analysis using transmission electron microscopy (TEM) was performed to elucidate further the morphological changes induced by capsaicin or DHC after 6 h of treatment. Non-treated cells were seen with a normal appearance of the mitochondria and rough endoplasmic reticulum (rER) (Fig. 2B, a). In cells treated with capsaicin, most of the mitochondria remained intact. The rER was mostly intact, but some of the organelles were ballooned and were likely to become autophagosome vacuoles. It was observed that a vacuolar structure was surrounded by a double-membrane structure resembling the rER after capsaicin treatment (Fig. 2B, b). Consistent with the results of phase-contrast microscopy, large cytoplasmic vacuoles were observed in the DHC-treated cells. In the DHC-treated cells, two types of large vacuoles were observed—one type originated from the dilated rER and the other type originated from the mitochondria. Most of the rER were dilated like hollow tubes
or were ballooned at one end (arrowhead). Most of the mitochondria were also dilated and severely damaged as determined by breakage of cristae, and ultimately remained as hollow structures or structures lined with an electron dense material that appeared as broken cristae (Fig. 2B, c, arrows). These findings suggest that the cytoplasmic vacuoles may have originated from the rER and mitochondria. Damage of both cell organelles was much greater after treatment with DHC than with capsaicin.

**Classic ER stressors induce the UPR and autophagy in WI38 cells.** To investigate if conventional ER stress inducers elicit the UPR as well as autophagy in WI38 cells, cells were treated with 3 μm tunicamycin (TM), 0.5 μM thapsigargin (TG), 1 μM A23187, 1 μM MG132 and 200 μM DHC. The level of ER-stress-related proteins, as well as the level of LC3II protein as an autophagy marker, was determined by immunoblot analysis. As shown in Figure 3, treatment with all of the ER stressors and DHC upregulated expression of Bip and IRE1, and activated the ER-stress-mediated apoptotic proteins Chop and caspase-7. In particular, conversion of LC3I to LC3II was markedly induced in cells treated with TG and MG132, and the level of
conversion was comparable to that in cells treated with DHC. These results indicate that DHC may induce ER-stress and autophagy through a similar signaling pathway as the pathway induced by the classic ER stressors TG and MG132.

**DHC induces a greater extent of the UPR as compared to the use of capsaicin.** To determine the effect of capsaicin or DHC on the UPR, the expression of ER-stress-related proteins was examined. As shown in Figure 4, DHC treatment markedly increased the protein level of IRE1 at 1 h and the level further increased with time. Treatment with capsaicin also caused upregulation of IRE1 at 1 h and remained elevated during treatment, but the extent of upregulation was much smaller than as seen with DHC-treated cells. Another ER-stress sensor, PERK, is autophosphorylated and then phosphorylates the downstream target eIF2α. Treatment of cells with DHC strongly induced phosphorylation of eIF2α at 1 h and then the level of phosphorylation gradually decreased. For cells treated with capsaicin, phosphorylation of eIF2α began at 1 h and peaked at 6 h after treatment. Expression of ATF4, a downstream target of eIF2α, was upregulated in DHC-
and capsaicin-treated cells. Expression of Bip, an important target of ATF6, was markedly upregulated within 1 h of DHC treatment and the level of the protein remained elevated up to 12 h. Treatment with capsaicin was able to induce upregulation of Bip expression. These results indicate that DHC is a more potent agent to induce ER-stress than capsaicin.

The effects of DHC and capsaicin on ER-stress-mediated apoptosis. As mentioned above, when ER functions are severely damaged, apoptosis is induced to remove damaged cells. As Chop and caspase-12 (or caspase-4) are associated with ER-stress-mediated apoptosis, the expression levels of Chop, and caspase-4, caspase-7, and caspase-3 were analyzed with immunoblots. As shown in Figure. 5A, the basal level of Chop in WI38 cells was barely detected. Chop was induced within 1 h after DHC treatment and the level of the protein peaked at 6 h and then still remained elevated up to 12h. Expression of Chop was also induced within 1 h after capsaicin treatment, and the level of the protein increased in a time-dependent manner, but the expression level was much lower as compared to the level in cells treated with DHC. In humans, ER-stress-mediated apoptosis is regulated by caspase-4 and is independent of the
mitochondrial pathway. Treatment of cells with DHC caused highly sustained activation of caspase-4 as was evident by the determination of the level of its cleaved form, p19. Treatment with capsaicin also activated caspase-4 and caspase-7 but the activation level of both caspases was relatively low as compared to the level of caspase-4 and caspase-7 in DHC-treated cells. Activation of caspase-4 elicited the downstream target caspase-3 activation, and the activation level of caspase was higher in DHC-treated cells than in capsaicin-treated cells. These results indicate that the level of ER-stress correlates with ER-stress-mediated apoptosis.

To evaluate the susceptibility of cells treated with capsaicin or DHC to ER-stress, cells were treated with 50–300 µM capsaicin or DHC for 18 h and then the expression of ER-stress-related proteins was analyzed with immunoblots. As shown in Figure 5B, treatment with capsaicin or DHC induced concentration-dependent upregulation of IRE1, Bip, Chop, and phosphorylated eIF2a. In 300 µM DHC-treated cells, phosphorylated eIF2a markedly downregulated. However, the upregulation of these proteins was much higher in DHC-treated cells. These results indicate that DHC is a more potent inducer
of ER-stress than capsaicin in WI38 cells.

**Capsaicin and DHC induce ER-stress-mediated autophagy.** Recent findings indicate that ER-stress is a potent inducer of autophagy (Ogata et al., 2006). We examined if treatment of cells with capsaicin and DHC induce autophagy. When WI38 cells transfected with GFP-LC3 were treated with 200 μM DHC, re-localization of GFP-LC3 was observed at 4 h after DHC treatment, and the effect was completely blocked by treatment with the autophagy inhibitor 3MA. Pretreatment with BF1, an inhibitor of fusion between the autophagosome and lysosome, caused aggregation of GFP-LC3 and the aggregation was observed as large green florescent dots (Fig. 6A). Treatment with capsaicin also induced re-localization of GFP-LC3 (data not shown). To confirm further the formation of autolysosomes by DHC treatment, transfected cells with GFP-LC3 were treated with 200 μM DHC and were then stained with lysotracker Red. In the merged figure, overlapping was observed between the green and red staining (Fig. 6B). Next, the induction of autophagy by DHC and capsaicin was analyzed at the protein level. Conversion of LC3 from cytoplasmic LC3I to the membrane bound LC3II form occurred at 1 hr after treatment with capsaicin or DHC and the
membrane bound LC3II form still remained elevated until 24 h. However, the conversion level of LC3 II protein was much higher in cells treated with DHC than in capsaicin-treated cells (Fig. 6C). To demonstrate if the induction of LC3II protein was regulated by an autophagy gene, cells were transfected with siRNA directed against Atg5 and a control (non-specific) siRNA, and the cells were then treated with 200 μM DHC for 6 h. The use of the control siRNA did not affect the expression level of Atg5 after DHC-induction. However, transfection with Atg5 siRNA significantly reduced the Atg5 protein level as compared to the level in DHC-treated cells and subsequently lead to the reduction of LC3II (Fig. 6D).

To examine if DHC-induced autophagy is mediated by ER-stress, an siRNA directed against the Ire1 gene was utilized as the level of IRE1 was significantly increased after DHC treatment. Transfection with a control siRNA did not affect the level of IRE1 protein after DHC-induction. Knockdown of the Ire1 gene almost completely downregulated expression of IRE1 induced by DHC as determined by immunoblot analysis, and the knockdown of Ire1 further caused the downregulation of expression of Chop and LC3II. Interestingly, the
knockdown of *Ire1* enhanced caspase-3 activation compared to capsaicin alone treatment (Fig. 6E). These results indicate that DHC-induced autophagy in WI38 cells is linked to the ER-stress pathway and that DHC-induced autophagy may be regulated by IRE1.

**Roles of MAPK in the ER-stress and autophagy induced by DHC.** Previous studies have reported that MAPK has an important role in ER-stress-mediated autophagy (Ogata *et al.*, 2006; Urano *et al.*, 2000). Autophagy has also been shown to be regulated by the ERK 1/2 pathway (Zhu *et al.*, 2007; Aoki *et al.*, 2007). To define the involvement of MAPK in capsaicin- and DHC-induced autophagy, cells were treated with 200 μM capsaicin or DHC and harvested as indicated in Figure 7A. A time-course study of MAPK phosphorylation demonstrated that JNK, ERK and p38 were rapidly activated by treatment of cells with DHC or capsaicin. However, the extent of phosphorylation of the three kinds of MAPKs was greater in DHC-treated cells than in capsaicin-treated cells. Activation of ERK1/2 peaked at 0.5 h after capsaicin treatment and recovered to a baseline level within 1 h; for DHC-treated cells, the phosphorylation level of ERK1/2 markedly increased at 0.5 h and peaked at 1 h.
and was then highly sustained until 4 h. The profile of JNK1/2 activation was similar to that of ERK activation with the use of both DHC and capsaicin. There was no difference in the profile and extent of p38 activation in cells treated with capsaicin and DHC.

In order to investigate the role of MAPKs for ER-stress and autophagy induced by DHC, as well as by the classic ER stress inducers TG and MG132. Treatment with TG and MG132 showed a similar pattern as with DHC for the expression of ER-stress-related proteins and LC3 II; see Figure 3. Cells were pretreated with MAPK inhibitors (PD98059 for ERK and SP600125 for JNK) for 30 min. The cells were then continuously treated with 200 μM DHC for 1 h. As shown in Figure 7B, treatment with PD98059 failed to downregulate the expression of IRE1 and Chop induced by treatment of cells with DHC, but treatment with SP600125 significantly down-regulated the level of the proteins and eventually decreased the level of LC3II protein. Treatment with PD98059 failed to inhibit the activation of ER-stress-related proteins, but significantly reduced the DHC-induced LC3II protein level. Treatment with SP600125 alone or PD98059 alone did not affect on the expression level of IRE1, Chop, and
LC3II compared to those of non-treated control cells (data not shown).

To confirm the regulation of DHC-induced autophagy by JNK/ERK, cells were transfected with GFP-LC3 and then cells were pretreated with PD98059 and SP600125 for 30 min prior to treatment with 200 μM DHC for 4 h. In non-treated cells, GFP-LC3 showed diffuse staining with green florescence in the cytoplasm with a basal level of FGP-LC3 dots. However, DHC treatment severely induced re-localization of GFP-LC3 that was significantly blocked by treatment with SP600125 and PD98059 (Fig. 7C). These results indicate that DHC can induce IRE1-dependent and IRE1–independent autophagy through JNK and ERK activation, respectively.

**Blocking of DHC-induced autophagy increases apoptotic cell death that is completely inhibited by the pan-caspase inhibitor zVAD.** As autophagy is involved in both cell survival and cell death, we next attempted to define the role of autophagy induced by DHC treatment of cells. For this purpose, cells were treated with 5 mM 3MA and 40 μM zVAD for 30 min and were continuously treated with 200 μM DHC for 18 h. The treated cells were then observed by phase-contrast microscopy. As shown in Figure 8A, DHC
treatment caused cell shrinkage and resulted in a small number of rounding and floating cells. Treatment with 3MA, with or without DHC, enhanced the number of floating cells. However, when cells were pretreated with zVAD before the addition of DHC or DHC plus 3MA, the presence of floating cells was not observed. To investigate further the effect of autophagy blockage on the modulation of cell death, we measured caspase-3 activity that is a marker to distinguish apoptotic cell death from autophagy. The effect of 3MA on autophagy blocking was confirmed by the use of immunoblot analysis for the LC3II protein level (Fig. 8B). As shown in Figure 8C, caspase-3 activation was significantly increased by treatment of cells with DHC and was seen with more than a 2.5-fold increase in the level of the protein in the presence of 3MA. When cells were treated with 3MA without DHC, a 1.7-fold increase in caspase-3 activation was seen as compared to the untreated cells. Pretreatment of cells with 40 μM zVAD completely blocked the caspase-3 activation induced by DHC and 3MA plus DHC; the number of PI positive cells showed a 4-fold increase after treatment with DHC as compared to the untreated cells. However, treatment of cells with 3MA and DHC markedly increased the number of PI
positive cells by almost 5-fold as compared to treatment with DHC alone. The number of PI positive cells induced by DHC and DHC plus 3MA treatment was reduced to the level determined in untreated cells with the use of zVAD (Fig. 8D). These results indicate that blocking of DHC-induced autophagy can enhance apoptotic cell death.

**PI3k inhibitors induce autophagy response to capsaicin in an ERK regulation manner.** The PI3K/Akt/mTor/p70 pathway has an important role in autophagy induction (Aoki et al., 2007). In a time course study (Fig. 9A), the phosphorylation of Akt and mTor, a downstream target of Akt, were rapidly induced by capsaicin treatment. On the contrary, DHC treatment delayed the phosphorylation of Akt and mTor. Therefore, we examined the possibility that the Akt/mTor pathway is involved in autophagy induction using capsaicin-treated cells. To downregulate the Akt activation, cells were treated with the class PI3K inhibitors LY294002 (50 μM) or wortmannin (1 μM) for 30 min, then further treated with 200 μM capsaicin for 1 h. Treatment with both PI3K inhibitors completely blocked the phosphorylation of Akt and mTor, thereby resulting in upregulation of the LC3 II conversion. Interestingly, pretreatment
with LY294002 and wortmannin markedly increased the level of phosphorylated ERK as compared to treatment with capsaicin alone (Fig. 9B). Next, we examined whether ERK can regulate DHC-induced autophagy using ERK siRNA. Transfection with a control siRNA did not affect the level of phosphorylated ERK after DHC-induction. Knockdown of the ERK gene markedly downregulated LC3II conversion induced by DHC as determined by immunoblot analysis (Fig. 9C). These results indicate that activation of ERK is also associated with autophagy induction via the inhibition of the PI3K/Akt/mTor pathway by treatment with DHC.
Discussion

The ideal chemopreventive drug will kill malignant cells but not normal cells. In this context, the use of capsaicin has attracted attention as an excellent chemopreventive compound because of its selective cytotoxicity only in malignant cells (Zhang et al., 2003; Lo et al., 2005; Sánchez et al., 2006). Previous studies have suggested that capsaicin can activate differential pathways that can contribute to cell survival or cell death depending on the cell type, i.e., a normal or malignant cell. In the present study, we have shown that WI38 cells were more sensitive to DHC than capsaicin. The differential susceptibility of the cells to both compounds is associated with ER-stress-mediated apoptosis/autophagy that is regulated by the extent of JNK/ERK activation. Capsaicin and DHC caused an increase in the levels of Akt phosphorylation, suggesting that the ER-stress response induced by both compounds seems to be involved in cell survival. Therefore, the UPR and autophagy could play an important role in the prosurvival effect of capsaicin.

DNA damage induces DNA repair pathways or proapoptotic pathways that are regulated by p53. With a low amount of DNA damage, p53 induces cell...
cycle progression from G1 to S phase. However, extensive DNA damage induces apoptosis (Vousden and Lu, 2002). Capsaicin treatment induced p53-dependent p21 expression. Despite a high level of activation and accumulation of p53, DHC treatment did not affect on p21 expression up to 6 h, rather induced activation of caspase-7 and caspase-3. Although we did not show direct evidence for a downstream effect of p53 after DHC treatment, it is possible to speculate that DHC-induced p53 upregulation can regulate downstream pathways that triggers cell death rather than cell cycle arrest.

Recent studies have suggested the involvement of p53 in the autophagic signaling (Periyasam-Thanadan et al., 2008; Tasdemir et al., 2008). In our present study, knockdown of p53 markedly downregulated the level of LC3 II induced by DHC treatment (Supplementary Information, Fig.1), suggesting that p53 may be regulate DHC-induced autophagy.

It has reported that the use of capsaicin as an agonist of the transient receptor potential vanilloid 1, localized in the plasma membrane and ER membrane, caused ER-stress and cell death in HEK293 kung cells (Han et al., 2007; Thomas et al., 2007), suggesting that the effect of capsaicin on
cytotoxicity might be caused by ER-stress. In WI38 cells, the classic ER stress inducers elicited the UPR by the activation of Bip, IRE1 and Chop. Particularly, LC3II was markedly upregulated by treatment with TG and MG132, and a similar pattern was observed in DHC-treated cells, suggesting that DHC can induce ER-stress and autophagy via the same signaling pathway as the pathway induced by the ER-stress inducers. DHC-induced ER-stress was determined by the formation of massive cytoplasmic vacuoles. Under stress, cytoplasmic vacuolization represents the formation of dilated cytoplasmic organelles such as the ER, mitochondria and autophagosomes (Corcelle et al., 2006; Tiwari et al., 2006). The ultrastuctural findings indicated that DHC caused severe damage to mitochondria, as evidenced by the breakage of cristae. Cytoplasmic vacuoles in DHC-treated cells originated from the dilated rER and damaged mitochondria. However, in capsaicin treated cells, most of the mitochondria and rER showed a normal structure, but some of the rER were seen with dilation. Marked morphological differences between capsaicin-treated and DHC-treated cells can caused by the extent of ER-stress, as determined by the measured activation levels of Bip, IRE 1, eIF2A, ATF4, Chop, and caspase-
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4 as compared to cells treated with capsaicin, indicating that DHC can elicit a higher level of ER-stress response than capsaicin.

The process of autophagosome formation depends on several autophagy proteins (Shintani and Klionsky, 2004). By translational modification of LC3, LC3II (16 kDa) localizes exclusively in the autophagosomal membranes and have used as an autophagy marker (Kabeya et al., 2000). In this study, GFP-LC3 dots were observed in WI38 cells after DHC treatment. Pretreatment of cells with the autophagy inhibitor 3MA caused disappearance of the GFP-LC3 dots. Another autophagy inhibitor, BF1, induced formation of large green fluorescence punctae due to the accumulation of LC3II protein. The ultrastructural analysis showed that some cytoplasmic organelles were surrounded by dilated rER membranes, and ultimately, autophagosomes (Fig. 2B, b). DHC treatment upregulated Atg5 and knockdown of Atg5 downregulated Atg5 and LC3II, suggesting that DHC activates the autophagy pathway in WI38 cells.

Recent findings suggest that ER-stress is essential in autophagy (Ogata et al., 2006; Yorimitsu et al., 2006; Hoyer-Hansen and Jaattela, 2007). However, it
is not still clear if ER-stress-mediated autophagy contributes to prosurvival or prodeath. ER-associated protein degradation (ERAD) is the primary degradation system for misfolded proteins in the ER (Meusser et al., 2005). When the amount of unfolded protein in the ER lumen exceeds the ERAD capacity, cells are required to utilize an alternative pathway to mitigate ER-stress, and the best candidate for such a pathway is autophagy. One possible explanation for the prosurvival effect of autophagy is due to its ability to remove effectively misfolded proteins in the ER (Ogata et al., 2006; Ding et al., 2007). Furthermore, blocking of ERAD elicited autophagy and mitigation of ER-stress and ultimately protection of cells, suggesting that autophagy can substitute for ERAD function and protect cells against ER-stress. However, the differential effects of ER-stress on cell protection in cancer cells and normal cells have demonstrated. Autophagy induced by classic ER-stress inducers contributed to the mitigation of ER-stress and ultimately cell survival in HCT116 human colon cancer cells and DU145 prostate cancer cells, but the same stimuli induced cell death in normal human colon cells and non-transformed murine embryonic fibroblasts (Ding et al., 2007). With the UPR, autophagic activity was observed
after treatment with capsaicin or DHC that was regulated by the IRE1, suggesting that DHC-induced autophagy is linked by ER-stress. Furthermore, knockdown of IRE1 enhanced caspase-3 activation. Furthermore, when DHC-induced autophagy was blocked by 3MA treatment, the number of apoptotic cells and the level of caspase-3 activation were increased and these effects were completely inhibited by ZVAD treatment. Therefore, autophagy induced by capsaicin and DHC has a role in cell survival.

The PERK-elF2a and IRE1–JNK signaling pathways are known to regulate ER-stress-induced Autophagy (Ogata et al., 2006; Fujita et al., 2007). In DHC-treated cells, the activation of JNK/ERK was a rapid and highly sustained, and pharmacological blockade of JNK downregulated IRE1, Chop and LC3II induced by DHC treatment as well as by TG and MG132 treatment, suggesting that DHC can induce ER stress-mediated autophagy which might be regulated by JNK. In neuronal cell death, ERK-dependent autophagy has an important role (Zhu et al., 2007; Aoki et al., 2007). By contrast, ERK activation causes disturbance of the fusion between autophagosomes and lysosomes and ultimately results in inhibition of cell death by autophagy (Corcelle et al., 2006).
In the present study, despite ERK activation, DHC treatment induced conversion of LC3 and overlapping of lysotracker Red and green GFP-LC3 punctae, as well as pharmacological blockade or knockdown of ERK downregulated the level of LC3II. The regulation of autophagy by ERK was further confirmed by using PI3K inhibitors. PI3K inhibitors completely inactivated Akt and mTor and ultimately upregulated LC3II accompanied by ERK activation, indicating that ERK might be involved in autophagy induction via downregulation of Akt pathway. Therefore, DHC-induced ERK activation is involved in an autophagy-signaling pathway that is independent of the JNK pathway which contribute the differential selectivity between capsaicin and DHC. Although, in the current study, we did not determine at what step of the autophagy signaling pathway ERK is involved, the transient downregulation of Akt phosphorylation in DHC-treated cells may have a synergistic effect with the ER-stress pathway for autophagy induction, by which DHC can induce a higher level of autophagy than capsaicin. Capsaicin and DHC can also elicit survival signals through Akt phosphorylation, whether it is associated with ER-stress or not.
In the present study, although we have not investigated the effects of capsaicin treatment on malignant cells, the distinct effect of capsaicin on cell survival/death in normal and malignant cells can be caused by the activation of ER-stress and is currently being investigated. Our findings indicate that WI38 cells are more sensitive to DHC than capsaicin and the effect was associated with a high sensitivity to ER-stress-mediated apoptosis and autophagy. Both signaling pathways are regulated by JNK/ERK activation. To the best of our knowledge, this study provides the first evidence for an ER-stress response in capsaicin- and DHC-treated normal cells.

**Acknowledgments**

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**References**


Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. *Am J Pathol* **171**: 513-524.


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Footnotes

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Legends for Figures

Figure 1. Effects of DHC and capsaicin on cell proliferation and cell cycle arrest in WI38 cells. (A) Cells were treated with the indicated concentrations of capsaicin or DHC for 24 h, and viability was determined using an MTT assay. The data are expressed as the mean ± SD of the average from three independent experiments performed in triplicate. open circles, capsaicin; closed circles, DHC. *P < 0.05. (B) Cells were treated with 200 μM of DHC or capsaicin up to 24 h, and were harvested and lysed. The lysates were analyzed for p53 (Do-1), phospho-p53 (ser 15) and p21. β-actin was used as a loading control. (C) Cells were treated with 200 μM DHC or capsaicin for 18 h. The percentage of the apoptotic cells and cell cycle profile were measured by flow cytometry as described in the Materials and Methods. Sub-G1, apoptotic DNA fragmentation. The data are representative from three independent experiments.

Figure 2. Morphological changes induced by treatment with capsaicin and DHC in WI38 cells. (A) DHC induced massive cytoplasmic vacuolization. Cells were treated with 200 μM DHC and vacuole formation was examined by phase-
contrast microscopy. X200 (B) Electron micrographs of WI38 cells at 6 h treated with DMSO, 200 μM capsaicin or 200 μM DHC. (a) The control cells showed intact structures in the mitochondria and rER. (b) In the capsaicin-treated cells, most of the mitochondria (arrows) and rER (arrowheads) are intact. A vacuolar structure is surrounded with the double-membrane structure resembling the rER (star). (c) In DHC-treated cells, various size of vacuoles were seen. Some mitochondria were dilated and broken cristae lined following to the mitochondrial membrane (arrows). rER-resembling tubular structure ballooned at one end (arrowheads). Bar, 200 nm.

Figure 3. Induction of ER-stress-related proteins by classic ER stress inducers in WI38 cells. Cells were treated with 3 μm tunicamycin (TM), 0.5 μM thapsigargin (TG), 1 μM A23187, 1 μM MG132 and 200 μM DHC. The level of ER-stress-related proteins, as well as LC3II protein as an autophagy marker, was determined by immunoblot analysis.

Figure 4. Induction of ER-stress-related proteins by capsaicin and DHC in WI38
cells. Cells were treated with 200 µM of capsaicin or DHC for up to 12 h and were harvested. The level of IRE1, p-eIF2a, ATF4, and Bip was determined by immunoblot analysis.

Figure 5. Differential effects of capsaicin and DHC on ER-stress-mediated apoptosis in WI38 cells. (A) After cells were treated with 200 µM of capsaicin or DHC up to 24 h, lysates were prepared and analyzed for Chop, caspase-4, -7 and -3 by immunoblotting. β-actin was used as a loading control. * Nonspecific bands. (B) Cells were treated with 50, 100, 200, or 300 µM capsaicin or DHC for 18 h. The level of IRE1, p-eIF2a, Bip, and Chop was determined by immunoblot analysis.

Figure 6. DHC induced autophagy in WI38 cells. (A) Transfected cells with GFP-LC3 were pretreated with either 3MA (5 mM) or BaF1 (100 nM) for 1 h and were continuously treated with 200 µM DHC for 6 h. DHC treatment induced massive punctae of GFP-LC3, and the effect was completely inhibited by treatment with 3MA. Pretreatment of BaF1 further accumulated GFP-LC3 in
punctae as compared to cells treated with DHC due to blocking the formation of autolysosomes. (B) BaF1 inhibits formation of autolysosomes after treatment with DHC. Transfected cells with GFP-LC3 were pretreated with either BaF1 (100 nM) or vehicle (DMSO) for 1 h, treated with 200 µM DHC for 6 h, and then labeled with Lysotracker Red. In the resulting images obtained by the use of fluorescence microscopy (X400), the green fluorescence indicates LC3 localized in autophagosomes and the red fluorescence indicates the Lysotracker Red lysosomal stain. A merged image is also shown. (C) Capsaicin or DHC induced conversion of LC3 from the LC3I form to LC3II form. The extent of LC3 conversion was greater after treatment of cells with DHC than with capsaicin. (D) Silencing of the atg5 gene induced downregulation of LC3II protein expression. Silencing of the atg5 gene was confirmed by immunoblot analysis. Transfection with nonspecific (NS) siRNA did not affect expression of Atg5 as compared to the parental control cells, whereas knockdown of atg5 gene markedly attenuated the level of Atg5 protein. Cells transfected with control or atg5-specific siRNA were treated with DHC (200 µM) for 6 h and LC3II protein expression was determined by immunoblotting. Induction of LC3II
expression by DHC treatment was markedly downregulated in cells transfected with \textit{atg5}-specific siRNA as compared with control cells transfected with nonspecific RNA. (E) Silencing of the \textit{ire1} gene downregulated Chop and LC3II protein expression and enhanced caspase-3 activation. Transfection with nonspecific (NS) siRNA did not affect expression of \textit{ire1} as compared to the control cells. Cells transfected with control or \textit{ire1}-specific siRNA were treated with DHC (200 µM) for 6 h and then Chop, LC3II, and caspase-3 were determined. The immunoblots shown are representative of at least three independent experiments.

Figure 7. Activation of MAPK by capsaicin and DHC in WI38 cells. (A) Cells were treated with 200 µM of capsaicin or DHC for up to 4 h, harvested, and then analyzed the extent of phosphorylation of ERK, JNK and p38 by immunoblot analysis. (B) The effect of JNK and ERK on ER-stress and autophagy induced by DHC treatment. Cells were treated with SP600125 (10 µM) and PD98059 (10 µM) for 30 min and were then continuously treated with vehicle (DMSO), DHC (200 µM), TG (0.5 µM) or MG132 (1 µM) for 1 h,
harvested. The level of IRE1, Chop, LC3 and β-actin were determined by immunoblot analysis. DM, DMSO; PD, PD98059; SP, SP600125. (C) Transfected cells with GFP-LC3 were pretreated with vehicle, SP600125 (10 μM) and PD98059 (10 μM) for 30 min and treated with 200 μM DHC for 6 h. DHC treatment induced massive punctae of GFP-LC3, and the effect was completely inhibited by treatment with SP600125 and PD98059.

Figure 8. The effect of 3MA and zVAD treatment on DHC-induced death of WI38 cells. (A) Cells were pretreated with 3MA (5 mM) or zVAD (40 µM) for 1 h before the addition of DHC (200 μM). After 18 h, cells were observed by the use of phase-contrast microscopy. Representative photomicrographs for each treatment (X200). (B) Pretreatment with 3MA markedly attenuated DHC-induced LC3 conversion. Cells were pretreated with 3MA (5 mM) for 1 h before the addition of 200 μM DHC for 18 h, harvested, and levels of LC3II. (C) The effect of the blocking of autophagy or apoptosis on DHC-induced caspase-3 activation in WI38 cells. Cells were treated as described in (A), and caspase-3-like activity was measured as described in Materials and Methods. DHC-
induced caspase-3-like activation was enhanced by the pretreatment of cells with 3MA, and caspase-3-like activation was completely blocked by pretreatment with zVAD. Results are expressed as the mean ± SD of the fold-increase in activity as compared with non-treated controls from three independent experiments. **P < 0.01. (D) Cells were treated as described in (A) and were stained with Hoechst 33342 (5 μg/ml) and propidium iodide (1 μg/ml). Cells with fragmented or condensed DNA were counted as apoptotic cells. Values are expressed as the mean ± SD of the average percentage increase over non-treated control cells from three independent experiments. **P < 0.01.

Figure 9. Activation of Akt/mTor by capsaicin and DHC and role of ERK on autophagy induction. (A) Activation of Akt by capsaicin and DHC. Cells were treated with 200 μM of capsaicin and DHC, harvested at the indicated time points on the figure, and the lysate were subjected to immunoblotting for analysis the level of phosphorylation of Akt and mTor. The activation of Akt and mTor delayed in DHC-treated cells than in capsaicin-treated cells. (B) Inhibition of the PI-3K pathway enhances the capsaicin-induced autophagy level and is
dependent on ERK activation. Cells were treated with wortmannin (1 μM) and LY294002 (20 μM) for 30 min and were further treated with 200 μM capsaicin for 1 h. Cells were then harvested and the extent of phosphorylation of AKT, mTor, ERK and LC3II was determined. (C) Knockdown of ERK downregulated the level of LC3II protein. Cells were transfected with ERK-specific siRNA or nonspecific (NS) siRNA and treated with DHC (200 µM) for 2 h and then analyzed ERK and LC3II by immunoblotting. Transfection with nonspecific (NS) siRNA did not affect expression of ERK as compared to the control cells.
Figure 1

A

Viability (% of control) vs Concentrations (μM)

B

P-p53 (ser15)
p53 (Do-1)
p21
β-actin

Control
Capsaicin
DHC

C

Cell Cycle Analysis

Sub-G1: 2.02
G1: 47.2
S: 16.3
G2M: 25.01

Sub-G1: 1.99
G1: 56.27
S: 15.73
G2M: 24.28

Sub-G1: 6.31
G1: 51.11
S: 16.38
G2M: 26.91
Figure 2

A

B
Figure 3

[Image of a gel showing protein bands for Bip, IRE-1, Chop, Cleaved caspase-7, LC3 II, and β-actin under various conditions.]
Figure 4

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Figure 5

A

Chop
Cleaved caspase-7
Cleaved caspase-3
β-actin

Capsaicin DHC
0 1 3 6 12 24

B

DMSO Capsaicin DHC
0 50 100 200 300 50 100 200 300

IRE1 Bip Chop P-eIF2α β-actin
Figure 6AB

A

DMSO  DHC

3MA + DHC  BF 1 + DHC

B

GFP-LC3  Lysotracker Red  Merge
Figure 6C-E
Figure 7

A

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B

Control | DHC | TG | MG132
---------|-----|----|------
DM | SP | PD | DM | SP | PD | DM | SP | PD
IRE 1   |     |     |     |     |     |     |     |     |
Chop    |     |     |     |     |     |     |     |     |
LC3     |     |     |     |     |     |     |     |     |
β-actin |     |     |     |     |     |     |     |     |

C

DMSO | DHC
--- | ---

SP600125+DHC | PD98059+DHC
Figure 8

A

Control  DHC  3MA

3MA+DHC  zVAD+DHC  zVAD+3MA+DHC

B

Cont  DHC  3MA

LC3 II  β-actin

C

D

Caspase-3 like activity (% of control)

Number of apoptotic cells

Cont  DHC  3MA

zVAD  DHC  3MA

**  **  **
Figure 9

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B

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