Endoplasmic Reticulum Stress-Mediated Autophagy/Apoptosis Induced by Capsaicin (8-Methyl-N-vanillyl-6-nonenamide) and Dihydrocapsaicin is Regulated by the Extent of c-Jun NH2-Terminal Kinase/Extracellular Signal-Regulated Kinase Activation in WI38 Lung Epithelial Fibroblast Cells

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ABSTRACT

Endoplasmic reticulum (ER) stress causes cell survival or death, which is dependent on the type of cell and stimulus. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) and its analog, dihydrocapsaicin (DHC), induced caspase-3-independent/-dependent signaling pathways in WI38 lung epithelial fibroblasts. Here, we describe the molecular mechanisms induced by both chemicals. Exposure to capsaicin or DHC caused induction of p38, p21, and G2/M arrest. DHC induced massive cellular vacuolization by dilation of the ER and mitochondria. Classic ER stress inducers elicited the unfolded protein response (UPR) and up-regulation of microtubule-associated protein 1 light chain-3 (LC3) II. DHC induced ER stress by the action of heavy chain-binding protein, IRE1, Chop, eukaryotic initiation factor 2α, 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. Blocking of DHC-induced autophagy by 3MA enhanced apoptotic cell death that was completely inhibited by treatment of cells with benzyl-oxcarbonyl-Val-Ala-Asp-fluoromethyl ketone. Knockdown of Ire1 down-regulated the DHC-induced Chop and LC3II and enhanced caspase-3 activation. DHC induced rapid and high-sustained c-Jun NH2-terminal kinase (JNK)/extracellular signal-regulated kinase (ERK) activation, but capsaicin induced transient activation of JNK/ERK. The JNK inhibitor SP600125 down-regulated the expression of IRE1, Chop, and LC3II induced by DHC, thapsigargin, and MG132 [N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal]. Pharmacological blockade or knockdown of ERK down-regulated LC3II. Capsaicin and DHC induced Akt phosphorylation, and the phosphatidylinositol 3-kinase inhibitors, wortmannin and LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride], 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.

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; DHC, dihydrocapsaicin; ER, endoplasmic reticulum; UPR, unfolded protein response; ATF, activating transcription factor; eIF2α, eukaryotic initiation factor 2α; ERAD, ER-associated degradation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; zVAD, benzyl-oxcarbonyl-Val-Ala-Asp-fluoromethyl ketone; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PI, propidium iodide; GFP, green fluorescent protein; LC3, microtubule-associated protein 1 light chain-3; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase; rER, rough endoplasmic reticulum; TG, thapsigargin; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; 3MA, 3-methyladenine; PD98059, 2′-amino-3′-methoxyflavone; PI3K, phosphatidylinositol 3-kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; BaF1, bafilomycin A1; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone; Bip, heavy chain-binding protein; IRE1, mosiul requiring 1; PERK, RNA-dependent protein kinase-like endoplasmic reticulum eIF2α kinase.

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 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 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 p38, p21, and G2/M arrest. DHC induced massive cellular vacuolization by dilation of the ER and mitochondria. Classic ER stress inducers elicited the unfolded protein response (UPR) and up-regulation of microtubule-associated protein 1 light chain-3 (LC3) II. DHC induced ER stress by the action of heavy chain-binding protein, IRE1, Chop, eukaryotic initiation factor 2α, 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. Blocking of DHC-induced autophagy by 3MA enhanced apoptotic cell death that was completely inhibited by treatment of cells with benzyl-oxcarbonyl-Val-Ala-Asp-fluoromethyl ketone. Knockdown of Ire1 down-regulated the DHC-induced Chop and LC3II and enhanced caspase-3 activation. DHC induced rapid and high-sustained c-Jun NH2-terminal kinase (JNK)/extracellular signal-regulated kinase (ERK) activation, but capsaicin induced transient activation of JNK/ERK. The JNK inhibitor SP600125 down-regulated the expression of IRE1, Chop, and LC3II induced by DHC, thapsigargin, and MG132 [N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal]. Pharmacological blockade or knockdown of ERK down-regulated LC3II. Capsaicin and DHC induced Akt phosphorylation, and the phosphatidylinositol 3-kinase inhibitors, wortmannin and LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride], 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.

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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 analog, 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, X-box-binding protein-1 (XBPI), and activates several UPR target genes. PERK phosphorylates eIF2α to block translation. Activation of ATF6 increases transcription of ER chaperones, including BiP/GRP78, which 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 (Meusser et al., 2005). As a consequence, 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) and induction of Chop (Morishima et al., 2002; Rao 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, whereas 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 and are 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 seem 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. In contrast, autophagy that was induced by the same chemicals contributed to cell death in nontransformed cells (Ding et al., 2007a). 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 analog DHC.

Materials and 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-methyladenine, and 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Benzyl-oxcarbonyl-Val-Ala-Aspfluoromethyl ketone (zVAD) and caspase-3 substrate (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide) were purchased from BIONOL Research Laboratories (Plymouth Meeting, PA) and Calbiochem (San Diego, CA), respectively. Other chemicals used were of the purest grade available from Sigma-Aldrich.

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 to 1 x 10⁵ cells/ml. A 200-μl sample of the cell suspension was seeded onto a 48-well plate. After culturing over-night, 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 at 570 nm using an enzyme-linked immunosorbent assay microplate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA).

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 ice-cold PBS buffer and resuspended in 1 ml of PBS buffer containing 500 μg/ml propidium iodide (PI). At least 10,000 events were analyzed by a FACScan (BD FacsCalibur and CellQuest software (Macintosh, Facstan); BD Biosciences, San Jose, CA). The percentage of nuclei with hypodiploid content was evaluated as apoptotic cells and also calculated as percentage of cells in each cell cycle phase (G1/G0, 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 postfixed 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, Japan).

Transfection. Dr. Xiao-Ming Yin (University of Pittsburgh School of Medicine, Pittsburgh, PA) 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'-CGUCGGCGCCUGGGAAAUU-3' and 5'-AAUUCGGCGCCUGGGCAG-3'), Atg5 (5'-GGACGAAUUCACUUGUUAU-3' and 5'-AACAGGUUGAAUUUCGUCC-3'), ERK (5'-GCAUGAAGGACAUUGCGUCAU-3' and 5'-UAAGACGAAUUGCUAUUGC-3'), or control siRNA for enhanced green fluorescent protein (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 NaN3, 2 mM phenylmethylsulfonyl fluoride, 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 polyvinylidene difluoride membrane and then immunoblotted with the corresponding antibodies. Anti-Atg8/LC3 antibody was obtained from Absent (San Diego, CA). 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 Inc. (Danvers, MA). Antibodies against Chop, ATF4, ATF6, PERK, Atg5, β-actin, caspase-3, and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-caspase-4 was obtained from Abcam plc (Cambridge, UK). Immobilized proteins were incubated with goat anti-mouse IgG and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and signals were detected using a chemiluminescence kit (GE Healthcare, Chalfont St. Giles, 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 ± S.D.

**Results**

**Antiproliferative Effect of Capsaicin and DHC on WI38 Cells.** Capsaicin and DHC constitute over 90% 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 Fig. 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 determine whether the cytotoxic effect by capsaicin or DHC is associated with cell cycle arrest, the expression of proteins that regulate cell cycle progression was analyzed on immunoblots. Cells were treated with 200 μM capsaicin or DHC up to 24 h. Because 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 and induced p53 accumulation that remained elevated up to 24 h. The level of p53 phosphorylation after 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-h period (Fig. 1B). To determine further whether 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 population 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 the 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 was performed to elucidate further the morpholog-
ical changes induced by capsaicin or DHC after 6 h of treatment. Nontreated cells were seen with a normal appearance of the mitochondria and rough endoplasmic reticulum (rER) (Fig. 2Ba). 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. 2Bb). 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 rERs were dilated like hollow tubes or were ballooned at one end (arrowhead). Most of the mitochondria were 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. 2Bc, 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 whether conventional ER stress inducers elicit the UPR and autophagy in WI38 cells, cells were treated with 3 μM tunicamycin, 0.5 μM thapsigargin (TG), 1 μM A23187 (Ding et al., 2007a), 1 μM MG132, and 200 μM DHC. The level of ER stress-related proteins and the level of LC3II protein as an autophagy marker were determined by immunoblot analysis. As shown in Fig. 3, treatment with all of the ER stressors and DHC up-regulated 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 with 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 Compared with 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 Fig. 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 up-regulation of IRE1 at 1 h and remained elevated during treatment, but the extent of up-regulation 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 up-regulated in DHC- and capsaicin-treated cells. Expression of Bip, an important target of ATF6, was markedly up-regulated 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 up-regulation 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. Because Chop and caspase-12 (or caspase-4) are associated with ER stress-mediated apoptosis, the expression levels of Chop and caspase-4,-7, and -3 were analyzed with immunobLOTS. As shown in Fig. 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 12 h. 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 compared with 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 -7, but the activation level of both caspases was relatively low compared with the level of caspase-4 and -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 to 300 µM capsaicin or DHC for 18 h, and then the expression of ER stress-related proteins was analyzed with immunobLOTS. As shown in Fig. 5B, treatment with capsaicin or DHC induced concentration-dependent up-regulation of IRE1, Bip, Chop, and phosphorylated eIF2α. In 300 µM DHC-treated cells, phosphorylated eIF2α was markedly down-regulated. However, the up-regulation 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 whether treatment of cells with capsaicin and DHC induces autophagy. When WI38 cells transfected with GFP-LC3 were treated with 200 µM DHC, relocation 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 bafilomycin A1 (BaF1), an inhibitor of fusion between the autophagosome and lysosome, caused aggregation of GFP-LC3, and the aggregation was
observed as large green fluorescent dots (Fig. 6A). Treatment with capsaicin also induced relocalization 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 h 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 whether the induction of LC3II protein was regulated by an autophagy gene, cells were transfected with siRNA directed against Atg5 and a control (nonspecific) 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 compared with the level in DHC-treated cells and subsequently led to the reduction of LC3II (Fig. 6D).

To examine whether DHC-induced autophagy is mediated by ER stress, an siRNA directed against the Ire1 gene was utilized because 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 down-regulated expression of IRE1 induced by DHC as determined by immunoblot analysis, and the knockdown of %Ire1% further caused the down-regulation of expression of Chop and LC3II. It is interesting that the knockdown of %Ire1% enhanced caspase-3 activation compared with 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 (Urano et al., 2000; Ogata et al., 2006). Autophagy also has been shown to be regulated by the ERK1/2 pathway (Aoki et al., 2007; Zhu et al., 2007). To define the involvement of MAPK in autophagy in capsaicin- and DHC-induced autophagy, cells were treated with 200 μM capsaicin or DHC and harvested as indicated in Fig. 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.

The roles of MAPKs for ER stress and autophagy induced by DHC and by the classic ER stress inducers were investigated next. Treatment with TG and MG132 showed a similar pattern as with DHC for the expression of ER stress-related proteins and LC3 II (see Fig. 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 Fig. 7B, treatment with PD98059 failed to down-regulate 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 with those of nontreated 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 before treatment with 200 μM DHC for 4 h. In nontreated cells, GFP-LC3 showed diffuse staining with green fluorescence in the cytoplasm with a basal level of FGP-LC3 dots. However, DHC treatment severely induced relocalization of LC3 and LC3II, and treated with 200 μM capsaicin or DHC for up to 4 h, harvested, and then analyzed for the extent of phosphorylation of ERK, JNK, and p38 by immunoblot analysis. B, 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 and harvested. The levels 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 punctate of GFP-LC3, and the effect was completely inhibited by treatment with SP600125 and PD98059.

Fig. 7. Activation of MAPK by capsaicin and DHC in WI38 cells. A, cells were treated with 200 μM capsaicin or DHC for up to 4 h, harvested, and then analyzed for the extent of phosphorylation of ERK, JNK, and p38 by immunoblot analysis. B, 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 and harvested. The levels 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 punctate of GFP-LC3, and the effect was completely inhibited by treatment with SP600125 and PD98059.
GFP-LC3 that was significantly blocked by treatment with SP600125 and PD98059 (Fig. 7C). These results indicate that DHC can induce IRE1-dependent and -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. Because 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 Fig. 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, which is a marker to distinguish apoptotic cell death from autophagy. The effect of 3MA on autophagy blockage was confirmed by the use of immunoblot analysis for the LC3II protein level (Fig. 8B). As shown in Fig. 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 compared with 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 compared with the untreated cells. However, treatment of cells with 3MA and DHC markedly increased the number of PI-positive cells by almost 5-fold compared with 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 PI3K/Akt, was 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 down-regulate 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 up-regulation of the LC3 II conversion. It is interesting that pretreatment with LY294002 and wortmannin markedly increased the level of phosphorylated ERK compared with 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 down-regulated 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, which is regulated by the extent of JNK/ERK activation. Capsaicin and DHC caused an increase in the levels of Akt phosphorylation and LC3II conversion. 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 the 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, it induced activation of caspase-7 and -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 up-regulation can regulate downstream pathways that trigger cell death rather than cell cycle arrest. Recent studies have
suggested the involvement of p53 in the autophagic signaling (Periyasamy-Thanadan et al., 2008; Tasdemir et al., 2008). In our present study, knockdown of p53 markedly down-regulated the level of LC3 II induced by DHC treatment (Supplemental Fig. 1), suggesting that p53 may be regulated by DHC-induced autophagy.

It has been 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. In particular, LC3II was markedly up-regulated by treatment with TG and MG132, and a similar 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 (Corcellé et al., 2006; Tiwari et al., 2006). The ultrastructural 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- and DHC-treated cells can be caused by the extent of ER stress, as determined by the measured activation levels of Bip, IRE 1, eIF2α, ATF4, Chop, and caspase-4 compared with 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 has been 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, BaF1, induced formation of large green fluorescence punctate because of 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 up-regulated Atg5, and knockdown of Atg5 down-regulated 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; Hayer-Hansen and Jäättelä, 2007). However, it is not still clear whether ER stress-mediated autophagy contributes to pro-survival or prodeath. 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., 2007b). 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 been reported. 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 nontransformed murine embryonic fibroblasts (Ding et al., 2007a). 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-eIF2α 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, highly sustained, and pharmacological blockade of JNK down-regulated IRE1, Chop, and LC3II induced by DHC treatment and 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 (Aoki et al., 2007; Zhu et al., 2007). In contrast, ERK activation causes disturbance of the fusion between autophagosomes and lysosomes and ultimately results in inhibition of cell death by autophagy (Corelle 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 punctate, and pharmacological blockade or knockdown of ERK down-regulated 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 up-regulated LC3II accompanied by ERK activation, indicating that ERK might be involved in autophagy induction via down-regulation of the Akt pathway. Therefore, DHC-induced ERK activation is involved in an autophagy signaling pathway that is independent of the JNK pathway, which contributes to 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 down-regulation 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 capsaiacin, 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 capsaiacin- and DHC-treated normal cells.

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References


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