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이 논문을 의학박사학위신청 논문으로 제출함.

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목 차

도목차

ER Stress-Mediated Autophagy/Apoptosis Induced by Capsaicin and Dihydrocapsaicin in WI38 Cells

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Endoplasmic reticulum (ER) stress can cause cell survival or cell death depending on the type of cell and stimulus. Capsaicin and its analogue dihydrocapsaicin (DHC) induced caspase-3 independent/dependent signaling pathways in WI38 fetal lung fibroblast. Here I describe the molecular mechanisms of the capsaicin and DHC-induced signaling pathways in WI38 cells.

Capsaicin and DHC caused upregulation of p53 and $p21^{waf/cip}$. Morphologically, DHC induced massive cellular vacuolization by dilation of the ER and mitochondria. Classic ER stress inducers elicited unfolded protein response (UPR) as well as upregulation of LC3II. DHC induced potent ER stress by the action of Bip, IRE1, Chop, eIF2a and caspase-4 activation; to a lesser degree by capsaicin. Treatment of 3-methyladenine (3MA) and bafilomycin A1 (BaF1) blocked DHC –induced upregulation of LC3 II and redistribution of GFP-LC3. Knockdown of *Ire1* downregulated Chop and LC3II induced by DHC. DHC induced rapid and high-sustained JNK/ERK activation, but capsaicin caused transient activation of JNK/ERK. The JNK inhibitor SP600125, thapsigargin and MG132 downregulated IRE1, Chop, and LC3 II induced by DHC. The ERK inhibitor PD98059 downregulated only LC3II. Blocking of DHC-induced autophagy by 3MA induced a high level of apoptotic cell death that was completely inhibited by the pancaspase inhibitor zVAD. Capsaicin and DHC increased the level of Akt phosphorylation with time, and the phosphatidylinositol 3-kinase (PI3K) inhibitors, wortmannin and LY294002, caused the induction of autophagy via ERK activation. The results indicate that the differential responses of capsaicin and DHC in cell protection are caused by the degree of the UPR and autophagy that are both regulated by the extent of JNK1/2 and ERK1/2 activation.

Introduction

Capsaicinoids are a complex of pungent ingredients found in the red pepper of the genus *capsicum* and representative compounds include capsaicin and dihydrocapsaicin (DHC) that are included in over 80% of pepper extracts. It has known that capsaicin selectively induces apoptotic cell death in malignant cells, but not in normal cells. The selectivity of capsaicin is ascribed to the sensitivity of the plasma membrane electron transport (PMOR) system to capsaicin in tumor cell lines and transformed cells (Morre *et al*., 1995; Macho *et al*.,1999), indicating that reactive oxygen species (ROS) have a role in capsaicin-induced apoptosis. In another study, exposure to capsaicin induced apoptosis in *r*as-transformed (H-ras MCF10A) human breast epithelial cells, but not in its parental MCF10A cells, and apoptosis was regulated by 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 by capsaicin are still unclear. Furthermore, these previous studies have underscored the prosurvival effect of capsaicin on normal cells. Therefore, it is of interest to explore the effect of capsaicin on normal cells for a use as a chemopreventive agent. In our previous report, capsaicin as well as its analogue DHC was shown to induce caspase-3 independent programmed cell death, autophagy, in HCT116 human colon cancer cells and MCF-7 human breast cancer cells. However, in WI38 normal fetal lung fibroblasts, it was found that DHC, but not capsaicin, greatly induced caspase-3 activation. I thus investigated the molecular mechanisms underlying the effects of capsaicin and DHC on cell survival

and cell death in WI38 cells.

The endoplasmic reticulum (ER) performs several functions including protein folding and trafficking, and the regulation of intracellular calcium. Upon disruption of the ER functions by accumulation of unfolded or misfolded proteins in the ER lumen, cells trigger unfolded protein response (UPR) as a self-protective mechanism (Schröder and Kaufman, 2005; Ma and Hendershot, 2004). The UPR enhances a protein folding activity through the upregulation of ER chaperones and inhibition of protein synthesis. Under normal conditions, the ER-resident stress sensor proteins IRE1, PERK and ATF6 reside in inactive forms by binding to Bip/GRP78. However, ER-stress causes the release of the ER-stress sensors from Bip/GRP78 and the transfer of downstream signals to the cytoplasm. Autophosphorylated IRE1 activates the downstream target XBP1 and in turn, activates several UPR target genes. Activated PERK phosphorylates eiF2a that reduces the protein load of the ER by blocking translation. Activation of ATF6 leads to increased transcription of ER chaperones, including BiP/GRP78 that is involved in stress mitigation. In addition, the UPR activates the ER-associated degradation (ERAD) system to maintain ER homeostasis. 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). Consequently, the UPR contributes to the reduction of ER-overload and to protection of the 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 in mice and caspase-4 in humans, and in turn,

activation of caspase-9, caspase-7 and caspase-3 in a cytochrome c independent manner (Nakagawa *et al*., 2000; Rao *et al*., 2001; Morishima *et al*., 2002). Chop is also involved in ER stress-mediated apoptosis (Zinszner *et al*., 1998; Oyadomari and Mori, 2004).

Autophagy is a cellular defense mechanism that occurs from the through degradation of cytoplasmic constituents and recycling of cellular constituents. Starvation-induced autophagy is known to have an important role in cell survival, while excessive autophagy can trigger cell death (Baehrecke, 2005; Codogno and Meijer, 2005). During autophagy, cytoplasmic constituents are sequestered into double-membrane vesicles (autophagosomes) which then fuse with lysosomes to form autolysosomes; eventually, the contents of the vesicles are degraded by lysosomal hydrolases. Autophagic cell death is thus characterized by the accumulation of vacuoles (Klionsky and Emr, 2000).

Accumulating evidence suggests that ER stress is linked to autophagy (Bernales *et al*., 2006; Ogata *et al*., 2006; Yorimitsu *et al*., 2006; Kouroku *et al*., 2007). 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 chemically induced 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 classic ER stress inducers could mitigate ER stress and ultimately cause cell protection. By contrast, autophagy that was induced by the same chemicals contributed to cell death in non-transformed cells (Ding *et al*., 2007a). However, it is still unclear whether ER-stress-mediated autophagy is involved in cell survival or cell death.

For a better understanding of the capsaicin-induced signaling pathway in normal cells, we have investigated the molecular mechanisms induced by capsaicin and its analogue DHC in WI38 cells. As the UPR response against ER stress is a cytoprotective mechanism, I have investigated whether the distinct signaling pathways induced by capsaicin and DHC in WI38 cells are associated with ER stress. I found that differential responses to ER stress were caused following treatment with capsaicin and DHC, and the responses were due to the extent of MAPK JNK/ERK activation. Capsaicin transiently activated JNK/ERK that contributed to cell survival. However, a sustained and high level of JNK/ERK activation triggered a signaling pathway that lead to cell death through ER stress-mediated apoptosis. Capsaicin and DHC also caused autophagy induction that seemed to counteract ER stress-mediated cell death. Therefore, dual roles of ER stress in cell survival and cell death might be regulated by the differential activation of MAPK. To the best of my knowledge, this study provides the first evidence for a critical step that leads to cell survival or cell death by ER stress in capsaicin and DHC-treated normal cells.

Materials and Methods

Cell cultures and chemicals. WI38 normal fetal lung fibroblasts 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% CO_2 - 95% air-humidified incubator. The compounds 3-metyladenine and MTT [3-(4,5-dimetylthiazole-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 × 10^5 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 at 570 nm using an ELISA microplate reader (Perkin-Elmer, Waltham, MA USA).

Measurement of caspase 3-like activity. In brief, a caspase-3 substrate (Ac-DEVD-pNA) was added to the cell lysates in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, pH 7.4) and was incubated for 4 h at 37 ^oC. Fluorescence signals were measured at 400/510 nm

excitation/emission, respectively.

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, Japan).

Flow cytometry and quantification of apoptotic cells. Cells were harvested and washed twice with cold PBS buffer. After fixation in 70% ethanol for 30 min at 4 ^{o}C, cells were washed with cold PBS buffer, and resuspended in 1 ml PBS buffer containing 500 µg/ml propidium iodide (PI). A FACScan (BD FacsCalibur and CellQuest software (Macintosh, Facstation; Becton Dickinson, Franklin Lakes, NJ USA) analyzed at least 10,000 events. The sub-G1 fraction (apoptotic) was estimated by gating hypodiploid cells of a non-treated control as a reference to compare with treated cells. Apoptotic cells were quantified after staining with Hoechst 33342 (5 µg/ml). Four independent fields were selected at random and cells with condensed or fragmented nuclei were counted.

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 *Ire 1* (5'-CUGCCCGGCCUCGGGAUUU-3' and 5'-AAAUCCCGAGGCCGGGCAG -3'), *Atg5* (5'-GGACGAAUUCCAACUUGUU-3' AND 5'-AACAAGUUGGAAUUCGUCC-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 Na_3VO_4, 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, phosphor-p53, mTor, and phosphor-mTor were purchased from Cell Signaling Technology (Danvers, MA USA). Anti-Chop and antibodies against ATF4, ATF6, PERK, Atg5, β-actin 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 WI 38 cells. To examine the cytotoxicity of DHC and capsaicin, WI38 cells were treated for 24 h with various concentrations of DHC and capsaicin as indicated in Fig. 1A, and cell viability was measured using an MTT assay. At 200 μM, the effect of capsaicin on WI38 was minimal, but DHC significantly decreased the viability, and it further decreased at 400 μM.

To examine the decrease of viability by both compounds is associated with cell cycle arrest, the expression of proteins that regulate cell cycle progression were analyzed by immunoblotting. Cells were treated with 200 μM capsaicin or DHC up to 24 h. As p53 is involved in cell cycle arrest and apoptosis, p53 and $p21^{waf/cip1}$, a downstream target of p53, were analyzed. A small but significant elevation of phosphorylation of p53 at serine 15 was rapidly induced, but gradually decreased after 6 h. Accumulation of p53 (Do-1) and upregulation of cyclin- dependent kinase $p21^{waf/cip1}$ were observed at 1 h, and the levels remained elevated up to 24 h. The level of p53 phosphorylation following DHC treatment was markedly increased in a time-dependent manner. Despite of accumulation of p53 (Do-1), DHC did not affect on the level of $p21^{waf/cip1}$ accumulation up to 6 h, but greatly increased after that (Fig.1B).

Next, to examine the apoptosis-inducing effect of DHC and capsaicin in WI38 cells, cells were treated with 200 μM capsaicin or DHC for times, up to 24 h. In DHC treated cells, caspase-3-like activity increased in a time dependent manner and reached a peak level at 12 h, whereas capsaicin induced significantly caspase-3 activation at 1 h,

but the level of caspase-3 decreased after 1 h and remained at a control level (Fig. 1C). To further determine whether DHC and capsaicin induce apoptotic cell death, cells were treated with 200 μM DHC and capsaicin for 24 h and performed a flow cytometric analysis after PI staining. In capsaicin-treated cells, percentage of sub-G1 portion representing apoptotic cells slightly increased. However, a sub-G1 content of DNA significantly increased by DHC treatment. These results indicate that differential effects of cell protection can be induced by treatment with capsaicin and DHC in WI38 cells.

DHC, but not capsaicin, 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, vacuoles were first observed, and nuclear detachment from the nuclear membrane in many cells was observed (Fig. 2B b, arrowheads). The number of vacuoles increased with time and peaked at 12 h. After 12 h, most of cells shrank seen with distinctive large vacuoles, and the cells ultimately died (Fig. 2A). Very few cytoplasmic vacuoles were seen in capsaicin-treated cells.

Ultrastructural analysis using transmission electron microscopy (TEM) was performed to elucidate further the morphological changes induced by capsaicin and DHC at 6 h of treatment. Control cells were seen with a normal appearance of the nucleus, mitochondria and rough endoplasmic reticulum (rER) (Fig. 2B, a). In cells treated with capsaicin, most of 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

the 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 DHC-treated cells, two types of large vacuoles were observed—one type originated from the dilated rER, the other type originated from the mitochondria. Most of the rER were dilated like hollow tubes or were ballooned at one end. 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 electron dense material that appeared as broken cristae (Fig. 2B, c). 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 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 Fig.3, 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 LC3 I 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 and 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. Capsaicin also caused upregulation of IRE1 at 1 h and the protein was maintained at a relatively high level during treatment, but the extent of upregulation was much smaller than seen with DHC-treated cells. Another ER stress sensor, PERK, is autophosphorylated and then phosphorylates the downstream target eIF2a. Treatment of cells with DHC strongly induced phosphorylation of eIF2a at 1 h and then the level of phosphorylation gradually decreased. For cells treated with capsaicin, phosphorylation of eIF2a began at 1 h and peaked at 6 h after treatment. Expression of ATF4, a downstream target of eIF2a, 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 could also induce upregulation of Bip expression. These results indicate that DHC is more potent in the induction of 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, levels of Chop, and caspase-4 and its downstream target caspase-7, and–3 were analyzed by immunoblotting. As shown in Fig. 5, the basal level

of Chop in WI38 cells was barely detected. Expression of Chop was significantly 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 then expression 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 also induced a high level of caspase-4 and caspase-7 activation at 1 h as determined by the presence of its cleaved form (p20) and then remained elevated up to 24 h. Cleaved forms (p18/16) of caspase-3 were detected at 6 h following DHC treatment. Treatment of cells with capsaicin activated caspase-4 and caspase-7, but relatively low as compared to those of DHC-treated cells and did not lead caspase-3 activation up to 24 h. These results indicate that the level of ER stress correlates with ER stress-mediated apoptosis.

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 capsaicin and DHC induce autophagy, When WI38 cells transfected with GFP-LC3 were treated with 200 μM DHC, relocalization 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 it was observed as large green florescence dots (Fig. 6A). Treatment with capsaicin also induced relocalization of GFP-LC3 (data not shown). To confirm further the formation

of autolysosomes by DHC, 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 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 I hr after treatment with capsaicin or DHC and still remained elevated until 24 h. However, the conversion level of LC3II protein was much higher in cells treated with DHC than in capsaicin-treated cells (Fig. 6C). To demonstrate whether the induction of LC3II protein is regulated by an autophagy gene, cells were transfected with siRNA against *Atg5* and a control siRNA, and cells were then treated with DHC for 6 h. The use of 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 then lead to the reduction of LC3II (Fig. 6D).

To examine whether DHC-induced autophagy is mediated by ER stress, we used siRNA against *Ire1* gene as the level of IRE1 was significantly increased after DHC treatment. Transfection with control siRNA did not affect the level of IRE1 protein after DHC-indution. Knockdown of *Ire1* gene almost completely downregulated expression of IRE1 induced by DHC as determined by immunoblot analysis, which further caused the downregulation of expression of Chop and LC3 II (Fig. 6E). These results indicate that DHC-induced autophagy in WI38 cells are linked to the ER stress pathway, and may be regulated by IRE1.

Roles of MAPK in ER stress and autophagy induced by capsaicin and 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 was also 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 of capsaicin and DHC and harvested as indicated in Figure 7A. A time-course study of MAPK phosphorylation showed that JNK, ERK and p38 were rapidly activated by treatment of cells with DHC and capsaicin. However, the extent of phosphorylation of the three kinds of MAPK 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 control level within 1 h, while in DHC-treated cells, the phosphorylation level of ERK1/2 was 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 MAPK in ER stress and autophagy induced by DHC, as well as by the classic ER stress inducers TG and MG132 (which showed a similar pattern as DHC in the expression of ER stress-related proteins and LC3 II; Fig. 3), cells were pretreated with MAPK inhibitors (PD98059 for ERK, SP600125 for JNK) for 30 min. The cells were then continuously treated with 200 μM DHC for 1 h. As shown in Figure 7B, PD98059 failed to downregulate the expression of IRE1 and Chop induced by treatment of cells with DHC, but SP600125 significantly

downregulated the level of the protein and eventually decreased the level of LC3 II protein. PD98059 failed to inhibit the activation of ER sress-related proteins, but significantly reduced the DHC-induced LC3II protein level. 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 non-treated control 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 relocalization of GFP-LC3 that was significantly blocked by 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. For this purpose, cells were treated 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 also 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 for distinguishing apoptotic cell death from autophagy. The effect of 3MA on autophagy blocking was confirmed by immunoblot analysis for the LC3 II 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 presence of 3MA. When cells were treated with 3MA without DHC, a 7-fold increase in caspase-3 activation was seen as compared to the untreated control. 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 by DHC compared to the control. 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 was reduced to the control level by zVAD (Fig. 8D). These results indicate that blocking of DHC-induced autophagy can enhance apoptotic cell death.

PI3k inhibitors induce the autophagy response to capsaicin in a ERK regulation manner. The PI3K/Akt/mTor/p70 pathway has an important role in autophagy induction (Arico *et al*., 2001; Takeuchi *et al*., 2005; Aoki *et al*., 2007). In a time course study (Fig. 9A), the phosphorylation of Akt and mTor, a downstream of Akt, were rapidly induced by capsaicin treatment. At 1 h of treatment, DHC did not affect on the level of Akt phosphorylation, rather decreased, and it caused dephosphorylation of mTor. After 3 h of DHC treatment, however, the phosphorylation level of Akt and mTor showed an increase with time. To examine the possibility that the Akt/ mTor pathway is

involved in autophagy induced by treatment of cells with capsaicin and DHC, capsaicin was used as a compound does not significantly affect the phosphorylation of Akt and LC3II protein after 1 h treatment. To examine whether the Akt/ mTor pathway is involved in in autophagy induced by treatment of cells with capsaicin, cells were treated with the class PI3K inhibitors LY294002 (50 μM) and wortmannin (1 μM) for 30 min, respectively. The cells were then further treated with 200 μM capsaicin for 1 h. Treatment with both PI3K inhibitors completely blocked the phosphorylation of Akt and its downstream target mTor, thereby resulting in upregulation of the expression of LC3II. Interestingly, pretreatment with LY294002 and wortmannin markedly increased the level of ERK phosphorylation as compared to treatment with capsaicin alone (Fig. 9B). These results indicate that activation of ERK is also associated with autophagy induction via the inhibition of the PI3K/Akt/mTor pathway by DHC.

Discussion

The ideal chemopreventive drug will kill malignant cells but not normal cells. In this context, capsaicin has attracted attention as an excellent chemopreventive compound because of its selective cytotoxicity only in malignant cells (Macho *et al*., 1999; Morre *et al*., 1995; Kang *et al*., 2003). Studies have demonstrated that capsaicin is capable of inducing apoptosis in several types of cancer cells (Zhang *et al*., 2003; Lee *et al*., 2004; Jung *et al*., 2001; Lo *et al*., 2005; Sánchez *et al*., 2006). These studies suggest that treatment of cells with 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. However, previous studies have underscored the molecular mechanisms underlying cell protection by capsaicin in normal cells. In the present study, we show that WI38 cells are more sensitive to DHC than capsaicin when both compounds are used at the same concentration. The differential susceptibility of the cells to both compounds is associated with ER stress-mediated apoptosis/autophagy that is regulated by the extent of MAPK JNK/ERK activation. Treatment of capsaicin and DHC to WI38 cells caused an increase of the levels of autophagy and Akt phosphorylation with time, suggesting that the ER stress response induced by capsaicin and DHC seems to be involved in the cell survival.

DNA damage causes induction of DNA repair program and proapoptotic program, which are regulated by p53. Under low degree of DNA damage, p53 induces cell cycle progression inhibitior $p21^{waf.cip21}$ and then control G1 to S phase entry. However, extensive DNA damage elicits apoptosis to demise the damaged cells (El-Deiry *et al*.,

1994; Vousden and Lu, 2002). We found that capsaicin induced p53-dependent p21 expression. Interestingly, despite high level of activation and accumulation of p53, DHC did not affect on the p21 expression, but induced a high level of caspase-7 and-3 activation. Although we did not show direct evidence for a downstream effect of p53 after DHC treatment, it is possible to speculate that p53 induction by DHC treatment can regulate downstream pathways which can trigger cell death rather than cell cycle arrest. Indeed, p53 regulates ER stress-mediated cell cycle arrest and apoptosis through the action of $p21^{waf.cip21}$, as well as the action of PUMA, NOXA, and bax (Li *et al*., 2006; Scorrano et al., 2003; Zhang *et al*., 2006), respectively. Furthermore, downregulation of $p21^{waf.cip21}$ is associated with autophagy induction (Yazbeck *et al*., 2008).Therefore, in this paper, we demonstrated that how autophagy or ER stress response is involved in the cell protection induced by capsaicin and DHC.

Previous studies have reported that capsaicin-induced cell death is associated with apoptosis in cancer cells as demonstrated by DNA fragmentation and caspase-3 activation, and ROS generation seems to be essential for triggering apoptosis (Morre *et al*., 1995; Jung *et al*., 2001; Zhang 2003; Lee *et al*., 2004; Lo *et al*., 2005). However, we did not observe ROS generation by treatment of cells with capsaicin and DHC but rather a decreased basal ROS level was seen up to 3 h after treatment (data not shown), suggesting that ROS generation may be dependent on cell type. Recent studies have demonstrated that the use of capsaicin as an agonist of the transient receptor potential vanilloid 1 (TRPV1 that is localized in the plasma membrane and ER membrane) caused ER stress and cell death in lung cells as well as protein synthesis inhibition in

HEK293 cells (Han *et al*., 2007; Thomas *et al*., 2007). These events were due to disturbance of Ca^{2+} homeostasis in the ER lumen, suggesting that the effect of capsaicin on cytotoxicity might be caused by ER stress.

ER stress can cause cell survival and cell death (Nakagawa *et al*., 2000; Rao, 2001; Morishima, 2002). Disruption of ER function induces accumulation of unfolded/misfolded proteins in the lumen and subsequently induces the UPR. The UPR reduces the protein load of the ER through an increase of protein folding activity, inhibition of protein synthesis and degradation of misfolded/unfolded proteins. ER stress can be induced pharmacologically by use of the Ca+ pump inhibitor TG, the N-linked glycosylation inhibitor TM, the calcium ionophore A23187 and the proteosome inhibitor MG132 (Rutkowski and Kaufman, 2004). In WI38 cells, ER stress inducers caused the UPR by the activation of Bip, IRE1 and Chop. Particularly, expression of LC3 II, an autophagy marker, was markedly upregulated in cells treated with TG and MG 132, and in DHC-treated cells, a similar pattern of the upregulation of the UPR-related proteins was observed. These results suggest that DHC can induce ER stress and autophagy via the same signaling pathway as the pathway induced by classic ER stress inducers. DHC-induced ER stress could be determined by the formation of massive cytoplasmic vacuoles. Under stress, cytoplasmic vacuolization represents the formation of dilated cytoplasmic orgarnelles such as the ER and mitochondria as well as an autophagosome (Ding *et al*., 2007b; Corcelle *et al*., 2006; Denoyelle *et a*l., 2006; Tiwari *et al*., 2006). Our ultrastuctural findings indicated that treatment of cells with 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.

Morphologically, a marked difference between capsaicin and DHC-treated cells was observed and we thus compared the expression level of ER stress-related proteins by both compounds. The treatment of cells with capsaicin and DHC induced the UPR through the activation of Bip, IRE 1, eIF2A, and ATF4. However, the level of the UPR was much greater in cells treated with DHC than with capsaicin as indicated by the expression level of ER-stress related proteins. ER stress-mediated apoptosis is known to be related with Chop induction as well as with caspase-12 (or caspase-4) activation (Oyamadori and Mori, 2004). Treatment of cells with DHC caused a high level of Chop induction and caspase-4 activation compared to those of cells treated with capsaicin, indicating that DHC was able to induce a higher level of ER stress response than capsaicin. This finding was further confirmed by varying the concentration of capsaicin. When cells were treated with 100–400 μM capsaicin for 24 h, the induction level of Chop after treatment with 300 μM capsaicin for 24 h was comparable that of the Chop level induced by 200 μM DHC for 6 h (data not shown). When the ER functions are severely disturbed, the cells trigger apoptosis to protect the organism (Rao *et al*., 2004).

The process of autophagosome formation depends on several autophagy proteins, and expansion of the autophagosome is mediated by two ubiquitin-like conjugation systems, Atg8/LC3-PE (LC3 II) and Atg12-Atg5 (Shintani and Klionsky, 2004). LC3 I (18 kDa) is cleaved by Atg4 to produce LC3 II (16 kDa), which is localized exclusively

in the autophagosomal membranes. Previous studies have used GFP-LC3 as an autophagy marker (Kabeya *et al*., 2000; Mizushima *et al*., 2001). In this study, GFP-LC3 dots were observed in WI38 cells after DHC treatment. Pretreatment of cells with 3 MA, a blocker of autophagy at the sequestration step, caused disappearance of the GFP-LC3 dots. Moreover, treatment with BaF1, an autophagy inhibitor at the maturation step, induced formation of large green fluorescence punctae due to accumulation of LC3 II protein in the autophagosomal membranes, as has been previously reported (Yamamoto *et al*., 1998). Our ultrastructural analysis shows that some cytoplasmic organelle was surrounding by the dilated rER membrane, autophagosome (Fig. 2B, b). The Atg5-Atg12 complex conjugates with Atg16 and is involved in the extension of an autophagosome. DHC treatment upregulated expression of Atg5; however, knockdown of *Atg5* failed to upregulate expression of Atg 5 and LC3 II by DHC. Therefore, our results strongly suggest that DHC activates the autophagy pathway in WI38 cells.

Recent findings suggest that ER stress is essential in autophagy induction (Bernales *et al*.,2006; Ogata *et al*., 2006; Yorimitsu *et al*., 2006; Ding *et al*., 2007a; Kouroku *et al*., 2007; Hoyer-Hansen and Jaattela, 2007). However, it is still not still clear if ER stress-mediated autophagy contributes to prosurvival or prodeath. ERAD is the primary degradation system for misfolded proteins in the ER lumen (Meusser *et al*., 2005). Autophagy, a bulk cellular degradation system, is activated under deprivation of nutrients or growth factors and is involved in the degradation of the long-lived proteins and cell organelles. When the amount of unfolded protein in the ER lumen exceeds the

ERAD capacity, cells are required to utilize an alternative pathway to mitigate the ER stress, and the best candidate for such a pathway is autophagy (Friedlander *et al*., 2000; Spear and Ng, 2003). One possible explanation for the prosurvival effect of autophagy is due to its ability to remove effectively misfolded proteins in the ER lumen (Teckman and Permutter, 2000; Bernales *et al*., 2006; Ogata *et al*., 2006; Ding *et al*., 2007a). Furthermore, blocking of ERAD leads to 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 (Ding *et al*., 2007b; Fujita *et al*., 2007). However, a recent study showed differential effects of ER stress on cell protection in cancer cells and normal cells: autophagy induced by classic ER stress inducers contributed to 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, 2007a). As with the UPR, we could observe autophagic activity after treatment with capsaicin and DHC. Treatment of cells with DHC induced rapidly and high sustained autophagy, which was then followed by caspase-3 activation. In contrast, the capsaicin-induced autophagy was relatively low compared to the LC3 II protein level induced by DHC. I demonstrated that autophagy induced by DHC treatment of cells was regulated by the ER stress sensor protein IRE1, as seen by knockdown of *Ire 1*. This finding suggests that DHC-induced autophagy is linked by ER stress. When DHC-induced autophagy was blocked by treatment of cells with 3MA, the number of apoptotic cells and the level of caspase-3 activation were increased and these effects

were completely inhibited by treatment of cells with zVAD. Therefore, autophagy induced by capsaicin and DHC has a role in cell survival against ER stress.

The signaling pathways underlying ER stress-mediated autophagy/apoptosis seem to depend on cell types and stimulations. The PERK-eIF2a and IRE1 –JNK signaling pathways are known to regulate ER stress-induced autophagy (Ogata *et al*., 2006; Fujita *et al*., 2007; Kouroku *et al*., 2007). Treatment of capsaicin and DHC caused activation of MAPK, especially JNK1/2 and ERK 1/2. In capsaicin-treated cells, phosphorylation of JNK/ERK was at a relatively low level and transient, but in DHC-treated cells, a rapid and highly sustained phosphorylation of JNK/ERK was seen. Treatment of cells with the JNK inhibitor SB60025 downregulated the expression of IRE1, Chop and LC3II induced by DHC as well as by TG and MG132, but ERK inhibitor PD98053 downregulated only the expression of LC3II, suggesting that DHC-induced autophagy could be regulated by differential pathways with JNK and ERK, respectively. In neuronal cell death, ERK-dependent autophagy is known to have an important role (Castro-Obregon *et al*., 2004; Zhu *et al*., 2007; Aoki *et al*., 2007). By contrast, ERK activation caused disturbance of the fusion between autophagosomes and lysosomes and ultimately resulted in inhibition of cell death by autophagy (Corcelle *et al*., 2006). However, in the present study, DHC treatment induced conversion of LC3 from the cytosolic LC3I form to the membrane-bound LC3II form, as demonstrated by transfection with GFP-LC3 and immunoblot analysis. To examine whether the enhancing effect of DHC on upregulation of LC3II was a result of inhibited fusion between an autophagosome and lysosome, we used Lysotracker Red to examine

lysosome localization in GFP-LC3-expressing WI38 cells. In contrast to a previous study (Corcelle *et al*., 2006), the red-stained lysosomes overlapped with the green GFP-LC3 punctate localized to autophagosomes, and BaF1 inhibited the effect. Furthermore, an ERK inhibitor blocked the upregulation of the LC3II protein level and GFP-LC3 dots.

Akt/PKB functions as a critical regulator of cell survival and proliferation, and inhibition of the PI3K/Akt/mTor signaling pathway triggers autophagy (Arico *et al*., 2001; Song et al., 2005; Takeuchi *et al*., 2005; Aoki *et al*., 2007). Treatment of cells with DHC transiently downregulated at early time, but capsaicin treatment rapidly increased of the level of Akt/PKB phosphorylation and further increased with time. We thus examined whether the blocking of the PI3K/AKt/mTor pathway could enhance autophagy in an ERK-regulated manner. We used inhibitors for PI3K including wortmannin and Ly294002. Although wortmannin and Ly294002 are known as inhibitors of autophagy (Blommaark *et al*., 1997; Aki *et al*., 2003), but other studies have reported the compounds act as autophagy inducers (Eskelinen *et al*., 2002; van der Poel *et al*., 2003; Takeuchi *et al*., 2005). At the concentrations used in our study, wortmannin and Ly294002 completely inactivated Akt and mTor, and ultimately upregulated expression of LC3II accompanied by ERK activation. Our results with those of a previous study (Aoki *et al*., 2007) indicate that ERK might be involved in autophagy induction through the PI3K/Akt/mTor/ signaling pathway. Therefore, capsaicin-induced ERK activation could be involved in an autophagy-signaling pathway that is independent of the JNK pathway. However, in the current study, we did

not determine at what step of the autophagy signaling pathway ERK is involved. In the context of our data, I speculate that the differential responses of ER stress on the cell protection by capsaicin and DHC are caused by the followings: the transient downregulation of Akt phosphorylation in DHC treated cells may have synergistic effect with ER stress pathway on autophagy induction, by which DHC can induce higher level of autophagy than capsaicin. The other one is that the treatment of capsaicin and DHC can elicit survival signals through acting Akt/PKB phosphorylation, whether it is associated with ER stress or not.

In the present study, although I 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 level of ER stress and is currently being investigated. The findings indicate that WI38 cells are more sensitive to DHC treatment than to treatment with capsaicin and the effect was associated with its high sensitivity to ER stress-mediated apoptosis and autophagy. The signaling pathway of DHC-induced autophagy occurring under ER stress shares some ER stress pathways and seems to counteract ER stress-mediated apoptosis. Both signaling pathways are regulated by JNK/ERK activation.

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Capsaicin 및 dihydrocapsaicin의 형질내세망 스트레스에 의한 autophagy/apoptosis

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형질내세망 스트레스는 세포와 자극의 종류에 따라 세포를 살리거나 죽게 할 수 있다. capsaicin과 이의 유사체인 dihydrocapsaicin (DHC)은 WI38 태아 폐 섬유모세포주에서 caspase-3 의존성 및 비의존성 신호전달계를 활성화 시켰다. 저자는 WI38 세포에서 capsaicin과 DHC에 의해 초래되는 신호전달체계의 분자기전에 관하여 연구하였다.

Capsaicin과 DHC는 p53과 $p21^{waf/cip}$의 상승을 초래하였다. 형태학적으로 DHC는 형질내세망과 미토콘드리아의 팽윤에 의해 심한 세포의 공포화가 초래되었다. 전통적인 형질내세망 스트레스 유도물질은 자가탐식의 표식자인 LC3의 증가는 물론 unfolded protein response (UPR)를 일으킨다. DHC는 Bip, IRE1, Chop, eIF2a와 caspase-4의 활성화에 의하여 강력한 형질내세망 스트레스를 초래하였으나, capsaicin의 경우는 이에 비하여 약하였다. 자가탐식 억제제인 3-methyladenine (3MA)과 bafilomycin A1 (BaF1)는 DHC에 의한 LC3 II의 상승과 GFP-LC3의 재분포를 억제하였다. Ire1을 siRNA로 knockdown 시켰을 때 DHC에 의하여 초래되는 Chop과 LC3 II를 감소시켰다. DHC는 빠르고 지속적으로 JNK/ERK를 활성화 시켰으나, capsaicin은 일시적으로 활성화 시켰다. JNK 억제제 SP600125는 thapsigargin, MG132 및 DHC에 의한 IRE1, Chop과 LC3 II 증가를 감소시켰으나, ERK 억제제 PD98059는 단지 LC3 II의 증가만을 감소시켰다. 3MA에 의한 DHC 유발 자가탐식(autophagy) 차단은 현저한 세포고사를 일으켰고, 이런 반응은 pancaspase inhibitor인 zVAD에 의하여 완전히 억제되었다. Capsaicin과 DHC는 시간경과에 따라 세포증식 및 보호에

관련된 단백질인 Akt 인산화를 증가시켰고, phosphatidylinositol 3-kinase (PI3K) 억제제인 wortmannin과 LY294002는 ERK 활성화를 통한 자가탐식을 일으켰다.

이상의 결과는 capsaicin과 DHC가 세포보호에 있어 반응정도가 다른 것은 JNK1/2와 ERK1/2의 활성화 정도에 따라 조절되는 UPR과 자가탐식의 정도에 기인한다는 것을 의미한다.

Figure legends

Figure 1. Effects of DHC and capsaicin on cell proliferation and caspase-3-like activation in WI38 cells. (A) Cells were treated with the indicated concentrations of capsaicin and 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 circle, capsaicin; closed circle, DHC. (B) Cells were treated with 200 μM of DHC and capsaicin up to 24 h, and were harvested and lysed. The lysates were analyzed for p53 (Do-1), phospho-p53 (ser 15) and $p21^{waf/cip1}$ by immunoblotting using the corresponding antibodies. β-actin was used as a loading control. Immunoblots shown are a representative of at least three independent experiments. (C) The lysates prepared as described above were measured for caspase-3-like activity as described in Materials and Methods. The data are expressed as the mean ± SD of the average from three independent experiments performed in triplicate. open bar, capsaicin; closed bar, DHC. $*P < 0.05$. (D) Cells were treated with 200 μM DHC or capsaicin for up to 24 h. The percentage of apoptotic cells was measured by flow cytometry as described in the Materials and Methods. Sub-G1 representing apoptotic DNA fragmentation was designated as M_1 in the data. Results are expressed as the mean ± SD of fold-increase compared to the non-treated control 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 (magnification, ×400). (B) Electron micrographs of WI38 cells at 6 h treated with DMSO, 200 μM capsaicin and 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 (arrowheads) and rER (arrow) are intact. A vacuolar structure is surrounded with the double-membrane structure resembling the rER (star). (c) In DHC-treated cells, various sizes of vacuoles were seen. Some

mitochondria were dilated and broken cristae lined following to the mitochondrial membrane (star). rER-resembling tubular structure ballooned at one end (arrow). Bar, 200 nm.

Figure 3. Induction of ER strss-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. β-actin was used as a loading control. Immunoblots shown are representative of at least three independent experiments.

Figure 4. Induction of ER strss-related proteins by capsaicin and DHC in WI38 cells. Cells were treated with 200 uM of capsaicin and DHC for up to 12 h and were harvested. The level of IRE1, p-eIF2a, ATF4, ATF 6, and Bip was determined by immunoblot analysis. β-actin was used as a loading control.

Figure 5. Differential effects of capsaicin and DHC on ER stress-mediated apoptosis in WI38 cells. After cells were treated with 200 μM of capsaicin and 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.

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 (magnification, 200×), 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 and 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 after downregulation of LC3 II protein expression. For cells transfected with *atg5*-specific siRNA, 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 LC3 II protein expression was determined by immunoblotting. Induction of LC3 II expression by DHC treatment was markedly downregulated in cells transfected with *atg5*-specific siRNA as compared with control cells transfected with nonspecific RNA. (E) Silencing of the *Ire1* gene downregulated Chop and LC3 II protein expression. Cells were transfected with *Ire1*-specific siRNA and silencing of the *Ire1* gene was confirmed by immunoblot analysis. Transfection with nonspecific (NS) siRNA did not affect expression of *Ire1* as compared to the parental control cells, whereas knockdown of *Ire1* gene expression markedly attenuated the level of Chop and LC3 II. Cells transfected with control or *Ire1*-specific siRNA were treated with DHC (200 μM) for 6 h and then Chop and LC3 II protein expression was determined by immunoblotting. The DHC-induced LC3 II conversion was almost completely downregulated in cells transfected with *Ire1*-specific siRNA as compared with control cells transfected with nonspecific RNA. The immunoblots shown are representative of at least three independent experiments. β-tubulin was used as a loading control.

Figure 7. Activation of MAPK by capsaicin and DHC in WI38 cells. (A) Cells were treated with 200 μM of capsaicin and DHC for up to 4 h, harvested, and then analyzed the extent of phosphorylation of ERK, JNK and p38 by immunoblot analysis. β-actin was used as a loading control. (B) The effect of MAPK 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, 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. Immunoblots shown are representative of at least three independent experiments. (C) Transfected cells with GFP-LC3 were pretreated with vehicle, SP600125 (10 μM) and PD98059 (10 μM) for 30 min and were further 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 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 completely 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 LC3 II. (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 controsl from three independent experiments. $^{**}P < 0.005$. (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. $^{**}P < 0.005$.

Figure 9. Activation of Akt/PKB by capsaicin and DHC, and role of ERK on autophagy induction. (A) Activation of Akt/PKB 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 Akt/ PKB phosphorylation. (B) Inhibition of the PI-3K pathway enhances the capsaicin-induced autophagy level and is dependent on ERK activation. Cells were treated with wortmannin (1μM) 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 LC3 II was determined by immunoblot analysis. β-actin was used as a loading control. The immunoblots shown are representative of at least three independent experiments.

Fig. 1A

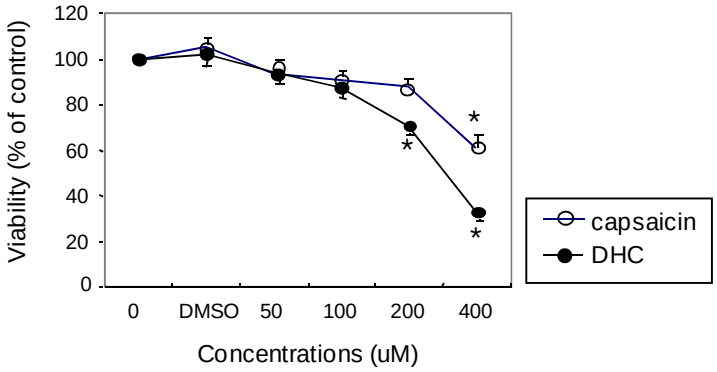

Viability (% of control)
120
100
80
60
40
20
0
0
DMSO
50
100
200
400
Concentrations (uM)
*
*
*
capsaicin
DHC

Fig. 1B

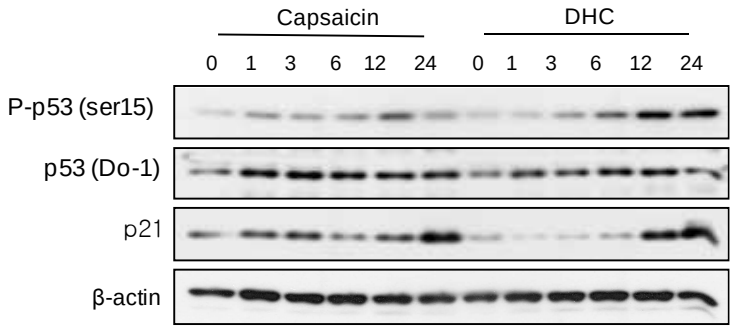

Capsaicin
DHC
0 1 3 6 12 24 0 1 3 6 12 24
P-p53 (ser15)
p53 (Do-1)
p21
β-actin

Fig. 1C

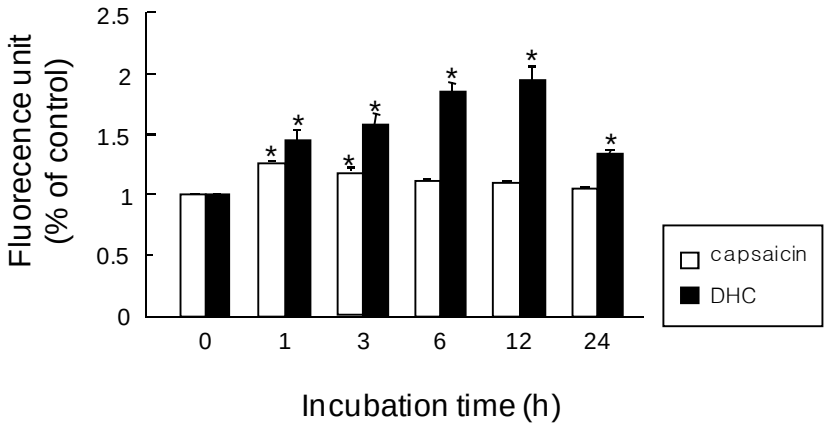

Fluorecence unit
(% of control)
2.5
2
1.5
1
0.5
0
0
1
3
6
12
24
Incubation time (h)
capsaicin
DHC

Fig. 1D

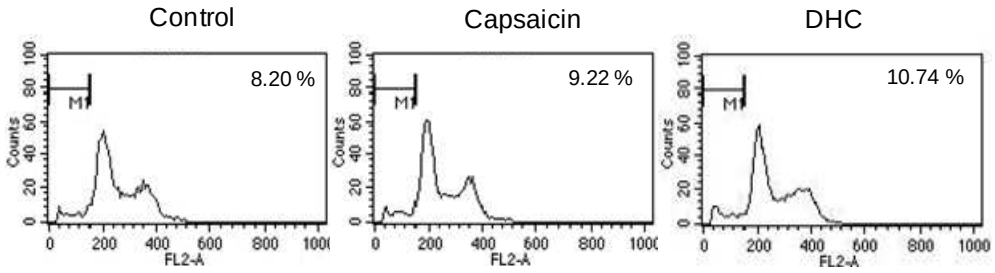

Control
Capsaicin
DHC
8.20 %
9.22 %
10.74 %
M1
Counts
FL2-A

Fig. 2A

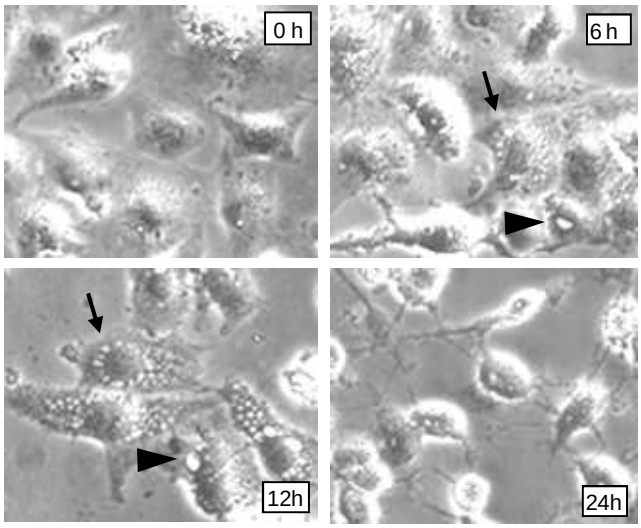


Fig. 2B

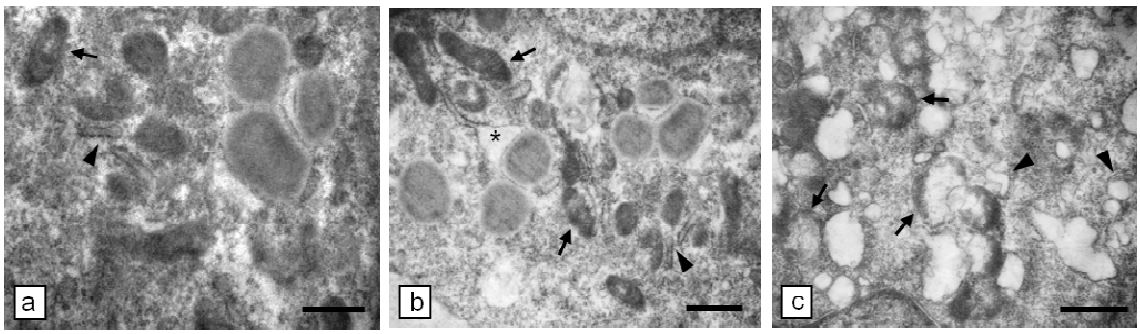


Fig. 3

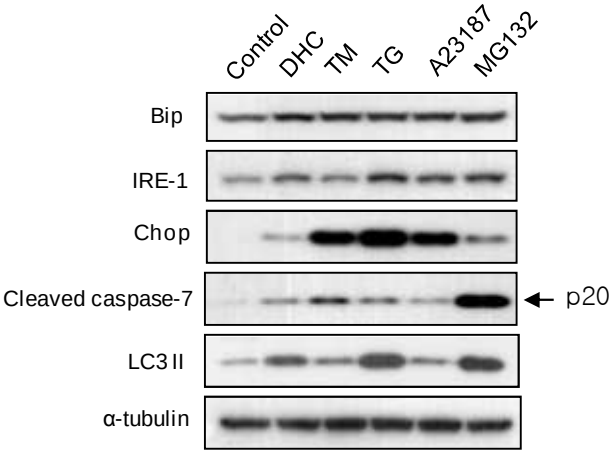

Control
DHC
TM
TG
A23187
MG132
Bip
IRE-1
Chop
Cleaved caspase-7
p20
LC3 II
α-tubulin

Fig. 4

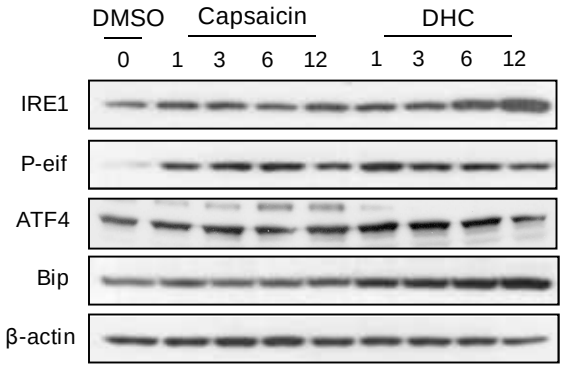

DMSO
Capsaicin
DHC
0
1
3
6
12
1
3
6
12
IRE1
P-eif
ATF4
Bip
β-actin

Fig. 5

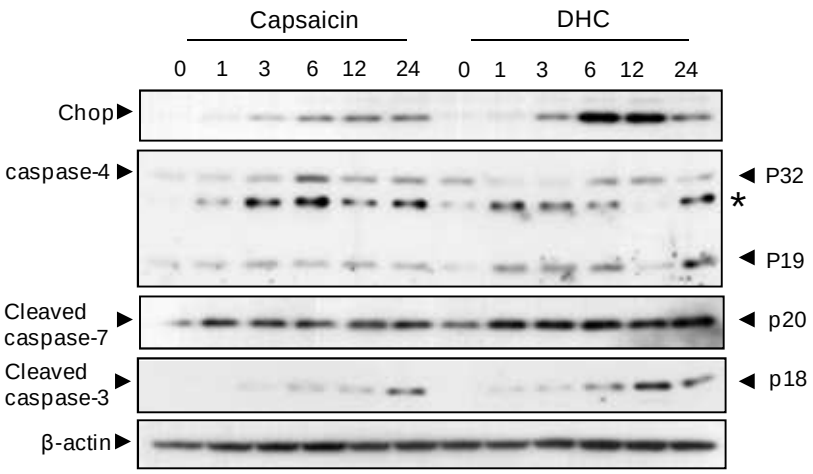

Capsaicin
DHC
0 1 3 6 12 24 0 1 3 6 12 24
Chop
caspase-4
P32
*
P19
Cleaved caspase-7
p20
Cleaved caspase-3
p18
β-actin

Fig.6A

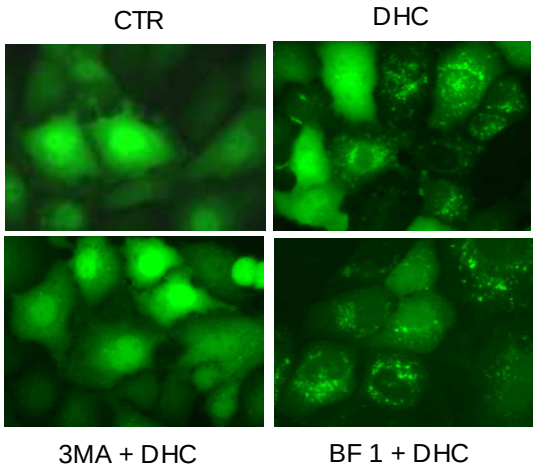


Fig.6B

Fig.6C

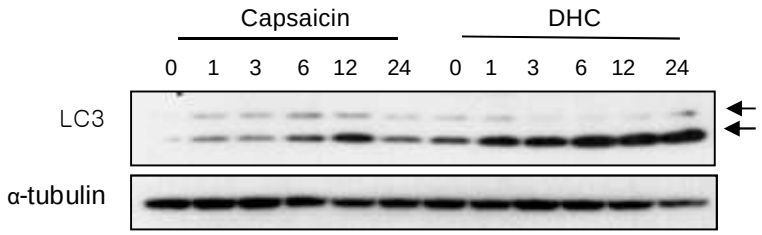


Fig.6D

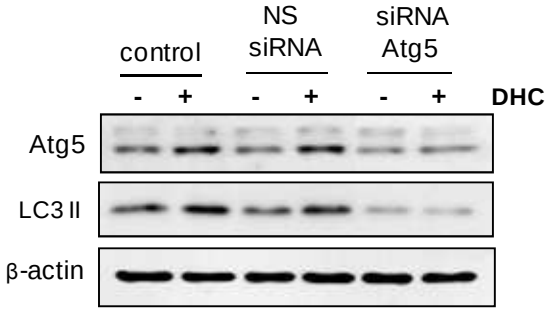


Fig.6E

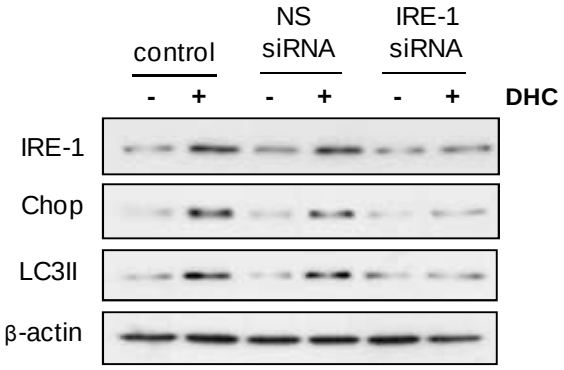


Fig.7A

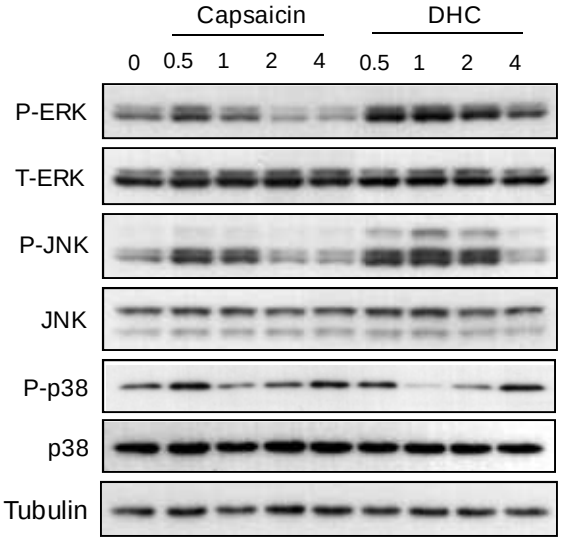


Fig.7B

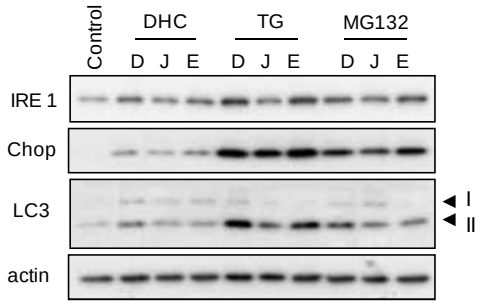


Fig.7C

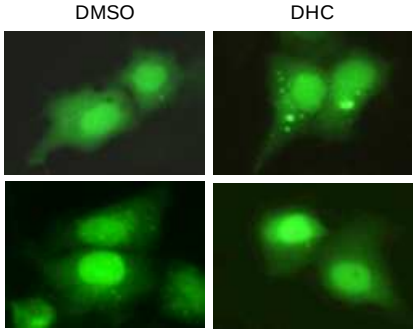


Fig. 8A

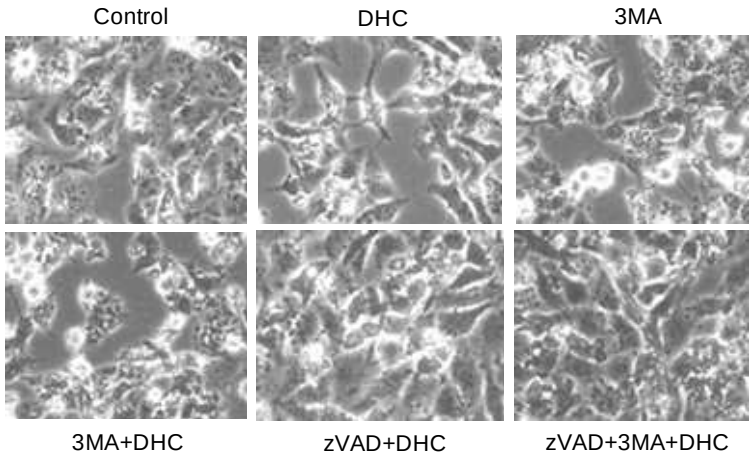


Fig.8B

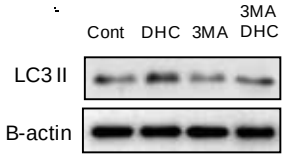


Fig.8C

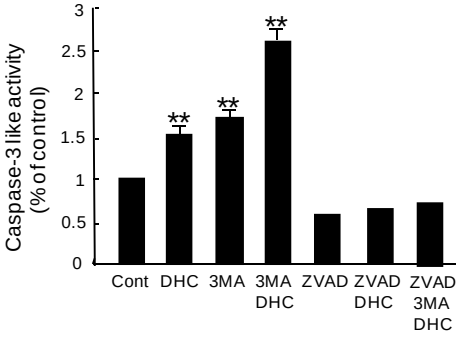


Fig.8D

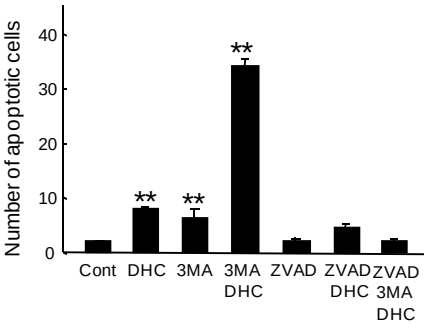


Fig.9A

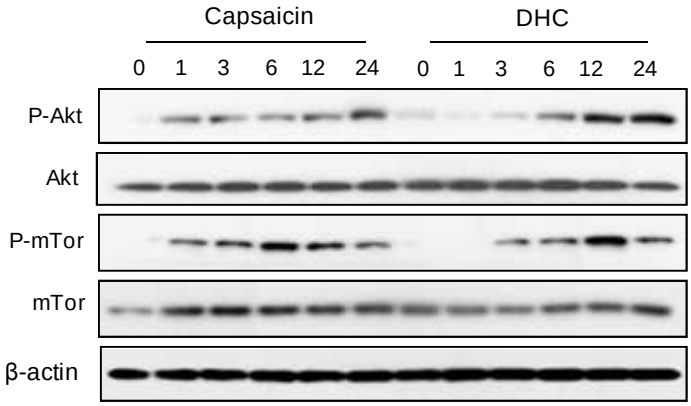
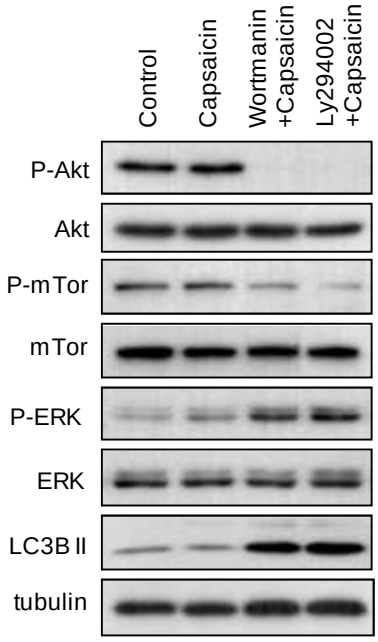

Capsaicin
DHC
0 1 3 6 12 24 0 1 3 6 12 24
P-Akt
Akt
P-mTor
mTor
β-actin

Fig.9B


Control
Capsaicin
Wortmanin +Capsaicin
Ly294002 +Capsaicin
P-Akt
Akt
P-mTor
mTor
P-ERK
ERK
LC3B II
tubulin