

Autophagy Induction by Capsaicin in Malignant Human Breast Cells Is Modulated by p38 and Extracellular Signal-Regulated Mitogen-Activated Protein Kinases and Retards Cell Death by Suppressing Endoplasmic Reticulum Stress-Mediated Apoptosis^S

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ABSTRACT

In our previous study, we showed that capsaicin induces autophagy in several cell lines. Here, we investigated the molecular mechanisms of capsaicin-induced autophagy in malignant (MCF-7 and MDA-MB-231) and normal (MCF10A) human breast cells. Capsaicin caused nonapoptotic cell cycle arrest of MCF-7 and MDA-MB-231 cells but induced apoptosis in MCF10A cells. In MCF-7 and MDA-MB-231 cells, capsaicin induced endoplasmic reticulum (ER) stress via inositol-requiring 1 and Chop and induced autophagy, as demonstrated by microtubule-associated protein 1 light chain-3 (LC3) conversion. Autophagy blocking by 3-methyladenine (3MA) or bafilomycin A1 (Baf1) activated caspase-4 and -7 and enhanced cell death. In MCF-7 and MDA-MB-231 cells, p38 was activated for more than 48 h by capsaicin treatment, but extracellular signal-regulated kinase (ERK) activation decreased after 12 h, and LC3II levels continuously increased. Furthermore, treatment

with 3MA markedly down-regulated capsaicin-induced p38 activation and LC3 conversion, and Baf1 completely down-regulated ERK activation and led to LC3II accumulation. In addition, pharmacological blockade or knockdown of the p38 gene down-regulated Akt activation and LC3II levels but did not affect ERK, and pharmacological blockade or knockdown of the ERK gene up-regulated LC3II induction by capsaicin. Knockdown of inositol-requiring 1 down-regulated p38-Akt signaling. In MCF10A cells, capsaicin did not elicit p38 activation and LC3 conversion and caused the sustained activation of caspase-4. Collectively, capsaicin-induced autophagy is regulated by p38 and ERK; p38 controls autophagy at the sequestration step, whereas ERK controls autophagy at the maturation step, and that autophagy is involved in the retardation of cell death by blocking capsaicin-induced ER stress-mediated apoptosis in MCF-7 and MDA-MB-321 cells.

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the principal pungent ingredient found in hot red chili peppers of the genus *Capsicum*, is consumed worldwide as a food additive. Capsaicin is believed to be a chemopreventive because of its

ability to induce apoptosis in several malignant cell lines (Lee et al., 2000, 2004; Mori et al., 2006; Tuoya et al., 2006). However, capsaicin may also have carcinogenic and tumorigenic properties (Surh and Lee, 1996), because several in vivo and epidemiological studies have shown a significant correlation between capsaicin intake and gastric cancer (Toth et al., 1984; Agrawal et al., 1986). This apparent contradiction suggests that the mechanism of capsaicin cytotoxicity requires clarification.

Apoptosis not only plays a crucial role during tissue development and homeostasis but is also involved in a wide range

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ABBREVIATIONS: UPR, unfolded protein response; ER, endoplasmic reticulum; IRE1, inositol-requiring enzyme 1; LC3, microtubule-associated protein 1 light chain-3; MAPK, mitogen-activated protein kinase; 3MA, 3-methyladenine; Baf1, bafilomycin A1; ERK, extracellular-regulated kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase; siRNA, short interfering RNA; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; PD98059, 2'-amino-3'-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; EB1089, seocalcitol.

of pathologies (Fadeel et al., 1999). Apoptotic cells have morphologically defined nuclear features that include chromatin condensation and DNA fragmentation (Wyllie et al., 1980). The apoptotic process occurs via two distinct signaling pathways, known as the tumor necrosis factor/Fas-receptor and mitochondrial pathways. Cascade activation of caspases plays a critical role in both pathways, which converge at the "executioner" molecule caspase-3 (Wolf and Green, 1999). Furthermore, caspase-activated DNase, which is activated by caspase-3, is responsible for the DNA fragmentation that is characteristic of apoptotic nuclear changes (Samejima et al., 2001).

Autophagy is a self-protective cellular mechanism that provides energy through the degradation and recycling of cytoplasmic constituents. Thus, autophagy may help cell survival, particularly under conditions of starvation or growth factor withdrawal, but excessive autophagy may trigger cell death (Baehrecke, 2005). During autophagy, portions of the cytoplasm are sequestered into double-membrane vesicles referred to as autophagosomes, which then fuse with lysosomes to form single-membrane autolysosomes. The contents of the autophagolysosomes are ultimately degraded by lysosomal hydrolase. Autophagic cells are thus characterized by the accumulation of vacuoles (Klionsky and Emr, 2000).

The unfolded protein response (UPR) is another protective cellular response. The endoplasmic reticulum (ER) performs several functions, which include protein-folding, trafficking, and intracellular calcium concentration regulation. When ER functions are disrupted, the UPR is triggered to increase cell survival during ER stress (Schröder and Kaufman, 2005). Three resident proteins of the ER membrane act as UPR sensors, namely, inositol-requiring 1 (IRE1), pancreatic ER-kinase-like ER kinase, and activating transcription factor 6, and the activations of their downstream signals inhibit protein translation and facilitate protein degradation, thus reducing ER overload to counteract ER stress. However, sustained or severe ER stress induces apoptosis, and the demise of damaged cells is mediated by the activation of an ER membrane-resident caspase 4 (caspase-12 in mice) (Moriyama et al., 2002).

Mitogen-activated protein kinase (MAPK) family members, including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38, have been reported to be involved in ER stress and autophagy. ER stress may lead to apoptosis or cell survival through the activation of JNK or p38 (Zhang et al., 2001; Ding et al., 2007a; Hamamura et al., 2009), and accumulated evidence suggests that ER stress is connected to autophagy (Bernales et al., 2006; Yorimitsu et al., 2006; Ding et al., 2007b). When the amounts of unfolded proteins exceed the capacity of the ER-associated degradation system, the ER can trigger alternative degradation systems, such as autophagy, to ensure cell survival. LC3 conversion was consistently induced by pancreatic ER-kinase-like ER kinase/eIF2 α -mediated ER stress caused by thapsigargin or polyglutamine 72 repeats (Kouyama et al., 2007), and in other studies, it was suggested that the IRE1-JNK signaling pathway is essential for autophagy (Ogata et al., 2006; Ding et al., 2007b). p38 has been reported to be involved in autophagy induction (Comes et al., 2007; Corcelle et al., 2007; Casas-Terradellas et al., 2008; Tang et al., 2008).

However, the nature of the coupling between ER stress and p38 during autophagy process is not understood.

During our previous studies, capsaicin was found to be able to induce autophagy in MCF-7 malignant breast epithelial cells but to induce ER stress-mediated apoptosis in MCF10A nonmalignant breast cells (Oh et al., 2008; Lee et al., 2009). Thus, the aims of the present study were to investigate the role of the selective induction of autophagy by capsaicin in malignant breast cells and to elucidate the molecular mechanisms responsible in nonmalignant and malignant breast cells. We show that p38 MAPK is required for capsaicin-induced autophagy at the sequestration step of autophagosome formation, and that this is further regulated by ERK at the maturation step of autophagosome. Capsaicin-induced p38 signaling pathway is regulated by IRE1 and demonstrate that the IRE1-p38 signaling pathway plays a key role in capsaicin-induced autophagy.

Materials and Methods

Cell Cultures and Chemicals. MCF-7 and MDA-MB-231 human breast cancer cells and nonmalignant human breast MCF10A cells were maintained in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum, 50 μ g/ml penicillin, and 50 μ g/ml streptomycin at 37°C in a 5% CO₂/95% air-humidified incubator. When required, cells were seeded on 60-mm dishes and exposed to chemicals after culturing overnight. 3-Methyladenine and bafilomycin A1 were obtained from Sigma-Aldrich (St. Louis, MO); 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride (Ly294002), wortmannin, 2'-amino-3'-methoxyflavone (PD98059), and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) were from Cell Signaling Technology (Danvers, MA); and Mitotracker Red and ER-Tracker Blue-White DPX were from Invitrogen (Carlsbad, CA). The other chemicals were of the purest grade available and were purchased from Sigma-Aldrich.

Cytotoxicity Assays. The viabilities of cultured cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assays. In brief, cells were suspended in complete media at a concentration of 10⁵ cells/ml, and samples (200 μ l) of the cell suspensions were seeded onto 48-well plates and cultured overnight. Cells were then exposed to chemicals for 24 h. After 4 h of incubation with MTT (0.5 mg/ml), media were removed, and 200 μ l of DMSO was added to dissolve the formazan crystals produced. Absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay microplate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA). For Trypan blue assays after treatment, floating and adherent cells were collected, centrifuged, and stained with 0.4% trypan blue for 5 min at room temperature. The numbers of trypan blue-positive (dead) and -negative (alive) cells were counted on a hemocytometer under a microscope. Cell viabilities are expressed relative to those of untreated controls.

Flow Cytometric Analysis. Cells were harvested and washed twice with ice-cold PBS buffer. After fixing in 70% ethanol for 30 min at 4°C, they were washed with ice-cold PBS buffer and resuspended in 1 ml of PBS buffer containing 500 μ g/ml propidium iodide. At least 10,000 events were analyzed using a FACScan (FACStation; BD Biosciences, San Jose, CA) using BD FACSCalibur and CellQuest software (using a Macintosh computer; Apple Computer, Cupertino, CA). Apoptotic cell percentages were calculated by determining the number of cells with hypodiploid nuclei.

Transfection. Cells were transfected with short interfering RNA (siRNA) corresponding to human *Ire 1* (5'-CUGCCCGGC-CUCGGGAUUU-3' and 5'-AAAUCCCGAGGCCGGGCAG-3'), ERK (5'-GCA AUGACCAUAUCUGCUA-3' and 5'-UAGCA-

GAUUAUGUCAUUGC-3'), p38 (Santa Cruz Biotechnology, Santa Cruz, CA), or control siRNA for enhanced green fluorescent protein (Ambion, Austin, TX) using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol. After incubation for 4 h, media were exchanged with complete medium containing 10% serum and antibiotics. The cells were then incubated for an additional 48 h and treated with capsaicin as detailed in the figure legends.

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 phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin. Lysates were then centrifuged, and protein contents were quantified. Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (12–15%), and separated proteins were transferred to polyvinylidene difluoride membranes and then immunoblotted with corresponding antibodies. Anti-rabbit polyclonal atg8/LC3 was obtained from Absent (San Diego, CA). Chop and ERK2 were purchased from Santa Cruz Biotechnology, and Akt, p-Akt, p38, p-p38, ERK1/2, p-ERK, and IRE1 were from Cell Signaling Technology. Anti-caspase-4 was obtained from Abcam (Cambridge, UK). β -Actin was obtained from Sigma-Aldrich. The immobilized proteins were incubated with goat anti-mouse IgG and goat anti-rabbit IgG (Santa Cruz Biotechnology), and signals were detected using a chemiluminescence kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Statistical Analysis. The Student's *t* test was used to determine the significances of differences between treatments and respective controls. Values are expressed as means \pm S.D.

Results

The Cytotoxic Effects of Capsaicin on Malignant and Nonmalignant Breast Cells. In previous studies, capsaicin was found to kill malignant cells via apoptosis. Here, to examine the effect of capsaicin, we used MTT assays to measure the viabilities of malignant MCF-7 and MDA-MB-231 and nonmalignant MCF10A human breast epithelial cells that had been exposed to capsaicin for 24 h at different concentrations. The cytotoxic effect of capsaicin was found to be much higher in MCF10A cells than in both malignant cell lines (Fig. 1A). Fluorescence-activated cell sorting analysis revealed that capsaicin treatment (250 μM) did not affect the apoptotic sub- G_1 population of MCF-7 and MDA-MB-231 cells; instead, it induced cell cycle arrest in the S or G_0/G_1 phase. In contrast, capsaicin markedly increased the percentage of sub- G_1 MCF10A cells (Fig. 1B); the apoptosis of MCF10A cells was confirmed by Hoechst 33342 staining (Fig. 1C). After capsaicin treatment, the nuclei of MCF10A cells were fragmented or condensed, whereas those of MCF-7 and MDA-MB-231 cells were intact. These findings suggest that capsaicin elicits different pathways in normal and malignant cells. Although the viabilities of the two malignant cell lines decreased in response to capsaicin treatment, they did not exhibit apoptotic characteristics. Therefore, we next examined the molecular mechanisms triggered by capsaicin in malignant cells.

Capsaicin Treatment Induced ER Stress but Not ER Stress-Mediated Apoptosis in MCF-7 and MDA-MB-231 Cells. In cells under ER stress caused by various stimuli, UPR is triggered as a cytoprotective mechanism. In our previous study, MCF10A cells elicited UPR response to capsaicin via IRE1, Bip, Chop, and caspase-4 activation (Lee et al., 2009) (Supplementary Fig. 1). To determine whether UPR is

induced in malignant breast cells by capsaicin, we examined the expressions of UPR-related proteins in MCF-7 and MDA-MB-231 cells. As shown in Fig. 2A, in MCF-7 cells, capsaicin treatment increased the protein levels of IRE1 and Chop with time, and using the same protocol, similar results were obtained for MDA-MB-231 cells (Fig. 2B). Severe ER stress leads to ER stress-mediated apoptosis, which in mammals is mediated by caspase-4, and thus, we examined the activations of caspase-4 and -7 after treating MCF-7 and MDA-MB-231 cells with capsaicin by Western blotting. The expression pattern of procaspase-4 is different in these two cell lines, but the band of the active form of caspase-4 at p19kDa was not observed after more than 24 h of treatment, and although caspase-7 was activated after 12 and 24 h of treatment in MCF-7 and MDA-MB-231 cells, respectively, this was not marked. These findings indicate that capsaicin induces UPR in malignant breast cells but that this is not enough to elicit ER stress-mediated apoptosis or that this is blocked by another signaling pathway.

Capsaicin Treatment Induced High Levels of Autophagy in Malignant Breast Cells but Limited Levels in Nonmalignant Cells. Autophagy is a cytoprotective mechanism against extracellular stress (Klionsky and Emr, 2000). To determine whether capsaicin induces autophagy, which is characterized by the conversion of LC3I (cytosolic form) into LC3II (autophagosome membrane-bound form), LC3II was analyzed by Western blotting. It is noteworthy that the level of LC3II induced within 0.5 h after capsaicin treatment was markedly elevated in a time-dependent manner in both MCF-7 and MDA-MB-231 cells. However, in MCF10A cells, the extent of LC3 conversion was very limited, and only a faint band of LC3II was observed after protracted exposure of membrane on the film (Fig. 3A). Autophagy induction by capsaicin in MCF-7 was also investigated using the pharmacological autophagy inhibitors 3MA and BaF1. Pretreatment of cells with 3MA markedly down-regulated the conversion of LC3I into LC3II, as evidenced by a decrease in LC3II protein levels, but when cells were pretreated with BaF1, LC3II protein accumulated to a greater extent (Fig. 3B). To determine how autophagy contributes to cell death, MCF-7 cells were observed by phase-contrast microscopy, and cell viabilities were determined using MTT assays. In MCF-7 cells, capsaicin treatment caused cellular vacuolization and shrinkage in some cells, and treatment with 3MA alone resulted in a small number of rounding cells and severe cell shrinkage, which was enhanced by cotreatment with capsaicin. On the other hand, treatment with BaF1 alone induced many big cytoplasmic vacuoles, and cotreatment with BaF1 and capsaicin caused severe cell shrinkage and large numbers of rounded and floating cells (Fig. 3F). Pretreatment with both autophagy inhibitors induced cell death at levels comparable with those seen in capsaicin-treated cells (Fig. 3D). Furthermore, capsaicin treatment in the presence of 3MA or BaF1 significantly reduced cell viability compared with cells treated with capsaicin only. Similar cell morphology and viability results were observed in MDA-MB 231 cells treated with capsaicin and/or the two autophagy inhibitors (Fig. 3, E and G).

Next, to investigate whether cell death elicited by autophagy blocking is related to the activations of caspase-4 or -7, caspase activation was examined by Western blotting. Pre-

treatments with 3MA or BaF1 followed by capsaicin were found to markedly activate caspase-4 and -7 in both MCF-7 and MDA-MB-231 cells (Fig. 3, B and C). These data indicate that capsaicin-induced autophagy in MCF-7 and MDA-MB-231 may be involved in the protection of cells against severe ER stress.

Capsaicin Induced the Activations of MAPK p38 and ERK. In our previous study, capsaicin was found to activate MAPK in WI38 cells (Oh et al., 2009). First, to investigate the activation of MAPK by capsaicin in breast cell lines, cells were treated with 250 μ M capsaicin for up to 24 h. A time course study of MAPK phosphorylation in MCF-7 and MDA-MB-231 cells showed that p38 and ERK were rapidly activated at 0.5 h and that these activations increased with time (Fig. 4A). However, in MCF10A cells, levels of phosphorylated p38 (p-p38) decreased after 1 h of treatment and then remained at basal level over 24 h, whereas levels of phosphorylated ERK (p-ERK) increased slightly at 0.5 h, peaked at 6 h, and then decreased (Fig. 4A). However, JNK was not activated in MCF-7 or MCF10A cells (data not shown). These observations indicate a possible involvement of p38 and ERK during capsaicin-induced autophagy.

To examine the relationship between autophagy and p38 or ERK activation in more detail, the three types of cells were treated with capsaicin for up to 48 h (Fig. 4B). In MCF-7 and MDA-MB-231 cells, p38 activation was sustained after 24 h of capsaicin treatment, but ERK activation subsided after 24 h of treatment. The level of LC3II continuously increased over 48 h in both cell lines. In contrast, in MCF10A cells, the level of p-p38 decreased under basal level and was undetect-

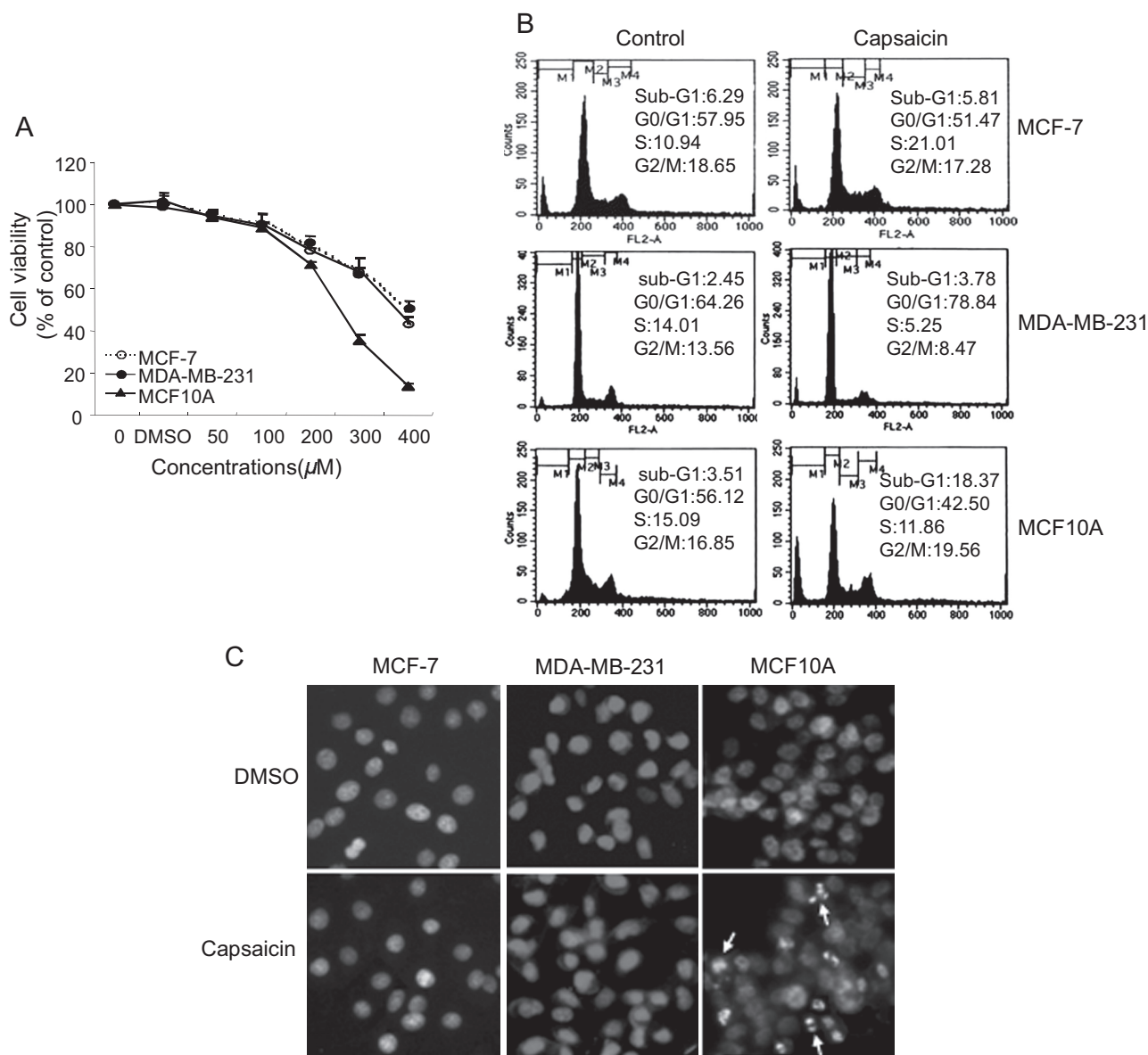


Fig. 1. Effects of capsaicin on the viabilities and apoptosis of MCF-7, MDA-MB-231, and MCF10A cells. A, cells were treated with the indicated concentrations of capsaicin for 24 h, and viabilities were determined using MTT assays. Data are expressed as the means \pm S.D. of three independent experiments performed in triplicate. B, cells were treated with 250 μ M capsaicin for 24 h. Percentages of apoptotic cells were measured by flow cytometry, as described under *Materials and Methods*. The sub-G₁ population represents apoptotic DNA fragmentation. C, after treatment with 250 μ M capsaicin for 24 h, MCF-7 and MCF10A cells were stained with Hoechst 33342 and analyzed under a fluorescence microscope (original magnification, 200 \times , Olympus 1X71; Olympus, Tokyo, Japan). Arrows indicate condensed or fragmented MCF10A nuclei. Data shown are a representative of three separate experiments.

able at 48 h. p-ERK was also undetectable at 24 h, and the level of LC3II was very low.

In our previous study, capsaicin induced the down-regulation of antiapoptotic Bcl2 protein in MCF10A cells (Lee et al., 2009), and thus, we examined the effect of capsaicin on Bcl2 protein expression in MCF-7 and MDA-MB-231 cells. It is noteworthy that Bcl2 protein levels increased in a time-dependent manner in both cell lines after capsaicin treatment (Supplementary Fig. 2) and continued to increase at 24 h compared with control cells. In contrast, in MCF10A cells, Bcl2 levels continuously decreased over 48 h compared with control cells (Fig. 4B). These results led us to examine the correlation between p38 or ERK activation and cell death. The three cell lines were treated as described in the legend of Fig. 4B, and dead cells were quantified using the trypan blue exclusion method. After 48 h of capsaicin treatment, the percentages of dead MCF-7 and MDA-MB-231 cells were 21 and 19.6%, respectively, compared with control cells. In MCF10A cells, 35 and 40% of the cells were dead after 24 and 36 h of exposure compared with control cells, and almost all were dead at 48 h (Fig. 4C). These findings suggest that association between p38/ERK or Bcl2 induction and autophagy.

Effects of p38 and ERK Activation on Capsaicin-Induced Autophagy Process. Recent studies have demonstrated the involvements of MAPK p38 and ERK signaling pathways in autophagy (Corcelle et al., 2006; Tang et al., 2008; Chiu et al., 2009). In the present study, long-term (48 h) treatment with capsaicin caused the sustained activation of p38 and inhibited ERK activation and markedly increased LC3II levels (Fig. 4B). Thus, to determine how p38 and ERK contribute to capsaicin-induced autophagy, we used the autophagy inhibitor 3MA, an inhibitor for sequestration step of autophagosome, and BaF1, an inhibitor for maturation step of autophagosome. MCF-7 and MDA-MB-231 cells were treated as described in the legend of Fig. 3, B and C, and p-p38, p-Akt, and p-ERK levels were determined by Western blotting. In MCF-7 cells, pretreatment with 3MA before capsaicin markedly attenuated p-p38 and p-Akt compared with capsaicin-treated cells, but did not affect capsaicin-induced p-ERK levels (Fig. 5A), and led to reduced LC3II levels (Fig. 3D). However, 3MA treatment without capsaicin markedly increased p-ERK level. In contrast, capsaicin treatment in the presence of BaF1 had no effect on the capsaicin-induced p-p38 and p-Akt levels, but activation of ERK was completely

blocked by capsaicin treatment in the presence of BaF1, and that led to LC3II accumulation (Fig. 3D). Similar results were obtained for MDA-MB-231 cells (Fig. 5B). In terms of the autophagy steps blocked by 3MA and BaF1, these observations suggest that p38 regulates autophagosome formation at the sequestration step and that ERK is involved in the maturation step of autophagy process.

p38-Regulated Autophagy via Akt Activation. In the Fig. 5, A and B, p38 activated upstream of Akt by capsaicin treatment. Thus, to determine the order of p38 and ERK involvement in autophagy process, we used pharmacological inhibitors to investigate the roles of MAPK p38 and ERK in capsaicin-induced autophagy. Cells were pretreated with SB203580 or PD98059 (both MAPK inhibitors) for 30 min and then treated with capsaicin for 6 h. SB203580 (a p38-specific inhibitor) completely down-regulated the phosphorylation of Akt, and eventually reduced LC3II levels. On the other hand, although PD98059 (an ERK-specific inhibitor) completely blocked ERK activation, it had no effect on capsaicin-induced Akt activation or on LC3II level (Fig. 6A). Similar data were obtained in MDA-MB-231 cells (Supplementary Fig. 3A). Therefore, these findings suggest that p38 induces autophagy via Akt activation and that ERK is involved in the autophagy process downstream of Akt.

To further investigate the roles of p38 and ERK in the capsaicin-induced autophagy process, p38 and ERK-specific siRNA were used to knock down the p38 or ERK genes. Transfection of MCF-7 cells with p38 siRNA down-regulated the level of capsaicin-induced p-p38 and p-Akt and reduced LC3II conversion compared with parental or nonspecific siRNA-transfected control cells. However, knockdown of p38 did not affect capsaicin-induced p-ERK level (Fig. 6B). Similar results were obtained in MDA-MB-231 cells (Supplementary Fig. 3B). Furthermore, the transfection of MCF-7 cells with ERK siRNA attenuated the level of p-ERK induced by capsaicin treatment, and consequently increased LC3II conversion compared with parental or nonspecific siRNA-transfected control cells. However, knockdown of ERK did not affect the level of p-Akt induced by capsaicin treatment (Fig. 6C). Similar results were obtained for MDA-MB-231 cells (Supplementary Fig. 3C). These results indicate that p38 regulates autophagy upstream of Akt and ERK regulates autophagy downstream of Akt, suggesting that the ERK is involved in the autophagosome maturation by p38 and that the p38 signaling pathway is essential for capsaicin-induced autophagy.

The UPR Sensor IRE1 Controlled the Capsaicin-Induced p38-Akt Pathway. In response to ER stress, IRE1 plays two roles: it activates Xbp1, a transcriptional factor for UPR genes, and it recruits tumor necrosis factor receptor-associated factor 2 and apoptosis signal-regulating kinase, thereby activating the JNK MAPK pathway. However, capsaicin activated p38 and ERK but not JNK in MCF-7 cells (data not shown). To examine whether a relation exists between IRE1 and p38 or ERK activation by capsaicin, the IRE1 gene was knocked down using IRE1-specific siRNA. IRE1 knockdown was found to reduce the phosphorylations of p38 and Akt and eventually to reduce LC3II conversion (Fig. 7). However, knockdown of IRE1 gene had no effect on p-ERK level induced by capsaicin treatment; rather, it increased. These findings indicate that IRE1 regulates capsa-

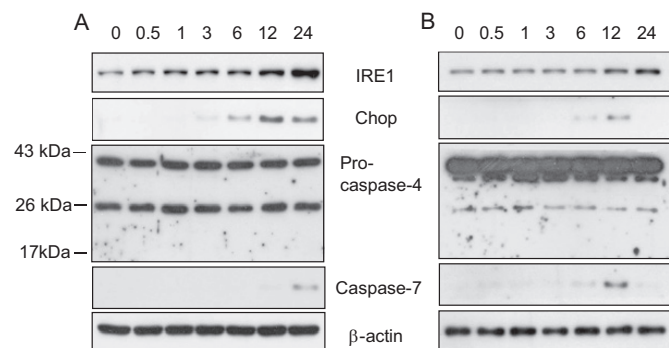


Fig. 2. Capsaicin induces ER stress but not ER stress-mediated apoptosis in MCF-7 and MDA-MB-231 cells. MCF-7 (A) and MDA-MB-231 (B) cells were treated with 250 μ M capsaicin for up to 24 h, harvested, and lysed. Western blot analyses were performed for IRE1, Chop, caspase-4, and caspase-7; β -actin was used as the loading control.

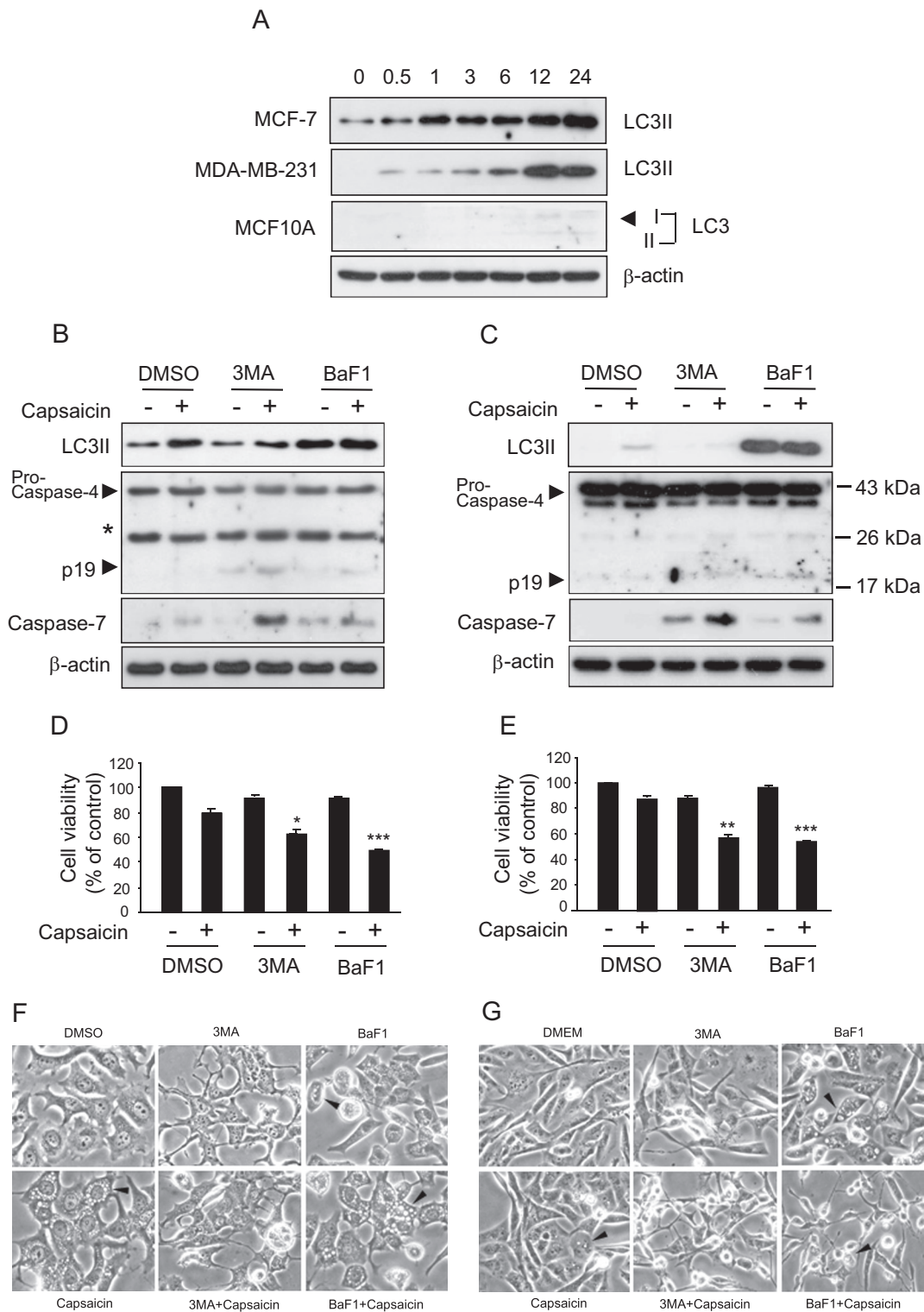


Fig. 3. Autophagy induction by capsaicin and the effects of autophagy inhibitors on cell death. **A**, cells were treated with 250 μ M capsaicin for up to 24 h, harvested, and lysed. Western blotting was used to quantify LC3II expressions in MCF-7, MDA-MB-231, and MCF10A cells. MCF-7 (**B**) and MDA-MB-231 (**C**) cells were pretreated with 3MA (10 mM) or BaF1 (100 nM) for 30 min, 250 μ M capsaicin was added for 6 h, cells were harvested, and LC3 flux was examined by Western blotting. LC3II levels were found to be markedly down-regulated by 3MA and up-regulated by BaF1. Blocking of autophagy activated caspase-4 and -7. MCF-7 (**D**) and MDA-MB-231 (**E**) cells were pretreated as described in **B** and treated with capsaicin for 24 h, and cell viabilities were determined using MTT assays. Pretreatment with 3MA or BaF1 greatly enhanced capsaicin-induced cell death. Values are expressed as means \pm S.D. of average percentages versus nontreated control cells from three independent experiments. ***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.01$. **F** and **G**, cells were treated as described in **D** and **E**, and cell morphologies were observed under a phase-contrast microscope (Olympus 1X71). Representative photomicrographs (original magnification, 200 \times) of the cells treated as described in **B** and **C**. Arrows indicate vacuolated cells.

icin-induced autophagy through a p38-Akt signaling pathway.

Malignant Breast Cells Were Much More Sensitive to Autophagy Induction by Capsaicin than Nonmalignant MCF10A Cells. We wondered whether the autophagy and inhibition of ER stress-mediated apoptosis induced by capsaicin in MCF-7 and MDA-MB-231 cells were involved in the delayed cell death and considered that MCF10A non-transformed breast cancer cells with an apoptotic response to

capsaicin might be less sensitive to the autophagy induced by capsaicin. To test this hypothesis, we examined cell death in MCF-7, MDA-MB-231, and MCF10A cells. First, we used trypan blue exclusion to measure cell death in the three cell types of cells after treating them for 24 h with increasing concentrations of capsaicin (50–400 μ M). Cell death in MCF-7 and MDA-MB-231 cells occurred only at basal levels in the presence of up to 200 μ M capsaicin, whereas MCF10A cell death reached almost 20% after treatment with 50 μ M

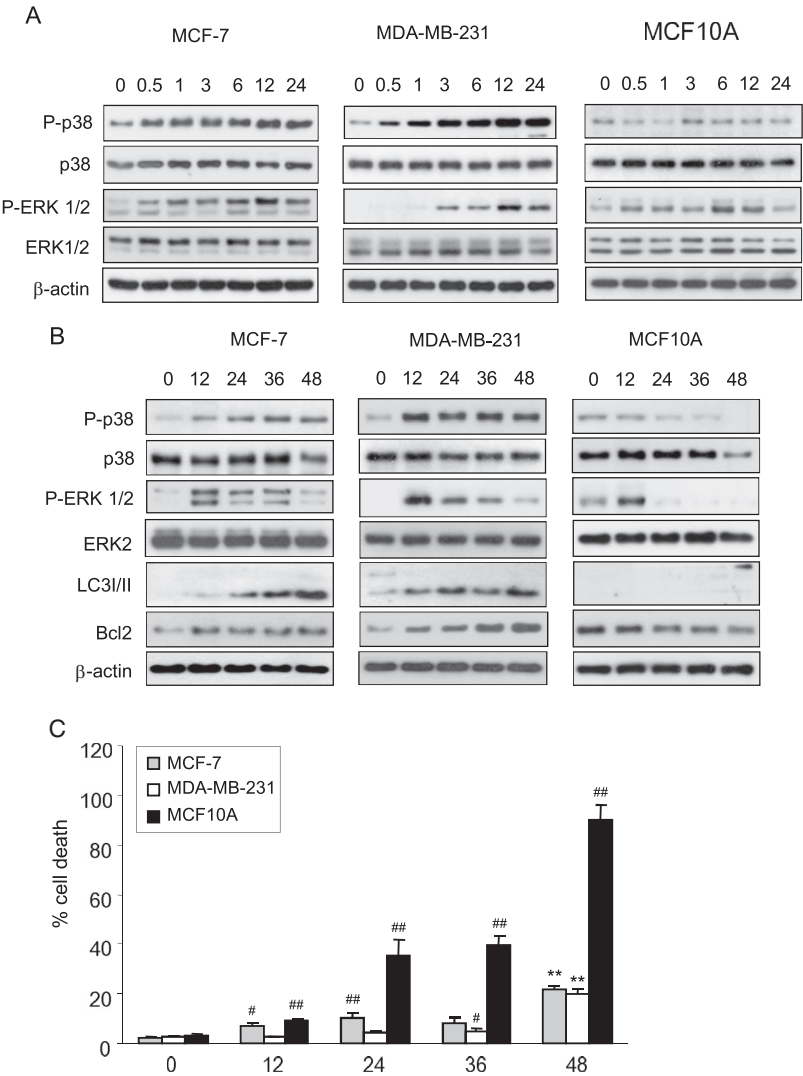


Fig. 4. Activations of MAPK p38 and ERK by capsaicin in MCF-7, MDA-MB-231, and MCF10A cells. A, cells were treated with 250 μ M capsaicin for up to 24 h, harvested, and lysed. p38, p-p38, ERK, and p-ERK levels were determined by immunoblotting. B, cells were treated with 250 μ M capsaicin for up to 48 h and harvested every 12 h, lysed, and p38, p-p38, ERK, p-ERK, LC3II, and Bcl2 levels were determined by immunoblotting. C, cells were treated as described in B, and cell viabilities were determined by trypan blue exclusion as described under *Materials and Methods*. The data shown are percentages of the total numbers of cells counted. Values are expressed as means \pm S.D. of percentages determined by three independent experiments. **, $P < 0.001$; #, $P < 0.05$; ##, $P < 0.005$.

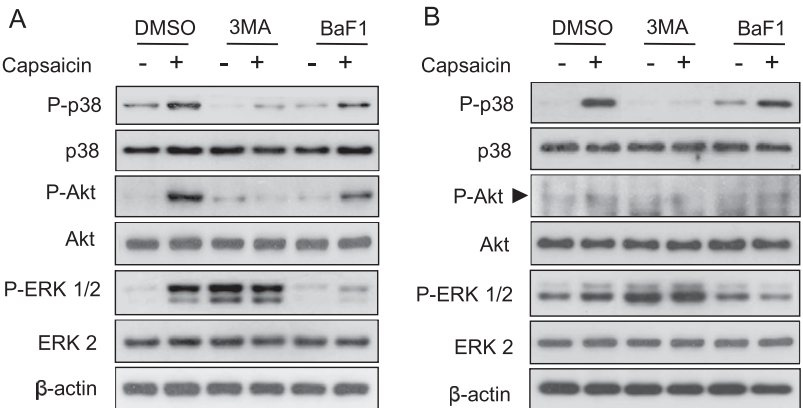


Fig. 5. Effects of autophagy inhibitors on the activations of p38 and ERK. MCF-7 (A) and MDA-MB-231 (B) cells were pretreated with 3MA (10 mM) or BaF1 (100 nM) for 30 min before adding 250 μ M capsaicin for 6 h. Cells were then harvested, and p38, p-p38, Akt, p-Akt, ERK, and p-ERK levels were analyzed by Western blotting. The immunoblots shown are representative of at least three independent experiments.

capsaicin, and this increased further as the capsaicin concentration was increased. At a capsaicin concentration of 400 μM , 45, 31, and 85% of MCF-7, MDA-MB-231, and MCF10A cells, respectively, died (Fig. 8A).

Next, we examined ER stress and autophagy response in the three types of cell lines after capsaicin treatment. Cells were treated with various concentrations of capsaicin for 12 h as described and analyzed for the expressions of ER stress-related proteins. In MCF-7 and MDA-MB-231 cells, levels of the ER stress-related proteins IRE1 and Chop increased in a dose-dependent manner. However, no activation of caspase-4 was observed, even at a capsaicin concentration of 400 μM . Furthermore, the antiapoptotic Bcl2 protein was found to be up-regulated in MCF-7 and MDA-MB-231 cells after treatment. In MCF10A cells, increases in the levels of IRE1 and Chop were observed after treatment with 300 or 400 μM capsaicin, and the cleaved form of caspase-4 was even observed after treatment with 50 μM capsaicin. Furthermore, Bcl2 were found to decrease (Fig. 8B).

We next compared p38 or ERK activation and LC3II levels in the three cell lines after treatment with capsaicin as described in Fig. 8B. In MCF-7 cells, p-p38 levels remained high, but p-ERK levels reduced in cells treated with 300 μM capsaicin and further decreased in 400 μM -treated cells. Furthermore, the conversion of LC3I into LC3II increased with concentration (Fig. 8C). Similar results were observed in MDA-MB-231 cells (Fig. 8C). However, capsaicin did not elicit the marked phosphorylations of p38 and ERK in

MCF10A cells. The conversion of LC3I to LC3II was observed after exposing film for an extended time, which suggests that MCF10A cells are less sensitive to capsaicin-induced autophagy than malignant MCF-7 or MDA-MB-231 cells and that capsaicin is able to induce the apoptosis of MCF10A cells. To compare protein expressions in three types of cell lines, all experiments were performed at the same times using the same chemicals, Western blotting systems, antibodies and their antibody dilutions, antibody incubation times, film exposure times, and so forth.

Discussion

It has been reported that MAPKs are involved in the regulation of autophagy (Corcelle et al., 2006; Tang et al., 2008; Chiu et al., 2009). In our previous study, autophagy induced by dihydrocapsaicin, an analog of capsaicin, was found to be regulated through JNK or ERK in WI38 lung epithelial fibroblast cells (Oh and Lim, 2009). Furthermore, MCF10A cells were found to elicit ER stress-mediated apoptosis via caspase-4 activation in response to capsaicin (Lee et al., 2009). In the present study, we found that malignant breast cells are highly sensitive to autophagy induction but that nonmalignant MCF10A cells did not promote autophagy induction. The capsaicin-induced autophagy is regulated by an interaction between p38 and ERK in MCF-7 and MDA-MB-231 cells; p38 regulates the sequestration of autophagosome and ERK promotes autolysosome formation, and p38 activa-

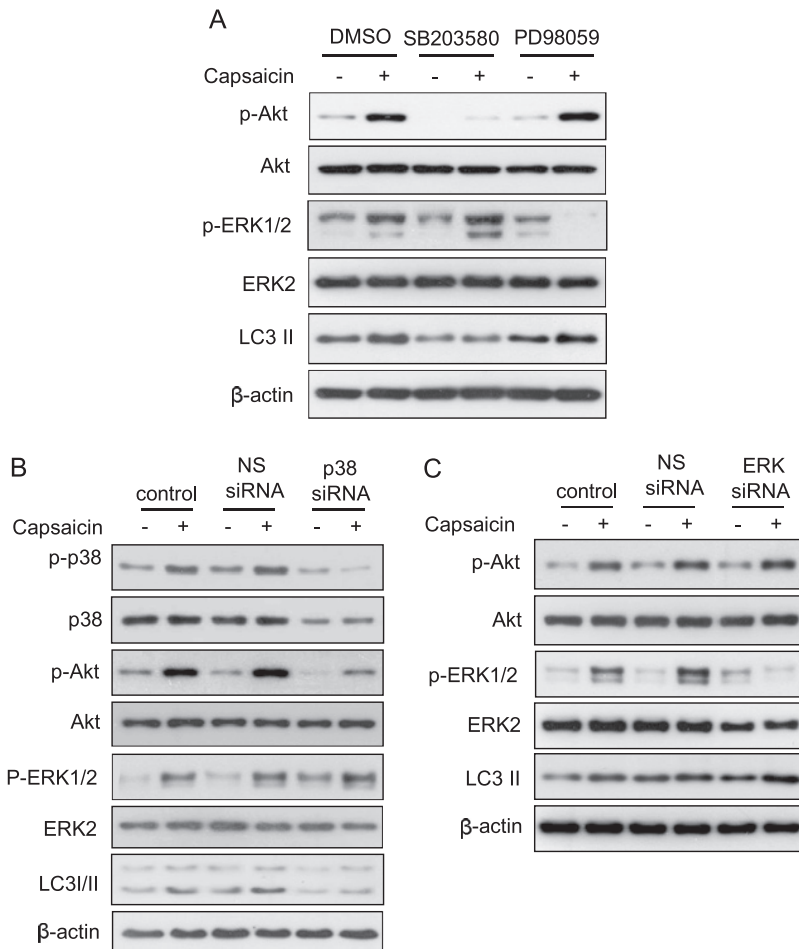


Fig. 6. Effects of p38 and ERK on capsaicin-induced autophagy. **A**, MCF-7 cells were pretreated with SB203580 (10 μM) or PD98059 (10 μM) for 30 min before 250 μM capsaicin was added for 6 h. Cells were then harvested and lysed, and lysates were analyzed for p38, p-p38, Akt, p-Akt, ERK, p-ERK, and LC3II by immunoblotting. **B**, knockdown of the p38 gene blocked capsaicin-induced autophagy. Cells transfected with nonspecific (NS) siRNA or p38-specific siRNA were treated with capsaicin (250 μM) or DMSO for 6 h, and lysates were analyzed by immunoblotting. **C**, knockdown of the ERK gene enhanced the induction of autophagy. Cells transfected with NS siRNA or ERK-specific siRNA were treated with capsaicin (250 μM) or DMSO for 6 h, and lysates were analyzed by immunoblotting. The immunoblots shown are representative of at least three independent experiments.

tion is mediated by IRE1, which indicates that capsaicin induces ER stress-mediated autophagy. Capsaicin had an antiproliferative effect and arrested the cell cycle at the S or G₀/G₁ phase without inducing apoptosis in MCF-7 and MDA-MB-231 cells.

However, when autophagy was blocked in both cells, capsaicin elicited the activation of caspase-4 and caspase-7 and enhanced cell death. Therefore, our data show that malignant breast cancer cells respond to capsaicin through autophagy and that enhanced cell survival, or at least an attenuation of the cell death response, is achieved by suppressing ER stress-mediated apoptosis.

Although blocking cell cycle progression in cancer cells might be of great advantage in terms of inhibiting tumor growth, cell cycle arrest after DNA damage allows time for DNA repair before cell cycle progression (Singh et al., 2002). However, excessive DNA damage should elicit the demise of damaged cells through apoptosis, which is important for preventing the cancer progression (Vousden and Lu, 2002). Accordingly, if apoptosis is not properly induced, damaged cells containing unrepaired DNA may proliferate and ultimately lead to tumorigenesis or carcinogenesis. Previous studies have reported that capsaicin causes DNA damage in human

endothelial cells, in SNU-1 Korean stomach cancer cells, and even in MCF-7 cells (Richeux et al., 2000; Tuoya et al., 2006), the latter of which lack the gene encoding caspase-3, an activator of caspase-activated DNase, which is responsible for DNA fragmentation (Samejima et al., 2001). However, in the present study, capsaicin at 250 μ M did not affect the sub-G₁ population in MCF-7 or MDA-MB-231 cells. Tuoya et al. (2006) found that capsaicin-induced DNA laddering was completely abrogated by a broad-spectrum caspase inhibitor, but they did detect DNA smearing on agarose gels, which could have been caused by pyknotic nuclei (Zamai et al., 2004). Autophagic cells do not exhibit DNA laddering, but partial chromatin condensation and nuclear pyknosis is considered to be a characteristic of these cells (Gozuacik and Kimchi, 2004). Therefore, it seems that MCF-7 cells might respond to capsaicin via some unknown pathway involved in cell survival or cell death. When we compared the cytotoxic effects of capsaicin on malignant breast cells (MCF-7 and MDA-MB-231 cells) with that on the nonmalignant breast cell line MCF10A, its cytotoxicity was found to be much higher in MCF10A cells than in both malignant breast cells, and this cytotoxicity was found to be caused by apoptosis. In the present study, MCF10A cells were found to respond to capsaicin-activated caspase-4 in a time- and dose-dependent manner, as was observed in our previous study (Lee et al., 2009). Although UPR is involved in the cell survival, severe or sustained ER stress response can induce ER stress-mediated apoptosis, and that is mediated by caspase-4/12 (Morishima et al., 2002; Rao et al., 2002). It is noteworthy that in MCF-7 and in MDA-MB-231 cells, capsaicin treatment increased the levels of the UPR-related proteins IRE1 and Chop but did not activate caspase-4. Furthermore, these cells did not exhibit any apoptotic signs by fluorescence-activated cell sorting analysis and Hoechst DNA staining. Therefore, our results suggest that capsaicin elicits different signaling pathways in normal and malignant cells.

Autophagy functions as a self-protective cellular response to stress, and thus, a defect in the process can promote oncogenesis (Kroemer and Jäättelä, 2005; Mathew et al., 2007). MCF-7 cells contain a monoallelic deletion of *beclin 1*, an autophagy-associated tumor suppressor gene, which might make them less sensitive to autophagy induction (Liang et al., 1999). Indeed, Høyer-Hansen et al. (2005) found that the ectopic expression of *beclin 1* in MCF-7 cells increased autophagic activity and sensitized the cells to seocalcitrol (EB1089), a vitamin D analog that causes cell death and inhibits cell growth.

However, the present study evidenced that capsaicin induces autophagy by various methods in MCF-7 and MDA-MB-231 cells. Furthermore, blocking of autophagy enhanced capsaicin-induced cell death. A recent study reported that oridonin-induced autophagy is a prerequisite of apoptosis, which suggests that autophagy and apoptosis are interconnected in MCF-7 cells (Cui et al., 2007). In our experimental setting, caspase-4 was activated when capsaicin-induced autophagy was pharmacologically blocked in MCF-7 and MDA-MB-231 cells, and this activation seemed to be related to increased cell death. Therefore, our data suggest that capsaicin-induced autophagy is involved in the suppression of ER stress-mediated apoptosis, and that may cause inhibition of cell death in malignant breast cells.

MAPK p38 and ERK have been shown to be involved in

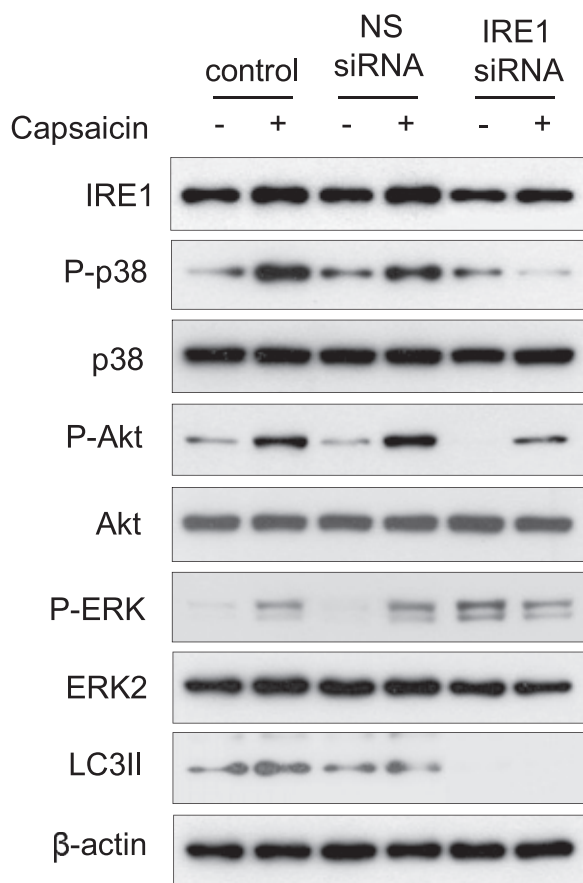


Fig. 7. IRE1 regulates capsaicin-induced autophagy through p38 MAPK. Cells transfected with nonspecific (NS) siRNA or IRE1-specific siRNA were treated with capsaicin (250 μ M) or DMSO for 6 h, and lysates were analyzed for p38, p-p38, Akt, p-Akt, ERK, p-ERK, and LC3II by immunoblotting. The results obtained confirmed silencing of the IRE1 gene by IRE1-specific siRNA. Furthermore, silencing of the IRE1 gene down-regulated p38 and Akt and ultimately down-regulated LC3II, but the p-ERK level increased. The immunoblots shown are representative of at least three independent experiments.

autophagy and ER stress signaling pathways. In neuronal cell death, ERK-dependent autophagy is known to play an important role (Aoki et al., 2007). In contrast, ERK activation was found to inhibit the formation of autolysosomes and ultimately to result in cell death inhibition by autophagy (Corcelle et al., 2006). The role of p38 in the regulation of autophagy also has been described previously. The Mograbi group reported that pentachlorophenol, an activator of ERK, inhibited autophagy through the activation of p38 in Sertoli cells (Corcelle et al., 2007). Furthermore, pharmacological blockage or genetic knockdown of p38 caused autophagy and death in colon cancer cells (Comes et al., 2007). In contrast, p38 activation achieved by Alexander disease-mutant glial fibrillary acidic protein accumulation-induced autophagy in astrocytes (Tang et al., 2008). In the present study, the activation of p38 was sustained for more than 24 h in MCF-7 and MDA-MB-231 cells, whereas ERK activation abruptly decreased after 12 h of treatment and highly accumulated LC3II over 48 h of treatment. Thus, we considered that the activation of p38 by capsaicin involves autophagy induction but that the constitutive activation of p38 can block ERK activation and ultimately cause accumulation of LC3II. Our belief was further confirmed by pharmacological inhibitors of autophagy. 3MA completely blocked capsaicin-induced p38 activation, suggesting that 3MA inhibits autophagosome formation by inactivating p38. Because BaF1 inhibits fusion between autophagosomes and lysosomes, autophagosome

formation is not inhibited by BaF1. BaF1 consistently had no effect on capsaicin-induced p38 activation but completely blocked the capsaicin-induced ERK activation, which suggests that ERK activation is required for the formation of autolysosome. Function of ERK in the autophagy process was further evidenced by using pharmacological blockade or genetic knockdown of ERK: enhanced the capsaicin-induced conversion of LC3I to LC3II, indicating that ERK is involved in the maturation of autophagy.

In MCF-7 and MDA-MB-231 cells, we found that sustained activation of p38 for more than 24 h by capsaicin treatment attenuated ERK activation, and that caused accumulation of LC3II. It is noteworthy that in both cells, down-regulation of p-p38 by 3MA or SB203580 or p38 siRNA enhanced capsaicin-induced p-ERK level. Furthermore, capsaicin treatment to MCF10A cells transiently activated ERK, did not elicit a marked activation of p38, and did not effectively promote LC3 conversion. Therefore, our present data of p38 and ERK in three types of cells suggest the relationship between ERK and p38 in the autophagy process. However, in the present study, we did not define the role of ERK during blocking of p38 activation by 3MA, SB203580, p38 siRNA, and IRE1 siRNA. IRE1 activation and the Akt-ERK pathway have been reported to play critical roles in cell survival in the presence of ER stress (Hu et al., 2004; Ogata et al., 2006; Lin et al., 2007). IRE1 forms a complex with tumor necrosis factor receptor-associated factor 2 and recruits apopto-

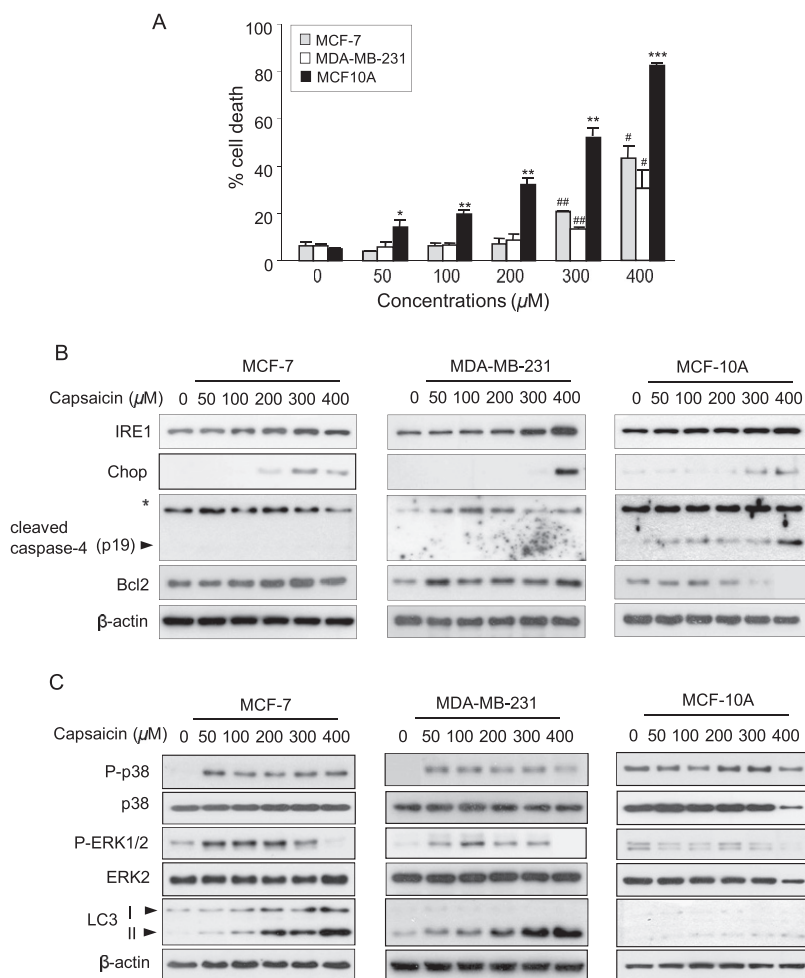


Fig. 8. The ER stress and autophagy responses of malignant breast cells to capsaicin were much more sensitive in those of nonmalignant breast cells. **A**, MCF-7 and MDA-MB-231 cells were much more resistant to capsaicin than MCF10A cells. Cells were treated with increasing concentrations of capsaicin for 24 h, and cell viability was determined using Trypan blue exclusion, as described under *Materials and Methods*. The data shown are percentages of the total number of cells. Values are expressed as means \pm S.D. of percentages from three independent experiments. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$; #, $P < 0.05$; ##, $P < 0.005$. **B** and **C**, MCF-7, MDA-MB-231, and MCF10A cells were treated as described in **A** for 12 h and then harvested and lysed. Lysates were then analyzed by immunoblotting. The immunoblots shown are representative of at least three independent experiments.

sis signal-regulating kinase and then activates JNK to promote apoptosis (Urano et al., 2000). Because capsaicin did not promote JNK in MCF-7 or in MCF10A cells (data not shown), we examined the involvement of p38 in capsaicin-induced IRE1 activation. Knockdown of IRE1 using siRNA was found to down-regulate p38-Akt signaling and LC3II level, which suggests that IRE1 might regulate the p38 signaling pathway to induce autophagy.

ER stress-mediated autophagy has been reported to be essential for cell survival under severe ER stress (Bernales et al., 2006), and MCF-7 cells respond to the conventional ER stressors thapsigargin and tunicamycin by inducing the Akt-ERK pathway, which assists cell survival by resisting ER stress-mediated apoptosis (Hu et al., 2004). Furthermore, the p38-Akt signaling pathway has been reported to play an important role in muscle differentiation (Cabane et al., 2004; Alisi et al., 2008). Collectively, these studies suggest that p38-Akt signaling is associated with cell survival. Indeed, in the present study, significant MCF-7 and MDA-MB-231 cell death was not induced by capsaicin at $>200 \mu\text{M}$. In both cells, treatment with different concentrations of capsaicin induced the sustained activation of p38, markedly increased LC3II levels, and up-regulated Bcl2. In contrast, in MCF10A cells, capsaicin did not promote p38 activation and LC3 conversion but induced the sustained activation of caspase-4 and the down-regulation of Bcl2 in a time- and dose-dependent manner and eventually caused apoptotic cell death. Taken together, these findings suggest that ER stress-mediated autophagy might be involved in cell survival or cell death retardation via p38 activation. However, we did not elucidate whether Bcl2 is related directly to cell survival in the present study.

In summary, the present study shows that capsaicin induces autophagy via ER stress response in MCF-7 and MDA-MB-231 cells and that this process is regulated by MAPK p38 and ERK and is involved in protecting cells against severe ER stress. However, in MCF10A cells, capsaicin was found to induce apoptosis via caspase-4 activation. These findings suggest that the targeting of autophagy genes may be of therapeutic benefit in cancers that are less sensitive to apoptosis-inducing agents.

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