Capsaicin Induces ATF4 Translation with Upregulation of CHOP, GADD34 and PUMA

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Primary Effusion Lymphoma (PEL) is a rare and aggressive B-lymphoma caused by Kaposi’s sarcoma-associated herpes virus (KSHV) infection that occurs in immunocompromised patients. PEL patients have a poor prognosis. KSHV modulates various cellular signaling pathways to maintain latent infection, and causes malignant conversion of host cells. We previously reported that capsaicin suppressed extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signaling and induced apoptosis in PEL. Generally, cellular stress such as nutrient starvation, oxidation and virus infection induce CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) expression by activating transcription factor 4 (ATF4), however endoplasmic reticulum (ER) stress induces CHOP expression by both ATF4 and ATF6. CHOP is associated with apoptosis induction and upregulates growth arrest and DNA damage-inducible protein 34 (GADD34) and p53 up-regulated modulator of apoptosis (PUMA) mRNA expression. In this study, we found a new mechanism in which capsaicin induces apoptosis via ATF4-CHOP-PUMA. Capsaicin promoted transcriptional activation of CHOP, which increased mRNA expression of GADD34 and PUMA, resulting in PEL apoptosis. Furthermore, capsaicin increased ATF4 protein levels by promoting ATF4 translation, not transcription, and had no effect on ATF6-dependent transcriptional activation. In sum, capsaicin promotes ATF4 translation and transcriptional induction of CHOP, which results in PUMA expression and apoptosis in PEL cells.

Key words primary effusion lymphoma; capsaicin; activating transcription factor 4; CCAAT/enhancer binding protein (C/EBP) homologous protein; apoptosis

INTRODUCTION

Primary effusion lymphoma (PEL) is classified as a non-Hodgkin’s B-cell lymphoma caused by Kaposi’s sarcoma-associated herpesvirus (KSHV) infection in immunocompromised patients, such as those with AIDS. Owing to the fact that conventional therapies including cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) therapy is ineffective to treat PEL, new treatment options are needed. The KSHV genome DNA is circularized in the nucleus of KSHV latently infected PEL cells. In latency, several viral proteins, such as LANA, v-FLIP, v-interleukin (IL)-6 and v-Cyclin are expressed in PEL cells and modulate signaling pathways including p53, Akt, mitogen-activated protein kinase (MAPK) and cytokine production, which contribute to the malignant phenotype of PEL.

Activating transcription factor 4 (ATF4), a basic-leucine zipper (bZIP) transcriptional factor, binds to cAMP response element (CRE) and amino acid response element (AARE). ATF4-mediated transcription is associated with development and responses against various cellular stress, such as amino acid starvation, hypoxia, oxidation, viral infection and endoplasmic reticulum (ER) stress. Although mRNA of ATF4 is constitutively expressed, translation is suppressed and only low levels of ATF4 protein are detectable in unstimulated cells. Cellular stress promotes ATF4 translation, not transcription, leading to the expression of both pro-survival and pro-apoptotic proteins, such as CHOP and growth arrest and DNA damage-inducible protein 34 (GADD34). GADD34 removes translational inhibition caused by cellular stress. CHOP is a transcriptional factor associated with initiating apoptosis, and facilitates mRNA expression of GADD34 and pro-apoptotic bcl family members Bim and p53 up-regulated modulator of apoptosis (PUMA).

We previously reported that capsaicin, an alkaloid contained in chili pepper, induced the transcriptional down-regulation of human IL-6 by suppressing extracellular signal-regulated kinase (ERK) and p38 MAPK signaling, and caused apoptosis in PEL. In addition, we confirmed that PEL growth could be suppressed by chemical inhibitors of ERK and p38 MAPK, respectively. In this study, we discovered a novel mechanism in which capsaicin induced ATF4 translation and apoptosis by inducing the expression of CHOP, GADD34 and PUMA in PEL.

MATERIALS AND METHODS

Reagents, Cell Lines, and Antibodies
Capsaicin (FUJIFILM Wako, Osaka, Japan), thapsigargin (Cayman Chemical, MI, U.S.A.), actinomycin D (FUJIFILM Wako) and cycloheximide (Merck, NJ, U.S.A.) were dissolved in dimethyl sulfoxide (DMSO). KSHV-positive PEL cell lines (BC2, BC3, HBL6 and JSC1) and a KSHV-negative B-cell line (Raji) were previously described. Primary antibodies used in this study included those against caspase-9, caspase-3 (Cell Signaling Technology, MA, U.S.A.), ATF-4, cyclin D1 and β-actin (Santa Cruz Biotechnology, TX, U.S.A.).

Quantitative RT-PCR (RT-qPCR) Cells were treated with 150 μM capsaicin for 3–12h. Then total RNA was extracted from harvested cells, and 80 or 160 ng total RNA
Table 1. Primers for Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>ATF4</td>
<td>5'-CCCTCCAACAACAGCAAGGA-3'</td>
<td>5'-ACCAAACAGGGAACATCCAAG-3'</td>
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<tr>
<td>ATF6</td>
<td>5'-GGAACTCAGGAGTGAGCTACAAG-3'</td>
<td>5'-AACCGCTCAACCTTCGAATG-3'</td>
</tr>
<tr>
<td>Bim</td>
<td>5'-CAGATATGCCGCCAGAGATAGT-3'</td>
<td>5'-ACATTCTGTGTTGGTCTTC-3'</td>
</tr>
<tr>
<td>BiP</td>
<td>5'-GGAAATTCCTGTCTCCTCCG-3'</td>
<td>5'-CAGGTGTCAGGGGATTCTTG-3'</td>
</tr>
<tr>
<td>CHOP</td>
<td>5'-GCCTGTATTGAGCAATGTT-3'</td>
<td>5'-TCTGGGAAAGTGGTGATG-3'</td>
</tr>
<tr>
<td>GADD34</td>
<td>5'-GAGGGCAGGAAGTCATATT-3'</td>
<td>5'-TCTCCCCCTGGTGTTTATCT-3'</td>
</tr>
<tr>
<td>GRP94</td>
<td>5'-TCTACAGAGACATGCTTCGACG-3'</td>
<td>5'-CTGACGGAGGCTTCCGT-3'</td>
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<tr>
<td>Bim</td>
<td>5'-ACCATTCGTGGGTGTTCTTC-3'</td>
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<tr>
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<td>5'-GGAAATTCCTGTCTCCTCCG-3'</td>
<td>5'-CAGGTGTCAGGGGATTCTTG-3'</td>
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<tr>
<td>CHOP</td>
<td>5'-GCCTGTATTGAGCAATGTT-3'</td>
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<td>PUMA</td>
<td>5'-ACCTCAACGCAACTGAGACG-3'</td>
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<td>sXBP1</td>
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<td>5'-GTAAGGCGAGGTCCCATG-3'</td>
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<td>uXBP1</td>
<td>5'-ACCTCAACGCAACTGAGACG-3'</td>
<td>5'-GTAAGGCGAGGTCCCATG-3'</td>
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<td>GAPDH</td>
<td>5'-GAGTCACCGGATTGTTCTCAG-3'</td>
<td>5'-GACAAGCTTCCCGTTCTCAG-3'</td>
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Fig. 1. (A) Capsaicin Increases mRNA Expression of CHOP and GADD34; (B) Capsaicin Enhances the Promoter Activity of CHOP Gene; (C) Capsaicin Upregulates mRNA Expression of Not Bim but PUMA in PEL Cells

(A) PEL (BC2, BC3, HBL6 and JSC1) and KSHV-negative Raji cells were treated with capsaicin (cap) for 3 h, and 80 ng of total RNA from harvested cells was subjected to RT-qPCR. Gray bars indicate vehicle (DMSO)-treated cells and black bars indicate cap-treated cells. The expression level of vehicle-treated Raji cells was defined as 1.0. (B) HeLa cells were transfected pGL3-CHOP (luciferase reporter plasmid containing the promoter region of CHOP gene) and pSV-β-GAL, and cells were treated with 150 µM cap or 0.1 µM thapsigargin (Tg; an ER stress inducer) for 15 h. The value of vehicle (DMSO)-treated cells was defined as 1.0. (C) Cells were treated with capsaicin for 12 h, and 160 ng of total RNA from harvested cells was tested by RT-qPCR. Gray bars indicate vehicle (DMSO)-treated cells and black bars indicate cap-treated cells. The value of vehicle-treated Raji cells was defined as 1.0.
was subjected to reverse transcription. The primer sets are shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The expression level of each gene was normalized to that of GAPDH.

Luciferase Reporter Assay HeLa cells (RIKEN BioResource Center, Saitama, Japan) were transfected with a luciferase promoter plasmid and pSV-β-GAL by calcium phosphate transfection. Then cells were treated with each chemical reagent for 15 h and were lysed in cold lysis buffer (20 mM Tris–HCl, pH 7.6, 0.1% NP40 and 1 mM dithiothreitol (DTT)) for luciferase and β-galactosidase assay. The luciferase activity divided by β-galactosidase activity in vehicle-treated cells was defined as 1.0. p5xATF6-pGL3 (5xATF6–binding site) was purchased from Addgene. pGL3-CHOP and pGL3-BiP (The promoter regions of CHOP and BiP genes, respectively) were kindly provided by Dr. K. Yoshida (Meiji University, Japan).

Statistical Analysis The standard deviation was determined by analyzing the data from at least three experiments and is indicated by error bars. The statistical significance between each group and the control was analyzed by one-way ANOVA followed by Dunnett’s test for multiple comparisons (Figs. 1B, 2A) or the two-tailed Student’s paired t-test (Figs. 1A, C). *p < 0.05, **p < 0.005 and ***p < 0.0005 indicate a statistical significance compared with vehicle-treated cells. ns, not significant. All data were analyzed by using GraphPad prism version 7 (GraphPad Software).

RESULTS AND DISCUSSION

We previously found that capsaicin suppressed ERK and p38 MAPK signaling and induced apoptosis in PEL cells. Furthermore, we reported that severe ER stress induced apoptosis in PEL cells, so we examined whether capsaicin upregulated ER stress responsible molecules. PEL and KSHV-uninfected cells were treated with capsaicin, and mRNA expression was evaluated by RT-qPCR (Fig. 1A). The mRNA expression of BiP, GRP94 and ATF4 were not increased by capsaicin treatment in PEL cells. Capsaicin weakly increased the mRNA expression of spliced XBP1 (sXBP1) and unspliced XBP1 (uXBP1). When uXBP1 mRNA is spliced by IRE1α, sXBP1 mRNA is produced and translated into XBP1 protein. The ratio of sXBP1/uXBP1 was modestly affected by capsaicin treatment. On the other hand, mRNA expression of CHOP and GADD34 were markedly increased in PEL cells and modestly increased in Raji cells by capsaicin. In particular, capsaicin had little effect on mRNA expression of ER stress responsible molecules including ATF4, whereas capsaicin induced mRNA expression of CHOP and GADD34. We also confirmed CHOP induction by capsaicin using a reporter assay.

HeLa cells were transfected with a luciferase plasmid containing the promoter region of the CHOP gene. In line with results observed in Fig. 1A, capsaicin increased CHOP promoter activity (Fig. 1B). Thapsigargin, a potent ER stress inducer, inhibits Sarcoplasmic/ER Ca2+-ATPase (SERCA) and induces CHOP upregulation. Furthermore, we evaluated whether capsaicin increased the expression of CHOP target
genes, Bim and PUMA. Cells were treated by capsaicin for 12 h, and mRNA expression was analyzed by RT-qPCR (Fig. 1C). Expression of PUMA was increased by capsaicin in all PEL cells. However, Bim expression was slightly increased in capsaicin-treated cells, except for HBL6 cells. Bim is known to be targeted by multiple transcription factors, such as FoxO3a, NF-Y and signal transducer and activator of transcription (STAT)-1, but its expression is suppressed by several survival pathways. Therefore, Bim expression might be tightly regulated by molecules involved in these pathways during capsaicin treatment. Consequently, capsaicin promoted transcriptional activation of CHOP and PUMA in PEL cells.

Next, we investigated the CHOP expression machinery upon capsaicin treatment. Both of ATF4 and ATF6 are major factors which induce CHOP expression. ATF4 translation, but not transcription, is promoted by cellular stress. ER membrane-associated ATF6 is cleaved by ER stress, and the N-terminal region of ATF6 translocates to the nucleus and functions as a transcription factor, which facilitates the expression of ER stress responsive molecules, such as BiP and xBP1. Therefore, we further investigated the effect of capsaicin on ATF4 and ATF6-mediated transcription. We examined whether capsaicin influenced ATF6-dependent transcriptional activity and the promoter activity of the BiP gene by a luciferase reporter assay (Fig. 2A). HeLa cells were transfected with luciferase plasmid containing either an ATF6 binding sequence (ERSE: ER stress response element) or the promoter of BiP gene and treated with capsaicin for 15 h. However, capsaicin did not have any effect. We next examined effect of capsaicin on the protein levels of ATF4 (Fig. 2B). Cells were treated with capsaicin for 6, 12 or 24 h and subjected to Western blotting analysis. Capsaicin increased ATF4 protein levels in all cells tested. We further confirmed cleavage (i.e., activation) of caspase-9 and -3 by capsaicin in PEL cells. On the basis of these results, we hypothesize that capsaicin promotes the ATF4-CHOP pathway and induces apoptosis in PEL cells, however capsaicin had little effect on ATF6 activation and ER stress. As an increase in ATF6 mRNA expression was detected in only JSC1 cells (Fig. 1A), not only ATF4 but also ATF6 might be associated with CHOP induction in JSC1 cells. Next, we elucidated the effect of capsaicin on transcription and translation of ATF4. HBL6 cells were co-treated with capsaicin and either the transcription inhibitor actinomycin D or the translation inhibitor cycloheximide (chx) (Fig. 2C). As a result, chx dramatically suppressed capsaicin-induced ATF4 expression, whereas actinomycin D had no effect. These results are supported by RT-qPCR data in Fig. 1 showing that capsaicin did not affect ATF4 mRNA expression. Cyclin D1 was used as a control for showing the effects of both inhibitors. These indicate that capsaicin increased ATF4 protein by facilitating translation or inhibiting degradation. Finally, we investigated whether capsaicin stabilized ATF4 (Fig. 2D). To induce ATF4 production, HBL6 cells were pre-treated with capsaicin for 6 h and re-suspended in fresh media containing chx. After 30 min, cells were treated with or without capsaicin for 2–8 h. Almost all ATF4 protein was degraded 2 h after chx treatment both with and without subsequent capsaicin treatment. These results indicate that the half-life of ATF4 was within 2 h, and capsaicin had little effect on ATF4 protein turnover. Taken together, capsaicin increased the amount of ATF4 protein by facilitating ATF4 translation.

ATF4 forms a heterodimer with other bZIP proteins, such as c-fos, c-jun, other ATFs and C/EBP family members including C/EBPα/β and CHOP. The binding partner of ATF4 is involved in determining transcriptional targets of transcriptional heterodimers including ATF4. ERK and p38 MAPK signaling enhances the expression of several bZIP proteins such as ATF1/2, c-fos, C/EBPβ and CHOP. We previously reported that capsaicin suppressed ERK and p38 MAPK signaling in PEL and KSHV-uninfected B cell lines, however the activation level of those signaling pathways was different in each cell line. Thus, we speculate that the observed differences in MAPK activation levels is related to the different expression levels of ATF4 and CHOP-targeted molecules, such as PUMA, GADD34, CHOP and Bim, in each respective B cell line.

In conclusion, capsaicin increases ATF4 protein levels by facilitating ATF4 translation from mRNA. Capsaicin induces expression of CHOP and GADD34, which are transcriptionally upregulated by ATF4. CHOP induces the transcriptional activation of PUMA, resulting in apoptosis induction in PEL cells. In light of these findings, capsaicin may be a new option for treating PEL.

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**Conflict of Interest** The authors declare no conflict of interest.

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