Abstract

Here, we investigated the potential mechanism of capsaicin-mediated apoptosis in pancreatic cancer cells. Capsaicin treatment phosphorylated c-Jun-NH₂-kinase (JNK); forkhead box transcription factor, class O (FOXO1); and BIM in BxPC-3, AsPC-1, and L3.6PL cells. The expression of BIM increased in response to capsaicin treatment. Capsaicin treatment caused cleavage of caspase-3 and PARP, indicating apoptosis. Antioxidants tiron and PEG-catalase blocked capsaicin-mediated JNK/FOXO/BIM activation and protected the cells from apoptosis. Furthermore, capsaicin treatment caused a steady increase in the nuclear expression of FOXO-1, leading to increased DNA binding. Capsaicin-mediated expression of BIM was found to be directly dependent on the acetylation of FOXO-1. The expression of CREB-binding protein (CBP) was increased, whereas SirT-1 was reduced by capsaicin treatment. Using acetylation mimic or defective mutants, our result demonstrated that phosphorylation of FOXO-1 was mediated through acetylation by capsaicin treatment. JNK inhibitor attenuated the phosphorylation of FOXO-1, activation of BIM, and abrogated capsaicin-induced apoptosis. Moreover, silencing FOXO1 by siRNA blocked capsaicin-mediated activation of BIM and apoptosis, whereas overexpression of FOXO-1 augmented its effects. Silencing Bim drastically reduced capsaicin-mediated cleavage of caspase-3 and PARP, indicating the role of BIM in apoptosis. Oral administration of 5 mg/kg capsaicin substantially suppressed the growth of BxPC-3 tumor xenografts in athymic nude mice. Tumors from capsaicin-treated mice showed an increase in the phosphorylation of JNK, FOXO-1, BIM, and levels of CBP, cleavage of caspase-3, PARP, and decreased SirT-1 expression. Taken together, our results suggest that capsaicin activated JNK and FOXO-1, leading to the acetylation of FOXO-1 through CBP and SirT-1. Acetylated FOXO1 induced apoptosis in pancreatic cancer cells through BIM activation. Mol Cancer Ther; 13(3); 1–12. ©2014 AACR.
increasing the phosphorylation of FOXO4 and reducing the protein levels of Bim (19). It is important to know whether phosphorylation of FOXO through oxidative stress is involved in Bim-induced apoptosis. Many histone deacetylase (HDAC) inhibitors were also found to induce apoptosis in tumor cells through Bim and Noxa, but the exact mechanism underlying this effect was not clear (20).

Capsaicin, a homovanillic acid derivative (N-vanillyl-8-methyl-nonenamide), has been used as spices in South Asian and Latin American countries (21–24). In our previous study, we have shown that capsaicin-induced apoptosis in pancreatic cancer cells was associated with the generation of reactive oxygen species (ROS) and persistent disruption of mitochondrial membrane potential without affecting the normal cells (25, 26). In the present study, we explored the mechanism of FOXO phosphorylation and acetylation by capsaicin in BxPC-3, AsPC-1, and L3.6PL pancreatic cancer cells.

Materials and Methods

Chemicals and antibodies

Capsaicin (purity > 99%), anti-actin, PEG-catalase, tiron, and nicotinamide were obtained from Sigma. The antibodies against Cl-caspase-3, Cl-PARP, JNK, p-JNK (Thr183/Tyr185), FOXO-1, p-FOXO-1 (Ser256), FOXO-3a, p-FOXO-3a (Ser253), BIM, p-BIM (Ser69), PI3K, p-PI3K (Tyr458), Akt, p-Akt (Ser473), 14-3-3 binding motif, CBP/p300, SirT-1, SirT-2, SirT-3, acetylated lysine, and acetylated CBP/p300 were purchased from Cell Signaling Technology. Lamin B was purchased from Santa Cruz Biotechnology, Inc. Apoptosis detection kit annexin V-fluorescein isothiocyanate (FITC) was procured from BD Biosciences, Inc. Alexa fluor 488 (green) and alexa fluor 594 (red)-conjugated goat-anti-rabbit secondary antibody and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen. SP60025, a specific JNK inhibitor, was procured from Cayman Chemicals.

Cell culture

Human pancreatic cancer cell lines, AsPC-1 and BxPC-3, were obtained from American Type Culture Collection in 2004, and L3.6PL was a generous gift from Dr. Ming-Hai Wang (Texas Tech University of Healthy Science Center, Amarillo, TX) in 2011. AsPC-1 and BxPC-3 cells were authenticated using short tandem repeat analysis by the core facility at the Cancer Center of Texas Tech University Health Sciences Center (Lubbock, TX). However, L3.6PL cells were not authenticated during the course of studies but now are being authenticated. Monolayer cultures of AsPC-1 and BxPC-3 cells were maintained in RPMI medium and L3.6PL was maintained in MEM medium supplemented with 10% FBS, PSN antibiotic mixture (10 ml/L), 2 mmol/L l-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 20% glucose. All the cultures were maintained at 37°C in a humidified chamber with 95% air and 5% CO2.

Transient transfection

The L3.6PL and AsPC-1 cells were transiently transfected with WT-FOXO-1 or FOXO-1 acetylation mutant (3KQ and 3KR) plasmid (a generous gift from Dr. Akiyoshi Fukushima, University of Tsukuba, Japan) using FuGENE-6 transfection reagent (Roche Diagnostics). Briefly 0.3 × 10⁶ L3.6PL and AsPC-1 cells were transfected with 2 μg WT FOXO-1, 1.2 μg of 3KQ (acetylation mimic) and 2 μg of 3KR (acetylation-defective mutant), respectively, using Opti-MEM serum-free medium. Cells were incubated with plasmid-FuGENE-6 mixture for 6 hours and after that, media were replaced with fresh RPMI media. Transfected cells were treated with dimethyl sulfoxide (DMSO) or 150 μmol/L capsaicin for indicated time periods.

Determination of BIM and CBP mRNA transcripts

To determine the effect of capsaicin on BIM and CREB-binding protein (CBP) gene transcription, BxPC-3 cells were treated with 150 μmol/L capsaicin for 24 hours. RNA was extracted from control and capsaicin-treated cells using Trizol RNA extraction reagent (Life Technologies, Inc.). RNA samples were prepared for reverse transcriptase PCR (RT-PCR) as described previously (27). The following primer sets were used: Bim-F, 5'-GAGAAGGTGA-CATTGCGAG-3', Bim-R, 5'-GACAATGTAACGTA-CAGTCG-3'; CBP-F, 5'-GGCGCTGTCAATCACAACCCC-3', CBP-R, 5'-CGTAGTCTCGACACAGTG-3'. RT-PCR was performed in 50 μL reactions using 100 ng RNA, 0.5 μmol/L of each primer, and an annealing temperature of 53°C for 40 cycles for BIM primer and 58°C for 40 cycles for CBP primer. The PCR products were separated on a 1.5% agarose gel, stained with 0.5 mg/mL ethidium bromide, and visualized under UV light.

Tumor therapy model

Tumor therapy experiments were performed as described by us previously (28, 29) with minor modifications. The use of athymic nude mice and their treatment was approved by the Institutional Animal Care and Use Committee, Texas Tech University of Health Science Center. All the experiments were carried out in strict compliance with their regulations. Exponentially growing BxPC-3 (1 × 10⁶) cells were injected subcutaneously into the right flanks of 20 athymic nude mice. When the tumors reached a size of approximately 70 mm³, mice were randomly segregated into two groups with 10 mice in each group. Test group of mice received 5 mg/kg body weight capsaicin in PBS by oral gavage every day for 35 days, whereas control mice received vehicle alone. Tumor volume and animal weights were taken as described previously (28, 29).

siRNA transfection

The AsPC-1 or BxPC-3 cells were transfected with FOXO-1 siRNA or BIM siRNA for 48 hours using siPORT NeoFX transfection reagent (Ambion INC.). Briefly, 0.3 × 10⁶ AsPC-1 or BxPC-3 cells were transfected with 60 nmol/L
of FOXO-1 siRNA or BIM siRNA using Opti-MEM serum-free medium. Cells were incubated with plasmid-siPORT mixture for 6 hours and after that, media were replaced with fresh RPMI media. Transfected cells were treated with 150 μmol/L capsaicin for indicated time period.

**Immunoprecipitation assay**

Immunoprecipitation assay was performed to examine the effect of capsaicin on the interaction of SirT-1, SirT-2, and CBP/p300, or acetylated lysine with FOXO-1 protein. Briefly, BxPC-3 cells were treated with DMSO or 150 μmol/L capsaicin for 24 hours, and whole-cell lysates were lysed using radioimmunoprecipitation assay buffer and immunoprecipitated with anti-SirT-1, anti-SirT-2, anti-CBP/p300, or anti-acetylated lysine or FOXO-1 antibody as described previously (29). The samples were immunoblotted with respective antibody.

**Electrophoretic mobility shift assay**

BxPC-3 cells were treated with DMSO or 150 μmol/L of capsaicin with or without transfection of WT-FOXO-1 for 24 hours. Nuclear fraction was isolated by nuclear extraction kit and FOXO-1 protein was captured using biotin-labeled FOXO-1-binding site oligomer 5'-CAAAACAACAAAA-CAACAAAAACA-3' and performed DNA-binding activity using commercially available kit from Panomics.

**Immunofluorescence assay**

BxPC-3 cells were plated on coverslips and allowed to attach overnight and then treated with 150 μmol/L capsaicin for 24 hours. Treated and untreated cells were fixed with acetone:methanol (1:1) mixture and blocked with goat-serum for 1 hour and incubated with FOXO-1 antibody overnight at 4°C. Immunofluorescence was detected by anti-rabbit immunoglobulin G (IgG) conjugated with DyLight 800 (red), DyLight 594 (green), and DAPI (blue). After four washing, coverslips were then mounted with antifade mounting reagents. Nuclei were stained with DAPI (blue) and the immunofluorescence was observed by a fluorescence microscope using oil immersion at ×60 magnification.

**Annexin-FITC apoptosis assay**

Apoptosis induction by capsaicin was assessed by annexin V/FITC by flow cytometry. About 0.3 × 10⁶ AsPC-1 or BxPC-3 cells were seeded in a 6-well plate and treated with 150 μmol/L of capsaicin for 24 hours after JNK inhibitor or BIM siRNA treatment. Apoptosis was determined using APOPTEST-FITC kit according FITC-Annexin V apoptosis detection kit (BD Biosciences) to manufacturer’s instructions and analyzed by Accuri C6 flow cytometer.

**Western blot analysis**

Cells were exposed to various concentrations or 150 μmol/L capsaicin for 24 hours or different time points and lysed on ice as described by previously (28, 29). In a separate experiment, cells were pretreated with PEG-catalase (500 U/mL), tiron (10 mmol/L), or LY294002 (10 μmol/L) for 1 hour followed by treatment with 150 μmol/L capsaicin for 24 hours. Whole-cell extracts were prepared as mentioned above. The tumors from control and capsaicin-treated mice were minced and lysed by the procedure described previously (28). The cell lysate was cleared by centrifugation at 14,000 g for 30 minutes. Cell lysate containing 10 to 80 μg protein was resolved by 6% to 12.5% SDS-PAGE and the proteins were transferred onto polyvinylidene fluoride membrane. After blocking with 5% nonfat dry milk in Tris-buffered saline, membrane was incubated with the desired primary antibody (1:1000 dilutions) overnight. Subsequently, the membrane was incubated with appropriate secondary antibody (1:2000 dilutions), and the antibody binding was detected by using enhanced chemiluminescence kit according to the manufacturer’s instructions. Each membrane was stripped and reprobed with antibody against actin (1:20000 dilutions) to ensure equal protein loading.

**Statistical analysis**

All statistical calculations were performed using GraphPad Prizm 5.0. ANOVA was used to test the statistical significance of difference between control and treated groups followed by Bonferroni post hoc analysis for multiple comparisons. P values less than 0.05 were considered statistically significant.

**Results**

**Capsaicin triggers apoptosis by activating JNK/FOXO/BIM cascade in pancreatic cancer cells**

In our previous study, we have demonstrated that capsaicin induced apoptosis in pancreatic cancer cells by causing ROS generation through mitochondrial complex-I and complex-III (25). But the exact mechanism by which capsaicin induced apoptosis was not clear. Previous study reported that mitogen-activated protein kinases get activated by oxidative stress and cause apoptosis by activating proapoptotic protein BIM in PC12 cells (15). Other studies reported the involvement of FOXO-3a in inducing cell death through BIM (2, 16–18). We hypothesized that capsaicin mediated ROS generation and apoptosis was through JNK/FOXO/BIM cascade. To determine the effect of capsaicin on JNK/FOXO/BIM cascade, BxPC-3, AsPC-1, and L3.6PL pancreatic cancer cells were treated with various concentrations of capsaicin for 24 hours or various time points. Our results reveal that capsaicin treatment increased the phosphorylation of JNK at Thr183/Tyr185, FOXO-1 at Ser256, and BIM at Ser69 but not FOXO-3a at Ser253 (Fig. 1A–C). Capsaicin treatment also increased BIM protein levels. Furthermore, cleavage of caspase-3 was observed by capsaicin treatment indicating apoptosis (Fig. 1A–C). In a time-dependent study, our results demonstrated that capsaicin treatment increased the phosphorylation of JNK, FOXO-1, and BIM as early as 2 hours and cleavage of caspase-3 at 8 hours (Fig. 1D), indicating that cell death induced by capsaicin required early phosphorylation of JNK, FOXO-1, and BIM to initiate apoptosis.
Capsaicin-mediated ROS generation activates JNK/FOXO/BIM but not PI3K/AKT cascade

To test whether ROS were involved in the activation of JNK/FOXO/BIM cascade, cells were pretreated with antioxidants, tiron, or PEG-catalase. Our results show that capsaicin failed to activate JNK/FOXO/BIM cascade and induce apoptosis when BxPC-3 cells were pretreated with tiron or PEG-catalase (Fig. 2A and B). These results suggest that capsaicin-induced apoptosis by JNK/FOXO/BIM cascade involved ROS as upstream regulator. Previous study reported that FOXO phosphorylation depends on two independent pathways. Phosphorylation of FOXO proteins in response to growth factor causes exclusion of FOXO protein from the nucleus by activated Akt (30). On the other hand, phosphorylation of FOXO protein in response to oxidative stress involving JNK imports FOXO into the nucleus. In our previous study, we have already reported that capsaicin-mediated ROS generation induces apoptosis in pancreatic cancer cells (25). To see whether capsaicin modulates the PI3K/Akt pathway, we treated BxPC-3 and AsPC-1 cells with various concentration of capsaicin for 24 hours. Our results show that capsaicin neither affected the phosphorylation of phosphoinositide 3-kinase (PI3K) and Akt nor the protein levels of PI3K and Akt (Fig. 2C), indicating that the PI3K/Akt pathway was not affected by capsaicin and not involved in the activation of FOXO-1. To further exclude the involvement of the PI3K pathway in capsaicin-mediated activation of FOXO-1, we treated BxPC-3 cells with PI3K-specific inhibitor, LY294002, before treatment with capsaicin. No significant change in the phosphorylation or expression of FOXO-1 or BIM was observed in the cells treated with LY-294002 and capsaicin together (Fig. 2D). Nonetheless, LY294002 alone reduced the phosphorylation of FOXO-1 and protein level of BIM (Fig. 2D). As shown in Fig. 2D, the increase in the phosphorylation of FOXO-1 by capsaicin treatment was blocked by LY294002. However, as compared with capsaicin treatment alone, combined treatment of capsaicin and LY294002 increased the cleavage of caspase-3 (Fig. 2D). These results indicate that LY294002 inhibits PI3K and further adds to capsaicin-induced apoptosis.

Capsaicin treatment causes nuclear retention of FOXO-1

FOXO proteins if sequestered in the nucleus are involved in the transcription of BIM. Because we observed an increase in BIM expression by capsaicin treatment, we next wanted to see whether FOXO was accumulated in the nucleus. Our results show that FOXO-1 levels steadily increased in the nuclear fraction of capsaicin-treated cells
No change was observed in the expression of FOXO-3a by capsaicin treatment (Fig. 3A). The nuclear retention of FOXO-1 was further confirmed by immunofluorescence. Our results show intensified staining of FOXO-1 (red) in the nucleus in response to the capsaicin treatment (Fig. 3B). Next, we wanted to see whether the retention of FOXO-1 in the nucleus led to increased DNA-binding activity. Our electrophoretic mobility shift assay (EMSA) shows that capsaicin treatment significantly increased the DNA binding of FOXO-1 in the nucleus (Fig. 3C). To confirm the increased DNA-binding activity of FOXO-1 in response to capsaicin treatment, BxPC-3 cells were transiently transfected with WT-FOXO-1 plasmid and then treated with capsaicin. Our results demonstrate that capsaicin treatment substantially increased the DNA-binding activity when compared with control (Fig. 3C). Previous studies suggested that once FOXO-1 is phosphorylated, it binds with 14-3-3 chaperone proteins, which export the complex from the nucleus resulting in the downregulation of BIM (28). To confirm the interaction of FOXO-1 and 14-3-3 proteins, we immunoprecipitated FOXO-1 and immunoblotted for 14-3-3 proteins. As expected, capsaicin treatment drastically decreased 14-3-3 binding with FOXO-1 protein indicating its retention in the nucleus (Fig. 3D).

**CBP-mediated FOXO-1 acetylation by capsaicin treatment and regulated by SirTs**

Stress-induced nuclear translocation initiates modification and regulation of FOXO proteins by acetylation. Transcription activity of FOXO is mediated through the acetylation of critical lysine residues by CREB-binding proteins (CBP/p300) and SirTs, respectively (1). To prove this, BxPC-3, AsPC-1, and L3.6PL cells were treated with various concentrations of capsaicin for 24 hours and analyzed by Western blotting. Our results show that capsaicin treatment substantially increased the expression of CBP and BIM (Fig. 4A), while expression of SirT-1, SirT-2, and SirT-3, which are the negative regulators of acetylation, were decreased (Fig. 4A) in all the cell lines. Furthermore, capsaicin treatment increased the mRNA levels of CBP and BIM (Fig. 4A), indicating an increase in CBP-mediated acetylation of FOXO proteins.
indicating that capsaicin-mediated increased protein levels of BIM and CBP in pancreatic cancer cells were transcriptionally mediated.

To further confirm the acetylation of FOXO-1, control and capsaicin-treated AsPC-1 cells were immunoprecipitated with anti-CBP/p300 or anti-acetylated lysine or anti-FOXO-1. Capsaicin treatment increased FOXO-1 acetylation (Fig. 4B). Our results also showed that capsaicin treatment significantly increased the levels of acetylated CBP/p300 in the cells immunoprecipitated with FOXO-1 (Fig. 4B), indicating that capsaicin caused FOXO-1 acetylation by recruiting CBP to FOXO-1. We next wanted to see the role of CBP-catalyzed acetylation of FOXO-1 in activating BIM protein levels. To prove this, we transfected BxPC-3 cells with CBP siRNA and treated with capsaicin. Our results revealed that CBP siRNA significantly decreased capsaicin-induced BIM expression, indicating that capsaicin-induced BIM expression was perhaps through CBP-mediated FOXO-1 acetylation (Fig. 4B, right).

Previous study reported that the transcriptional activities of FOXO proteins were regulated by CBP/p300-mediated acetylation and SirT-1–mediated deacetylation of FOXO (1). To confirm whether SirT-1 was involved in the acetylation of FOXO-1 in response to capsaicin treatment, AsPC-1 cells were treated with 150 µmol/L capsaicin for 24 hours and immunoprecipitated with anti-SirT-1, anti-SirT-2, or anti-FOXO-1 antibodies (Fig. 3C). Our results demonstrated that capsaicin treatment decreased the recruitment of SirT-1 and SirT-2 to FOXO-1 (Fig. 3C). These results indicated that capsaicin weakens the interaction of SirT-1/SirT-2 with FOXO-1 and increases the recruitment of CBP/p300, resulting in the acetylation of FOXO-1, which in turn causes induction of BIM. To confirm this hypothesis, BxPC-3 cells were treated with nicotinamide, which is a strong inhibitor of SirT. Nicotinamide treatment increased capsaicin-mediated BIM expression (Fig. 4C). These results indicated that SirT inhibition by nicotinamide synergistically increased capsaicin-mediated BIM expression. A significant increase in FOXO-1 was also observed in nicotinamide-treated cells (Fig. 4C, right). These results showed the involvement of SirT-1 in regulating the acetylation of FOXO-1, resulting in the increased expression of BIM.

**FOXO-1 acetylation plays critical role in FOXO-1 phosphorylation**

Our next step was to elucidate the sequences of events related to FOXO-1 posttranslational modification. To test
this, we transfected AsPC-1 cells with 3KQ plasmid. 3KQ is a FOXO-1–mutant (acetylation mimic) plasmid in which the Lys residues of all three CBP-dependent acetylation sites (Lys-242, Lys-245, and Lys-262) were replaced by glutamine. These substitutions mimic the constitutively acetylated form through neutralization of the positive
Charges (31, 32). Our results showed that 3KQ transfection increased the constitutive phosphorylation of FOXO-1 at Ser 256, which was further enhanced by capsaicin treatment (Fig. 4D). The acetylation mimic (3KQ) further increased capsaicin-mediated expression and phosphorylation of BIM at Ser 69 (Fig. 4D). To confirm these observations, we then transfected AsPC-1 cells with 3KR plasmid. 3KR is acetylation-defective mutant in which lysine residues (Lys-242, Lys-245, and Lys-262) were replaced by arginine residues (31). Our results revealed that 3KR (acetylation-defective mutant) blocked FOXO-1 phosphorylation by capsaicin treatment (Fig. 4D). BIM was not activated either by capsaicin treatment in AsPC-1 cells transfected with 3KR (Fig. 4D). These results clearly suggest that acetylation precedes phosphorylation during posttranslational modification of FOXO-1 by capsaicin treatment in pancreatic cancer cells.

**Capsaicin-induced apoptosis and activation of JNK/FOXO/BIM cascade prevented by JNK inhibitor**

To confirm, whether capsaicin-mediated apoptosis was through the activation JNK/FOXO/BIM cascade, cells were treated with JNK inhibitor before capsaicin treatment. Our results showed that JNK inhibitor not only blocked the phosphorylation of JNK at Thr183/Tyr185, but also blocked the phosphorylation of FOXO-1 at Ser256 and BIM at Ser69 in BxPC-3 cells. In addition, JNK inhibitor also blocked capsaicin-mediated BIM expression and cleavage of caspase-3 (Fig. 5A). These results indicated that capsaicin-mediated activation of JNK/FOXO/BIM was responsible for inducing apoptosis in pancreatic cancer cells (Fig. 5A). These results were confirmed by apoptosis assay using flow cytometry, where JNK inhibitor significantly blocked capsaicin-induced apoptosis when compared with controls (Fig. 5A), indicating that JNK activation is required for capsaicin induced-apoptosis in pancreatic cancer cells.

**Involvement of FOXO-1 and BIM in capsaicin-mediated apoptosis**

To prove the involvement of FOXO-1 and BIM in capsaicin-induced apoptosis, BxPC-3 and AsPC-1 cells were transiently transfected with WT-FOXO-1 plasmid or FOXO-1 siRNA, respectively, and treated with 150 μmol/L capsaicin for 24 hours. Our results demonstrated that transfection of WT-FOXO-1 increased the phosphorylation and protein level of FOXO-1 in response to capsaicin treatment (Fig. 5B). Our results also showed that WT-FOXO-1 transfection increased the phosphorylation and protein level of BIM, indicating that capsaicin-mediated BIM activation was through FOXO-1 (Fig. 5B). On the other hand, FOXO-1 siRNA blocked the phosphorylation and protein levels of FOXO-1 and BIM in response to capsaicin treatment, indicating that activation of FOXO-1 regulates BIM and apoptosis (Fig. 5C). To prove the role of BIM in capsaicin-induced apoptosis, AsPC-1 cells were transfected with BIM siRNA and treated with 150 μmol/L capsaicin. Our results revealed that BIM siRNA blocked capsaicin-mediated increased expression of BIM and cleavage of PARP (Fig. 5D). These observations were confirmed by annexin V-FITC assay using flow cytometer, where BIM siRNA significantly prevented capsaicin-induced apoptosis, indicating that capsaicin-mediated activation of BIM causes apoptosis in pancreatic cancer cells. (Fig. 5D).

**Capsaicin suppresses the growth of BxPC-3 human pancreatic tumor xenografts**

To test the possibility that capsaicin treatment would suppress pancreatic tumor growth, BxPC-3 tumor xenografts were implanted in athymic nude mice. Once each mouse had palpable tumors, mice were randomized into two groups and treated group of mice was fed 5 mg/kg capsaicin every day and tumor growth was recorded periodically. Our results showed that oral gavage of 5 mg/kg every day drastically reduced the growth of the tumors starting day 15 of the treatment and continued till the end of the experiment (Fig. 6A). At day 35 of the treatment, tumor volume in the treated group was reduced by 76% as compared with control groups (233.48 ± 35.82 mm³ vs. 55.28 ± 12.73 mm³, n = 10; Fig. 6A). The average body weight of the control and capsaicin-treated mice did not change throughout the experiment, indicating no apparent systemic toxicity in capsaicin-treated mice (Fig. 6B). The average wet weight of the tumors dissected from capsaicin-treated mice was approximately 71% less than the weight of the tumors from the control mice (Fig. 6C). Right panel shows the size of control and capsaicin treated tumors to compare the efficacy of capsaicin.

**Capsaicin-mediated tumor growth inhibition was associated with activation of JNK/FOXO/BIM cascade**

We next aimed to investigate the mechanism and wanted to know whether capsaicin-mediated pancreatic tumor growth was through JNK/FOXO/BIM cascade. To confirm this hypothesis, tumors from control and capsaicin-treated mice were examined by Western blotting. In agreement with our in vitro data in BxPC-3 cells, phosphorylation of JNK at Thr183/Tyr185, FOXO-1 at Ser256, BIM at Ser69 was found to be much higher in the tumors of capsaicin-treated mice as compared with controls (Fig. 6D). Our results also demonstrated that capsaicin significantly decreased SirT-1 and increased CBP/p300 and BIM protein level in the tumors (Fig. 6D). The cleavage caspase-3 was also observed in capsaicin-treated tumors indicating apoptosis. Consistent with our in vitro results, we observed increased phosphorylation of JNK, FOXO-1, and BIM in the tumors from capsaicin-treated mice as compared with control tumors, indicating that activation of JNK/FOXO/BIM cascade was associated with the overall capsaicin-mediated tumor growth suppression in vivo.
Discussion

In our previous study, we have shown that capsaicin treatment suppressed the growth of pancreatic cancer cells by generating ROS (25, 26). However, the exact mechanism by which capsaicin induced apoptosis in pancreatic cancer cells was not clear. Our current results demonstrate a novel mechanism by which capsaicin induced apoptosis in pancreatic cancer cells. Our study showed that capsaicin-mediated ROS was associated with phosphorylation of JNK at Thr183/185 (26). Activation of JNK by stress signal has been shown to activate proapoptotic protein BIM (33). Other studies have shown apoptosis in cancer cells through the interaction of FOXO3a and BIM (2, 17, 18). FOXO4 is also reported to activate BIM and induce apoptosis (19). In the present study, our results clearly showed that capsaicin-mediated JNK activation; FOXO-1 phosphorylation, and acetylation were involved in BIM-mediated apoptosis in pancreatic cancer cells.
Furthermore, our results showed that capsaicin failed to activate JNK/FOXO/BIM cascade and apoptosis in the cells that were pretreated with antioxidants tiron or PEG-catalase, suggesting the involvement of ROS in the activation of JNK/FOXO/BIM cascade. A previous study reported that phosphorylation and acetylation of FOXO-1 by HDAC inhibitor induce apoptosis through BIM (34). In agreement, our study revealed a novel mechanism where capsaicin-mediated oxidative stress precedes acetylation and phosphorylation of FOXO-1 to activate BIM resulting in apoptosis.

Phosphorylation, ubiquitination, and acetylation are the three main well-defined posttranslational modifications of FOXO for regulating gene expression (8, 9, 35). In response to growth signals, FOXO proteins are phosphorylated by Akt. Akt-mediated phosphorylated FOXO translocates to the nucleus, associates with 14-3-3 chaperon protein, and gets out of the nucleus (2, 9, 35, 36). On the other hand, stress-induced nuclear translocation of FOXO protein associates with histone acetylase proteins such as CBP/p300 and PCAF. This interaction not only is an initial step in assembling the transcriptional activation FOXO, but also it leads to the acetylation of FOXO-1 (1). Acetylated and phosphorylated FOXO-1 then recruits SirT-1 in the nucleus, and this recruitment is dependent on oxidative stress (1). Akt-mediated regulation of FOXO was not observed in our model as capsaicin failed to modulate PI3K/Akt. Nevertheless, capsaicin treatment elicited stress response in pancreatic cancer cells resulting in the activation of JNK by phosphorylation at Thr183/Tyr185. Activated JNK further phosphorylated FOXO-1 at Ser256 translocating FOXO-1 into the nucleus resulting in increased DNA binding. In another study, we have shown that FOXO-1 translocation into the nucleus increased DNA-binding activity and transcription of proapoptotic gene BIM by benzyl isothiocyanate (28). Previous studies also reported that acetylation of FOXOs plays a key role in activating downstream targets of FOXO-1 (4, 11). Our
results indicated that capsaicin-mediated FOXO-1 translocation into nucleus increased FOXO-1 DNA binding and expression of proapoptotic gene BIM. Our studies also demonstrated the involvement of FOXO-1 and BIM in capsaicin-mediated apoptosis as FOXO-1 siRNA and BIM siRNA blocked apoptosis.

FOXO-1 acetylation was shown using CBP-mediated acetylation site mimic. Using acetylation mimic and acetylation defective mutants, our study further established that the acetylation of FOXO-1 in fact regulated the phosphorylation of FOXO-1 leading to the activation of BIM in pancreatic cancer cells. Our results are in agreement with the other published studies where acetylation enhanced FOXO3 and BIM activation (11) and acetylation of FOXO-1 increased the sensitivity to phosphorylation (37).

Previous studies have shown 85% to 95% absorption of capsaicinoids from the gastrointestinal tract. Significant metabolism was observed in the liver resulting in relatively low $C_{\text{max}}$ and shorter half-life (38). Interestingly, other published reports suggest that 0.5 to 4 mg/kg/d intake of capsaicinoids by humans (http://ec.europa.eu/food/fs/sc/scf/out120_en.pdf), which produces subtherapeutic capsaicin intake of capsaicinoids by humans (http://ec.europa.eu/food/fs/sc/scf/out120_en.pdf), which produces subtherapeutic capsaicin intake of capsaicinoids by humans.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K.C. Pramanik, N.M. Fofaria, P. Gupta, S.K. Srivastava


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.C. Pramanik, N.M. Fofaria, P. Gupta, S.K. Srivastava

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.C. Pramanik, N.M. Fofaria, P. Gupta, S.K. Srivastava

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.K. Srivastava

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