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Original Article

Capsaicin attenuates cell migration via SIRT1 targeting and inhibition to enhance cortactin and β-catenin acetylation in bladder cancer cells

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Abstract: We have studied the chemopreventive property of capsaicin, a major active component in chili pepper, and found that it exhibited apoptotic activity against various lines of cancer cells. Interestingly, accumulating data has revealed that, in addition to cytotoxicity, capsaicin also plays regulatory role on cell migration and invasion. However, its effect on cell migration is paradoxical and not completely understood. Here, we set out to elucidate the molecular events underlying capsaicin-inhibited cell migration in bladder cancer cells. Our results show that the capsaicin-reduced cell migration was associated with down-regulation of sirtuin 1 (SIRT1) deacetylase, possibly through proteasome-mediated protein degradation. More importantly, we employed a cellular thermal shift assay (CETSA) to demonstrate that there was a direct binding between capsaicin and SIRT1. The engagement with capsaicin and protein degradation diminished the deacetylase of SIRT1, which in turn, enhanced acetylation of cortactin and β-catenin to decrease MMP-2 and MMP-9 activation, resulting in cell migration impairment in bladder cancer cells.

Keywords: Capsaicin, cell migration, cellular thermal shift assay (CETSA), silent mating type information regulation 1 (Sirtuin 1, SIRT1)

Introduction

Based on the GLOBOCAN 2018 estimates of cancer and mortality produced by the International Agency for Research on Cancer, bladder cancer is listed the 10th most common type of cancer worldwide, with a projection of 549,000 new cases and 200,000 deaths [1, 2]. The incidence and mortality rates of bladder cancer in men are about four times higher than those of women, with Southern Europe having the highest incidence rates for bladder cancer in both genders [1]. Among those bladder cancer patients, approximately 75% are clinically categorized as non-muscle-invasive type, however, these patients often encounter tumor recurrence, causing their cancer to progress to muscle-invasive type, which is extremely aggressive and frequently advances to metastasis. The most common treatments of bladder cancer are mainly based on the tumor's clinical stage, such as surgical resection, and may combine with radiation or chemotherapy. However, despite the current treatments, the outcomes resulting in poor survival rates have made it not only important, but necessary to seek out better therapeutic strategies.

Capsaicin (8-methyl-*N*-vanillyl-6-noneamide) is a major ingredient of red chili pepper, which is also used as chemopreventative agent for its anti-cancer activity [3-5]. In most cases, capsaicin exerts its cytotoxic action by inducing apoptosis in cancer cells through various mechanisms [6-10]. Aside from its apoptotic activity, accumulative data also suggested that capsaicin might play a regulatory role on cell migration. For example, capsaicin is demonstrated to enhance cell migration in human corneal epithelial cells (HCEC) [11]. Similarly, low concen-

tration capsaicin promotes colorectal cancer cell migration and invasion by triggering production of reactive oxygen species [12]. Profound changes in cellular motility and speed were induced by capsaicin in MDCK-C7 epithelial cells through rearrangements of the cytoskeleton and tight junction proteins [13]. Furthermore, capsaicin-sensitive transient receptor potential channel (TRPV1) is shown to play a stimulatory role on cell migration in human hepatocellular carcinoma (HepG2) cells pre-treated with hepatocyte growth factor (HGF) [14]. The activation of TRPV1 by capsaicin, in another study, correlated with an attenuation in cell migration and invasion in human papillary thyroid carcinoma BCPAP cells, supported by a decrease in the expression of MMP-2 and MMP-9 [15]. Nevertheless, TRPV1 is not the sole target of capsaicin involved in cell migration regulation. Through the phosphatidylinositol 3-kinase/Akt/Rac1 signaling pathway, B16-F10 melanoma cell migration was inhibited by capsaicin [16]. Capsaicin also attenuates cell migration, invasion, and EMT effectively in cholangiocarcinoma cells by targeting Hedgehog signaling pathway [17]. In human fibrosarcoma cells, capsaicin reversed epidermal growth factor (EGF)-induced cell migration and invasion by down-regulating matrix metalloproteinase-9 through repressing AP-1 activation [18]. These studies reveal a paradoxical role of capsaicin on cell migration, possibly as a results of its different effect on various targets.

SIRT1 belongs to the sirtuin protein family of NAD^+-dependent deacetylases that target histones and nonhistone proteins, engaging in an array of cellular events responding to nutritional and environmental stresses [19-23]. However, its role in cell migration and invasion remains a matter of controversy as well. For instance, SIRT1 reduces epithelial-to-mesenchymal transition (EMT), which is important in the development of cancer metastasis through deacetylation of Smad4 and MMP-7 attenuation [24]. Similarly, SIRT1 suppresses the migration and invasion of gastric cancer by deacetylation and inhibition on c-JUN, resulting in down-regulation of ARHGAP5 expression [25]. On the contrary, liver tumor tissues and hepatocellular carcinoma lines with enhanced SIRT1 expression are closely correlated with higher invasion and metastatic potential by inducing EMT [26]. Formation of lamellipodium extension, an important feature at the leading edge of migrating cells, is regulated by SIRT1 via its participation in the maintenance of phosphatidylinositol-3,4,5-triphosphate (PIP3) level in melanoma cells [27]. Remarkably, SIRT1 collaborates with several key transcription factors, thus promoting EMT by diminishing epithelial markers and augmenting mesenchymal markers [28]. Evidently, high expression SIRT1 is correlated with poor clinical outcomes in many types of cancer, most likely contributed by its regulatory role on metastasis potential and cancer progression [26]. Given these ambiguous findings, it is important to identify the regulatory role of SIRT1, in particular, on capsaicin-mediated cell migration and invasion.

We have come across a relationship in which SIRT1 deacetylase is pivotal to capsaicin-induced cell death [29]. Moreover, multiple cancer phenotypes, including cell migration, were attenuated by capsaicin concurrent with SIRT1 downregulation in T24 bladder cancer cells [30]. To clarify the role of SIRT1 in capsaicin-inhibited cell migration, in this present study, we present evidence demonstrating that capsaicin not only induced SIRT1 degradation, but also engaged to this deacetylase protein, which in turn, enhanced acetylation status of cortactin and β-catenin to decrease MMP-2 and MMP-9 activation, resulting in an attenuation in cell migration.

Materials and methods

Materials

Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). Anti-SIRT1, anti-acetyl-β-catenin, anti-β-catenin, anti-Snail, anti-MMP-2, anti-MMP-9, and anti-N-cadherin antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-cortactin and anti-acetyl-cortactin antibodies were from Signalway Antibody, Inc. (College Park, MD, USA). The anti-β-actin antibody was from Millipore Corp. (Temecula, CA, USA). Anti-mouse and anti-rabbit IgG antibodies and other chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA), unless specified otherwise.

Cell culture

T24 human bladder carcinoma cells were grown in RPMI. Media were supplemented with 10% FBS, 100 units/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at a temperature of 37°C in a humidified atmosphere of 5% CO_2 in air, and the media were replaced every 2-3 days. Cells were either treated with different concentrations of capsaicin (dissolved in ethanol), as described in the text, or with the same volume of ethanol (vehicle control).

Wound healing migration assay

Migration of cells was measured by a wound healing assay in vitro. Briefly, cells were cultured in 6-well plates until there was the development of a monolayer. The monolayer was then scratched by a 200 μL pipette tip and incubated in fresh medium with different concentrations of capsaicin or same volume of ethanol. The width of the wound was then monitored at 0, 12, and 24 h after scratching and visualized by an inverted phase contract microscope. Data shown are representative of three independent experiments.

Boyden chamber assay

A Boyden chamber with filter inserts containing 8 mm pores (Neuro Probe, Inc., Gaithersburg, MD, USA) was used to measure cell invasion. Cells (5×10^5 cells/mL) treated with different concentration of capsaicin or same volume of ethanol in serum-free medium were added to the upper chamber of a transwell chamber. Medium supplemented with 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, the non-migrating cells were removed from the upper surface of the membrane by gently scrubbing with a cotton tipped swab. Cells that had moved to the lower surface of the membrane were fixed and stained with crystal violet. Five random fields were selected and photographed under an inverted microscope. Photographs were analyzed by using ImageJ software. Data shown are representative of three independent experiments.

Cellular thermal shift assay (CETSA)

Engagement between capsaicin and SIRT1 in cells was analyzed by CETSA. Samples were prepared from control cells and those exposed to the drug. For each set, 2×10^7 cells were seeded in a 10-cm cultured dish. After 24 h of culture, the cells were pretreated with 10 μM MG132 for 1 h, washed with PBS, treated with trypsin, and collected. Samples were centrifuged at 12,000 rpm for 2 min at room temperature. Afterwards, the pellets were gently resuspended with 1 mL of PBS and the samples were then centrifuged at 7,500 rpm for 3 min at room temperature. The pellets were resuspended with 1 mL of PBS containing 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 ng/ml leupeptin, and 10 mg/ml aprotinin. The samples were transferred to Eppendorf tubes, subjected to three freeze-thaw cycles; for each cycle, they were exposed to liquid nitrogen for 3 min, placed in a heating block at 25°C for 3 min, and vortexed briefly. The samples were then centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatants were transferred to new Eppendorf tubes. For the experimental sample set, capsaicin was added to a final concentration of 2 mM; for the control sample set, the same volume of vesicle solvent was added. The samples were heated at 37°C for 1 h and dispensed to 100 μL aliquots. Pairs consisting of one control aliquot and one from experimental aliquots were heated at 40°C, 43°C, 46°C, 49°C, 52°C, 55°C, 58°C, 61°C, or 67°C for 3 min. Finally, the samples were placed on ice and subjected to Western blot analysis using antisera to SIRT1.

Western blot analysis

Cell extracts were prepared in lysis buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 ng/ml leupeptin, 10 μg/ml aprotinin). Volumes of extract containing equal amounts of proteins (40 μg) were applied to SDS-PAGE gels, and resolved proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked, washed, and probed with primary antibody. After washing to remove unbound primary antibody, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 hours. The blots were washed again and developed using enhanced chemiluminescence (ECL) reagents, according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ, USA).

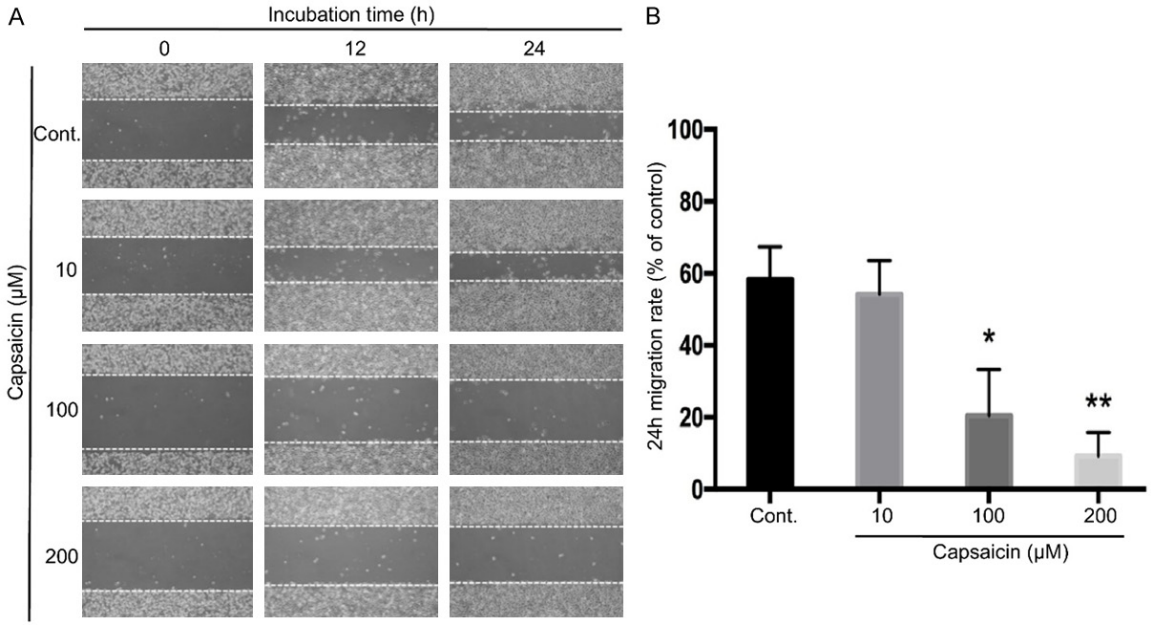

Figure 1. Effect of capsaicin on wound healing ability of T24 cells. Cell monolayer was wound by scratching with a pipette tip and then treated with different concentrations of capsaicin or vesicle as the control. (A) Wound closure was examined at 0, 12, and 24 h after scratching by inverted light microscopy. Representative images of three independent experiments are shown. (B) Quantitative analysis of wound closures in (A) is shown in the histogram. Values (means ± SEs) are from three independent experiments performed in at least triplicates (**P*<0.05, ***P*<0.01).

Statistics

All data are expressed as the means ± SEs of three independent experiments. The significance of differences between control and treatment groups was calculated using a one-way ANOVA, followed by Dunnett's test.

Results

Capsaicin suppresses cell motility in T24 bladder cancer cells

In our previous study, we have used cell impedance measurements to examine the effect of capsaicin on the migration of bladder cancer cells and found that 100 and 200 μM capsaicin effectively attenuated cell migration in T24 cells [30]. To continue our exploration on the mechanism underlying capsaicin-inhibited cell motility, we first validated the effect of capsaicin on cell migration determined by wound healing assays. Our results showed that the migration of T24 cells into the wounded area was distinctly reduced in the presence of capsaicin (100 and 200 μM) compared to the controls 12 h or 24 h after scratching (**Figure 1A**). However, the wound closure rate of those cells exposed to 10 μM capsaicin remained to be similar compared to the control. Dose-response effect of capsaicin on wound closure was observed in the treatment with 100 and 200 μM (**Figure 1B**).

Alternatively, cell migration was evaluated with transwell chamber system. We showed that 100 and 200 μM capsaicin reduced cells' migrating ability to lower chamber markedly (**Figure 2A**). Quantitative analysis also illustrated that capsaicin suppresses cell migration in a dose-dependent manner (**Figure 2B**).

Capsaicin downregulates SIRT1 expression through proteasome degradation

In our previous study, we also found that capsaicin decreased SIRT1 expression and concurrently reduced cell migration at 100 and 200 μM [30]. However, the molecular events to explain how SIRT1 suppression attenuates cell migration are largely undecided. In this present study, we again validated that the SIRT1 expression was indeed markedly reduced by 100 and 200 μM capsaicin in T24 bladder carcinoma cells (**Figure 3A**). Furthermore, using a cycloheximide-chase assay, we analyzed protein sta-

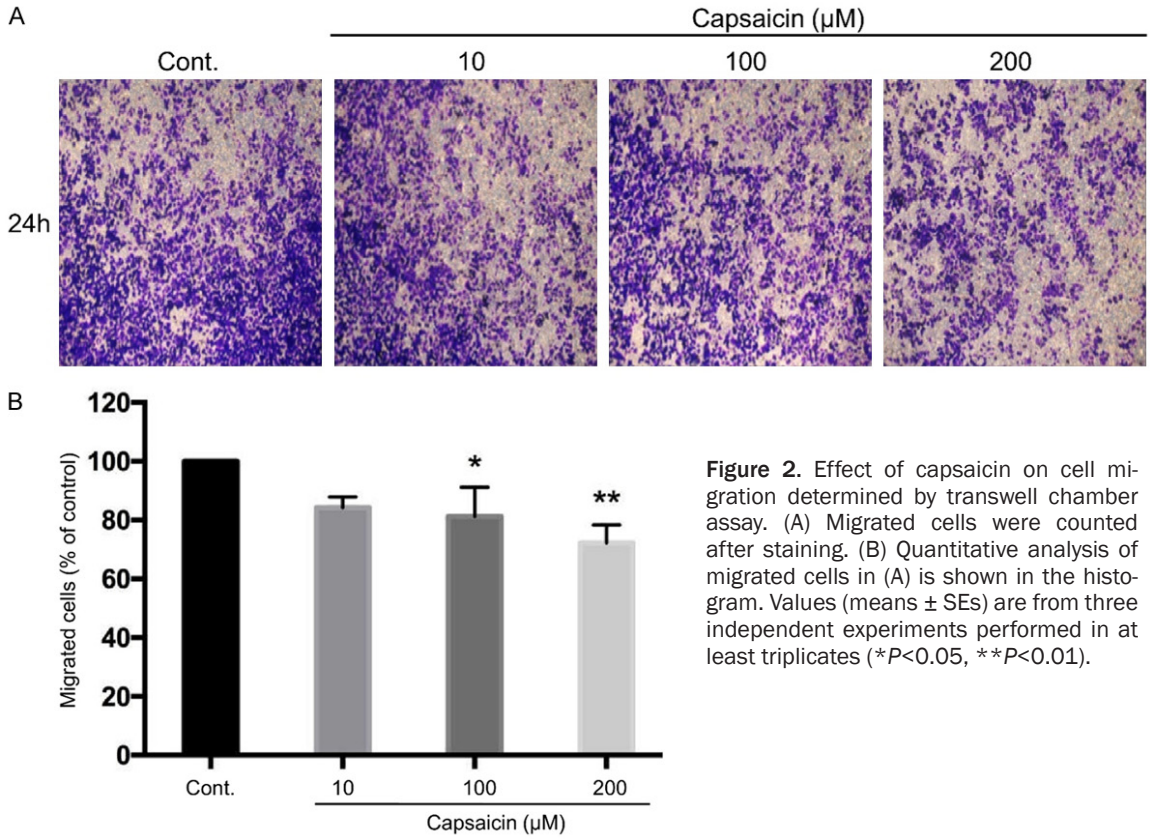

Figure 2. Effect of capsaicin on cell migration determined by transwell chamber assay. (A) Migrated cells were counted after staining. (B) Quantitative analysis of migrated cells in (A) is shown in the histogram. Values (means ± SEs) are from three independent experiments performed in at least triplicates (*P<0.05, **P<0.01).

bility and found that the half-life of SIRT1 was significantly decreased in T24 cells exposed to capsaicin for 6 h and longer (**Figure 3B**). The pre-treatment with proteasome inhibitor MG132 considerably increased the stability of SIRT1 expression in T24 cells exposed to capsaicin, suggesting that proteasome degradation is involved in the capsaicin-mediated SIRT1 down-regulation (**Figure 3C**).

Capsaicin engages to SIRT1 and suppresses its deacetylase activity to enhance acetylated cortactin and β-catenin leading to cell migration attenuation

We further investigated whether capsaicin engages SIRT1 deacetylase to contribute to the inhibition of cell migration in T24 bladder cancer cells. The cellular thermal shift assay (CETSA) is used to study ligand binding in cells based on the ability of ligand engagement to increase the stability of target proteins [31, 32]. Cells exposed to capsaicin seemed to delay the precipitation of SIRT1 at a higher temperature compared to the un-treated group, as illustrated in Western blot analysis using antibody against SIRT1 (**Figure 4A**). For CETSA, thermal melting curves can be plotted and the melting temperature (T_m; the temperature at which 50% of proteins are unfolded and rapidly precipitated by heat) are derived. We also plotted the thermal melting curves and calculated their T_m values. A marked shift in the thermal melting curves in the capsaicin-treated lysates of T24 cells compared to control lysates was observed. The T_m value significantly increased from 48.8°C (control) to 54.8°C (capsaicin-treated), indicating that capsaicin bound to the SIRT1 deacetylase protein and enhanced its thermal stabilization (**Figure 4B**).

As our CETSA results indicated that capsaicin indeed bound to SIRT1, we next analyzed whether capsaicin binding affects the enzymatic ability of SIRT1 deacetylase. Given that SIRT1-directed deacetylation of cortactin has recently shown to promote cell migration [33], we next examined whether cortactin acetylation level is altered by capsaicin in our system. Using Western blot analysis, our data indicated that acetylated cortactin was enhanced when exposed to capsaicin whereas

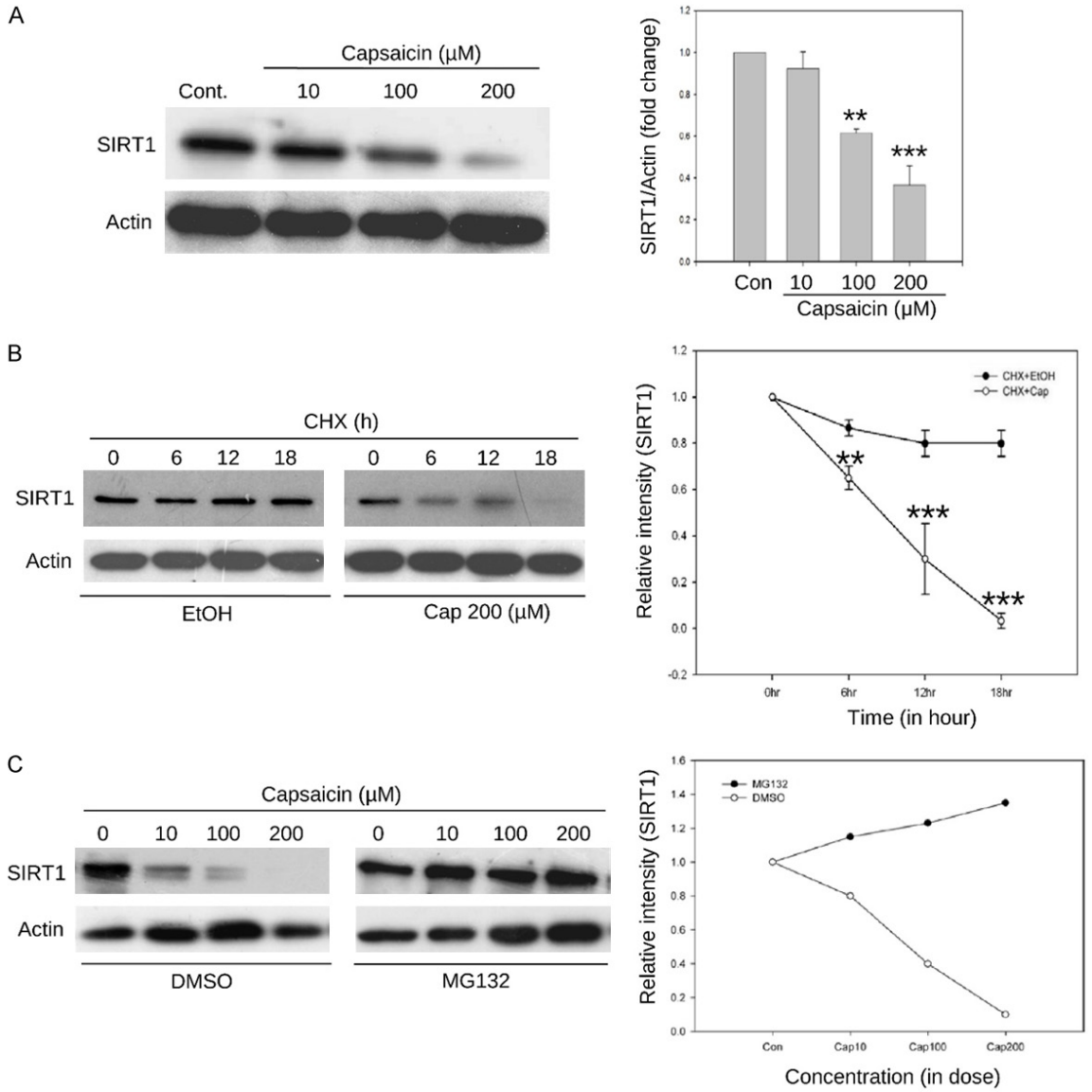

Figure 3. Capsaicin down-regulates SIRT1 expression through proteasome-mediated degradation. A. SIRT1 expression was markedly attenuated by capsaicin at 100 and 200 μM. B. Capsaicin at 200 μM reduced SIRT1 stability significantly starting at 6 h with cycloheximide chase assay. C. MG132 proteasome inhibitor reversed capsaicin-inhibited SIRT1 expression. Aliquots of cell lysates were separated by SDS-PAGE and analyzed for protein expression by Western blotting; β-actin was used as an internal control to monitor for equal loading. Representative images are shown from multiple independent experiments. Values (mean ± S.E.) are from at least three independent experiments (***P*<0.01, ****P*<0.001 for cells treated capsaicin vs. controls).

the total level of cortactin remained largely unchanged, possibly by the direct engagement of capsaicin and SIRT1 deacetylase protein (**Figure 5**). In addition, deacetylation of β-catenin by SIRT1 is correlated with β-catenin nuclear accumulation important for the regulation of cell migration, we also examined the acetylation status of β-catenin. Our results showed that, by inhibiting SIRT1 deacetylase activity, capsaicin increased β-catenin acetylation, which in turn decreased its nuclear accumulation and signaling pathways in T24 cells (**Figure 5**). Furthermore, a key transcription factor Snail in EMT regulation, mesenchymal marker N-cadherin, active form of MMP-2, and MMP-9 were all found to be diminished by capsaicin exposure in T24 bladder cancer cells (**Figure 5**). These various lines of evi-

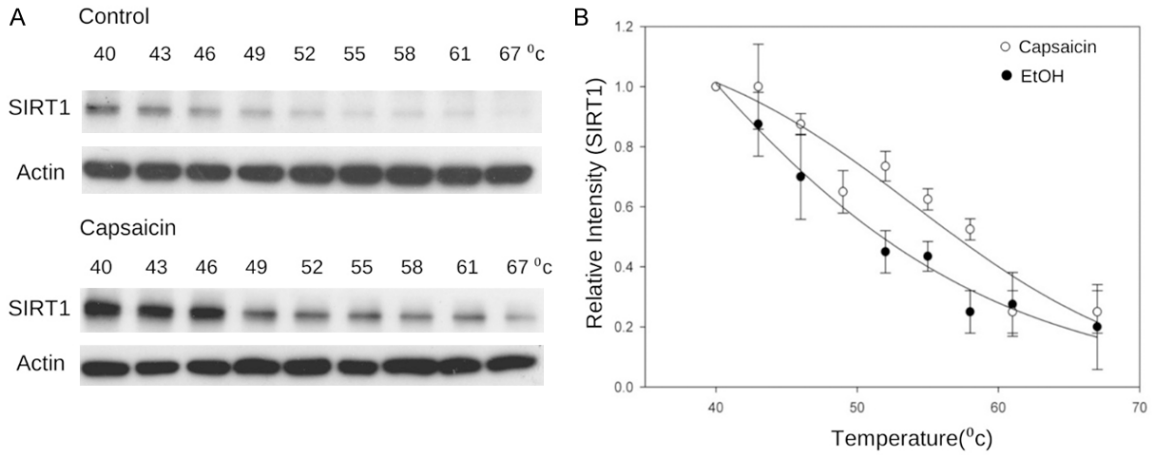

Figure 4. CETSA-based determination of binding between capsaicin and SIRT1. A. T24 cell lysates in the absence and presence of 2 mM capsaicin were separated by SDS-PAGE and analyzed by Western blotting. β-Actin was used as an internal control. The band intensities of SIRT1 were normalized with respect to the intensity at 40°C. Representative images are shown. B. CETSA curves of SIRT1 in T24 cells were plotted with or without capsaicin. The denaturation midpoints were determined using a standard process.

dence implicated that the binding of capsaicin to SIRT1 and its effect on deacetylase activity are important in capsaicin-attenuated cell migration.

Discussion

Tumor cell migration is a requirement for cancer metastasis and invasion and is commonly correlated with EMT, in which epithelial cells lose their morphology and adhesive properties and become more mesenchymal-like [34]. Considerable controversy has arisen over the role of capsaicin on cell migration. Capsaicin is shown to stimulate different TRPV channels that enhance calcium influx resulting a migratory phenotype [14, 35]. TRPV1 is the most-often mentioned protein targets of capsaicin that belongs to a member of the TRP family of non-selective cation channels [36]. Interestingly, in null-TRPV1 urothelial cancer cells, capsaicin promotes more aggressive gene phenotypes and invasiveness, which is totally opposite to its impact in TRPV1-overexpressing cells [37], supporting the contradictory role of capsaicin on cell motility. A significance of this study is that we provide evidence demonstrating that capsaicin suppresses cell motility and migration in T24 bladder cancer cells through targeting SIRT1 protein. On one hand, capsaicin reduced protein expression of SIRT1 via proteasomal degradation, thus inhibited the elevation of EMT as reported previously in hepatocellular carcinoma [26]. On the other hand, we demonstrated that capsaicin elicited an obviously different shift in the thermal melting curves of treated lysates versus untreated lysates, suggesting that capsaicin engaged with SIRT1 protein; hence, its deacetylase activity was inhibited, leading to enhanced acetylation status of cortactin and β-catenin. To the best of our knowledge, no previous work has shown that capsaicin engages with SIRT1 to achieve its inhibitory effects on cell migration.

Although SIRT1 regulates a host of cellular events through its deacetylase activity, including life span and metabolism, its role in cell migration remains vague. SIRT1 deacetylase interacts with the repeat region of cortactin leading to the deacetylation of cortactin, and the deacetylation/hypoacetylated of cortactin is associated with enhanced migratory ability in cancer cell model as well as breast tumor tissues [33]. Cortactin, a prominent Src substrate as well as a ubiquitous F-actin-binding protein, acts on cell migration regulation [38, 39]. Recent studies further potentiate a key role for cortactin in invasive tumor phenotypes, such as enhanced actin polymerization, impairment of down-regulation of epidermal growth factor receptor, and formation of podosomes and invadopodia [40]. Similarly, histone deacetylase 6 (HDAC6) can also deacetylate cortactin to affect cell motility through

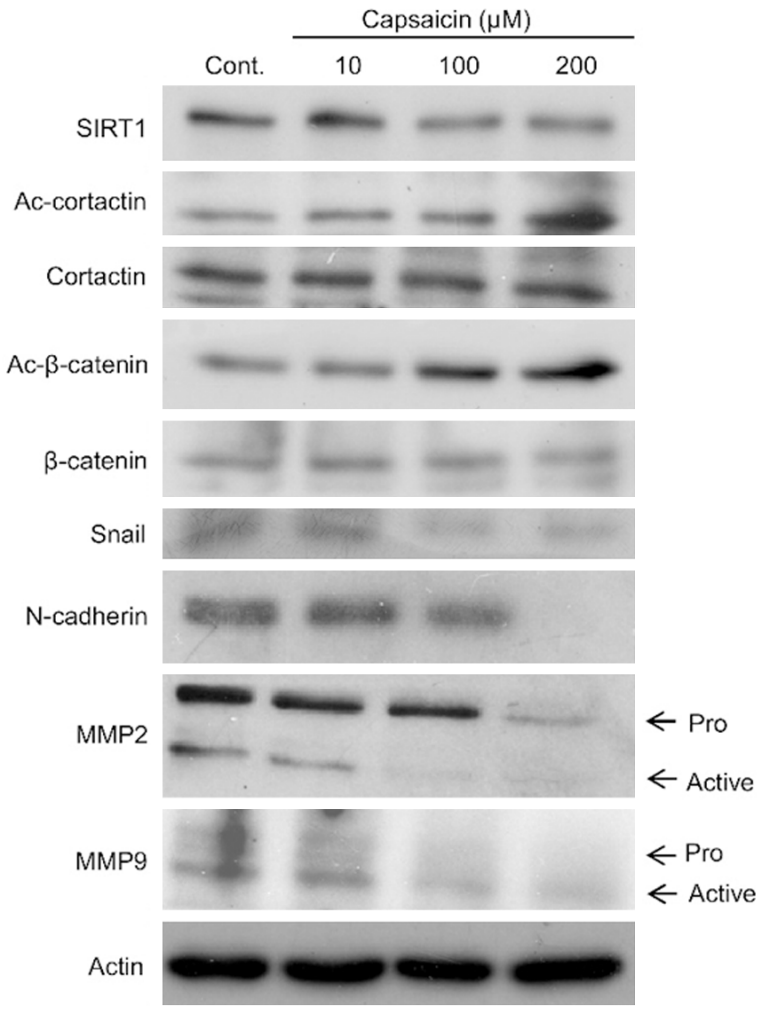

Figure 5. Effect of capsaicin on expression of key proteins involved in EMT and cell migration. T24 cells were treated with or without capsaicin for 24 h. Aliquots of cell lysates were separated by SDS-PAGE and analyzed by western blotting. β-actin was used as an internal control. Representative images are shown.

actin-dependent manner [41]. Most recently, acetylated cortactin is showed to decrease cell migration by reducing its binding to Keap1 [42]. Consistent with others, in this current study, we further demonstrated that capsaicin acts through SIRT1 to enhance cortactin acetylation and attenuate cell migration.

Moreover, capsaicin is found to inhibit β-catenin signaling due to the dissociation of β-catenin/TCF-1 complex, possibly through protein stability and/or their cellular localization [9, 43]. Interestingly, several studies have validated that deacetylation of β-catenin by SIRT1 is correlated with β-catenin nuclear accumulation for its transcriptional activity [44-46]. As nuclear accumulation of β-catenin is a recognized tumor marker, the deacetylation of β-catenin by it should not come as a surprise that SIRT1 is correlated with cancer phenotypes and tumor growth. Similarly, SIRT1 up-regulation induced by the carcinogen benzo[a]pyrene (B[a]P), was associated with the up-regulation of β-catenin, which drives cell migration, invasion, and even tumorgenesis [47]. All these lines of evidence agree that SIRT1 is tumor-promoting and associated with enhanced cell motility. Here, we validated that capsaicin down-regulated and suppressed SIRT1 deacetylase to increase β-catenin acetylation and diminish its nuclear accumulation, which in turn, inhibited cell migration.

Capsaicin, long considered a chemopreventive agent, may target different proteins and subsequently affect divergent cellular outcomes. Here, we present evidence demonstrating that capsaicin-induced SIRT1 suppression attenuates cell migration in human bladder cancer cells. We further elucidate that, for the first time, capsaicin directly engages with SIRT1 by CETSA, which in turn enhances acetylation of cortactin and β-catenin and decreases EMT, ultimately impairing cell migration. The findings of this study assist us in understanding the biological function of capsaicin, providing further evidence of the chemopreventative activity of capsaicin.

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Disclosure of conflict of interest

None.

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