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Low-concentration capsaicin promotes colorectal cancer metastasis by triggering ROS production and modulating Akt/mTOR and STAT-3 pathways

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Colorectal cancer (CRC), one of the most common human malignancies, is a major public health problem in the developed world. Capsaicin, widely used as a food additive and as an analgesic agent, is a major pungent ingredient of red pepper. Though capsaicin-induced apoptosis was previously reported in cancer cells, relatively little is known about the impact of capsaicin on other aspect of cancer cell behavior. In this study, we demonstrated that treatment with high-concentration of capsaicin (≥ 200 µM for SW480 and CT-26 cell lines; ≥ 25 µM for HCT116 cell line) inhibited CRC cell proliferation in a dose-dependent manner. In spite of no anti-proliferative effect, notably, low-concentration of capsaicin (100 µM for SW480 and CT-26 cell lines; 12.5 µM for HCT116 cell line) enhanced both migratory and invasive capability of these cells, which was validated by both *in vitro* and *in vivo* model. Further, we showed that 100 µM capsaicin induced epithelial-to-mesenchymal (EMT), up-regulated expression of MMP-2 and MMP-9, and activated Akt/mTOR and STAT-3 pathways in SW480 cells. Finally, we showed that capsaicin-induced metastasis of CRC cells was mediated by modulating reactive oxygen species (ROS) production. Our findings are considered as a significant step toward a better understanding of capsaicin-associated regulatory network on CRC cells.

Key words: colorectal cancer, capsaicin, ROS, EMT, metastasis

CRC, one of the most common human malignancies, is a major public health problem in the developed world [1]. Although about half of individuals with CRC could be cured by surgery, radiation therapy and/or chemotherapy 40 - 50% of patients had metastatic diseases and prognosis is poor with a 5 year survival < 10% [2]. Accumulating evidences showed that there is a direct link between dietary pattern and CRC incidence [3].

Capsaicin ($C_{18}H_{27}NO_3$, 8-methyl-N-vanillyl-6-nonenamide), widely used as a food additive or an analgesic agent, is a major pungent ingredient of red pepper and is the active component of chili peppers [4]. The association between capsaicin and carcinogenesis has long been investigated, which, however, reached paradoxical observations in different tumor types. It is reported that capsaicin displayed an anti-proliferative activity against lung cancer [5], prostate cancer [6] and pancreatic cancer [7]; nevertheless, capsaicin also acts as a co-carcinogen in the development of skin cancer [8]. Further, it is reported that capsaicin induced apoptosis in human breast cancer cell line through caspase-independent pathway [9]. However, it is also observed that capsaicin induced denervation of sensory neurons which promotes breast tumor metastasis to lung and heart [10]. Therefore, a more detailed study is still needed to characterize the impact of capsaicin on cancer cells.

In this study, we conducted a series of experiments that afford further insights into capsaicin-induced metastasis of CRC cells and the underlying mechanisms. We provide evidences suggesting that capsaicin induces CRC cell metastasis by modulating MMPs, Akt/mTOR and STAT-3 signaling pathways as well as ROS production.

Materials and methods

Agents. Capsaicin (M-2028) was obtained from Sigma (St. Louis, MO). Rabbit monoclonal anti-Akt (#4685) and mouse monoclonal anti-P-Akt (Ser473) (#4051) were obtained from Cell Signal Technology (Daners, MA, USA). Mouse monoclonal anti-MMP-2 (ab3158), Rabbit monoclonal anti-MMP-9 (ab38898), Mouse monoclonal anti-STAT-3 (ab119352), Rabbit monoclonal anti-P-STAT-3 (Y705) (ab30646), Rabbit monoclonal anti-mTOR (ab2732), Rabbit monoclonal anti-P-mTOR (phosphor-S2448) (ab51044), Mouse monoclonal anti-N-Cadherin (ab12221), Rabbit monoclonal anti-E-cadherin (ab1416), Rabbit monoclonal anti-Vimentin (ab20346) and Mouse monoclonal anti-β-actin (ab3280) were obtained from Abcam.

LY294002 (S1737) and AG 490 (S1509) were purchased from Beyotime (Haimen, China).

Cell culture. Human colorectal carcinoma cell line SW480 and HCT116, and murine colorectal carcinoma CT-26 cell line were purchased from American Type Culture Collection (ATCC, Rockville, MD). SW480 and HCT116 cells were maintained in DMEM supplemented with 10% FBS, 100U/mL penicillin and 100μg/mL streptomycin at 37°C under an atmosphere of 95% air and 5% CO_2. CT-26 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100U/ml penicillin and 100μg/mL streptomycin at 37°C under an atmosphere of 95% air and 5% CO_2.

Determination of cell viability. Cell viability was determined by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as reported previously [11]. MTT was obtained from Sigma (St Louis, MO, USA). MTT assay was conducted 48h after capsaicin treatment. The SW480 and CT-26 cells were incubated with 0.5 mg/mL MTT in fresh medium at 37°C for 4 h. All culture media were then removed and resuspended in 150 μL DMSO. Cell viability was assessed by colorimetric changes using a Galaxy Microplate Reader at 570 nm (BMG LabTech, Offenburg, Germany). Data were expressed as a percentage of untreated control cultures.

Cell migration and invasion assay. Cells were trpsinized, and 2.5×10^4 cells were plated on Boyden chambers either coated with 10 μg Matrigel (BD Biosciences, Sparks, MD) per well (for invasion assay) or uncoated (for migration assays) in the medium containing 1% fetal bovine serum. The medium containing 10% fetal bovine serum was added in the lower chamber, and served as chemoattractment. Capsaicin was added in both upper and bottom chambers at the indicated concentration. After proper time (20 h for migration assay; 72 h for invasion assay), the cells that had moved to the lower surface of the membrane were fixed with methanol and stained with crystal violet. Photographs of three randomly selected fields of the fixed cells were captured, and cells were counted.

Tumor xenograft model. *In vivo* metastasis assay was performed as previously reported [12]. Experimental procedures were approved by the Institutional Animal Care and Treatment Committee of Chengdu Medical College. For animal inoculation, 6-8 week-old BALB/c mice were obtained from the West China Experimental Animal Center of Sichuan University and maintained on a standard diet at room temperature. For the pulmonary metastatic model, CT-26 cells were pre-treated with or without 100 μM capsaicin for 48 h. Mice were then intravenously injected with 0.1 ml suspension containing 2×10^5 CT-26 cell on day 0. Mice were killed by cervical vertebra dislocation on day 15, and lungs were immediately harvested, weighed, and analyzed. The metastatic nodules of CT-26 cells were counted in each lung.

Western blot. Cells were lysed with RIPA buffer (50 mM Tris base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF). Proteins were quantified by DC protein assay kit (Bio-Rad, USA). Samples were then loaded onto a 10-15% gels, electrophoresed at 100 V (BioRad Hercules, CA, USA), and then transferred to PVDF membranes (Amersham Biosciences). After blocking at 37 °C for 2 h, the blots were probed by the primary antibodies at 4 °C overnight. After washing three times with TBS containing 0.1 % Tween 20, the blots were incubated with HRP-conjugated secondary antibody (diluted 1:10,000; Santa Cruz Biotechnology) for 2 h at room temperature. Finally, blots were incubated with enhanced chemoluminescence western blotting detection reagents (Amersham Pharmacia, Buckinghamshire, UK) and exposed to X-OMAT AR films (Eastman Kodak, Rocheser, NY, USA).

ROS assay. Accumulation of intracellular ROS was detected using 6-carboxy-2-7-dichloroXuorescein diacetate (DCFH-DA). DCFH-DA was obtained from (GENMED, GMS10016.2). After treatment, the cells were incubated with 100 μM DCFH-DA for 30 min. Cells were then rinsed with phosphate-buffered saline (PBS) for three times, and the fluorescent signal was measured by using a Galaxy Microplate Reader.

Statistical analyses. Comparisons between two groups were performed by Student's *t* test. Statistical significance was defined as $p<0.05$.

Results

Low-concentration capsaicin promotes migration and invasion of CRC cells. To determine the potential effects of capsaicin on colorectal cancer cells, SW480 cells and CT-26 cells were treated with varying concentrations of capsaicin for 48 h. Cell viability was then examined by MTT assay. As shown, treatment with 200-1600 μM capsaicin resulted in proliferation inhibition of both SW480 and CT-26 cells in a dose-dependent manner. However, for both two cell lines, no significant difference was observed between untreated cells and those cells treated with a low concentration of capsaicin (12.5-100 μM) (Fig. 1A-B).

To evaluate whether capsaicin has a role in regulating metastatic property of CRC cells, SW480 cells were treated with 100 μM capsaicin for 48 h, and migratory capability of SW480 was determined by transwell assay. As shown, the transwell assay revealed a 4-fold elevation in cell motility upon capsaicin treatment ($P < 0.01$) (Fig. 1C). We also measured the impact of capsaicin on the invasive capability of SW480 cells. As shown in Fig. 1D, the number of capsaicin-treated SW480 cells migrating in to the bottom chamber was 236.8

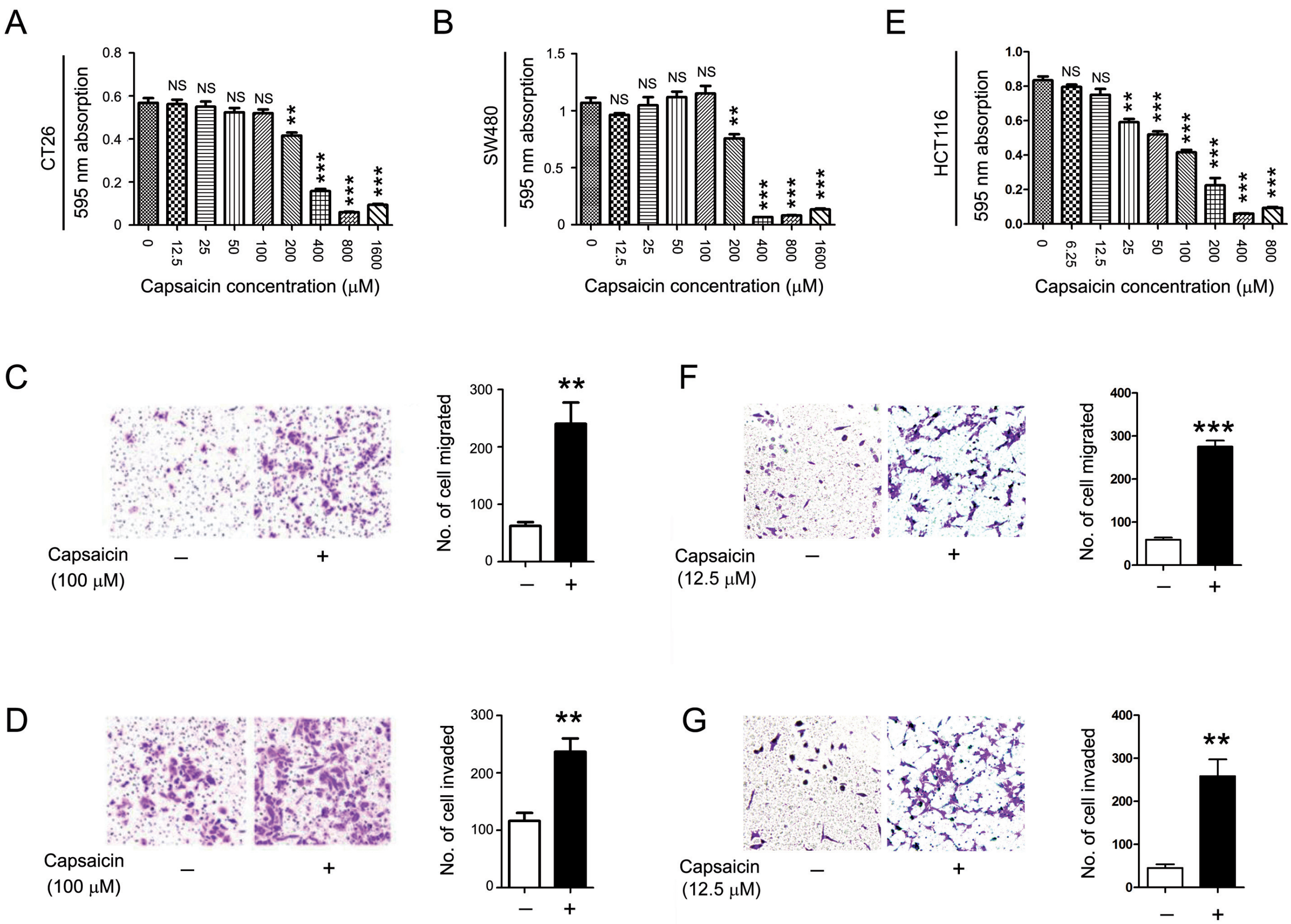

Figure 1. Low-concentration capsaicin induces migration and invasion in CRC cells *in vitro*
(A) CT-26 cells were treated with capsaicin at various doses for 48 h. Cell vitality was examined by MTT assay. High-concentration capsaicin (200-1600 μM) inhibited CT-26 cell proliferation. (B) SW480 cells were treated with capsaicin at various doses for 48 h. Cell vitality was examined by MTT assay. High-concentration capsaicin (200-1600 μM) inhibited SW480 cell proliferation. (C) SW480 cells were treated with 100 μM capsaicin for 48 h. Migratory capability was examined by transwell assay. Low-concentration capsaicin (100 μM) promoted SW480 cell migration. (D) SW480 cells were treated with 100 μM capsaicin for 48 h. Invasive capability was examined by Matrigel assay. Low-concentration capsaicin (100 μM) promoted SW480 cell invasion. (E) HCT116 cells were treated with capsaicin at various doses for 48 h. Cell vitality was examined by MTT assay. High-concentration capsaicin (25-800 μM) inhibited HCT116 cell proliferation. (F) HCT116 cells were treated with 12.5 μM capsaicin for 48 h. Migratory capability was examined by transwell assay. Low-concentration capsaicin (12.5 μM) promoted HCT116 cell migration. (G) HCT116 cells were treated with 12.5 μM capsaicin for 48 h. Invasive capability was examined by Matrigel invasion assay. Low-concentration capsaicin (12.5 μM) promoted HCT116 cell invasion.

± 23.32, in contrast, the number of untreated group was only 116.3 ± 14.21 ($P < 0.01$).

To support our findings, we further tested the effect of capsaicin on proliferation and motility of HCT116 cells. As shown in Fig 1E, proliferation inhibition was observed when the cells were treated with 25-800 μM capsaicin for 48 h. Notably, treatment with 12.5 μM capsaicin, which had no impact on cell proliferation, dramatically increased both migratory and invasive capability of HCT116 cells, revealed by transwell assay (Fig 1F, $P < 0.001$) and Matrigel invasion assay (Fig 1G, $P < 0.01$). These results suggested that low-concentration capsaicin conferred an aggressive phenotype on CRC cells.

To further validate the pro-metastatic effect of capsaicin on CRC cells *in vivo*, mice lung metastasis model was established. As shown, 100 μM capsaicin-treated CT-26 cells raised clearly increased lung metastatic nodules (Fig. 2A-C, $P < 0.01$) and elevated lung weight (Fig. 2D, $P < 0.01$) 15 days after injection, compared to the untreated mice. These data suggested that low-concentration capsaicin promotes metastatic property of CRC cells *in vivo*.

Low-concentration capsaicin induces EMT in CRC cells. EMT, which plays essential roles in development and wound healing, was recently considered as a key step toward cancer metastasis [13]. Next, of our particular interest, we

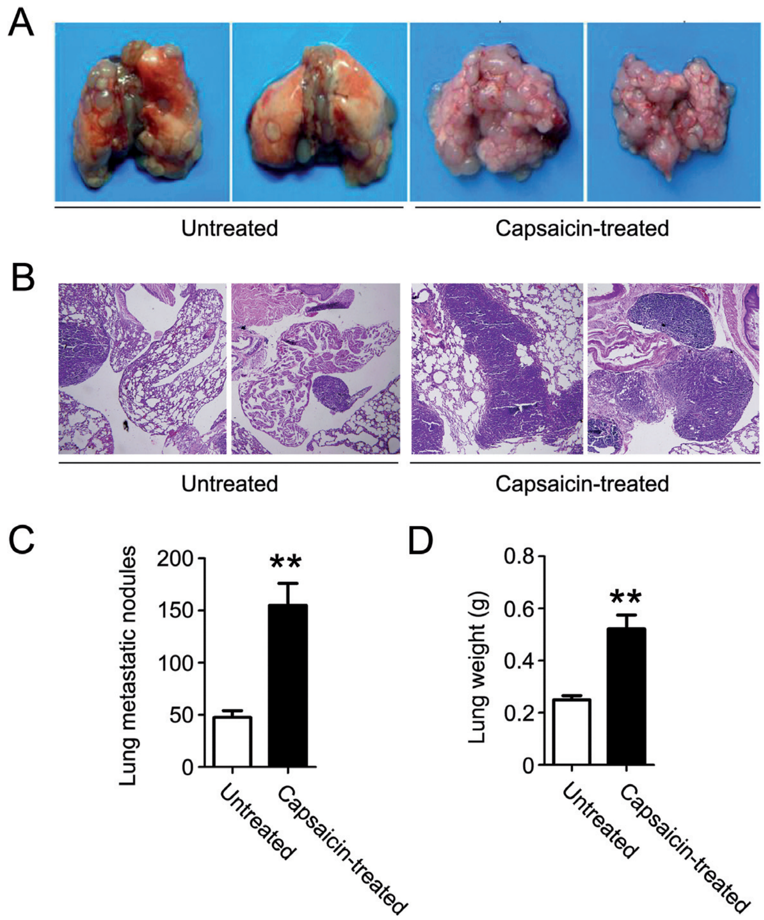

Figure 2. Low-concentration capsaicin induces migration and invasion in CRC cells *in vivo*
CT-26 cells were pre-treated with 100 μM capsaicin for 48 h and then intravenously injected in mice. Representative images of the lungs from each group (A) and representative image of H&E staining (B) are shown. Lung metastasis of CT-26 cells was determined by counting the lung metastatic nodules (C) and measuring the lung weight (D). The data showed that low-concentration capsaicin induces migration and invasion in CRC cells *in vivo*.

sought to determine whether low-concentration capsaicin induced EMT in CRC cells, by examining the expression level of a set of epithelial or mesenchymal markers. As shown in Fig. 3A, SW480 cells treated with 100 μM capsaicin showed a decrease in expression of epithelial marker E-cadherin and an increased in expression of mesenchymal marker Vimentin and N-cadherin. These results suggested that capsaicin treatment induced clear EMT in SW480 cells, which might contribute to capsaicin-induced metastatic phenotype of these cells.

Low-concentration capsaicin activates expression of MMP-2 and MMP-9. Emerging evidences had linked the elevated expression of MMP-2 and MMP-9, which are both proteolytic enzymes required for extracellular matrix degradation in a variety of physiological and pathologic processes, with tumor metastasis [14]. Therefore, we next examined the expression level of MMP-2 and MMP-9 upon capsaicin treatment. As results, both MMP-2 and MMP-9 were sharply accumulated in SW480 cells after treatment with 100 μM capsaicin for 48 h (Fig. 3B).

Low-concentration capsaicin activates Akt/mTOR and STAT-3 pathways. It is reported that both Akt/mTOR and STAT-3 pathways were upstream pathways controlling both EMT and expression of MMP-2 and MMP-9 [15-18]. Therefore, we wanted to determine whether PI3K/Akt and STAT-3 pathways were activated upon low-concentration capsaicin treatment. As shown, treatment with 100 μM capsaicin for 48 h markedly enhanced phosphorylation of both Akt (S473) and mTOR (S2448) in SW480 cells (Fig. 3C). Further, phosphorylation of STAT-3 at Tyr705, which promotes STAT-3 translocation into the nucleus, was significantly increased 48 h after treatment with 100 μM capsaicin (Fig. 3C). To investigate whether Akt/mTOR or/and STAT-3 pathways played a role in capsaicin-induced CRC cell metastasis, these two pathways were inhibited by LY294002 or AG490, respectively [19, 20]. As shown, treatment with LY294002 or AG490

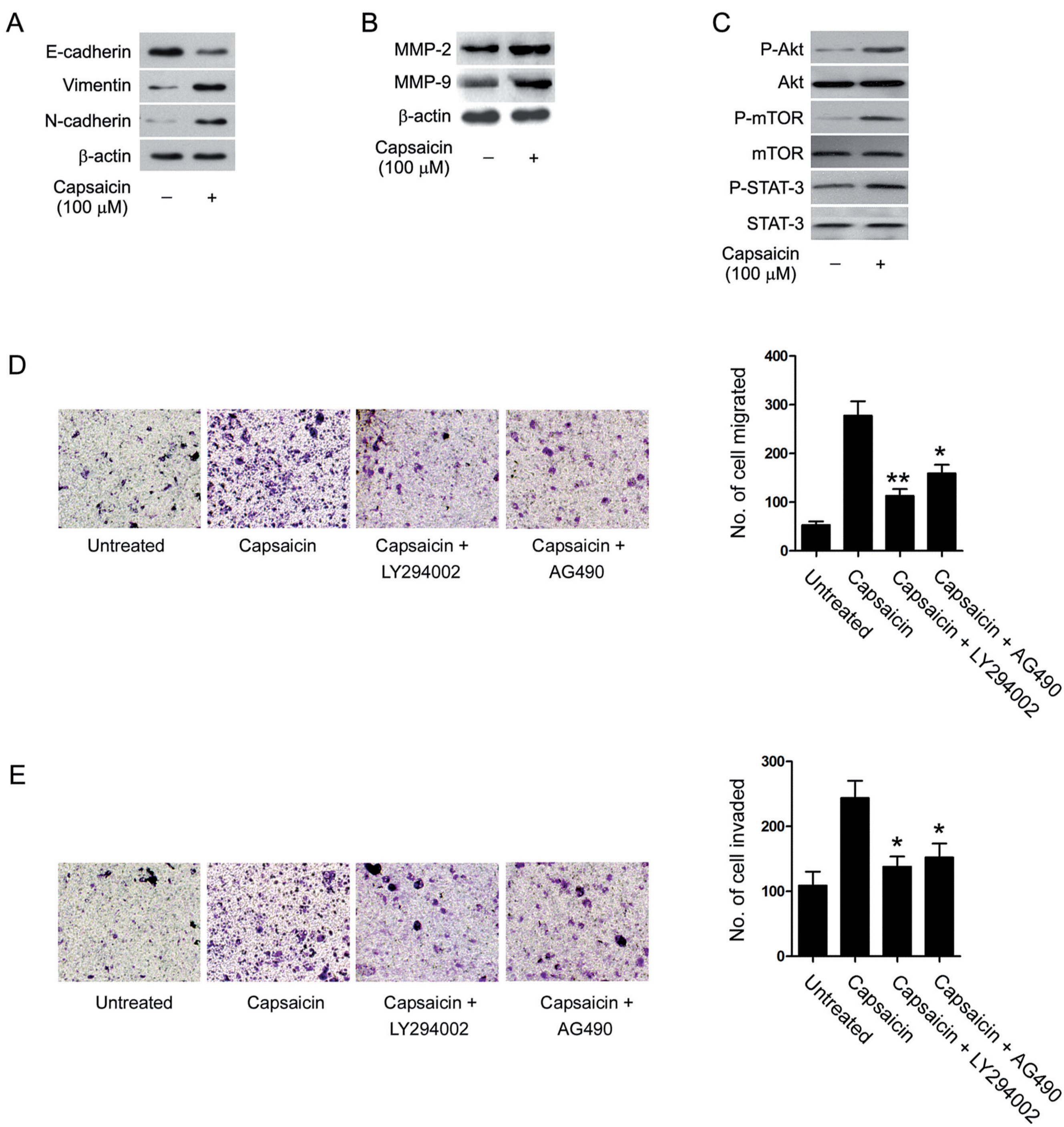

Figure 3. Low-concentration capsaicin induces metastasis of CRC cells by activating Akt/mTOR and STAT3 pathways

(A) SW480 cells were treated with 100 µM capsaicin for 48 h. Expression of E-cadherin, Vimentin and N-cadherin was examined by immunoblot. Treatment with 100 µM capsaicin repressed E-cadherin expression and enhanced expression of Vimentin and N-cadherin. (B) SW480 cells were treated with 100 µM capsaicin for 48 h. Expression of MMP-9 and MMP-2 was examined by immunoblot. Treatment with 100 µM capsaicin induced expression of MMP-2 and MMP-9. (C) SW480 cells were treated with 100 µM capsaicin for 48 h. Expression of phosphorylated Akt, mTOR and STAT-3 was examined by immunoblot. The data showed that treatment with 100 µM capsaicin activated both Akt/mTOR and STAT3 pathways. (D) SW480 cells were treated with 100 µM capsaicin alone or in presence of 20µM LY294002 or 10 µM AG490, respectively, for 48 h. Migratory capability of SW480 cells was examined by transwell assay. Inhibition of either Akt/mTOR or STAT3 pathway decreased migration of SW480 cells. (E) SW480 cells were treated with 100 µM capsaicin alone or in presence of 20µM LY294002 or 10µM AG490, respectively, for 48 h. Invasive capability of SW480 cells was examined by Matrigel invasion assay. Inhibition of either Akt/mTOR or STAT3 pathway decreased invasion of SW480 cells.

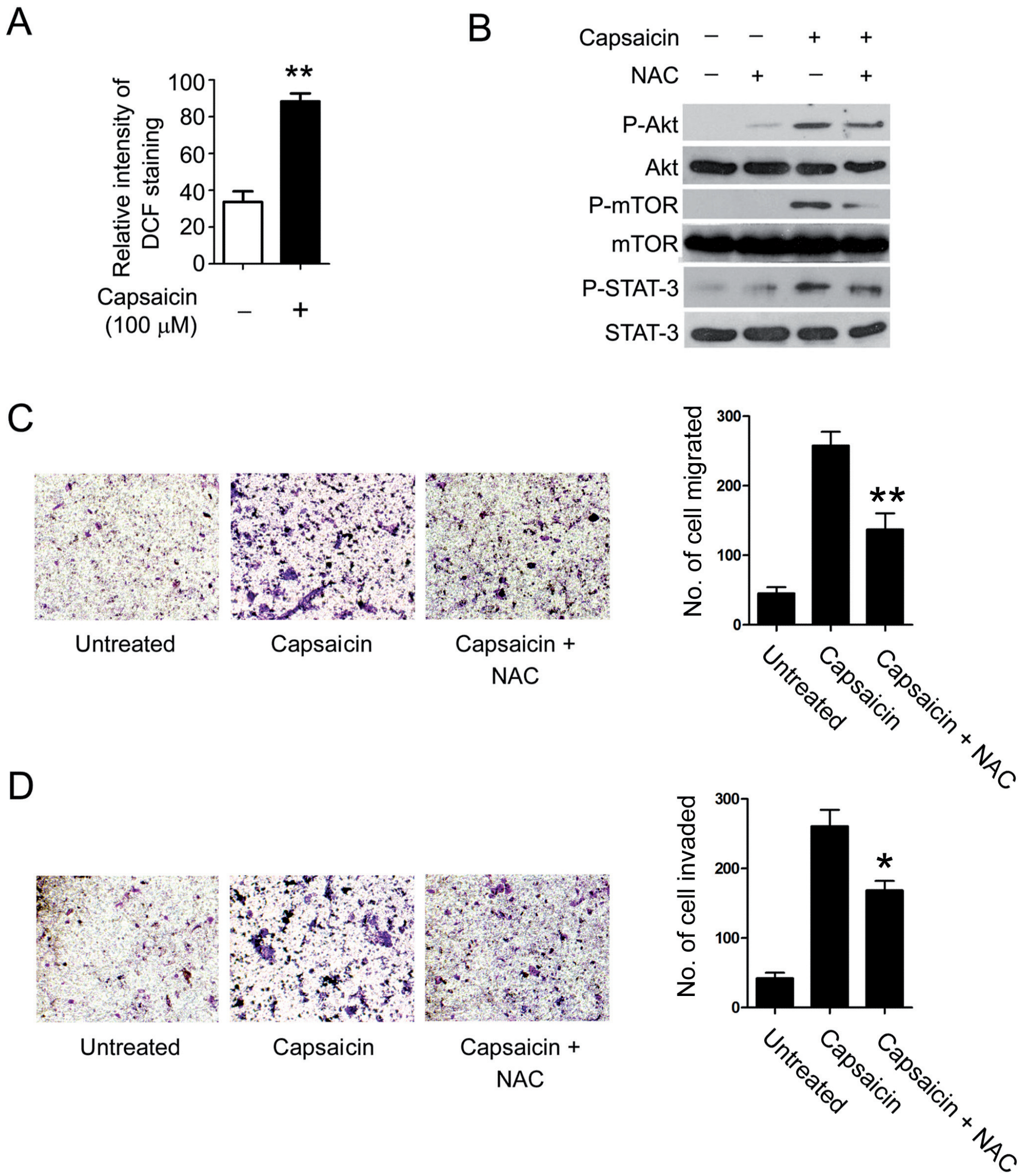

Figure 4. Low-concentration capsaicin-induced metastasis of CRC cells is mediated by triggering ROS production
(A) SW480 cells were treated with 100 μM capsaicin for 48 h. Intracellular ROS level was determined by DCF staining. Treatment with 100 μM capsaicin triggered ROS production in SW480 cells. (B) SW480 cells were treated with 100 μM capsaicin for 48 h in presence or absence of 10 mM NAC. Expression of phosphorylated Akt, mTOR and STAT-3 was examined by immunoblot. Treatment with NAC attenuated capsaicin-induced activation of Akt/mTOR and STAT3 pathways. (C) SW480 cells were treated with 100 μM capsaicin for 48 h in presence or absence of 10 mM NAC. Migratory capability was examined by transwell assay. Treatment with NAC decreased capsaicin-induced migration of SW480 cells. (D) SW480 cells were treated with 100 μM capsaicin for 48 h in presence or absence of 10 mM NAC. Invasive capability was examined by Mtrigel invasion assay. Treatment with NAC decreased capsaicin-induced invasion of SW480 cells.

markedly abolished capsaicin-induced migration (Fig. 3D) or invasion (Fig. 3E) in SW480 cells. These results suggested that PI3K/Akt and STAT-3 pathways were required for low-concentration capsaicin-induced CRC cell metastasis.

Low-concentration capsaicin-induced metastasis of CRC cells is mediated by modulating intracellular ROS. Recently, oxidative stress appeared to be involved in the regulation of various physiological and pathological processes, including tumor metastasis [21-23]. Thus we next set out to determine if ROS has a role in low-concentration capsaicin-induced metastasis of CRC cells. As shown in Fig. 4A, the level of intracellular ROS, detected by using cell-

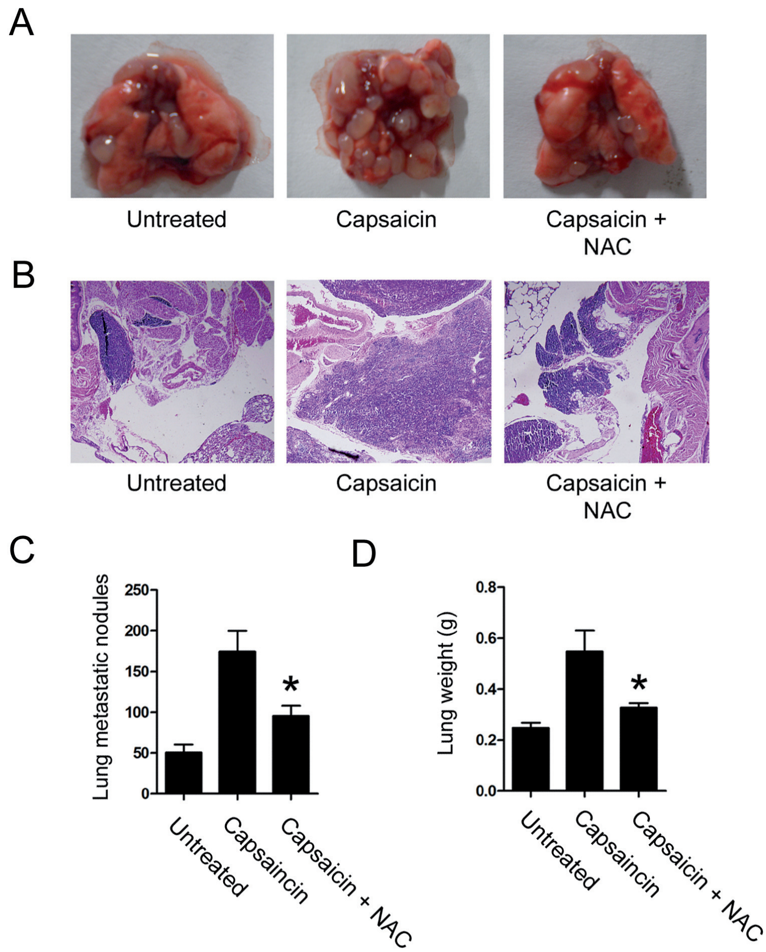

Figure 5. Inhibition of ROS production attenuates Low-concentration capsaicin-induced metastasis of CRC cells *in vivo*
CT-26 cells were pre-treated with 100 µM capsaicin in presence or absence of 10 mM NAC for 48 h and then intravenously injected in mice. Representative images of the lungs from each group (A) and representative image of H&E staining (B) are shown. Lung metastasis of CT-26 cells was determined by counting the lung metastatic nodules (C) and measuring the lung weight (D). The data showed that treatment with NAC decreased the capsaicin-induced metastatic potential of CT-26 cells *in vivo*.

permeable 2', 7'-dichlorofluorescin diacetate as a probe, were found to be over 2-fold higher in the SW480 cells treated with 100 µM capsaicin for 48 h, compared to the untreated cells ($P < 0.01$). Further we examined whether ROS was involved in capsaicin-induced metastasis of CRC cells. To this end, N-acetylcysteine (NAC), an antioxidant reagent, was used. As shown, both activation of Akt/mTOR and STAT-3 pathway induced by 100 µM capsaicin was attenuated upon treatment with NAC (Fig. 4B). Furthermore, treatment with NAC substantially abolished capsaicin-induced migratory (Fig. 4C, $P < 0.01$) and invasive (Fig. 4D, $P < 0.05$) potential of CRC cells. These observations were further supported by using mice lung metastasis model (Fig. 5A-B). As results, inhibition of ROS production by NAC also reduced capsaicin-mediated lung metastasis of CT-26 cells, shown by the decreased number of lung metastatic nodules and lung weight (Fig 5C-D). These results suggested that low-concentration capsaicin facilitated CRC cell metastasis by modulating intracellular ROS.

Discussion

Capsaicin was considered as an angiogenetic inhibitor due to its role in blocking VEGF-induced capillary-like tube formation of endothelial cells [24]. However, it is also documented that capsaicin possesses the capability to promote cancer metastasis [10]. In this study, we demonstrated that high-concentration capsaicin (≥ 200 µM for SW480 and CT-26 cell lines; ≥ 25 µM for HCT116 cell line) showed anti-proliferative activity in CRC cells in a dose-dependent manner. Interestingly, though low-concentration capsaicin did not affect cell proliferation, it dramatically enhanced both migratory and invasive capability of SW480 and HCT116 cells. Such pro-metastatic property of low-concentration capsaicin was further validated *in vivo* that 100 μM capsaicin-pretreated CT-26 cells formed more lung metastatic nodules compared to untreated cells. Therefore, our data suggested that capsaicin harbored both anti-proliferation and pro-metastatic effect on CRC cell, which were probably concentration-dependent. Further studies will be conducted to conform whether the dual role of capsaicin exits in other tumor types.

EMT is a biological process allowing epithelial cells to obtain a mesenchymal phenotype, including loss of epithelial marker E-cadherin and gain of mesenchymal marker Vimentin and N-cadherin. Recently, amounting evidences suggested EMT as a crucial step in tumor metastasis which enable tumor cell migrate and invade into the surrounding stroma and spread to a distant organ [13]. In this study, we demonstrated that low-concentration capsaicin induced EMT in SW480 cells, revealed by decreased expression of E-cadherin and elevated expression of both Vimentin and N-cadherin.

The collection of molecular factors that cooperate with each other to promote EMT is very continuously growing. Transforming growth factor-β (TGF-β) is one of the most relevant inducers of EMT and its pro-EMT activity is mediated by both Smad-dependent and -independent pathways [25]. Recently, a small but increasing number of studies provided clues pointing MMPs as potential regulator of EMT in both normal cells and cancer cells [26, 27]. In present data, we showed that two gelatinase, MMP-2 and MMP-9, were markedly upregulated in SW480 cells treated with 100 μM capsaicin. Further, we manifested that two MMPs regulatory signaling cascades, Akt/mTOR and STAT-3 pathway, were both activated in capsaicin-treated SW480 cells. Further, inhibition of either Akt/mTOR or STAT-3 pathway by chemical antagonists significantly retarded capsaicin-induced metastasis of SW480 cells. Therefore, it is reasonable to infer that low-concentration capsaicin induced CRC cell metastasis probably through activating Akt/mTOR and STAT-3 pathways as well as up-regulating MMP-2 and MMP-9.

It was generally believed that ROS exert cytotoxic and genotoxic effects by causing damage to lipids, proteins and DNA, because of their greater chemical reactivity with regard to oxygen [15, 21]. Recently, however, ROS was proposed to be involved in tumor metastasis as a second-messenger for regulation of diverse cellular processes. It is reported that CRC cells treated with hydrogen peroxide exhibited an enhanced migration and invasion by activating ITGB3 and STMN1 [28]. In current study, we demonstrated that low-concentration capsaicin induced significant accumulation of cellular ROS in CRC cells. Further, inhibiting ROS production by NAC, substantially blocked capsaicin-induced metastasis of CRC cells both *in vitro* and *in vivo*, suggesting a crucial role of ROS in capsaicin-mediated CRC cell metastasis.

Efforts have been made to explore the capsaicin-mediated regulatory network on CRC cells, but no rational signaling pathway has been established thus far. The data presented here provides new clues pointing MMPs, Akt/mTOR pathway, STAT-3 pathway and ROS (Fig. 6). Treatment with low-concentration capsaicin triggers oxidative stress in CRC cells, which lead to activation of Akt/mTOR and STAT-3 signaling pathway and subsequent overexpression of MMP-2 and MMP-9. Accumulation of extracellular MMPs induced EMT, and elevated cell migratory and invasive capability, conferring CRC cells with a metastatic phenotype.

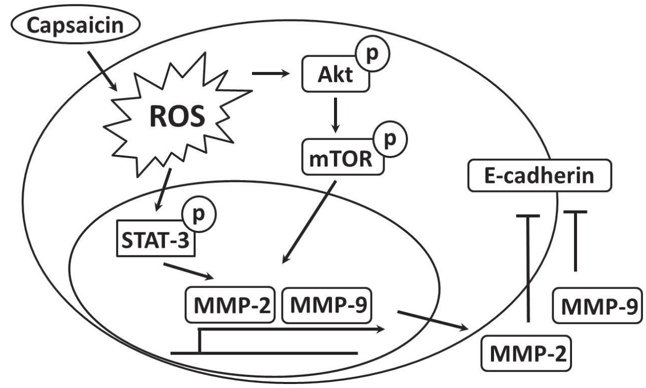

Figure 6. Schematic model of low-concentration capsaicin-induced metastasis in CRC cells. Schematic illustrating the potential role of low-concentration capsaicin in CRC metastasis. Treatment with low-concentration capsaicin promotes ROS production in CRC cells, which subsequently results in activation of Akt/mTOR and STAT-3 signaling pathway as well as overexpression of MMP-2 and MMP-9. Accumulation of extracellular MMPs suppresses expression of E-cadherin and elevated cell migratory and invasive capability, conferring CRC cell with a metastatic phenotype.

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