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Cocarcinogenic Effect of Capsaicin Involves Activation of EGFR Signaling but Not TRPV1

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Abstract

Epidemiologic and animal studies revealed that capsaicin can act as a carcinogen or cocarcinogen. However, the molecular mechanisms of the cancer-promoting effects of capsaicin are not clear. Here, we report that capsaicin has a cocarcinogenic effect on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–promoted skin carcinogenesis *in vivo* and is mediated through the epidermal growth factor receptor (EGFR), but not the transient receptor potential vanilloid subfamily member 1 (TRPV1). Topical application of capsaicin on the dorsal skin of 7,12-dimetylbenz(*a*)anthracene–initiated and TPA-promoted TRPV1 wild-type (WT) and TRPV1 knockout (KO) mice induced more and larger skin tumors in TRPV1/KO mice, suggesting a TRPV1-independent mechanism. Cyclooxygenase-2 (COX-2) was highly elevated by capsaicin treatment in tumors and murine embryonic fibroblasts from TRPV1/KO mice. Inhibitors of EGFR/MEK signaling suppressed TPA/capsaicin-induced COX-2 expression in TRPV1/KO cells, indicating that activation of EGFR and its downstream signaling is involved in COX-2 elevation. Capsaicin induced a further induction of TPA-increased COX-2 expression in EGFR/WT cells, but not in EGFR/KO cells. TPA/capsaicin cotreatment caused EGFR tyrosine phosphorylation and activated EGFR downstream signaling, including ERKs and Akt in EGFR/WT, but not EGFR/KO cells. Specific inhibition of EGFR and TRPV1 indicated that capsaicin-induced ERK activation in A431 cells was dependent on EGFR, but not TRPV1. Together, these findings suggest that capsaicin might act as a cocarcinogen in TPA-induced skin carcinogenesis through EGFR-dependent mechanisms. *Cancer Res; 70(17); 6859–69. ©2010 AACR.*

Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is a principal pungent ingredient of *Capsicum* fruits, which are widely consumed worldwide. It is used in topical creams and its role in carcinogenesis is controversial. Capsaicin induces the apoptosis of cancers cells, including myeloid leukemia (1), human hepatoma (2), and colon cancer (3). However, epidemiologic and animal experimental evidence suggests that capsaicin also acts as a carcinogen or cocarcinogen, particularly during the tumor promotion stage (4). Capsaicin from chili peppers promoted stomach and liver cancer in BALB/c mice (5) and also induced duodenal adenocarcinoma in mice (6). Epidemiologic studies suggested a positive association between the incidence of stomach cancer and the consumption of chili pepper–rich diets (7). Capsaicin enhanced the metastasis of murine breast cancer cells by reducing the expression of apoptosis-related genes (8) and induced LNCaP prostate cancer cell proliferation by increasing androgen receptor expression through the activation of ERKs and Akt (9). Thus, clarifying the underlying molecular mechanisms to reveal whether or not capsaicin exerts carcinogenic activities is important.

Transient receptor potential vanilloid subfamily member 1 (TRPV1) is a ligand-gated cation channel with limited selectivity for calcium ions. TRPV1 is activated by noxious stimuli including heat, acid, and vanilloid compounds such as capsaicin (10). Capsaicin is a selective agonist of TRPV1 and causes TRPV1 to be desensitized after activation. TRPV1 is widely expressed in primary afferent neurons and also in nonneuronal tissues, including skin keratinocytes, fibroblasts, liver, prostate, and bladder smooth muscle (11). Thus, studying the function of TRPV1 in a broader context than pain perception is relevant. Although the biological role of TRPV1 in cells other than primary sensory neurons is unclear, some studies indicated a role for TRPV1 in carcinogenesis. Increased expression of TRPV1 was detected in prostate, colon, and pancreatic cancers (12), whereas the expression of TRPV1 was found to decrease as bladder cancer and brain tumor malignancy progressed (13, 14), suggesting that TRPV1 might negatively control tumor progression. Our

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recent study also suggested that TRPV1 might act as a tumor suppressor in skin cancer (15).

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that mediates intracellular signaling in response to various extracellular stimuli. By binding with its ligands, EGFR activates intracellular signaling cascades including Ras/Raf/MEK/ERK and phosphoinositide-3-kinase/ Akt, and subsequently controls proliferation, migration, and apoptosis. EGFR is overexpressed in colorectal, pancreatic, lung, and non–small cell lung cancers (16). Aberrant regulation of EGFR activates downstream signals including ERKs and Akt resulting in increased tumor cell proliferation, survival, and invasiveness. Thus, modulation of EGFR signaling is key in preventing cancers. However, a role for capsaicin in the regulation of EGFR to mediate its carcinogenic effects has not been reported. Here, we show that capsaicin acts as a co-carcinogen in 12-O-tetradecanoylphorbol-13-acetate (TPA)– induced skin cancer and its action is mediated through EGFR-dependent mechanisms. Notably, TRPV1, which was shown to exert a tumor-suppressive effect in skin cancer, is not a primary target of capsaicin in skin tumor development.

Materials and Methods

Chemicals

Capsaicin, EGF, TPA, and anti–β-actin were from Sigma-Aldrich. Cell culture medium and supplements were from Life Technologies, Inc. AG1478, U0126, GM6001, PP2, and anti-TRPV1 were obtained from Calbiochem. Capsazepine and anti–cyclooxygenase-2 (COX-2) were purchased from Cayman. Antibodies against phosphorylated ERKs (Thr[202]/ Tyr[204]), total ERKs, phosphorylated Akt (Ser[473]), total Akt, phosphorylated tyrosine, and phosphorylated EGFR (Tyr[1068], Tyr[845], and Tyr[992]) were from Cell Signaling Biotechnology. Antibodies against EGFR and agarose-conjugated EGFR were from Santa Cruz Biotechnology. The neutralizing antibody against HB-EGF was purchased from R&D Systems, Inc. A chemiluminescence detection kit was from Amersham Pharmacia Biotech and a protein assay kit was obtained from Bio-Rad Laboratories.

Cell culture and transfection

TRPV1 or EGFR wild-type (WT) and knockout (KO) murine embryonic fibroblasts (MEF) and A431 human epidermoid carcinoma cells were cultured as described (15). EGFR/WT cells were grown to 50% confluence, and transfected with scramble (negative control) or siRNA-Src (Bioneer Inc., Korea) using LipofectAMINE 2000 (Invitrogen).

Western blot analysis

Cells were cultured to 70% confluence and starved in serum-free DMEM for 24 hours. The cells were then treated with capsaicin followed by TPA (20 ng/mL) at various times. Cell lysates were scraped and harvested according to procedures previously described (17), and membrane and cytosolic fractions were prepared as specified (15). SDS-PAGE and Western blotting were performed as described previously (15).

Immunoprecipitation

Cells were cultured to 70% confluence and starved in serum-free DMEM for 24 hours. Cells were then treated with 50 μmol/L of capsaicin, followed by 20 ng/mL of TPA for various times. Cells were disrupted with lysis buffer (18) and centrifuged at 14,000 rpm for 10 minutes by microcentrifuge. Lysates (1,000 μg) were immunoprecipitated with an antibody against agarose-conjugated EGFR overnight at 4°C. After washing, beads were resolved by SDS-PAGE and Western blot to assess the expression level of phosphotyrosine.

Reverse transcription-PCR analyses

Total RNA was extracted using Trizol reagent (Invitrogen) according to the instructions of the manufacturer. cDNA was synthesized using a reverse transcription system purchased from Promega, and amplified using i-Star Taq DNA polymerase obtained from iNtRON Biotechnology, Inc. (Korea). The sense and antisense primers cox-2 mRNA were 5′-GGGTTGCTGGGGGAAGAAATGTG-3′ and 5′-GGTGGCTGT-TTTGGTAGGCTGTG-3′, respectively, which yielded a 479-bp PCR product. The amplification conditions for COX-2 were 28 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 60 seconds, followed by one cycle for 5 minutes at 72°C. The sense and antisense primers $gapdh$ mRNA were 5′-CCCCTTCATTGACCTCAACTACATGG-3′ and 5-GCCTGCTTCACCACCTTCTTGATGTC-3′, respectively, which yielded a 690-bp PCR product. The amplification conditions for glyceraldehyde-3-phosphate dehydrogenase were 28 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds, followed by one cycle for 5 minutes at 72°C. The primers and amplification conditions for HB-EGF and amphiregulin were previously described and reverse transcription-PCR (RT-PCR) analysis was conducted following the same procedure as described (19).

In vivo mouse studies

TRPV1/KO B6, 129S4-Trpv1 mice, and wild-type (TRPV1/ WT) mice were purchased from The Jackson Laboratory. Mice were divided into eight groups each of TRPV1/WT and TRPV1/KO and matched by age and gender. All eight groups were initiated by topical application of 200 nmol of 7,12-dimethylbenz(a)anthracene (DMBA) in acetone. Two weeks later, TPA ± capsaicin or capsaicin-only treatment was begun and continued twice a week for 21 weeks. Groups 1, 3, 5, and 7 comprised wild-type (TRPV1/WT) mice and groups 2, 4, 6, and 8 were knockout (TRPV1/KO) mice. Groups 1 and 2 were treated with acetone only; groups 3 and 4 were treated with 17 nmol of TPA only; groups 5 and 6 were treated with 10 μmol of capsaicin followed 30 minutes later with 17 nmol of TPA; and groups 7 and 8 were treated with 10 μmol capsaicin only. All treatments were applied topically in acetone. Mice were weighed, photographed, and tumors measured once a week beginning when first measurable tumors (1 mm[3]) were observed (week 12).

Immunohistochemical analysis

Sections (5 μm thick) of formalin-fixed, paraffin-embedded tissue were cut, mounted on glass slides, deparaffinized, and

rehydrated. Antigen retrieval was performed by incubation with proteinase K solution. To eliminate endogenous peroxidases, slides were incubated in 3% hydrogen peroxide, followed by blocking treatment with 3% bovine serum albumin solution. Slides were incubated overnight with an affinity-purified primary antibody at 4°C and then developed using the Histostain Plus Kit (Zymed Laboratories Inc.). The reaction was visualized with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin (Sigma-Aldrich).

Statistical analyses

Where necessary, data are presented as means ± SD. The Student's t-test or one-way ANOVA was used for single statistical comparisons. A probability value of $P < 0.05$ was used as the criterion for statistical significance.

Results

Capsaicin induces TPA-induced skin carcinogenesis more strongly in TRPV1/KO mice

TRPV1/WT and KO mice were subjected to the well-known two-stage skin carcinogenesis experiment with DMBA/TPA. Coapplication of capsaicin with TPA to the dorsal surface of either TRPV1/WT or KO mice induced significantly more and larger skin tumors compared with TPA treatment alone (Table 1; Fig. 1A–C). Furthermore, TRPV1/KO mice developed more skin tumors than WT mice in response to cotreatment with TPA and capsaicin (Table 1; Fig. 1A–C). In contrast, treatment with only capsaicin or vehicle did not induce any skin tumors in either TRPV1/WT or KO mice (Table 1; Fig. 1C), indicating that capsaicin alone does not act as a carcinogen. All mice (100%) in both groups developed papillomas in response to TPA and capsaicin by 21 weeks (Fig. 1D), whereas ∼20% of TRPV1/WT mice versus 75% of TRPV1/KO mice developed tumors in response to TPA only (Table 1; ref. 15). These results suggest

that capsaicin might act as a cocarcinogen through TRPV1-independent mechanisms.

Capsaicin increases TPA-induced COX-2 expression more strongly in TRPV1/KO mice

Increased amounts of COX-2 are commonly found in both malignant tissues and tumors, including skin cancer (20). We examined COX-2 protein abundance in tumors extracted from mouse skin. Both TRPV1/WT and KO mice treated with capsaicin and TPA together exhibited higher COX-2 protein levels than groups treated with TPA alone (Fig. 2A), and tumors from TRPV1/KO mice expressed the highest level of COX-2 as a result of treatment with capsaicin and TPA together (Fig. 2A). Western blot results confirmed that tumor lysates from TRPV1/KO mice treated with capsaicin and TPA exhibited significantly higher levels of COX-2 than TRPV1/WT mice (Fig. 2B). Additionally, tumors from TRPV1/KO mice cotreated with capsaicin and TPA exhibited higher COX-2 expression levels compared with mice treated with TPA alone (Fig. 2C). These results suggest that capsaicin acts through a TRPV1-independent mechanism to induce COX-2 expression in the presence of TPA.

### Capsaicin increases TPA-induced COX-2 expression in TRPV1/KO cells

Genomic PCR and Western blot analysis verified that the TRPV1 gene and protein were only detected in TRPV1/WT MEFs (Fig. 3A). In addition, elevated COX-2 expression levels were seen in response to cotreatment with capsaicin and TPA in TRPV1/KO MEFs (Fig. 3B). To determine the signaling molecules involved in COX-2 upregulation by capsaicin and TPA, chemical inhibitors for EGFR and downstream MEK signals were used. AG1478 (an EGFR inhibitor) and U0126 (a MEK inhibitor) distinctly suppressed TPA/capsaicin-induced COX-2 expression in TRPV1/KO MEFs, suggesting a possible role of the EGFR pathway in capsaicin-induced COX-2 upregulation when TRPV1 is depleted (Fig. 3C).

Table 1. Final average papilloma volume and number per mouse at 21 wk

Treatment group	N	Average volume/ mouse (mm³)	SE	Average number/ mouse	SE
DMBA/WT-acetone	17	0.00	0.00	0.00	00.0
DMBA/KO-acetone	16	0.00	0.00	0.00	00.0
DMBA/WT-10 µmol capsaicin	17	0.00	0.00	0.00	00.0
DMBA/KO-10 µmol capsaicin	17	0.00	0.00	0.00	00.0
TRPV1/WT-TPA treated	16	2.23	1.36	0.31	0.18
TRPV1/KO-TPA treated	16	12.06*	4.47	1.75*	0.37
TRPV1/WT 10 µmol capsaicin/TPA	16	24.37[†]	5.41	7.94[†]	1.27
TRPV1/KO 10 µmol capsaicin/TPA	17	55.80*[†]	10.59	13.18*[†]	1.67

*Significantly greater average volume and number of papillomas in TRPV1/KO mice compared with TRPV1/WT mice ($P < 0.05$).
[†]Significantly greater average volume and number of papillomas in capsaicin-treated TRPV1/WT or TRPV1/KO mice compared with untreated TRPV1/WT or TRPV1/KO mice.
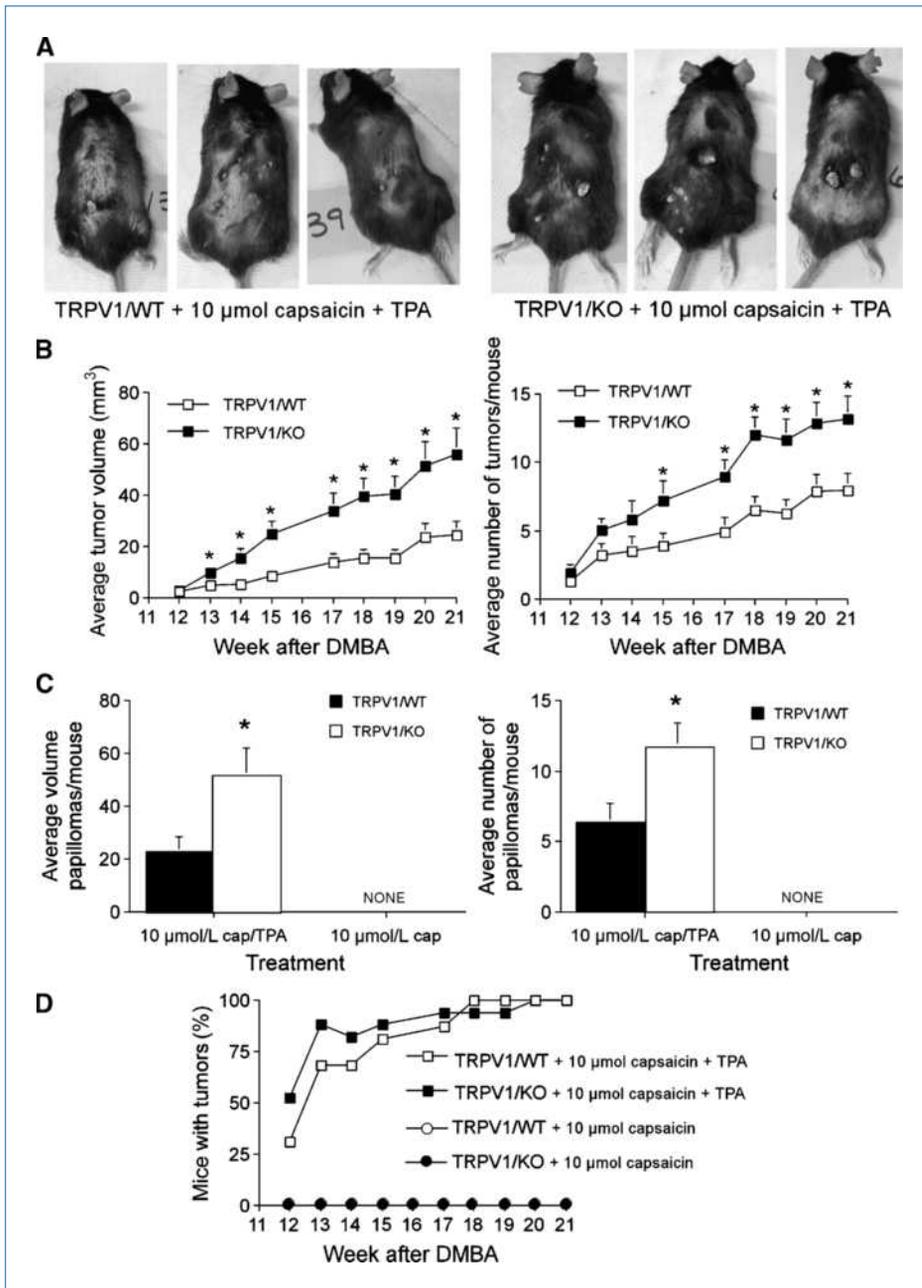
Figure 1. Capsaicin increases TPA-promoted skin carcinogenesis in TRPV1/KO mice. The shaved dorsal surface of each mouse was initiated by topical application of DMBA (200 nmol) in acetone. After 2 wk, mice were topically treated with capsaicin (10 μmol) in acetone followed 30 min later with TPA (17 nmol) in acetone or acetone only without TPA. A, representative photographs of papilloma development in TRPV1/WT (left) and TRPV1/KO (right) mice. B, average volume (left) and number (right) of papillomas developed per mouse in response to TPA and capsaicin cotreatment. C, final (21 wk) average volume (left) and number (right) of papillomas in mice treated with capsaicin and TPA or capsaicin only. B and C, *, $P < 0.05$, indicates a significant difference. D, percentage of TRPV1/WT and TRPV1/KO mice developing papillomas when treated with capsaicin and TPA or capsaicin only.

We then determined the inhibitory effect of AG1478 on the phosphorylation of MEK, one of the direct downstream substrates of EGFR, and that of U0126 on the phosphorylation of ERKs, a direct downstream substrate of MEK. TPA/capsaicin-induced phosphorylations of MEK and ERK were suppressed by AG1478 and U0126, respectively, suggesting that each inhibitor blocks its respective target in this cell system (Fig. 3D).

EGFR is involved in TPA/capsaicin-promoted COX-2 expression

We next compared the effect of capsaicin on TPA-induced COX-2 expression in EGFR/WT and KO MEFs. EGFR was only detected in EGFR/WT MEFs (Fig. 4A). Treatment with capsaicin alone did not induce COX-2 expression when EGFR was depleted (Fig. 4B). Similarly, TPA-induced COX-2 expression was only increased by capsaicin in EGFR/WT MEFs (Fig. 4C). To determine whether TPA-induced COX-2 expression is regulated by capsaicin at the transcriptional or posttranscriptional level, we measured cox-2 mRNA levels using RT-PCR. Capsaicin upregulated cox-2 mRNA expression more strongly than TPA alone, and the TPA/capsaicin-induced cox-2 mRNA level was higher in EGFR/WT cells than KO (Fig. 4D). These results indicate that cox-2 is upregulated by TPA/capsaicin treatment at the transcriptional level and
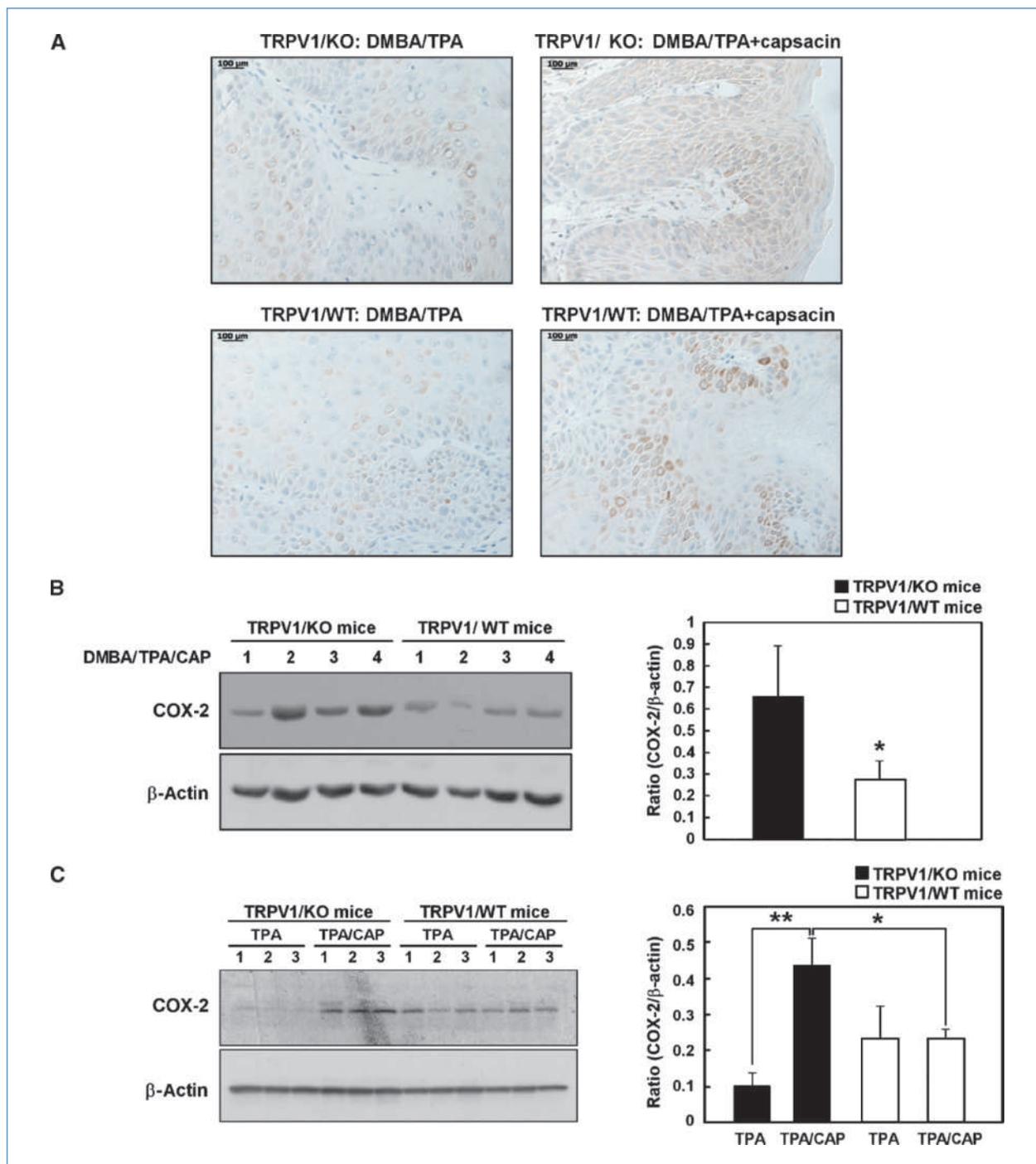
Figure 2. TPA and capsaicin cotreatment increases COX-2 protein abundance in TRPV1/KO mice. A, TPA-induced COX-2 expression is strongly induced by treatment with capsaicin in TRPV1/KO mice. Tumors were extracted from dorsal mouse skin and cut into 5-μm-thick paraffin-embedded sections. Sections were mounted on silane-coated slides and subjected to immunohistochemical analysis using anti–COX-2. Brown staining indicates expression of COX-2. B, cotreatment with TPA and capsaicin strongly induces COX-2 protein abundance in TRPV1/KO mice. Tumors were extracted from mouse dorsal skin and lysates were subjected to SDS-PAGE and COX-2 expression analyzed by Western blot (left). Densitometer analysis of each band was performed with ImageJ. *, $P < 0.05$, indicates a significant difference between each group (right). C, the abundance of COX-2 protein in TRPV1/KO mouse skin is increased by TPA and capsaicin cotreatment compared with TPA alone. Three mice were randomly chosen and dorsal skin lysates were subjected to SDS-PAGE and COX-2 abundance was analyzed by Western blot (left). Densitometer analysis of each band was performed using ImageJ (right). **, $P < 0.01$, indicates a significant difference between TRPV1/KO mice cotreated with TPA and capsaicin and TRPV1/KO mice treated with TPA alone. *, $P < 0.01$, indicates a significant difference between TRPV1/WT and TRPV1/KO mice cotreated with TPA and capsaicin. B and C, mice in each group were randomly numbered and the number of the mouse indicated.
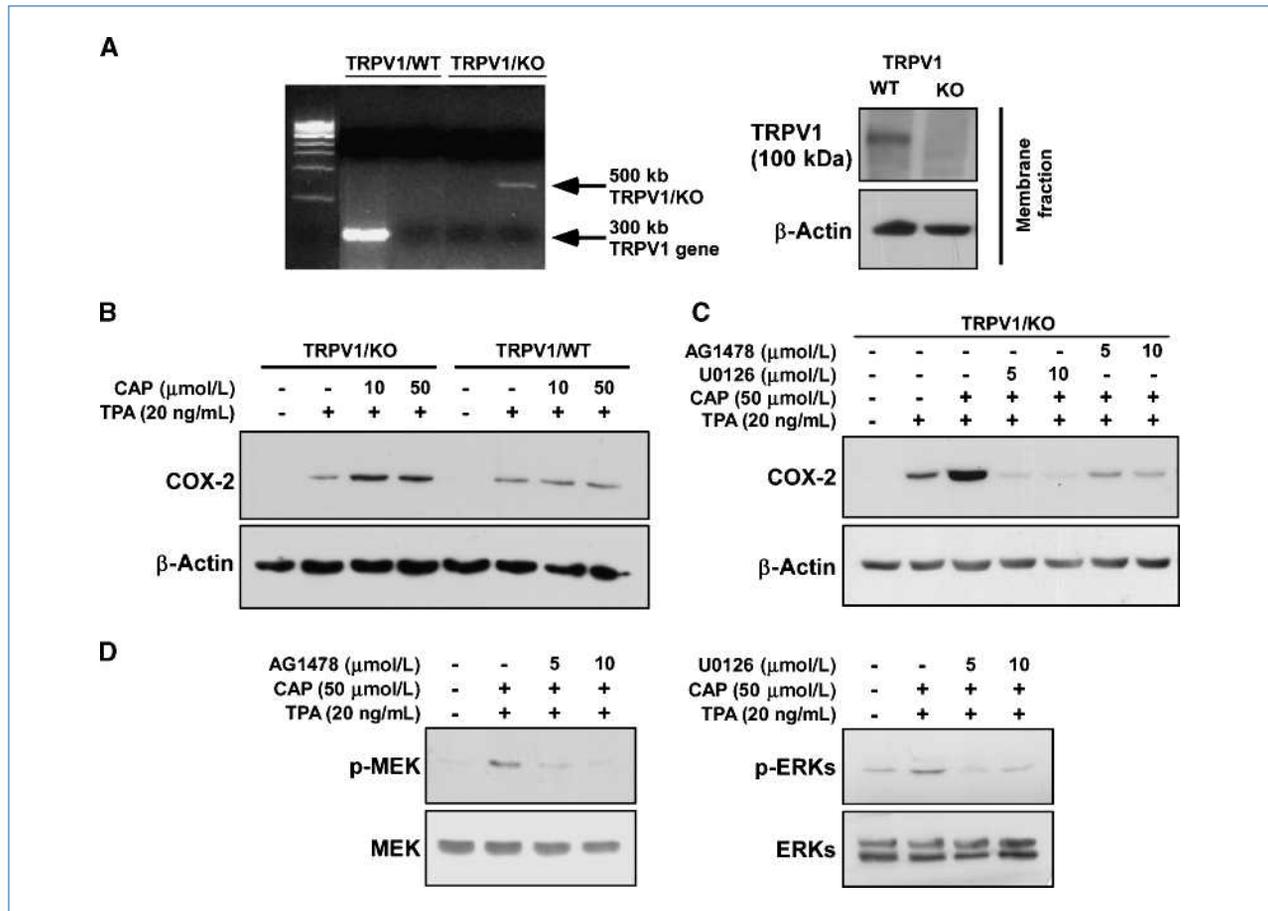
Figure 3. TPA and capsaicin cotreatment increases COX-2 expression in TRPV1/KO MEF. A, genomic PCR detection (left) and protein abundance (right) of TRPV1 in WT and KO MEFs. B, capsaicin induces strong TPA-promoted COX-2 expression in TRPV1/KO MEFs. TRPV1/KO and WT MEFs were treated with capsaicin (0, 10, or 50 μmol/L) for 30 min before TPA (20 ng/mL) treatment (4 h). Cells were lysed and COX-2 abundance analyzed by Western blot. C, COX-2 expression induced by cotreatment with TPA and capsaicin is mediated by activation of EGFR signaling in TRPV1/KO MEFs. TRPV1/KO MEFs were treated with AG1478 (an EGFR inhibitor) or U0126 (a MEK inhibitor) 30 min before treatment with capsaicin (50 μmol/L). At 30 min after capsaicin treatment, TPA (20 ng/mL) was added. Cells were lysed after 4 h and COX-2 protein abundance analyzed by Western blot. B and C, data shown are representative of two independent experiments with similar results. D, effects of AG1478 and U0126 on phosphorylation of downstream targets. TRPV1/KO MEFs were treated with AG1478 or U0126 30 min before treatment with capsaicin (50 μmol/L). At 30 min after capsaicin treatment, TPA (20 ng/mL) was added and cells were lysed 15 min later and phosphorylation of MEK (left) or ERKs (right) analyzed by Western blot.

support the idea that capsaicin-induced COX-2 expression is dependent on the EGFR pathway.

EGFR downstream signaling is sustainably activated in response to TPA and capsaicin only in the presence of EGFR
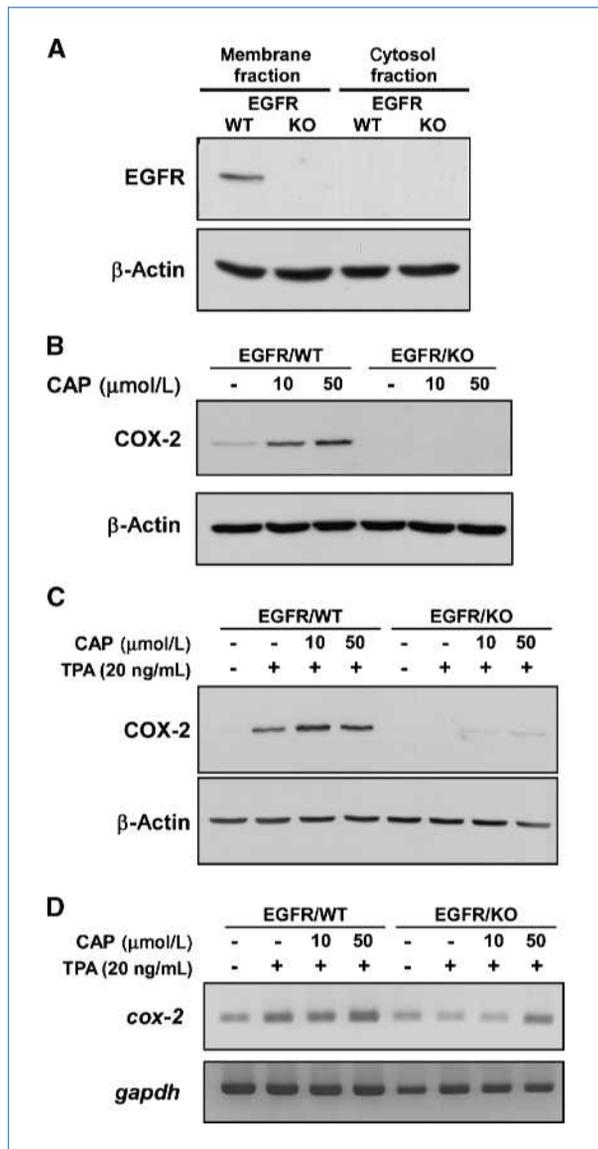
After the activation of EGFR, several intracellular signaling pathways, including ERK and phosphoinositide-3-kinase/Akt, are recruited to EGFR and subsequently activated (21). Cotreatment with capsaicin and TPA induced the phosphorylation of ERKs and Akt in EGFR/WT MEFs more strongly than in EGFR/KO MEFs (Fig. 5A). The more time that passed after cotreatment with TPA and capsaicin, the more strongly sustained was the phosphorylation of ERKs and Akt in EGFR/WT MEFs (Fig. 5B). In EGFR/KO cells, however, TPA/capsaicin treatment had little effect on ERKs or Akt phosphorylation,

indicating that EGFR plays a key role in capsaicin-induced activation of downstream intracellular signal pathways.

Activation of EGFR signaling in response to TPA and capsaicin is dependent on both EGFR and Src activation

Treatment with AG1478 or PP2 (an Src inhibitor) showed that sustained activation of ERKs and Akt was still dependent on EGFR activation but also on Src (Fig. 5C). Next, tyrosine phosphorylation of EGFR was determined by immunoprecipitating cells with anti-EGFR and subsequent immunoblotting with a phosphotyrosine antibody. Treatment with TPA and capsaicin together induced phosphorylation of EGFR at tyrosine residues (Fig. 5D, top). Because EGFR was reported to be activated intracellularly by Src kinase, we next examined the effect of PP2 on TPA/capsaicin-induced EGFR phosphorylation

and found that the TPA/capsaicin-induced EGFR phosphory-lation was suppressed by PP2 (Fig. 5D, bottom). Phosphoryla-tion of EGFR was also blocked by AG1478 (Fig. 5D, bottom). To further confirm the importance of Src for TPA/capsaicin-

induced signal activation, we examined the effect of siRNA-Src on TPA/capsaicin-induced COX-2 expression. Transfection of siRNA-Src resulted in the complete inhibition of COX-2 expression in response to TPA/capsaicin cotreatment, sug-gesting the important role of Src activation (Supplementary Fig. S1A and B).

Figure 4. EGFR is involved in TPA/capsaicin-induced COX-2 expression. A, EGFR is expressed only in EGFR/WT. Cells were lysed and membrane and cytosolic fractions separated and EGFR abundance determined by Western blot. B, treatment with capsaicin alone induces COX-2 expression only in EGFR/WT MEFs. EGFR/WT and KO MEFs were treated with capsaicin (0, 10, or 50 μmol/L) for 4 h and COX-2 protein abundance determined by Western blot. C, capsaicin enhances stronger TPA-promoted COX-2 expression in EGFR/WT MEFs. EGFR/WT and KO MEFs were treated with capsaicin (0, 10, or 50 μmol/L) for 30 min followed by TPA (20 ng/mL) for 4 h. Cells were lysed and COX-2 expression analyzed by Western blot. D, capsaicin strongly upregulates TPA-induced cox-2 mRNA expression in EGFR/WT cells. EGFR/WT and KO cells were cultured overnight and starved for 24 h. Cells were treated with capsaicin (50 μmol/L) for 30 min and then TPA (20 ng/mL) for 2 h. Cells were lysed and cox-2 mRNA levels were analyzed by RT-PCR.

Activation of the EGFR pathway in response to TPA and capsaicin is mediated through matrix metalloproteinase activation and EGF-like ligand production

Several studies showed that EGFR could be transactivated in the absence of its direct ligands through the activation of matrix metalloproteinase (MMP) and subsequent production of EGF-like ligands (22). We determined that inhibition of MMP activation by GM6001 (an MMP inhibitor) and the reduction of HB-EGF, an EGF-like ligand, by a neutralizing antibody, resulted in the inactivation of EGFR signaling (Supplementary Fig. S2A), suggesting the involvement of MMP-dependent EGF-like ligand production in capsaicin/TPA-induced EGFR transactivation. We next measured the production of HB-EGF and amphiregulin, a major EGF-like ligand induced by MMP activation, using RT-PCR. *Amphiregulin* was highly increased and *HB-EGF* was slightly induced by co-treatment with TPA/capsaicin from 15 minutes to 4 hours (Supplementary Fig. S2B). The induction of EGF-like ligands becomes potentiated at later times, suggesting the contributa-ble role of EGF-like ligands on the sustained activation of EGFR downstream signaling.

EGFR and downstream signaling are activated by capsaicin in A431 epidermoid carcinoma cells

To determine whether capsaicin truly activates EGFR and downstream signaling, we examined the effect of capsaicin in A431 human epidermoid carcinoma cells, which overexpress EGFR (23). Capsaicin treatment induced EGFR phosphoryla-tion at Tyr[992], which is important for the activation of down-stream ERKs and Akt for cell survival (Supplementary Fig. S3A). Through the activation of EGFR, capsaicin induces phosphorylation of downstream effectors including ERKs in A431 cells (Supplementary Fig. S3B). To reveal the mecha-nism involved in capsaicin-induced ERK phosphorylation, we used EGFR inhibitors and TRPV1 antagonists. The results indicated that capsaicin-induced ERK phosphorylation was dependent on EGFR activation, but independent of TRPV1 activation (Supplementary Fig. S3C). To further reveal whether capsaicin-induced EGFR activation was dependent on Src and MMP activations, we examined the effect of PP2 and GM6001. Capsaicin-induced EGFR phosphorylation was inhibited by PP2 or GM6001 (Supplementary Fig. S3D), suggesting that capsaicin induces EGFR phosphorylation through the activa-tions of Src and MMP.

Discussion

TRPV1 is involved in the regulation of pain pathways (24). Capsaicin initially activates TRPV1 and excites nociceptive neurons, leading to the perception of pain. However,
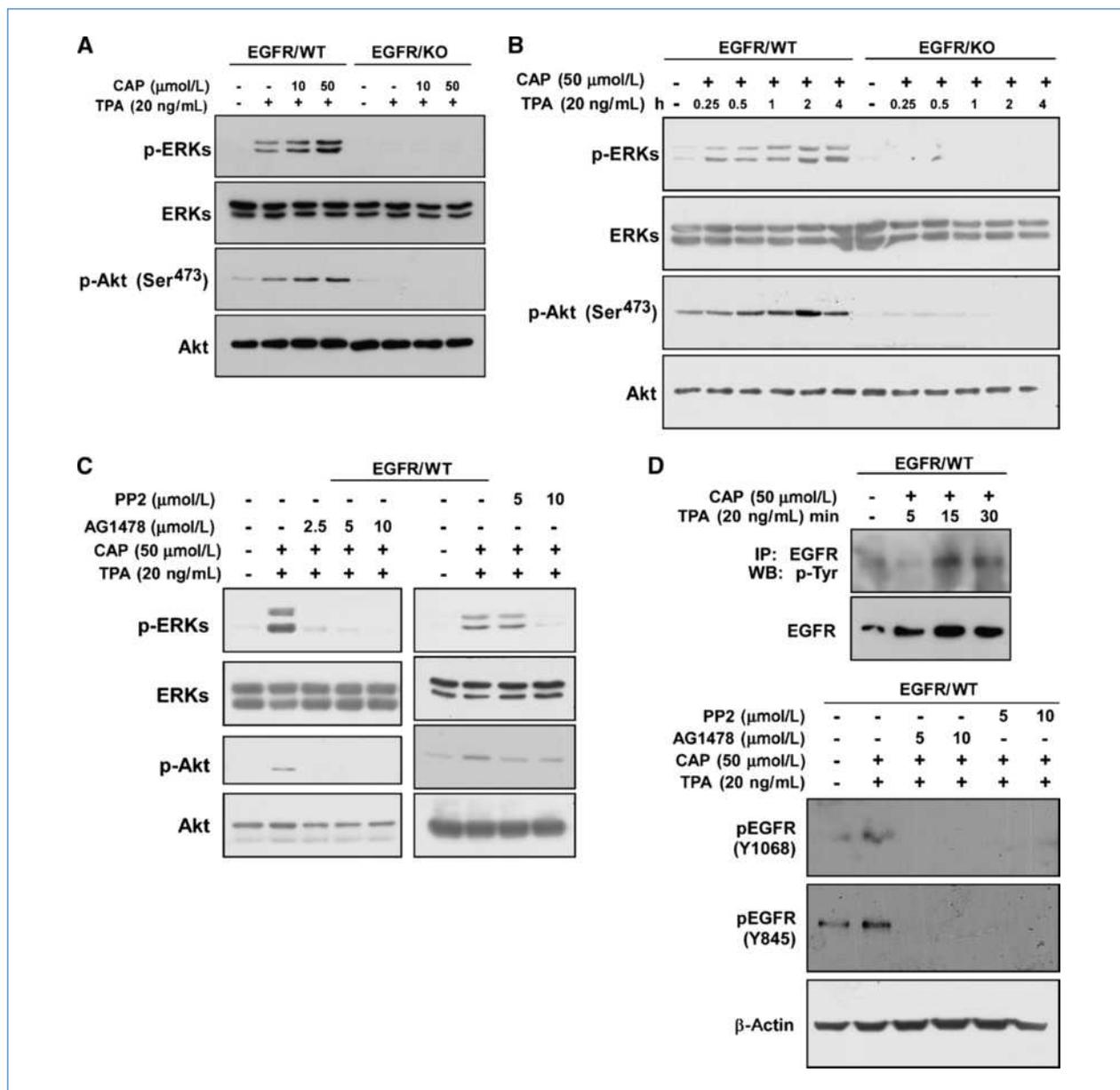
Figure 5. Comparison of EGFR downstream signals activated by TPA and capsaicin cotreatment in EGFR/WT and KO MEFs. A, TPA and capsaicin cotreatment induces sustained activation of ERKs and Akt dose-dependently only in EGFR/WT MEFs. Cells (1.2×10^6) were cultured overnight and starved for 24 h and then treated with capsaicin (0, 10, or 50 μmol/L) for 30 min followed by TPA (20 ng/mL) for another 4 h. Cells were lysed and the expression of each protein was analyzed by Western blot. B, cotreatment with TPA and capsaicin induces the phosphorylation of EGFR downstream targets, ERKs and Akt, time-dependently and more strongly in EGFR/WT MEFs. Cells (1.2×10^6) were cultured overnight and starved for 24 h and then treated with capsaicin (0, 10, or 50 μmol/L) for 30 min and then TPA (20 ng/mL). Cells were lysed and protein abundance was analyzed by Western blot. C, sustained phosphorylation of ERKs and Akt is dependent on EGFR and Src activation. Cells were treated with AG1478 (EGFR inhibitor) or PP2 (Src inhibitor) as indicated for 30 min followed by capsaicin (50 μmol/L). Cells were then treated with TPA (20 ng/mL) for 4 h. Cells were lysed and phosphorylation of ERKs and Akt was assessed by Western blot. D, capsaicin induces EGFR phosphorylation in combination with TPA (top). Cells were treated with capsaicin for 30 min followed by TPA (0, 5, 15, or 30 min). Cells were lysed, immunoprecipitated with anti-EGFR, and immunoblotted with anti-phosphorylated tyrosine. TPA/capsaicin-induced EGFR phosphorylation is dependent on Src activation (bottom). EGFR/WT MEFs were treated with AG1478 or PP2 30 min before capsaicin (50 μmol/L) treatment for 30 min, followed by TPA (20 ng/mL) for 15 min. Cells were lysed and phosphorylation of EGFR was analyzed by Western blot.

prolonged and repetitive exposure to capsaicin causes TRPV1 to become insensitive to noxious stimuli, resulting in desensitization (10). Thus, capsaicin-containing drugs have been developed to treat neuropathic pain through top-ical administration. Accumulating evidence suggests that re-peated or high-dose treatments of capsaicin could cause apoptosis of TRPV1-expressing cells, primarily through the elevation of intracellular calcium ion levels (25), indicating

that TRPV1-unresponsive or deficient states might be clinically important with exposure to capsaicin for a prolonged period. Because capsaicin-induced initial activation of TRPV1 is inevitably followed by desensitization, the possibility that capsaicin affects intracellular signals independently of TRPV1 cannot be excluded (26). TRPV1 expression was reportedly reduced in aged mice, an effect that corresponded with loss of noxious sensitivity in aging animals (27). Thus, to determine the effects and target proteins of capsaicin when TRPV1 is physiologically absent is important.

TRPV1 is distributed widely in many cell types other than sensory neurons (11). In skin, TRPV1 is also expressed in keratinocytes and fibroblasts (28). TRPV1 activation is involved in inflammatory responses in human keratinocytes through the release of inflammatory mediators, such as prostaglandin E_2 and interleukin-8 (28), and UV irradiation-increased MMP-1 is mediated by TRPV1 in keratinocytes (29). In contrast, keratinocytes were reportedly resistant to vanilloid-induced TRPV1 activation and subsequent calcium influx (30). In our previous studies, we confirmed that TRPV1 expression was depleted in skin cancer tissues, and lack of TRPV1 corresponded with increased incidence of mouse skin cancer development mediated by high levels of EGFR (15). These results indicated that agents activating EGFR signaling could lead to the induction of skin tumors in TRPV1/KO mice. In the present study, we found that capsaicin induced DMBA-initiated and TPA-promoted skin carcinogenesis more readily in TRPV1/KO mice than in WT mice, through the upregulation of COX-2 expression, and EGFR signaling was involved in capsaicin-induced COX-2 expression in the absence of TRPV1. TRPV1 has been considered as an inflammatory receptor based on the findings that nociception and proinflammatory mediators are evoked through the activation of TRPV1 (24). In addition, TRPV1 has been reported to be elevated in inflammatory diseases of specific tissues including colon (31), and several cancers such as prostate and pancreatic cancers (12). Recently, however, a paradoxical role of TRPV1 as both proinflammatory and protective has emerged (32). For example, TRPV1 reportedly mediates protection against chemically induced colitis (33).

EGFR is frequently overexpressed and abnormally activated in many types of cancers. EGFR has an extracellular ligand-binding domain, a single membrane-spanning domain, and a cytoplasmic catalytic domain. After ligands bind with the ectodomain of EGFR, these receptors form homodimers or heterodimers, leading to the autophosphorylation of tyrosine residues in the cytoplasmic domain and activation of the receptors' intrinsic kinase activity (34). Phosphotyrosine residues in the EGFR kinase domain recruit downstream effectors that contain Src homology-2 or phosphotyrosine-binding domains, resulting in the activation of signaling pathways including MEK/ERK, Akt, and Src. We found that capsaicin potentiated TPA-mediated ERKs and Akt phosphorylation only in EGFR-expressing cells through induction of EGFR phosphorylation. The activation of EGFR downstream signaling by TPA and capsaicin was dependent on EGFR and Src activation. Additionally, ectodomain shedding of HB-EGF by MMP was also involved in the TPA/capsaicin-mediated EGFR signaling cascades.

Evidence indicates that EGFR can be activated in the absence of its direct ligands. Cholesterol depletion by methyl-β-cyclodextrin was reported to induce EGFR phosphorylation without release of membrane-bound EGFR ligands, suggesting that ligand-independent EGFR activation can take place (35). One possible mechanism of ligand-independent EGFR activation is the direct phosphorylation of the EGFR kinase domain by intracellular mediators. Previously, the induction of c-Src was reported as a prerequisite of arsenic-induced EGFR and downstream ERK activation (36). We found that although an EGFR inhibitor blocked TPA/capsaicin-induced EGFR downstream signals, none of the inhibitors of MMP, Src, or HB-EGF completely interfered with the TPA/capsaicin-mediated EGFR downstream signals. These results suggest that other mechanisms, such as ligand-independent EGFR activation, might be involved.

Even though epidemiologic studies suggested a positive association between the consumption of capsaicin or chili pepper–rich diets and the incidence of cancers in humans, whether the EGFR activation caused by capsaicin is involved is not clear. Among many cancers, accumulating evidence focused on the causative effects of capsaicin-containing diets on stomach or gallbladder cancers (7, 37). Stomach cancer is a major cancer expressing high levels of EGFR. According to a recent human study in Chinese patients with gastric carcinoma, ∼42% of cases showed elevated expression levels of EGFR (38). Another study also revealed that EGFR overexpression was detected in patients with gastric carcinoma, which was associated with an unfavorable prognosis (39). Similarly, EGFR overexpression is frequently reported in patients with gallbladder cancer (40). Our present findings that EGFR activation induced by capsaicin in skin cancer leads to a cancer-promoting effect underlies the relevance of epidemiologic reports of capsaicin to the incidence of EGFR-overexpressed human cancers.

Spice-derived phytochemicals including capsaicin have suggested potential chemopreventive effects (41). The anticarcinogenic effects of capsaicin are achieved through various mechanisms, including apoptosis (42). A major mechanism by which capsaicin exhibits its anticarcinogenic effects might be the inhibition of NF-κB and signal transducers and activators of transcription 3 activation. Capsaicin was reported to block NF-κB activation induced by diverse agents including phorbol esters in leukemia cells (43) and in the two-stage skin carcinogenesis model (44, 45). Capsaicin was found to block signal transducers and activators of transcription 3 activation as well as to downregulate its dependent gene products including cyclin D1, Bcl-2, and survivin (46). Although our study indicates that capsaicin might act as a cocarcinogen in the DMBA/TPA-promoted skin cancer model, the anticarcinogenic potential of capsaicin cannot be excluded.

A recent study assessing the oncogenic potential of capsaicin revealed that dermal application of capsaicin did not lead to the induction of preneoplastic or neoplastic skin lesions in the Tg.Ac mouse model (47). Consistent with this result, our study showed that capsaicin alone did not cause skin carcinogenesis. Capsaicin was reported to cause a

burning sensation and skin irritation when applied topically and to induce the erosion of gastric mucosa and hepatic necrosis when administered in large amounts (48). Although capsaicin could induce cancer cell death, it can also kill normal neuronal cells (49). Although whether capsaicin has genotoxic and carcinogenic effects remains controversial, evidence supports these suspected adverse effects of capsaicin. We found that capsaicin might act as a cocarcinogen by inducing the sustained activation of EGFR downstream signals through activation of EGFR. Capsaicin might promote cancer cell survival in the absence of TRPV1 (14). Similar to capsaicin, tumor necrosis factor is suspected to both induce and reduce cancer cell growth, and was shown to commit cells to survival when stimulating EGFR transactivation mechanisms, indicating that EGFR could act as a molecular switch determining the antiapoptotic effect of tumor necrosis factor (50). Thus, capsaicin might commit tumors to survival and evade apoptosis in the absence of TRPV1 through the activation of EGFR. However, because capsaicin is also reported to induce proinflammatory signals through the activation of TRPV1 (28), the possibility that capsaicin induces inflammation and affects cancer development in a TRPV1-dependent manner cannot be excluded.

In summary, our data suggest that capsaicin has a cocarcinogenic effect in TPA-promoted skin carcinogenesis, and this effect was more pronounced in TRPV1/KO mice than in WT mice. Capsaicin induces the sustained activation of EGFR downstream signals through the induction of EGFR transactivation mediated through Src and MMP-dependent mechanisms. This observation has implications for understanding the role of EGFR in capsaicin-mediated survival of tumors.

Disclosure of Potential Conflicts of Interest

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Cocarcinogenic Effect of Capsaicin Involves Activation of EGFR Signaling but Not TRPV1

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