Research Article

Capsaicin, a Component of Red Peppers, Inhibits the Growth of Androgen-Independent, p53 Mutant Prostate Cancer Cells

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Abstract

Capsaicin is the major pungent ingredient in red peppers. Here, we report that it has a profound antiproliferative effect on prostate cancer cells, inducing the apoptosis of both androgen receptor (AR)-positive (LNCaP) and -negative (PC-3, DU-145) prostate cancer cell lines associated with an increase of p53, p21, and Bax. Capsaicin down-regulated the expression of not only prostate-specific antigen (PSA) but also AR. Promoter assays showed that capsaicin inhibited the ability of dihydrotestosterone to activate the PSA promoter/enhancer even in the presence of exogenous AR in LNCaP cells, suggesting that capsaicin inhibited the transcription of PSA not only via down-regulation of expression of AR, but also by a direct inhibitory effect on PSA transcription. Capsaicin inhibited NF-κ activation by preventing its nuclear migration. In further studies, capsaicin inhibited tumor necrosis factor-α–stimulated degradation of IκBα in PC-3 cells, which was associated with the inhibition of proteasome activity. Taken together, capsaicin inhibits proteasome activity which suppressed the degradation of IκBα, preventing the activation of NF-κB. Capsaicin, when given orally, significantly slowed the growth of PC-3 prostate cancer xenografts as measured by size [75 ± 35 versus $336 \pm 123 \text{ mm}^3$ ($\pm$SD); $P = 0.017$] and weight [203 ± 41 versus 373 ± 52 mg ($\pm$SD); $P = 0.0006$; capsaicin-treated versus vehicle-treated mice, respectively]. In summary, our data suggests that capsaicin, or a related analogue, may have a role in the management of prostate cancer. (Cancer Res 2006; 66(6): 3222-9)

Introduction

Prostate cancer is an increasingly prevalent health problem in the U.S., with >230,000 new cases and 29,000 deaths occurring as a result of the disease every year, thereby, constituting the most common male malignancy. Despite the prevalence of the disease, no successful, long-term therapies exist once the cancer has relapsed. The limited therapeutic options provide a strong stimulus for developing new therapeutic approaches.

Capsaicin, a homovanillic acid derivative (8-methyl-N-vanillyl-6-nonenamide), is an active component of the red pepper, genus *Capsicum*, used for many years in food additives and drugs (1). Topically, capsaicin has therapeutic value in a variety of neuropathic pain conditions including rheumatoid arthritis, cluster headaches, herpes zoster, and vasomotor rhinitis (2–5). Furthermore, capsaicin decreases the growth of human leukemic cells (6–8), gastric (9), and hepatic carcinoma cells *in vitro* (10). However, the mechanism of capsaicin-induced growth inhibition remains unclear, and the effect of capsaicin on the growth of human prostate cancer cells has not been studied.

Analysis of androgen-responsiveness of prostate cancer cells often uses prostate-specific antigen (PSA) as a surrogate marker. The 5′ upstream promoter and enhancer region of the PSA gene has several androgen response (AR) elements (ARE), and the expression of PSA is regulated by binding of the liganded AR to these AREs (11–13). A recent study revealed that the nuclear transcription factor κB (NF-κB) is constitutively activated in the androgen-independent prostate cancer cell lines PC-3 and DU-145, but not in the androgen-responsive LNCaP human prostate cancer cells (13). NF-κB is a generic term for a dimeric transcription factor formed by heterodimerization or homodimerization of a number of Rel family members (14). These transcription factors are activated by a wide range of stimuli, including DNA damage, free radicals, and cytokines, such as tumor necrosis factor-α (TNF-α; ref. 15). In unstimulated cells, NF-κB is maintained in an inactive state in the cytoplasm by complexing with members of the IκB inhibitory protein family, including IκBα and IκBβ (16). The interaction between NF-κB and IκBα prevents NF-κB's nuclear localization and DNA binding activity (15, 16). With activation, IκBα kinase (IKK) stimulates the ubiquitination of IκBα, resulting in its degradation by the 26S proteasome complex (17). NF-κB is then free to translocate to the nucleus and act as a transcription factor for a variety of genes, including cytokines, cell cycle regulatory proteins as well as antiapoptotic proteins (15, 16, 18). An earlier study noted that capsaicin may exert its anti-growth effects in human myeloid cells through inhibition of the activity of the transcription factor NF-κB by blocking the degradation of IκBα, thereby inhibiting the translocation of the p65 subunit of NF-κB to the nucleus (19).

In the present studies, we show that capsaicin has a profound inhibiting effect on the growth of prostate cancer cells *in vitro* and *in vivo*, inducing the apoptosis of both AR-positive and AR-negative prostate cancer cell lines. We also showed that capsaicin profoundly decreased the transcription of AR target genes.

Materials and Methods

Cells and compounds. LNCaP, PC-3, and DU-145 cells (American Type Culture Collection, Manassas, VA) were maintained at 37°C in 5% CO$_2$ in RPMI 1640 (Life Technologies, Rockville, MD) with 10% fetal bovine serum (FBS; Omega, Tarzana, CA). Capsaicin and capsazepine (Sigma Chemical Co., St. Louis, MO) were dissolved in 100% ethanol or 100% methanol, respectively. Ruthenium red (Calbiochem, La Jolla, CA), SB366791, resiniferatoxin (Alexis Biochemicals, San Diego, CA), and dihydrotestosterone (Sigma Chemical) were dissolved in 100% ethanol. TNF-α (Sigma Chemical)

was dissolved in PBS. For all compounds, the diluent was never present at >0.5% in the experiments; and control dishes having the same concentration of diluent had no detectable effect.

MTT and clonogenic assay in soft agar. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma Chemical) and clonogenic assays in soft agar were done as previously described (20, 21).

Measurement of apoptosis. Terminal deoxynucleotidyl transferase–mediated nick end labeling assay (TUNEL) was done for immunohisto-chemical detection and for the quantification of programmed cell death at the single cell level, based on labeling of DNA strand breaks, the *In situ* Cell Death Detection kit, POD (Roche, Indianapolis, IN) was used.

Cell cycle analysis. PC-3 cells were exposed to either increasing doses of capsaicin, or vehicle control (10% FCS in RPMI 1640 containing 0.1% or 0.2% ethanol) for 24 hours. Cells were fixed in 75% chilled methanol and stained with propidium iodine. Cell cycle status was analyzed on a Becton Dickinson Flow Cytometer (BD Biosciences, Franklin Lakes, NJ).

Western blotting. Cytoplasmic and nuclear components of the cells were extracted using CelLytic NuCLEAR Extraction kit (Sigma Chemical). Protein concentrations were determined using the Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's recommendation. Western blotting was done as previously described (20). The following primary antibodies were used: anti-AR (sc-7305), anti-Bax (sc-493), anti-hnRNP A1 (sc10030), anti-IκBα (sc847), anti-NF-κB p65 (sc-372-G), anti-p21 (sc-397), anti-p53 (sc126), and anti-PSA (sc-7638) from Santa Cruz Biotechnology (Santa Cruz, CA), and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Research Diagnostics (Flanders, NJ).

Real-time reverse transcription-PCR. Total cellular RNAs were isolated from cells using RNeasy mini kit (Qiagen, Valencia, CA) and cDNAs were synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's recommendation. Expression levels were determined using HotMaster Taq DNA Polymerase (Eppendorf, Westbury, NY) and SYBERGreen I (Molecular Probes, Eugene, OR) with the following primers: AR forward 5′-AGGATGCTCTACTT-CGCCCC-3′, AR reverse 5′-ACTGGCTGTACATCCGGGAC-3′, transient receptor potential vanilloid type 1 (TRPV1) forward 5′-AACTGGACC-ACCTGGAACAC-3′, TRPV1 reverse 5′-GCCTGAAACTCTGCTTGACC-3′. Reactions were done in triplicate using a iCycler iQ system (Bio-Rad Laboratories). Reaction products were visualized on ethidium bromide–stained agarose gels. For each sample, the amount of the target gene and reference gene (18S) was determined from standard curves.

Transfections and luciferase assays. The following promoter reporter constructs were used: PSA P/E-Luc, PSA enhancer E4-LUC, PSA enhancer E4 S-ALL-LUC, and ARE4-E4Lux (21). The NF-κB reporter construct (pGL3-NF-κB) containing four copies of NF-κB binding sequences were cloned into pGL3-basic plasmid (Promega, Madison, WI), a generous gift from Dr. Moshe Arditi (Cedars-Sinai Medical Center, Los Angeles, CA). Cells were transfected with the indicated plasmids using GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA) under serum-free conditions. A PhRLTK vector was included as an internal control for transfection efficacy. Following transfections, cells were incubated in RPMI 1640 with 10% charcoal-stripped FBS either with or without dihydrotes-tosterone and either with or without capsaicin for 24 hours, and were then collected with tissue lysis buffer (Promega). Luciferase activity of the cell lysates was measured by luminometry, and activities were normalized by β-galactosidase activities. Proliferation assays with IKKβ- and AR vector–transfected cells were done in either 6- or 12-well plates. Cells were plated on day 0 and grown in RPMI 1640 with 10% FBS at 37°C. Cells were cotransfected on day 1 with an IKKβ-expressing vector (kind gift from Prof. R. Gaynor, Eli Lilly, Indianapolis, IN) or an AR vector and pcDNA 3.1 neomycin-resistant vector (also used as control) with LipofectAMINE (Invitrogen) according the manufacturer's instruction. On day 2, G418 (Invitrogen) was added, and on day 3, capsaicin was added. Between days 10 and 12, colonies were stained with crystal violet (Sigma Chemical) and the growth-inhibitory effect was evaluated by counting the number of colonies.

Immunofluorescence. PC3 cells (p65 staining) and LNCaP cells (AR staining) were grown overnight on coverslips in six-well plates and then

incubated in 10^{-8} mol/L dihydrotestosterone (only LNCaP cells) $\pm$ 2×10^{-4} mol/L capsaicin for 4 and 12 hours. Cells were fixed in 100% methanol and incubated with the primary antibody against AR and p65 (Santa Cruz Biotechnology) at 1 μg/mL (in PBS with 5% bovine serum albumin and 0.6% Tween 20), at 4°C overnight. A secondary biotinylated antibody and RPE-conjugated streptavidin (DAKO A/S, Denmark) were used for visualization.

Proteasome function assay. PC3 cells were treated with capsaicin (2×10^{-4}, 24 or 48 hours), harvested, and a proteasome function assay was done as previously described (22). The chymotrypsin-like, trypsin-like, and PGPH activities of the proteasome were determined by the ability to degrade the appropriate fluorogenic substrates: Suc-LLVY-AMC (Sigma Chemical), Z-ARR-AMC (Calbiochem, Darmstadt, Germany), and Z-LLE-AMC (Sigma Chemical).

***In vivo* animal treatment protocol.** Three-week-old BNX *nu/nu* male mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were maintained in pathogen-free conditions and fed irradiated chow. PC-3 cells (5×10^6) in 0.1 mL of Matrigel (Collaborative Biological Products, Bedford, MA) were injected s.c. into the bilateral flanks of each mouse and treatment (5 mg/kg/d in 100 μL of PBS containing 0.3% ethanol per day) was started the next day and continued for 4 weeks. Five control mice received vehicle (100 μL PBS containing 0.3% ethanol per day). Both groups received the agent by gavage 3 days per week (Monday, Wednesday, and Friday). Tumor sizes were measured every week using the formula $A \times B \times C \times 0.5236$ (A, length; B, width; C, height; all measured in millimeters). The mice were sacrificed by carbon dioxide asphyxiation after 4 weeks and tumor weights were measured. Differences of tumor sizes and weights between mice in control and experimental groups at the end of the study were analyzed by Student's *t* test (statistical tests were two-sided).

Results

Effect of capsaicin on clonal proliferation and cell cycle of LNCaP, PC-3, and DU-145. We tested the effect of increasing doses of capsaicin on the clonogenic growth in soft agar of the prostate cancer cell lines LNCaP, PC-3, and DU-145. Capsaicin inhibited the clonal proliferation of each in a dose-dependent manner (Fig. 1*A*). At 5×10^{-4} mol/L, the growth of each cell type was completely inhibited. For cell cycle analysis, PC-3 cells were treated with either increasing doses of capsaicin or vehicle for 24 hours and examined by flow cytometry. The population of cells in the G_0/G_1 phase increased and those in S phase decreased in a capsaicin dose-dependent manner (Fig. 1*B*).

Capsaicin induces apoptosis in prostate cancer cells. Cells were exposed to increasing concentrations of capsaicin for 24 hours and apoptosis was measured by TUNEL assay. Capsaicin induced apoptosis in a dose-dependent manner, with the percentage of apoptotic cells ranging from 3% at 1×10^{-4} mol/L capsaicin to 75% at 5×10^{-4} mol/L capsaicin for PC-3 cells, and from 9% at 1×10^{-4} mol/L capsaicin to 93% at 5×10^{-4} mol/L in LNCaP cells (Fig. 3*C*).

Capsaicin modulates the levels of proteins associated with apoptosis and cell cycle in a time-dependent manner. To characterize the molecular mechanism of capsaicin-induced cell cycle arrest followed by apoptosis, we examined the levels of several apoptotic- and cell cycle–related proteins in capsaicin-treated (2×10^{-4} mol/L) LNCaP cells (Fig. 1*D*). Expression of p21 Waf1 protein increased after 6 hours, and levels of Bax and p53 increased after 3 and 12 hours exposure to capsaicin, respectively.

Effect of capsaicin on the transcriptional activity of androgen-responsive reporter constructs. PSA is an androgen-responsive gene produced by prostate epithelial cells. To investigate the effect of capsaicin on PSA transcription, androgen-sensitive LNCaP cells were transfected with a PSA promoter/enhancer-luciferase reporter vector (PSA P/E-Luc). These cells were incubated

in 10% charcoal-striped FBS RPMI 1640 either with or without dihydrotestosterone (1×10^{-8} mol/L) and either with capsaicin (2×10^{-4} mol/L) or diluent control for 24 hours. Luciferase activity was measured and the fold induction calculated relative to that measured in control cells (Fig. 2). Dihydrotestosterone increased reporter activity >60-fold compared with control LNCaP cells. Capsaicin completely inhibited this effect. These results indicate that capsaicin inhibited the ability of androgen to transactivate the PSA promoter/enhancer. Additional experiments used the luciferase-reporter construct containing either a 496-bp fragment of the PSA enhancer, containing six AREs or ARE4-E4Lux (multimerized four consensus AREs from the PSA promoter; ref. 23); luciferase activity was stimulated by dihydrotestosterone (1×10^{-8} mol/L), and capsaicin again completely inhibited this stimulation for each construct (data not shown).

Capsaicin reduces androgen-induced PSA and AR expression. Protein levels of PSA were examined in the androgen-sensitive prostate cancer cell line, LNCaP. Cells were incubated with 10^{-9} mol/L dihydrotestosterone either with or without capsaicin (0.5, 1, or 2×10^{-4} mol/L, 24 hours). As expected, PSA

expression was induced by dihydrotestosterone, which was blocked by capsaicin (10^{-4} mol/L; Fig. 3A). The expression of AR was also induced by dihydrotestosterone, and capsaicin (10^{-4} mol/L) inhibited this effect with levels of AR protein becoming undetectable in the presence of 2×10^{-4} mol/L capsaicin.

To determine whether capsaicin affected the transcription of AR, RNA expression levels of AR were measured by real-time reverse transcription-PCR. LNCaP cells were incubated with 10^{-9} mol/L dihydrotestosterone either with or without capsaicin (2×10^{-4} mol/L) for either 12 or 24 hours. Capsaicin inhibited dihydrotestosterone-induced RNA expression of AR within 12 hours (Fig. 3B).

To determine whether the inhibitory effect of capsaicin on PSA transcriptional activity in LNCaP cells was solely a result of down-regulation of AR protein levels, AR cDNA expression vector (pCMV-AR) was placed in LNCaP cells (Fig. 3C). These cells were also transfected with PSA P/E-Luc and cultured either with or without dihydrotestosterone (10^{-8} mol/L) and either with or without capsaicin (10^{-4} mol/L) for 24 hours. Cells were harvested and Western blot analysis and Luciferase assay were done.
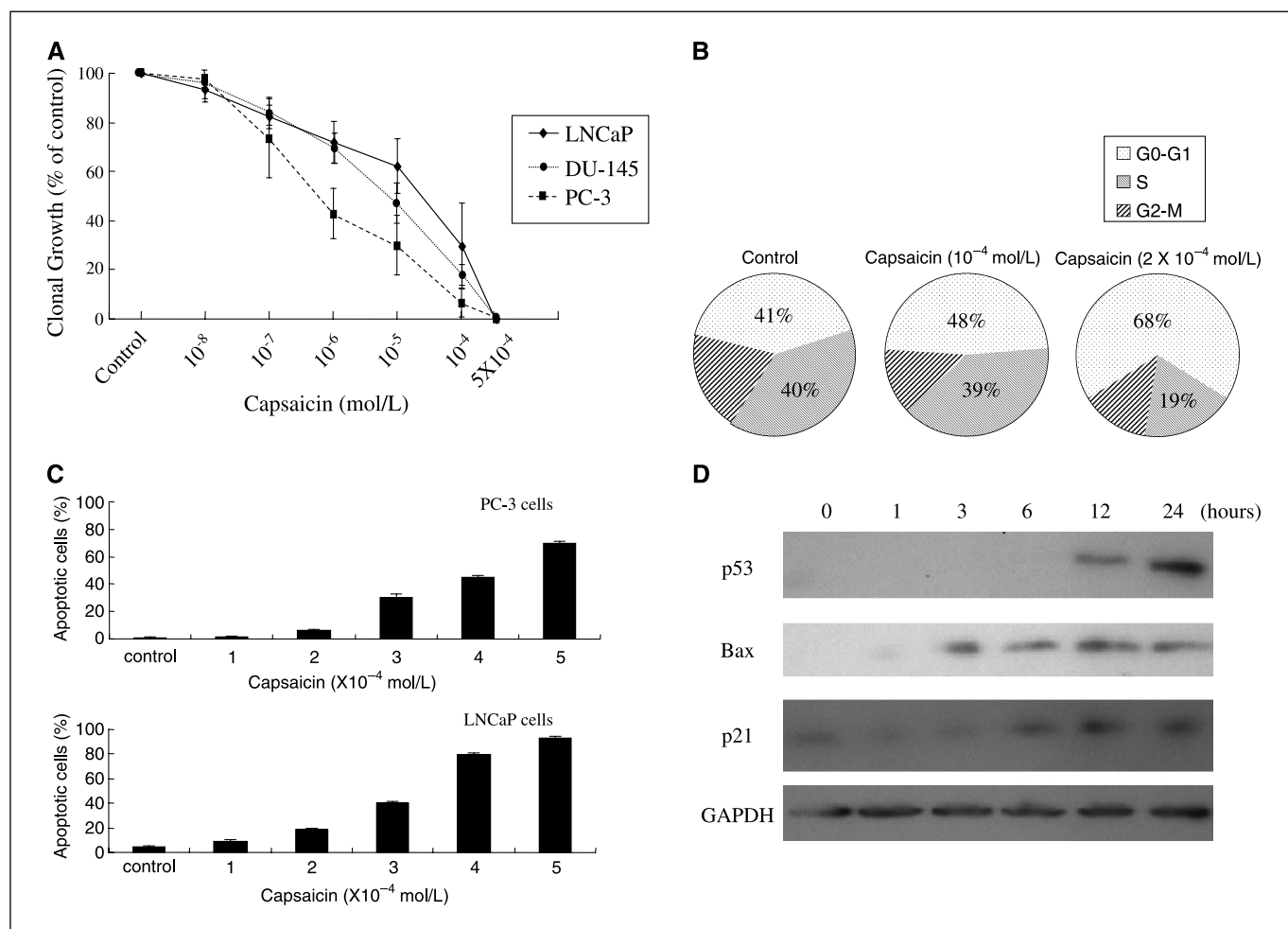
Figure 1. LNCaP and PC-3 prostate cancer cells: effect of capsaicin on their clonal proliferation, cell cycle, apoptosis, and related proteins. *A,* cells were cultured in soft agar with increasing amounts of capsaicin, colonies were counted (day 14) and expressed as a percentage of colonies relative to untreated control plates. *Points,* means of three independent experiments done in triplicate plates; *bars,* ±SD. *B,* PC-3 cells were exposed to increasing amounts of capsaicin for 24 hours, and cell cycle analysis was done. *C,* cells were cultured for 24 hours with increasing amounts of capsaicin. Apoptosis was measured by TUNEL assay and expressed as a percentage of apoptotic cells relative to untreated control PC-3 and LNCaP cells. *Columns,* means of three independent experiments; *bars,* ±SD. *D,* LNCaP cells were incubated with capsaicin (2×10^{-4} mol/L) for indicated times. Lysates were analyzed by Western blot for levels of p53, Bax, p21 Waf1, and GAPDH.
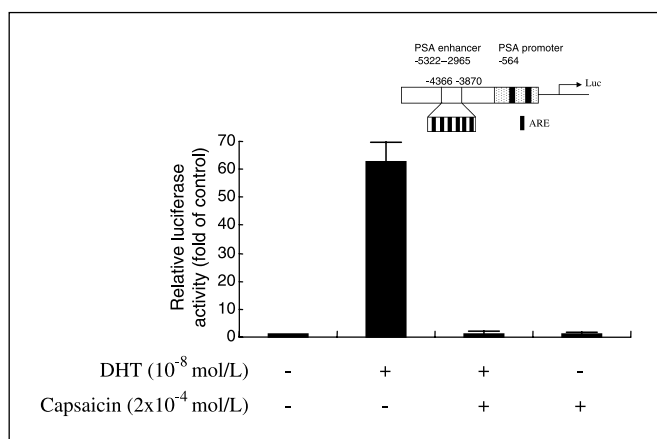
Figure 2. Effect of capsaicin on the transcriptional activity of the PSA promoter/enhancer in LNCaP cells. LNCaP cells were transfected with PSA enhancer-Luciferase (Luc) reporter [ARE sites, darkened rectangles (*top right*)] and cultured for 24 hours with dihydrotestosterone (10^{-8} mol/L) either with or without 2×10^{-4} mol/L of capsaicin. PhRLTK vector was cotransfected for normalization. *Columns,* means of three or more experiments; *bars,* ±SD.

Western blot analysis confirmed that up-regulation of AR occurred in pCMV-AR–transfected cells (data not shown). PSA P/E-Luc reporter activity increased ∼55-fold in the presence of exogenous AR and dihydrotestosterone. Capsaicin strongly inhibited this activation, suggesting that the ability of capsaicin to inhibit PSA transcriptional activity was not only via down-regulation of AR expression, but also by directly inhibiting the ability of ligand-activated AR to transcriptionally activate the PSA gene. Furthermore, the proliferation of these cells was assessed by performing colony-forming assays in the presence of capsaicin (10^{-5} mol/L, 2×10^{-4} mol/L). Capsaicin had the same potency to inhibit the growth of LNCaP cells when overexpressing AR (data not shown), suggesting that the growth-inhibitory effect of capsaicin is not mediated via down-regulation of AR expression.

In order to exert its transcriptional effects, AR accumulates in the nucleus. To investigate whether capsaicin affected dihydrotestosterone-induced nuclear accumulation of AR, the subcellular localization of AR was studied by immunofluorescence. Figure 3*D* shows the localization of AR in LNCaP cells after 12 hours of incubation either without dihydrotestosterone (left), with dihydrotestosterone (10^{-8} mol/L; center) or with both dihydrotestosterone (10^{-8} mol/L) and capsaicin (2×10^{-4} mol/L; right). In unexposed LNCaP cells, AR is already localized mainly to the nucleus (Fig. 3*D, left*). However, when cultured with dihydrotestosterone, nuclear localization becomes more prominent (Fig. 3*D, center*), an effect that could not be reversed by capsaicin (Fig. 3*D, right*), suggesting that the effect of capsaicin on dihydrotestosterone-induced ARE transcription is not mediated through inhibition of nuclear localization.

Effect of capsaicin on NF-κB promoter activity in PC-3 cells. Previous studies in murine myeloid leukemia cells suggested that capsaicin inhibits the activity of the transcription factor NF-κB by blocking the degradation of IκBα (19). NF-κB is constitutively activated in the androgen-independent prostate cancer cell lines PC-3, but not in the androgen-responsive LNCaP cells (13). In order to investigate whether the anti-growth effect of capsaicin on prostate cancer cells is mediated at least in part by NF-κB, PC-3 cells were transfected with NF-κB-Luc, which contains four copies

of NF-κB binding sites attached to the pGL3 luciferase reporter plasmid. Following transfections, cells were incubated with either capsaicin (2×10^{-4} mol/L) or diluent control for 24 or 48 hours (Fig. 4*A*). Luciferase activities were 61% and 81% lower in capsaicin-treated cells compared with control cells after 24 and 48 hours of culture, respectively.

Activation of NF-κB involves two important steps: (*a*) degradation of IκBα mediated by IKK, resulting in the release of NF-κB, and (*b*) nuclear translocation of activated NF-κB (15, 16). Initially, therefore, NF-κB status in the PC-3 nucleus was examined. Cells were incubated with capsaicin (2×10^{-4} mol/L) for either 2 or 4 hours. After 4 hours of culture with capsaicin, NF-κB levels in the nuclear extracts decreased 84%, whereas NF-κB levels in the whole cell extract decreased 28% of control, suggesting that nuclear translocation of NF-κB was inhibited (Fig. 4*B*). Subcellular localization of p65, either in the absence or presence of capsaicin (Fig. 4*C*, left and right panels, respectively), was also studied in these cells by immunofluorescence. Nuclear staining of p65 decreased when PC-3 cells were exposed to capsaicin, confirming that nuclear translocation of NF-κB was inhibited by the compound. Further studies sought to determine whether the inhibitory effect of capsaicin was due to the prevention of IκBα degradation. PC-3 cells, either untreated or pretreated with capsaicin (2×10^{-4} mol/L, 3 hours, 37°C), and then exposed to TNF (20 ng/mL), were assayed for the amount of cytoplasmatic IκBα by Western blot analysis (Fig. 4*D*). TNF treatment caused IκBα to disappear completely by 15 minutes. Pretreatment of these cells with capsaicin, however, prevented the degradation of IκBα. Also, PC-3 cells incubated with only with capsaicin (1 or 2×10^{-4} mol/L, 2 hours) had an increase in cytoplasmatic levels of IκBα (Fig. 4*E*). Taken together, these results suggest that capsaicin inhibits IκBα degradation in the cytoplasm.

Degradation of IκBα results from the phosphorylation of IκBα by IκB kinases, followed by its ubiquitination and degradation by the multicatalytic 26S proteasome (15). To determine whether capsaicin affected the proteasome activity of PC-3 cells, these cells were treated with capsaicin (2×10^{-4} mol/L, 24 or 48 hours), followed by proteasome functional assay. Chymotrypsin-like, trypsin-like, and PGPH proteasome activities were down-regulated by capsaicin (Fig. 5). Finally, to study if the anti-proliferative effect of capsaicin could be affected by the overexpression of IKKβ, PC-3 cells were transfected with an IKKβ expression vector. These cells exhibited significantly increased nuclear localization of p65 but did not affect their sensitivity to capsaicin compared with cells transfected with the control vector (data not shown). These findings suggest that the inhibiting effect of capsaicin on proliferation is not mediated through down-regulation of IKKβ and that the effect of capsaicin on IκBα degradation is still dominant over IKKβ expression.

The antiproliferative effect of capsaicin is not related to TRPV1. Capsaicin exerts its physiologic function in sensory neurons by intracellular binding to TRPV1 (24). Therefore, we studied whether the antiproliferative effect of capsaicin is related to TRPV1. Initially, TRPV1 was confirmed to be expressed in both PC-3 and LNCaP cells, using real-time reverse transcription-PCR (data not shown). To determine the involvement of TRPV1 in the antiproliferative action of capsaicin, LNCaP and PC-3 cells were cultured with the TRPV1 antagonists capsazepine (10^{-5} and 10^{-4} mol/L), ruthenium red (10^{-5} mol/L), or SB366791 (10^{-5} mol/L), either with or without capsaicin (10^{-7} to 2×10^{-4} mol/L) for 96 hours, and measuring cell viability by MTT

assay. These antagonists did not reverse the antiproliferative effect of capsaicin (data not shown), suggesting that the antiproliferative activity of capsaicin is distinct from its binding to TRPV1. We also studied the effect of the more potent vanilloid receptor agonist resiniferatoxin on LNCaP cells. Resiniferatoxin was ~10 times more potent in inhibiting proliferation compared with capsaicin. However, as was the case with capsaicin, the TRPV1 inhibitors ruthenium red and SB366791 were unable to affect the antiproliferative activity of capsaicin (data not shown).

Capsaicin inhibited human prostatic cancer xenografts. The effect of capsaicin on growth of human prostate cancer cells was studied in nude mice. PC-3 cells were injected into the flanks, and from the next day, capsaicin was given by gavage 3 days per week. Tumor volumes were measured weekly and all mice were killed at the end of the fourth week when tumors were dissected and weighted. Tumors in capsaicin-treated mice compared with those in vehicle-treated mice were statistically significantly smaller [75 ± 35 versus 336 ± 123 mm^3 (±SD), respectively; $P = 0.017$]. Prostate tumors weighed significantly less in experimental versus diluent control mice [203 ± 41 versus 373 ± 52 mg, respectively

($P = 0.0006$); Fig. 6A and B]. Mean body weights and hair coat as well as overall activity were similar in both groups at the completion of the experiment, suggesting that capsaicin had no major side effects on these mice (data not shown).

Discussion

Recently, capsaicin has been receiving attention as an anticancer agent because of its pharmacologic and toxicologic properties (6–10). This study shows that capsaicin inhibits the growth of prostate cancer cells including androgen-independent PC-3 tumors growing in mice without causing gross toxicity of the animals. These results suggest that capsaicin may have a role for the management of prostate cancer patients, even for those who are refractory to hormonal therapy.

We explored the molecular mechanisms of capsaicin-induced growth inhibition and apoptosis in prostate cancer cells. A recent study suggested that expression of wild-type p53 is necessary for capsaicin-induced cellular growth inhibition and apoptosis of myeloid leukemia cells (NB4) via apoptosis (8). In our study, however, capsaicin inhibited growth and induced apoptosis not
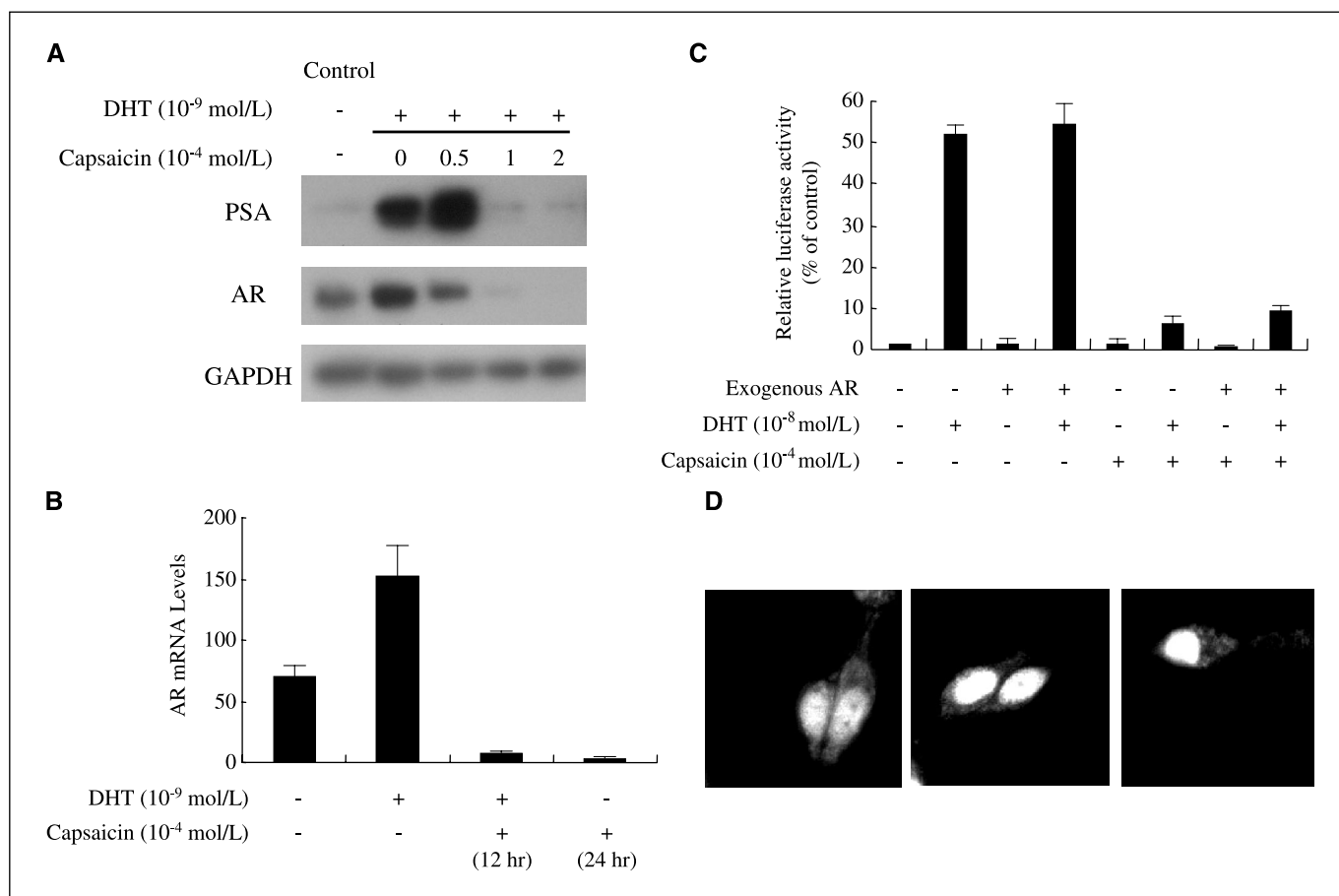
Figure 3. Effects of capsaicin on dihydrotestosterone-induced PSA and AR levels and on transcriptional activity of the PSA promoter/enhancer in AR-overexpressing LNCaP cells. *A,* LNCaP cells were cultured (10^{-9} mol/L dihydrotestosterone, 24 hours) either with or without capsaicin (0.5, 1, or 2 × 10^{-4} mol/L). Western blot was sequentially probed for levels of PSA, AR, and GAPDH. *B,* LNCaP cells were cultured in the presence of dihydrotestosterone (10^{-9} mol/L, 24 or 48 hours) either with or without capsaicin (2 × 10^{-4} mol/L). AR mRNA levels were determined by real-time reverse transcription-PCR. *Columns,* ratio of AR transcripts/18S transcripts and means of three experiments; *bars,* ±SD. *C,* LNCaP cells were transfected with PSA P/E-Luc and pCMV-AR, and cultured with dihydrotestosterone (10^{-8} mol/L) either with or without capsaicin (10^{-4} mol/L, 24 hours) and luciferase activity was determined. *Columns,* means of three or more experiments; *bars,* ±SD. *D,* subcellular localization of AR analyzed by immunofluorescence. LNCaP cells were cultured for 12 hours in either dihydrotestosterone (1 × 10^{-7} mol/L; *center*), dihydrotestosterone and capsaicin (2 × 10^{-4} mol/L; *right*), or without the addition of drug (*left*).
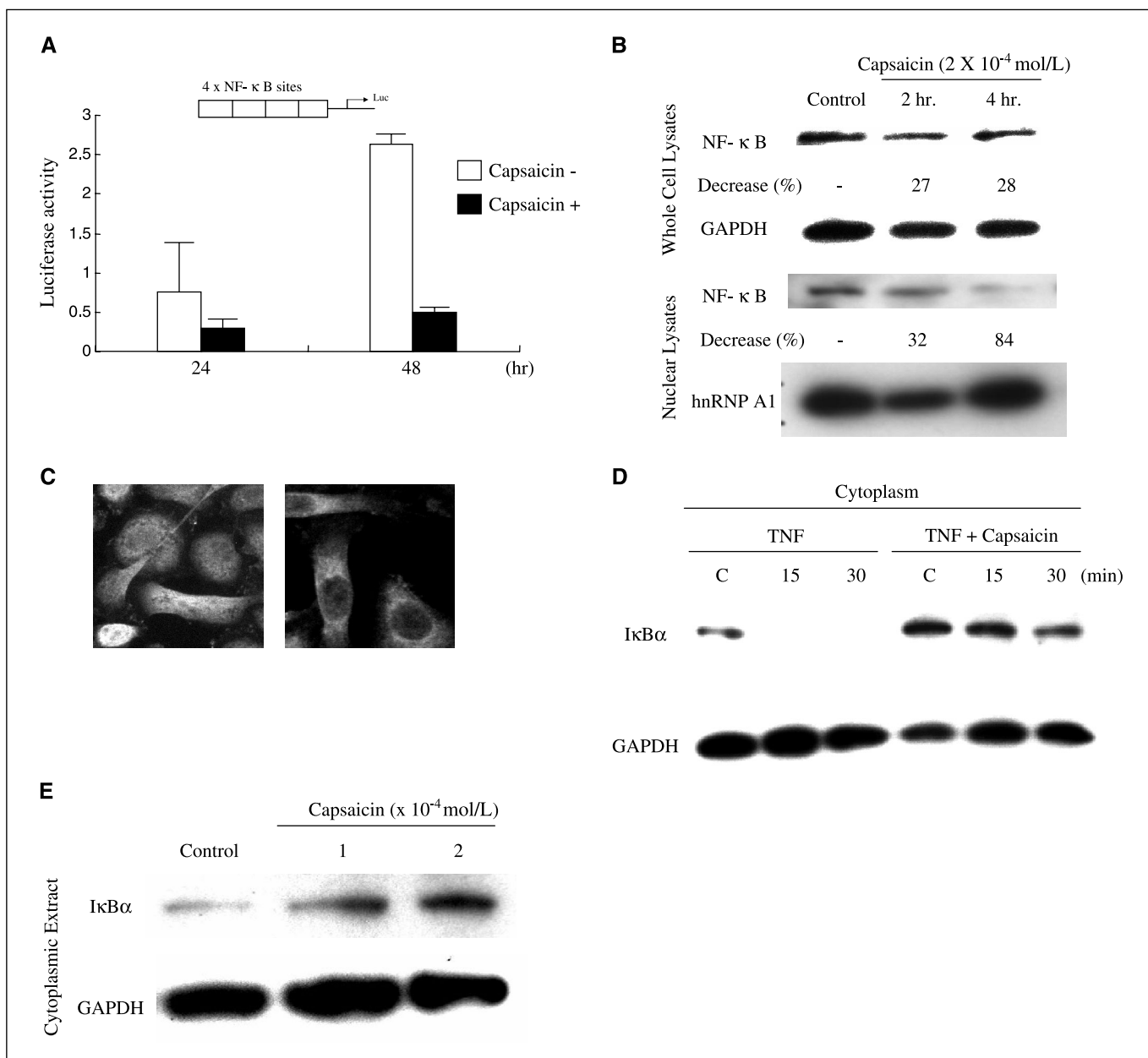
Figure 4. Capsaicin inhibits NF-κB promoter activity of PC-3 cells. *A,* PC-3 cells were transfected with NF-κB-Luc reporter gene. Following transfections, cells were incubated either with or without capsaicin (2 × 10^{-4} mol/L) for either 24 or 48 hours. *Columns,* means of three or more experiments; *bars,* ±SD. *B,* Western blot analysis of NF-κB expression in PC-3 cells. Cells were incubated with capsaicin (2 × 10^{-4} mol/L) for either 2 or 4 hours. Whole cell extract or nuclear proteins were assayed for expression of the p65 subunit of NF-κB and either GAPDH or hnRNP A1 (nuclear protein–specific control), respectively. *C,* PC-3 cells were examined for their subcellular localization of p65 after incubation either with (*right*) or without (*left*) capsaicin (2 × 10^{-4} mol/L, 4 hours). *D,* effect of capsaicin on TNF-induced degradation of IκBα in PC-3 cells. PC-3 cells, either untreated or pretreated with capsaicin (2 × 10^{-4} mol/L, 3 hours), were further incubated for either 15 or 30 minutes with TNF (20 ng/mL), and assayed by Western blot for IκBα in the cytoplasmic fraction. *E,* effect of capsaicin on IκBα in the cytoplasm. Cells were incubated with capsaicin (1 or 2 × 10^{-4} mol/L, 2 hours). Cytoplasmic proteins were harvested and assayed for expression of the IκBα and GAPDH.

only of wild-type p53 expressing LNCaP cells, but also p53-null PC-3 cells and p53-mutant DU-145 cells (25). Therefore, capsaicin could have *in vitro* and *in vivo* antiproliferative activity independent of p53 activity. Nevertheless, in the wild-type p53 expressing LNCaP cells, capsaicin induced p53, and its targets Bax and p21 in a time-dependent manner.

In further studies, we showed that capsaicin inhibited the NF-κB pathway. Previous studies have shown that the NF-κB signaling pathway is constitutively active in the hormone-independent prostate cancer cell lines, PC-3 and DU-145, but not in the hormone-responsive LNCaP cells (13). We confirmed that the NF-κB transcriptional activity is constitutively robust in PC-3 cells as shown by their stimulation of a reporter gene containing NF-κB binding sites. However, this activity of NF-κB was dramatically inhibited by capsaicin. We also showed that capsaicin inhibited the TNF-α–induced IκBα degradation in the cytoplasm, thus preventing the TNF-α–dependent translocation of NF-κB to the nucleus. Furthermore, IκBα protein levels increased in PC-3 cells cultured with capsaicin for 2 hours compared with the levels in untreated cells. Taken together, these results suggest that capsaicin either

stabilizes the IκBα and/or inhibits the IKK activity, resulting in the inhibition of IκBα degradation. In addition, activation of the NF-κB pathway is normally achieved by phosphorylation, polyubiquitination, and subsequent degradation of IκBα by the 26S proteasome (15). In general, proteasome activity correlates with a robust NF-κB signaling pathway in the cancer cells; and PC-3 cells are known to have high proteasome activity (26). We showed that capsaicin inhibited proteasome activity in PC-3 cells, suggesting that this inhibition of proteasome function stabilizes IκBα expression, preventing NF-κB nuclear localization in PC-3 cells. Overexpression of IKKβ, which is the main IKK kinase responsible for the phosphorylation of IκB, did not affect capsaicin sensitivity in PC-3 cells, suggesting that the effect of capsaicin on the NF-κB signaling pathway is not mediated through inhibiting IKK but rather through its effect on IκBα.

We also showed a profound effect of capsaicin on the levels of the AR-regulated PSA gene as well as AR itself in prostate cancer cells. Capsaicin profoundly inhibited protein levels of PSA and AR. Our reporter gene studies showed that capsaicin inhibited androgen activation of the AREs in the PSA regulatory region. Furthermore, reporter gene experiments revealed that even when AR was exogenously overexpressed in LNCaP cells, capsaicin strongly suppressed the PSA transcriptional activity, suggesting that capsaicin suppressed PSA transcription even when high levels of AR were present. Also, overexpression of AR did not affect the antiproliferative effect of capsaicin, suggesting that down-regulation of AR is not the decisive mechanism of action for capsaicin. By immunofluorescence, we also showed that capsaicin did not inhibit the dihydrotestosterone-induced nuclear translocation of AR. These results suggest that capsaicin acts neither through down-regulation of AR, nor by inhibiting the nuclear translocation of AR. The androgen-AR complex cooperates with various coregulators to modulate its target genes for proper function (27–30). Some of these coregulators contain E3 ligase activity, which regulate AR activity via the ubiquitin-proteasome pathway (27–30). Furthermore, inhibition of the proteasome suppresses AR transactivation and interaction between AR and AR coregulators (31). Our studies revealed that capsaicin suppressed proteasome activity, which may be involved in the disruption of AR activity.

Capsaicin was crucial to the discovery of TRVP1 and the therapeutic effects of the drug in pain disorders are mainly mediated through interaction between the drug and this receptor
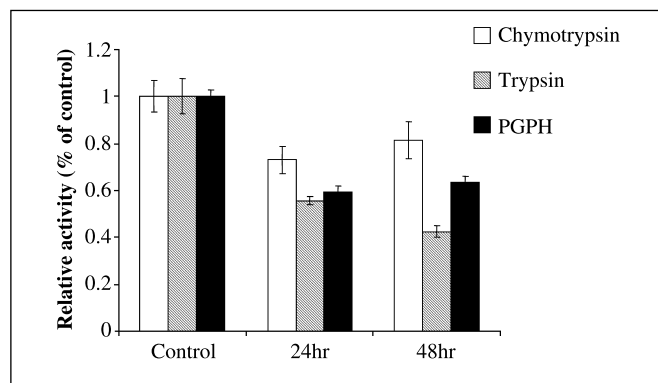

Figure 6. Capsaicin inhibits human prostate cancer cells growing in mice *in vivo*. PC-3 cells (5×10^6) were injected s.c. into bilateral flanks of 10 male BNX *nu/nu* mice, forming two tumors per mouse. Capsaicin (5 mg/kg/d; five mice) or diluent (five mice) was given by gavage, 3 days per week for 4 weeks. *A,* tumor sizes were measured weekly. *B,* after 4 weeks of therapy, tumors were dissected from all mice and weighted. All statistical tests were two-sided.

(25). However, our experiments using the three TRVP1-inhibitors capsazepine, ruthenium red, and SB366791, did not show any attenuation of the inhibitory activity of capsaicin. Also, concentrations of capsaicin used to exert the antiproliferative effects in cancer cells are considerably higher than those needed to affect TRVP1 (32). Taken together, our results indicate that the anticancer effects are not mediated through interaction with TRVP1, which is consistent with what others have reported (33). Alternative mechanisms of action that have been associated with capsaicin includes increased production of reactive oxygen species (34), increased NO production (35), and effects mediated through PPARγ (36).

In conclusion, we show that capsaicin has potent activity against not only androgen-sensitive, but also androgen-independent prostate cancer cells *in vitro* and *in vivo*. Given the clinical tolerability of capsaicin, a clinical trial of this agent seems appropriate in selected individuals with prostate cancer.

Acknowledgments

Figure 5. Proteasome activity following exposure to capsaicin. PC3 cells were cultured with capsaicin (2×10^{-4} mol/L), harvested at the indicated times and chymotrypsin-like, trypsin-like, and PGPH activities were assayed using appropriate fluorogenic peptides as described in Materials and Methods.
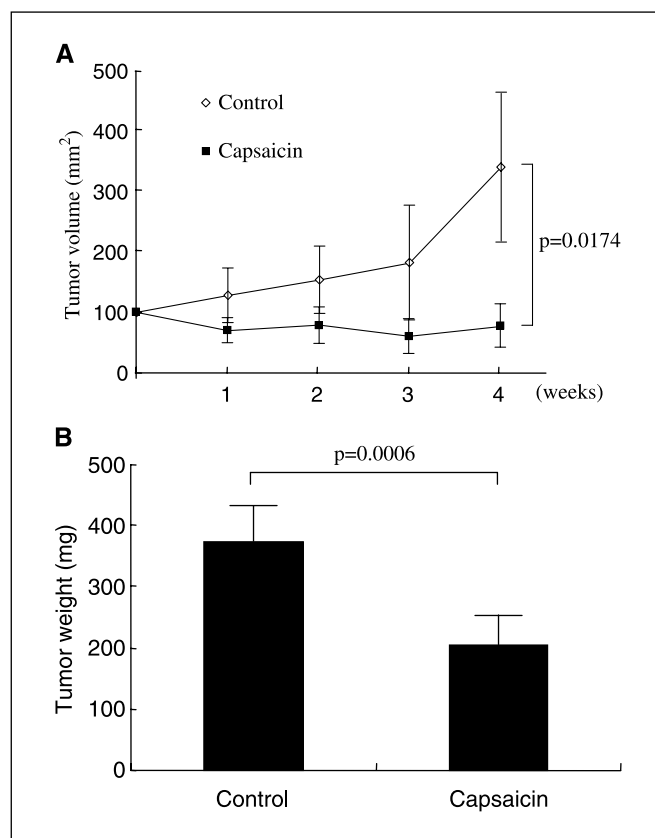
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