



## Capsaicin, a component of red peppers, induces expression of androgen receptor via PI3K and MAPK pathways in prostate LNCaP cells

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### ABSTRACT

**In this study, capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) induced an increase in the cell viability of the androgen-responsive prostate cancer LNCaP cells, which was reversed by the use of the TRPV1 antagonists capsazepine, I-RTX and SB 366791. In further studies we observed that capsaicin induced a decrease in ceramide levels as well as Akt and Erk activation. To investigate the mechanism of capsaicin action we measured androgen (AR) receptor levels. Capsaicin induced an increase in the AR expression that was reverted by the three TRPV1 antagonists. AR silencing by the use of siRNA, as well as blocking the AR receptor with bicalutamide, inhibited the proliferative effect of capsaicin.**

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### 1. Introduction

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is a highly potent active ingredient of hot red and chilli peppers that belong to the plant genus *Capsicum* (Solanaceae) [1,2]. It has been found to act on the capsaicin-sensitive afferent nerves and it has been extensively studied in vivo and in vitro for the treatment of migraine, chronic cough, overactive bladder and diabetes [3,4] and as a potent analgesic [5,6] and anti-cancer agent [7]. Many of the capsaicin actions are mediated by the transient receptor potential vanilloid 1 (TRPV1) receptor, which is a non-selective membrane cation channel that is activated not only by capsaicin but also by noxious heat, acid and by recently described endogenous lipids [8]. Numerous investigations have been conducted to determine the role of capsaicin and TRPV1 in cell proliferation, but the results are conflicting. Many studies have reported that capsaicin exerts anti-cancer effects by causing cell cycle arrest and inducing apoptosis in vitro in many different human cancers [9–11]. The involvement of TRPV1 in such anti-proliferative effects of capsaicin has not always been demonstrated, and in most cases, capsaicin induces apoptosis by a receptor-independent mechanism [12]. In contrast, capsaicin has been shown to significantly reduce liver injury and hepatic apoptosis [13] and to increase DNA synthesis in vitro [14]. The effect elicited by capsaicin may depend on cell

type, doses used [14] as well as the length of the treatment. Long-term treatments of rodents with capsaicin beyond 90 days, as well as high doses, have been shown to be toxic and potentially carcinogenic [15,16] whereas small amounts of capsaicin display few or no deleterious effects [16]. In previous studies, our group has demonstrated the role of capsaicin against the androgen-independent prostate cancer cell line PC-3 in vitro and in vivo [17,18]. The anti-proliferative effect of capsaicin in PC-3 cells was receptor-independent and mediated by de novo synthesis of ceramides [18]. However, the effect of capsaicin in androgen-sensitive prostate cells has not been studied before. Normal prostate cells have androgen-dependent growth mediated by the androgen receptor (AR), a ligand-mediated transcription factor that belongs to the superfamily of steroid receptors [19]. At initial stages, prostate cancer cells depend on androgens for growth and therefore androgen pathway blockage is the standard therapy for the treatment of prostate cancers. However, in spite of initial sensitivity, prostate cancers move more or less quickly towards an androgen-independent stage characterized by resistance to such therapy. It is therefore essential to understand what drives the progression to androgen-independence and identify the molecular markers for both the early androgen-dependent and the advanced androgen-independent prostate cancer stages.

In this study, we investigated the effect of capsaicin on proliferation and signaling pathways of prostate androgen-sensitive LNCaP cells. We found that capsaicin induced an increase in cell viability through TRPV1, PI3K/Akt, ERK and AR activation.

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## 2. Materials and methods

### 2.1. Reagents

Capsaicin and capsazepine were purchased from Tocris (Bristol, UK). The antagonist SB 366791 was from Sigma (St. Louis, MO, USA) and I-RTX from Alexix (Grünberg, Germany). Bisindolylmaleimide (BIM), Fumonisin B<sub>1</sub> and PD 98059 were obtained from Calbiochem (Darmstadt, Germany). LY-294,002 was purchased from Alexix (Grünberg, Germany). Bicalutamide was kindly provided by AstraZeneca (Cheshire, UK). Anti-phospho-p42/44 MAP Kinase and Phospho-Akt (Thr308) were purchased from Cell Signaling Technology (Beverly, MA, USA), and anti-phospho-ERK (E-4), anti-phospho-STAT3 and STAT3 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human androgen receptor monoclonal antibody was supplied by Becton Dickinson Biosciences (Erembodegem, Belgium). Other products were from Sigma (St. Louis, MO, USA).

#### 2.1.1. Cell culture

Lymph node carcinoma of the prostate (LNCaP) cells were purchased from ATCC (CRL-1740) and were cultured as previously described [17]. LNCaP cells from between the 5th and the 25th passage in vitro were used to conduct the studies.

#### 2.1.2. Cell viability assays

Cells were plated in 24-well plates at 20000 cells per well. Prostate cells were treated with the indicated stimuli and further incubated at 37 °C for 48 h. Cell viability was estimated directly on culture plates by MTT assay (Sigma, St. Louis, MO, USA).

#### 2.1.3. Lipid extraction and analysis

Cell pellets were resuspended in 0.6 ml distilled water, and disrupted at 4 °C by brief sonication. Lipids were extracted with chloroform/methanol and ceramide content was determined using *E. coli* diacylglycerol kinase (Calbiochem, Darmstadt, Germany) and [<sup>32</sup>P]γ-ATP (6000 Ci/mmol; Perkin-Elmer, Barcelona) as previously reported [20]. Radioactive ceramide-1-phosphate was isolated by thin-layer chromatography using chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, by volume) as the developing solvent. Products of this reaction were quantified and expressed as a percentage of the value observed before treatment.

#### 2.1.4. Western-blot analysis

Cultured cells were lysed into a lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol) containing 5 μg/ml leupeptin, 5 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride, and disrupted by sonication. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA). Western blotting was done as previously described [17].

#### 2.1.5. RNA silencing

LNCaP cells were transfected with siRNA duplexes corresponding to human androgen receptor gene (5'-UCCCAAGCCAUCGUAG-ATT-3', Invitrogen, Life Technologies Co., Carlsbad, CA). The purified siRNA duplexes were transfected to the cells with the lipofectamine reagent (Invitrogen Co., Carlsbad, CA), in MEM medium as recommended by the manufacturer and the final siRNA concentration was 100 nM. 24 h after transfection, cells were placed into fresh RPMI 1640 medium supplemented with 10% FBS. At the indicated time points after transfection, cells were used for MTT assays or Western blot.

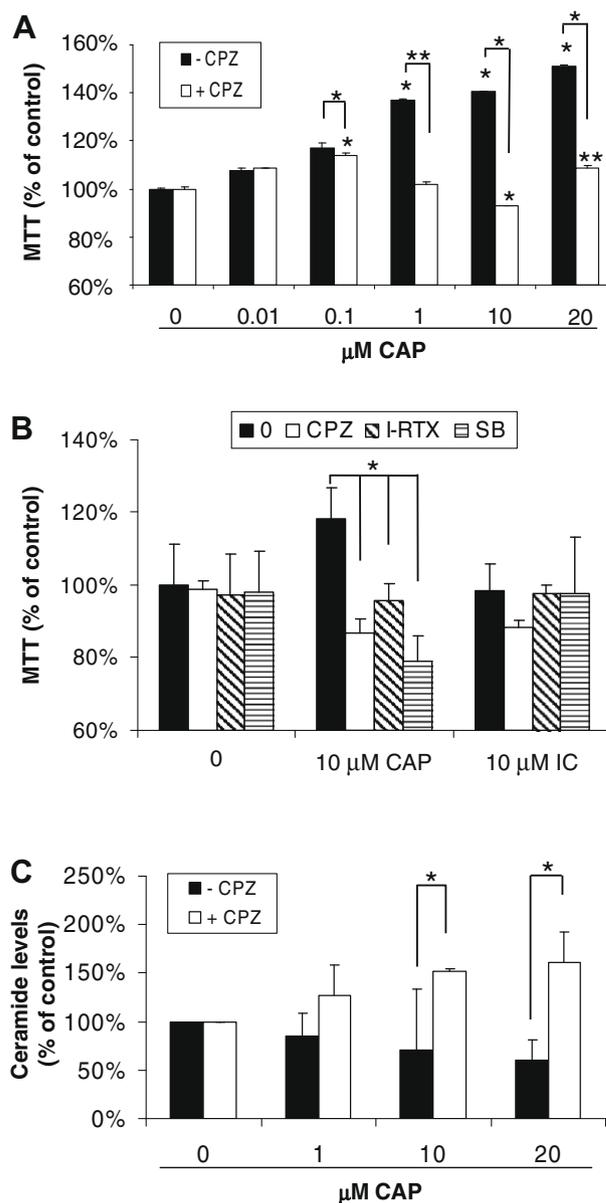
### 2.1.6. Statistical analysis

Data are presented as the mean ± S.D. of the number of experiments indicated. Statistical comparisons among groups were made with a Student's *t*-test and the difference was considered to be statistically significant when the *P* value was <0.05.

## 3. Results

### 3.1. Induction of LNCaP cell proliferation by capsaicin

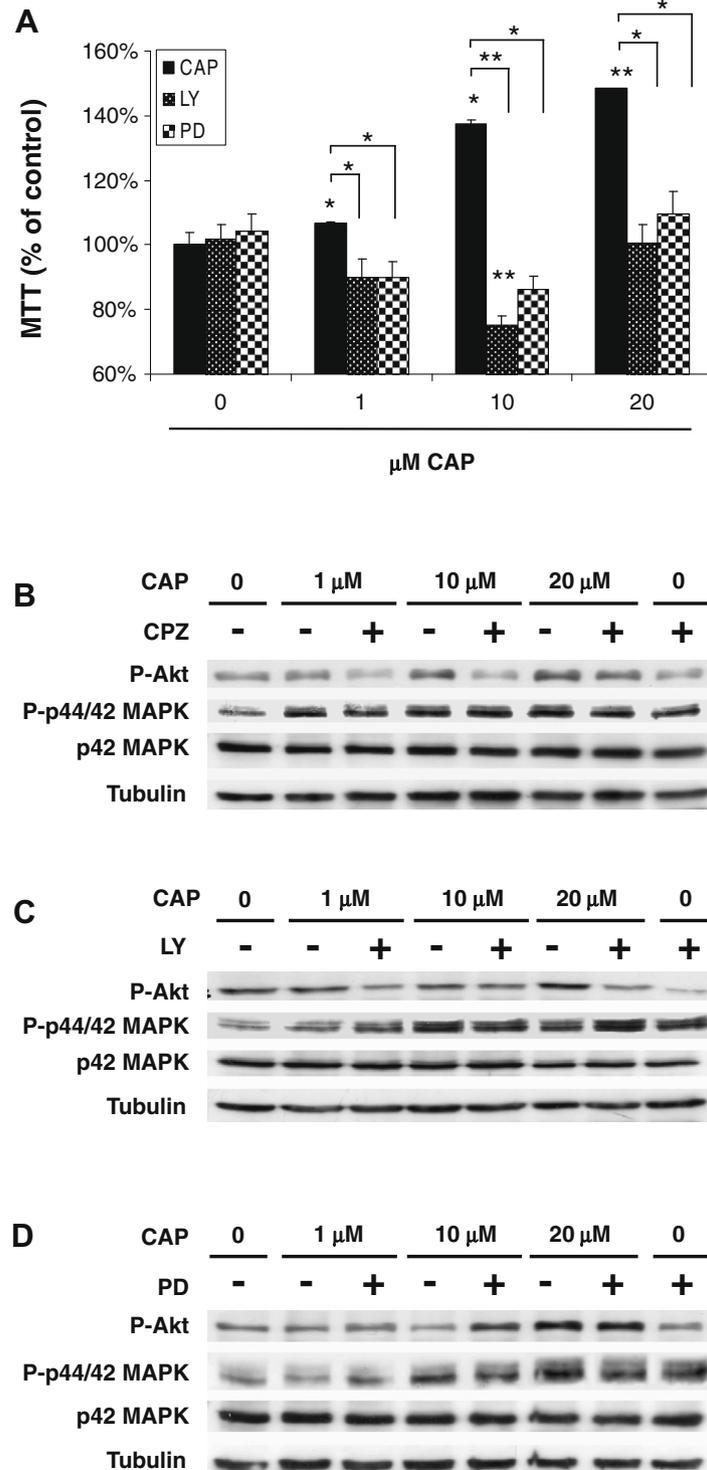
We first examined the effect of the prototypic vanilloid receptor agonist, capsaicin, on the androgen-sensitive prostate cancer LNCaP cell line and we found surprisingly that capsaicin caused a concentration-dependent increase in cell viability (Fig. 1A). This



**Fig. 1.** Capsaicin effects on cell viability. LNCaP cells were incubated with various doses of capsaicin (CAP) in the presence or absence of 1 μM of the TRPV1 antagonist capsazepine (CPZ). (A) Cell viability was assessed by MTT test. (B) Effect of different TRPV1 antagonists (1 μM CPZ; 10 μM SB 366791; 100 nM I-RTX) and TRPM8 agonist (icilin, 10 μM IC) on capsaicin-induced cell viability. (C) Ceramide levels were determined using diacylglycerol kinase and are expressed as a percentage of the value observed before treatment. The *P*-values for statistical analyzes calculated by Student's *t*-test are: \**P* < 0.01; \*\**P* < 0.05.

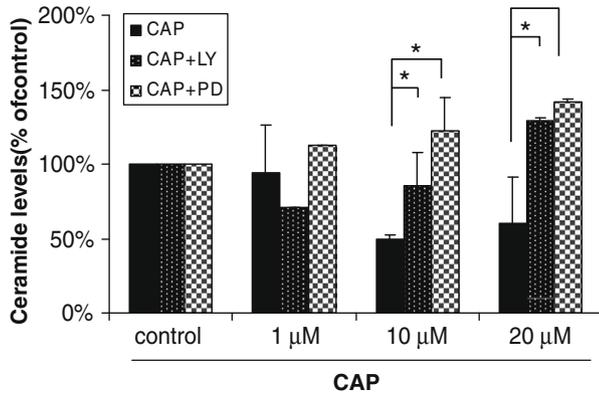
effect was reversed by the vanilloid receptor antagonist, capsazepine, at 1  $\mu\text{M}$  (Fig. 1A), suggesting a receptor-dependent mechanism. To confirm the involvement of the TRPV1 receptor, we used the compound SB 366791 which exhibits potent competitive antagonistic activity in human receptors and has a better selective profile than capsazepine [21]. SB and I-RTX, a classical TRPV1 antagonist [21], inhibited the pro-survival effect induced by capsaicin, suggesting a receptor-dependent effect (Fig. 1B). Capsazepine has also been described as a TRPM8 antagonist. To rule out the involvement of the channel TRPM8, we used the specific and potent TRPM8 agonist, icilin [22,23]. As shown in Fig. 1B, icilin did not have any effect on LNCaP cell viability.

Results obtained with capsaicin in LNCaP cells contrasted with our previous results showing receptor-independent cell death



**Fig. 2.** Activation of the PI3K and ERK pathways by capsaicin in LNCaP cells. Cells were incubated with indicated doses of capsaicin in the presence or absence of 1  $\mu\text{M}$  capsazepine, 1  $\mu\text{M}$  LY 294002 (LY) and 100 nM PD 98059 (PD). (A) Cell viability assessed by MTT test. The *P*-values for statistical analyzes calculated by Student's *t*-test is  $P < 0.05$ . (B–D) Western blots for Akt, p44/42 MAPK, total MAPK and tubulin.

promotion by capsaicin in androgen-insensitive PC-3 cells [17]. Therefore, in order to ascertain discrepancies obtained between PC-3 cells and LNCaP cells, we analyzed different signaling pathways.



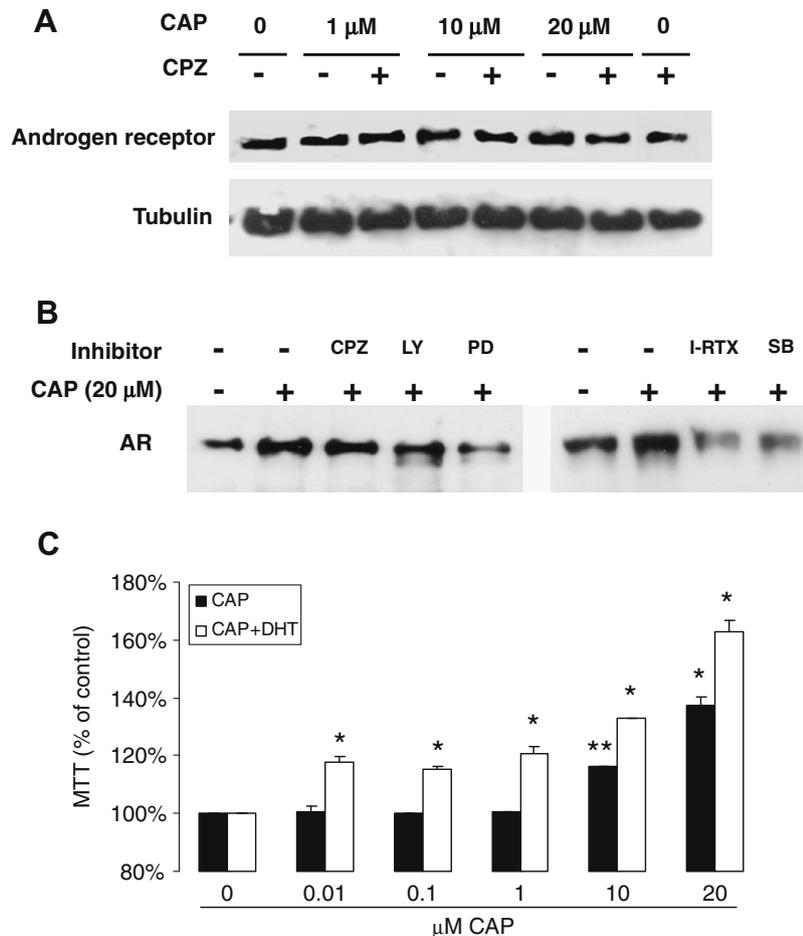
**Fig. 3.** Involvement of the PI3K and ERK pathways in the capsaicin-induced ceramide decrease. LNCaP were incubated with 1 μM of capsaicin in the presence of 1 μM LY294002 or 100 nM PD 98059. Ceramide levels were determined using diacylglycerol kinase and are expressed as a percentage of the value observed before treatment (\*,  $P < 0.05$ ).

### 3.2. Capsaicin inhibits ceramide production

We have previously shown that capsaicin promotes prostate PC-3 cell death through de novo synthesis of ceramide [18], so we measured intracellular levels of ceramides in LNCaP cells treated with capsaicin. In concordance with the results obtained for cell viability we found that capsaicin induced a dose-dependent reduction of ceramide production (Fig. 1C). This effect was also receptor-dependent, as confirmed by the increase in ceramide production when the inhibitor capsazepine was added (Fig. 1C).

### 3.3. Capsaicin activates PI3K and ERK pathways

To determine which mechanisms were activated by capsaicin, additional experiments using various signaling pathway inhibitors were performed. We observed a reversion of the capsaicin-induced proliferation effect with the PI3K inhibitor LY 294002 (LY) at 1 μM, and the MEK inhibitor PD 98059 (PD) at 100 nM (Fig. 2A). To corroborate that PI3K and ERK kinases were activated by capsaicin in LNCaP cells, we detected the phosphorylated forms of Akt and 44/42 MAPK, which are their respective downstream substrates. The results show that capsaicin induced a dose-dependent increase in the phosphorylated forms of Akt and p44/42 MAPK, which was reverted by the antagonist capsazepine at 1 μM (Fig. 2B) To confirm PI3K and MEK activation and to elucidate the relationship between pathways, Western blots were performed in the presence of the inhibitors LY and PD (Fig. 2C and D). As seen in Fig. 2C, Akt



**Fig. 4.** Capsaicin induces androgen receptor expression in LNCaP cells. (A) Western blot of AR in cells incubated with the indicated doses of capsaicin in the presence or absence of 1 μM capsazepine. (B) Western blot of AR in cells incubated with 20 μM capsaicin in the presence or absence of 1 μM LY294002 or 100 nM PD 98059, 10 μM SB 366791 or 100 nM I-RTX. (C) Cell viability of cells treated with capsaicin in the presence or absence of 2 μM bicalutamide (BIC). The  $P$ -values for statistical analyzes are: \* $P < 0.01$ ; \*\* $P < 0.05$ .

phosphorylation was blocked by the PI3K inhibitor LY, confirming the activation of the PI3K pathway by capsaicin. However, p44/42 MAPK phosphorylation was not inhibited by LY, suggesting that ERK pathway activation was not dependent on PI3K. In the same way, p44/42 MAPK phosphorylation was blocked by the MEK inhibitor, PD, whereas it was not modified by LY (Fig. 2D). This result suggests that activation of the PI3K and ERK pathways by capsaicin in LNCaP cells are independent phenomena that are activated through the TRPV1 receptor.

To test whether the capsaicin-induced stimulation of ceramide was dependent on PI3K or MEK activation, we preincubated LNCaP cells with LY or PD at the concentrations used above. Fig. 3 shows that pre-treatment of cells with both inhibitors reversed the capsaicin-induced decrease in intracellular ceramide. This effect was observed at various doses of capsaicin, and suggests that capsaicin activates PI3K and p44/42 MAPK pathways to inhibit ceramide production and to induce increased cell viability.

### 3.4. Role of the androgen receptor

To gain some insight into the capsaicin-induced cell viability increase taking place in LNCaP cells, we measured androgen receptor (AR) levels using an immunoblot approach. The data show that capsaicin induced a dose-dependent increase in AR, which was consistent with its proliferative activity (Fig. 4A). The induction of AR expression was mediated by the TRPV1 receptor, as confirmed by the inhibition by capsazepine at all the capsaicin doses tested (Fig. 4A) and by SB 366791 and I-RTX (Fig. 4B). Both the PI3K inhibitor LY and the MEK inhibitor PD reduced the in-

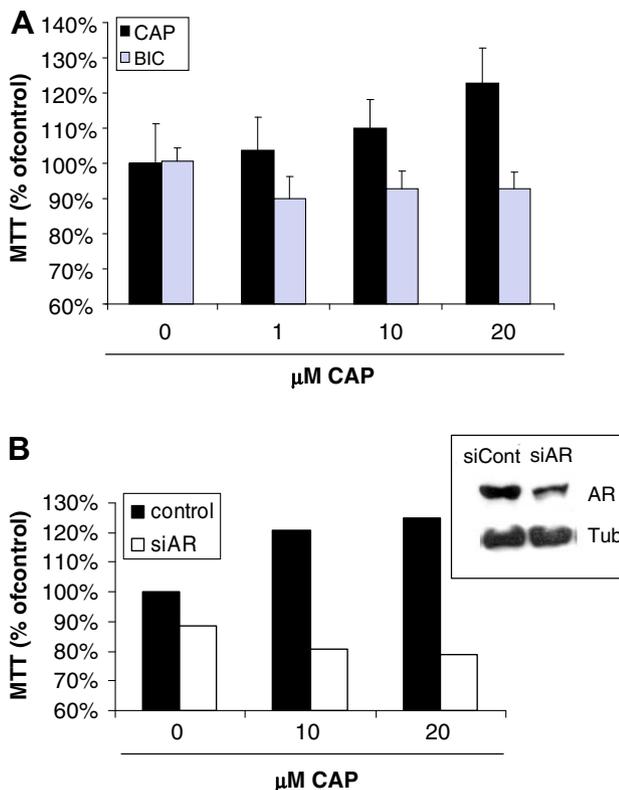
crease in AR expression (Fig. 4B) suggesting that activation of these signaling pathways occurred prior to androgen receptor induction. To investigate the role of the androgen receptor in the capsaicin-promoted cell viability increase, we incubated LNCaP cells with capsaicin in the presence of the competitive AR antagonist bicalutamide. The results shown in Fig. 5A indicate that bicalutamide inhibits the increase in cell viability induced by capsaicin.

To assess the role of AR in the proliferative action of capsaicin, we silenced the expression of this receptor with small interfering RNA (siRNA). An immunoblot experiment confirmed that AR was drastically suppressed after siRNA-AR transfection for 72 h, dropping to  $20 \pm 7\%$  of the control scrambled RNA detected in the Western-blot assay (Fig. 5B, insert). In cells transfected with specific AR siRNA, capsaicin did not induce an increase in cell viability, confirming that the AR receptor is essential for capsaicin-induced cell proliferation in the androgen-sensitive LNCaP cells.

## 4. Discussion

We have previously reported that the androgen-sensitive prostate cancer LNCaP cell line expresses the TRPV1 receptor [24]. In the present study capsaicin was found to increase the viability of the androgen-sensitive LNCaP cell line in a TRPV1 receptor-dependent way. We demonstrated that the proliferative action of capsaicin was concomitant with a reduction in intracellular ceramide levels and was mediated by the PI3K/Akt and ERK pathways. The reduction of ceramide levels by capsaicin is consistent with its effect on cell viability. The intracellular effects of ceramide are pleiotropic, but for the most part growth-inhibiting [25]. Besides, reduction of ceramide levels has been reported to attenuate cell death in several instances, probably due to its conversion into ceramide-1-phosphate, which is mitogenic. The mechanism whereby capsaicin promotes a reduction in ceramide levels and its possible metabolism into phosphorylated mediators was not tackled in this study but will be approached in further research.

Activation of the PI3K and ERK pathways by capsaicin has previously been demonstrated to occur in dorsal root ganglion neurones [26] and in human hepatoma HepG2 cells [27] through activation of the capsaicin receptor TRPV1. Recently, several groups reported that Akt is involved in the pain hypersensitivity induced by intradermal injection of capsaicin in rats [28–30]. In prostate cells, activation of the PI3K pathways regulates the transcriptional activity of the androgen receptor as well as its expression, cooperating with the AR in tumor development [31]. As the vanilloid capsaicin-induced activation of the PI3K/Akt pathway, we investigated whether capsaicin regulated AR expression and activity in the androgen-responsive prostate LNCaP cell line. We found that capsaicin induced a dose-dependent increase in AR expression. Moreover, we demonstrated that capsaicin induced its pro-survival effect through the AR receptor, since AR silencing with siRNA reduced the effect of capsaicin. The fact that the AR antagonist bicalutamide inhibited the pro-survival effect of capsaicin provides additional evidence that the effect of capsaicin is via AR activation. Studies performed by Mori et al. [32] showed that capsaicin inhibited the growth of LNCaP cells and the DHT-induced expression of AR. However, the doses of capsaicin used in that study were higher than ours and, as the authors pointed out, higher than those needed to affect TRPV1. Also, overexpression of AR did not affect the anti-proliferative effect of capsaicin, suggesting that downregulation of AR was not the decisive mechanism of action for capsaicin [32]. Since the proliferative effect of DHT in LNCaP cells is so strong, there lies the possibility that the effect of capsaicin in the presence of DHT may be different to that of capsaicin alone.



**Fig. 5.** Induction of cell viability by capsaicin is mediated by the androgen receptor. (A) Cell viability of LNCaP cells incubated with different doses of capsaicin in the presence of 2  $\mu$ M bicalutamide. (B) Cells were transfected with specific AR siRNA or control scrambled RNA for 48 h followed by incubation with capsaicin for an additional 48 h. Cell viability was then assayed by MTT. Insert, Western blot of AR in cells transfected with siRNA or control scrambled RNA.

We have previously shown that capsaicin induces apoptosis in the androgen-resistant prostate PC-3 cell line by a TRPV1-independent pathway [17,18]. However, the involvement of TRPV1 in cell growth inhibition in androgen-resistant prostate cells is far from being elucidated. Melck et al. [33] showed that arvanil, a structural hybrid between capsaicin and anandamide and an agonist for both cannabinoid and TRPV1 receptors, inhibited the prolactin-induced prostate DU-145 cell growth, which was blocked by capsazepine. These results suggested a role for TRPV1 in the anti-proliferative effect of arvanil on this androgen-resistant prostate cell line. However, the effect of arvanil was only observed in the presence of PRL and not alone [33]. Although the mechanism of TRPV1 activation remains elusive, recent data suggest that it complexes with the high affinity neurotrophic receptor TrkA, which regulates TRPV1 activity [34]. The effect of arvanil in prostate DU-145 cells observed by Melck et al. could be due to the TrkA down-regulation induced by arvanil and could explain the discrepancies with our results. Also, the different effects of capsaicin on cellular proliferation and on metabolic activities may be dependent on dose, term of treatment, and the stage and androgen sensitivity of the targeted prostate cell. The opposite effects on cell proliferation induced by capsaicin in LNCaP cells and in PC-3 cells can be explained if the action of capsaicin in prostate cells is dependent on different signaling pathways and on androgen sensitivity. This seems to be the case since in LNCaP cells the effect of capsaicin is mediated by the TRPV1 and androgen receptor whereas in PC-3 cells capsaicin induces apoptosis in a receptor-independent way. The role of TRPV1 in LNCaP cells is not surprising and is common to other transient receptor potential (TRP) receptor family members. It has previously been reported that TRPV8 is expressed in LNCaP cells and that it is required for cell survival [35]. Moreover, recent research demonstrated differential TRPM8 localization and the functional dependence on AR activity of human prostate epithelial cells and on their differentiation status [36]. Our results highlight the importance of androgen sensitivity in the response of prostate cancer cells to capsaicin. The results have clinical relevance since TRPV1 desensitisation by intravesical capsaicin administration is currently used for bladder incontinence [37]. However, desensitisation may become obsolete when non-toxic, potent TRPV1 antagonists become available. A more thorough understanding of the mechanism of capsaicin action could potentially lead to the development of new therapeutic approaches not only for bladder disorders but also for prostate cancer.

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