Amino Acids (2010) 38:1505–1513
DOI 10.1007/s00726-009-0366-0

ORIGINAL ARTICLE

Icaritin induces growth inhibition and apoptosis of human prostatic smooth muscle cells in an estrogen receptor-independent manner

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Received: 14 July 2009 / Accepted: 9 October 2009 / Published online: 30 October 2009

Abstract Icaritin has selective estrogen receptor (ER) modulating activity. ERs are expressed in the prostate stroma, and estrogens have an important role in the pathology of benign prostatic hyperplasia (BPH). However, the impact of icaritin on BPH was not studied. Human prostatic smooth muscle cells (PSMCs) were treated with 0–100 μM icaritin, also using 10 μM ICI182780 as a specific ER antagonist. The effects on cell growth and apoptosis were determined by cell counting and sandwich-enzyme-immunoassay. Western blotting was employed to illustrate the possible mechanisms. Cell growth was strongly inhibited by icaritin, and this was accompanied by an augmented apoptosis. Few changes in icaritin-induced growth inhibition and apoptosis were observed after pre-treatment in the presence of ICI182780. Consistent with growth inhibition and apoptosis induction, icaritin decreased cyclin D1 and CDK4 expression and increased Bax/Bcl-2 ratio in human PSMCs. Furthermore, icaritin induced sustained phosphorylation of extracellular signal-regulated kinase (ERK) in human PSMCs. PD98059, a specific ERK inhibitor, blocked the activation of ERK by icaritin and abolished the icaritin-induced growth inhibition and apoptosis. The results indicate that icaritin reduces growth and induces apoptosis in human PSMCs via ERK signaling pathway without involvement of ERs.

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Keywords Icaritin · Growth · Apoptosis · Extracellular signal-regulated kinase · Prostatic smooth muscle cell

Introduction

The stromal compartment of the prostate plays a major role in the pathogenesis of benign prostatic hyperplasia (BPH), which is one of the most common diseases in elderly men (Roehrborn 2008; Untergasser et al. 2005). In hyperplastic tissue the fraction of stromal tissue is significantly increased (Roehrborn 2008; Untergasser et al. 2005; Deering et al. 1994). Smooth muscle cells (SMCs) are one of the main cellular components of prostatic stroma (Shapiro et al. 1992). In human BPH tissue as well as in animal BPH models there is an increase in the fibromuscular portion of the gland (Shapiro et al. 1992; Robinette 1988). Recently, interest was focused on the reduced cell death theory in the prostate, and it has been suggested that an imbalance between cell apoptosis and cell proliferation may result in the development of BPH (Roehrborn 2008; Claus et al. 1997).

Primary treatment options for BPH include conventional pharmaceuticals (α-blockers and 5-α reductase inhibitors), herbal therapy, and surgical procedures. The use of phytotherapy for BPH is widespread (Dedhia and McVary 2008). Icaritin, a prenylflavonoid derivative from Chinese herbs of Epimedium genus, may have various activities, including neuroprotective effects (Wang et al. 2007), stimulation of neuronal and cardiac differentiation (Wang et al. 2009b; Wo et al. 2008; Zhu and Lou 2005), prevention of steroid-associated osteonecrosis (Zhang et al. 2009), enhancement of osteoblastic and suppression of osteoclastic differentiation and activity (Huang et al.

2007a), and inhibition of human prostate carcinoma PC-3 cell growth (Huang et al. 2007b) (possibly involving the estrogen receptor (ER) signaling (Wang et al. 2007) and mitogen-activated protein kinase (MAPK) signaling (Wang et al. 2007; Wang et al. 2009b; Wo et al. 2008)). However, there are no reports on activity of icaritin against BPH.

The purpose of the current study was to investigate the effect of icaritin on the growth and apoptosis regulation of human prostatic SMCs. The mechanism of action and signal transduction occurring after the challenge to icaritin also were examined.

Materials and methods

Reagents

Icaritin was prepared according to the existing protocol from icariin (Liu et al. 2005). Icariin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). PD98059 was purchased from Calbiochem Corp. (San Diego, CA, USA). ICI182780 and dimethylsufoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 phenol red-free medium was purchased from Gibco BRL (Burlington, Ontario, Canada). Charcoal/dextran treated fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Primary antibodies of β-actin, extracellular signal-regulated kinase (ERK), p-ERK, p38, p-p38, c-jun N-terminal Kinase (JNK), and p-JNK were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Primary antibodies to cyclin D1, CDK4, Bcl-2, and Bax were purchased from Santa Cruz Biotechnology Inc. (Waltham, MA, USA). Anti-mouse, and -rabbit IgG horseradish peroxidase (HRP)-conjugate antibodies were also purchased from Santa Cruz Biotechnology Inc.

Cell cultures

Human prostatic tissue explants were managed and cultured cells were obtained as previously described (Guh et al. 1998). Isolated human PSMCs were identified by accepted criteria. The cultured cells exhibited positive immunofluorescence staining for smooth muscle α-actin but negative immunostaining for epithelial cytokeratins (data not shown). Isolated cells were cultured in RPMI 1640 phenol red-free medium (Gibco, Rockvile, MD, USA), supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37°C.

Analysis of human PSMC growth

The effect of icaritin on PSMC growth was determined through cell counting. The cells were seeded into 24-well plastic plates at a density of 1×10^5 cells/well and allowed to attach for 24 h ahead of treatments. To determine whether the effect of icaritin on human PSMC growth is dose-dependent, the cells were cultured in medium with gradient concentrations of icaritin (0–100 μM) for 72 h in the presence of 10% FBS. Cells were also cultured with or without 100 μM icaritin, for 1–5 days. To study the effects of ER and MAPK inhibitors, cells were pretreated with the anti-estrogen ICI182780 (10 μM) or with the MAPK-kinase inhibitor PD98059 (10 μM) for 3 h prior to icaritin (100 μM) treatment for 72 h. The cells were then harvested by trypsinization, and resuspended in 2.5 ml of phosphate-buffered saline (PBS). Triplicate samples from each well were counted with a hemocytometer under a phase-contrast microscope.

Cell apoptosis measurement

Apoptosis was assessed directly by measurement of cytoplasmic nucleosomes (i.e., DNA complexed with histone in the cytoplasm) using a Cell Death Detection ELISA Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany), according to the kit protocol. This kit allows a specific determination of mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysates (Tang et al. 2007; Wang et al. 2009a; Xie et al. 2007, 2008). Briefly, cells were plated at a density of 1×10^5 cells/well in 24-well plates and allowed to attach for 24 h followed by treatment with vehicle or 10–100 μM icaritin for 72 h in the presence of 10% FBS. To study the effects of inhibitors, attached cells were pretreated with ICI182780 (10 μM) or PD98059 (10 μM) for 3 h prior to icaritin (100 μM) treatment for 72 h in the presence of 10% FBS. The cell layers were rinsed with PBS and extracted with 0.5 ml of lysis buffer after 30 min of incubation at 4°C. The cell lysates were then centrifuged for 10 min at 15,000 rpm, and aliquots of the supernatant were tested for apoptosis using the Cell Death Detection Kit.

Cells were also cultured in six-well plates at a density of 2×10^5 cells/well and allowed to attach for 24 h followed by treated with vehicle or 100 μM icaritin for 72 h in the presence of 10% FBS. Treated cells were labeled using the nucleic acid-binding dye mix of 100 μg/ml acridine orange and 100 μg/ml ethidium bromide (Sigma Chemical Company) in PBS. The cells were examined by fluorescence light microscopy.

Protein extraction and western blotting

The human PSMCs were plated at a density of 4×10^5 cells/well in 6-well plates and allowed to attach for 24 h followed by treatment with vehicle or 10–100 μM icaritin for 72 h in the presence of 10% FBS. The cells were then harvested at 4°C and lysed on ice in Triton lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide, 10 mM EDTA, 10 μg/ml aprotinin and 1 μg/ml aminoethylbenzenesulfonyl fluoride). Cell monolayers were also treated with 100 μM icaritin for 5–120 min, washed quickly with cold PBS containing 5 mM of EDTA and 0.1 mM of Na_3VO_4, and lysed with a buffer consisting of 20 mM of Tris–HCl (pH 7.5), 150 mM of NaCl, 1% Triton X-100, 10 mM of NaH_2PO_4, 10% glycerol, 2 mM of Na_3VO_4, 10 mM of NaF, 1 mM of ABSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Total protein present in each lysate was quantified by the Bradford protein assay. Total protein (40 μg lysate per lane) was subjected to gel electrophoresis, and the proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were blocked for 1 h in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBS). Primary antibodies were added in 5% milk, and the blots were incubated overnight at 4°C. The blots were then washed three times for 5 min with 10 ml TBS and incubated with the anti-mouse and -rabbit IgG HRP-conjugated secondary antibodies (1:2,500) in 5 ml TBS with gentle agitation for 1 h at room temperature. Then the blots were washed three times for 5 min each with TBS, exposed to a chemiluminescent detection using the SuperSignal West Pico Substrate (Pierce, Rockford, USA), and scanned to X-ray film.

Statistical analyses

SPSS 13.0 was used for the statistical analyses. Data are presented as the mean ± SD. Comparisons were made using one-way ANOVA. All experiments were repeated at least three times, and representative experiments are shown.

Results

Effects of icaritin on growth and apoptosis of human PSMCs

Cell counting was performed to examine possible effects of icaritin on cell growth of human PSMC cultures. As shown in Fig. 1, the effect of icaritin on the human PSMCs was dose-dependent at concentrations from 10 to 50 μM.

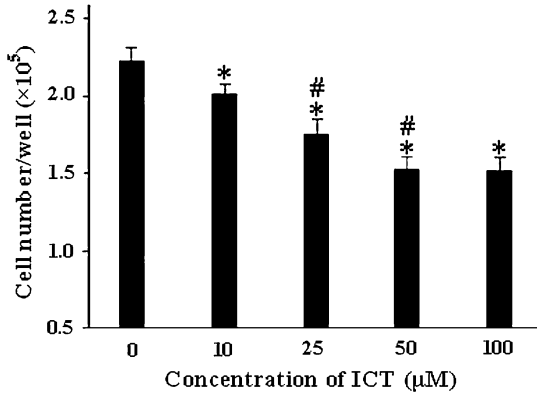

Fig. 1 Growth of human PSMCs treated with different concentrations of icaritin (ICT) for 72 h. The *bars* represent the mean cell count ± SD ($n = 4$). $^*p < 0.05$, versus the control group; $^{\#}p < 0.05$, versus the preceding group

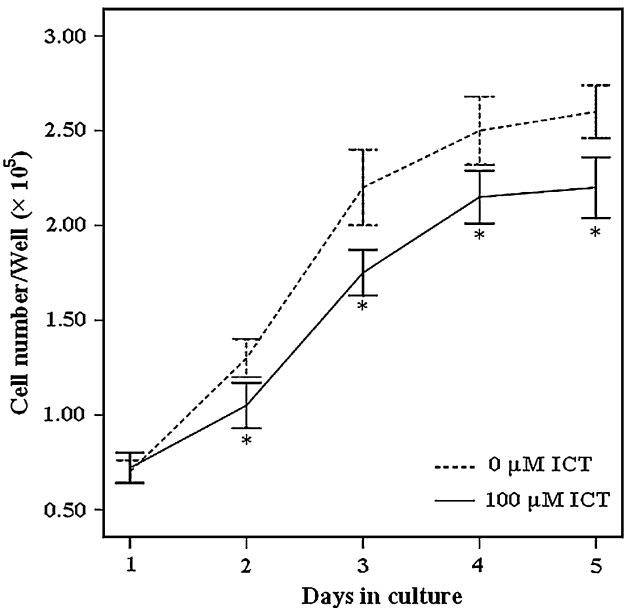

Fig. 2 Cell count for growth of human PSMCs treated with or without 100 μM of icaritin (ICT) on different days. The *bars* represent the mean ± SD ($n = 4$). $^*p < 0.05$, versus the control group

Figure 2 shows that the average numbers of the control and 100 μM icaritin-treated cells were very similar on Day 1. On Days 2–5, the average cell numbers in the icaritin-treated group were significantly lower than those in control cultures. These results indicated that icaritin has a growth-inhibiting effect on the human PSMCs.

After 72 h of incubation, apoptosis at 10–100 μM icaritin was larger than that of vehicle-treated cells, showing massive and consistent dose dependence (Fig. 3a). Figure 3b shows that 25 or 100 μM icaritin-treated PSMCs exhibit more apoptotic cells with yellow chromatin in condensed or fragmented nuclei than that of vehicle-treated cells.

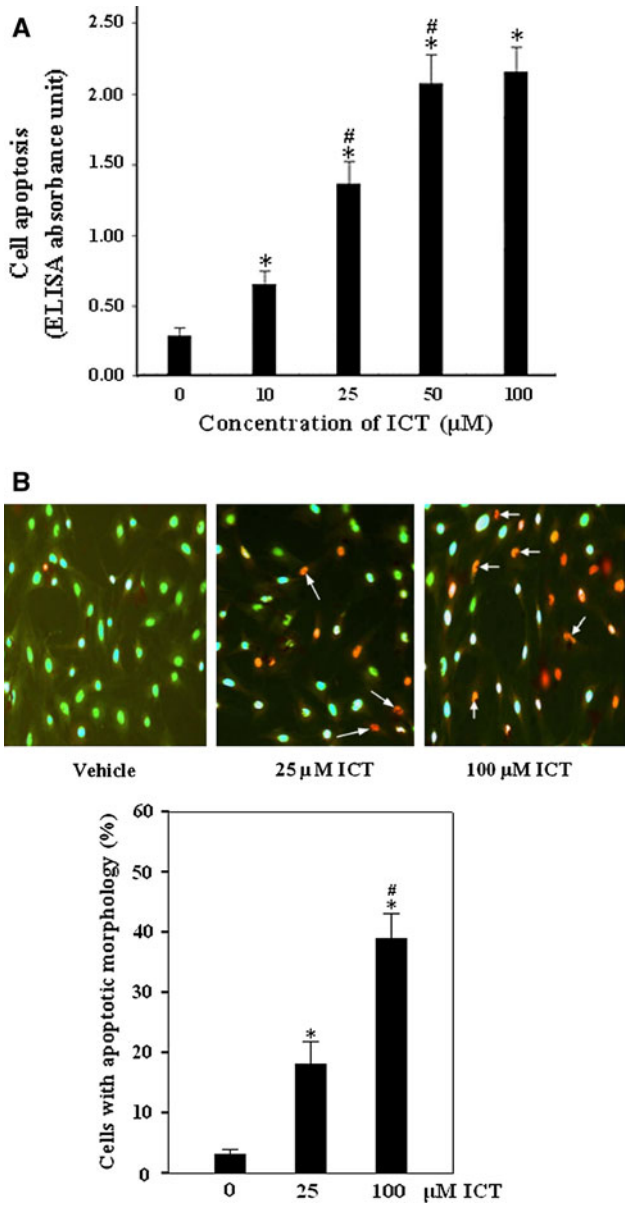

Fig. 3 Effect of icaritin (ICT) on apoptosis of human PSMCs. **a** Cells were exposed to different concentrations of ICT for 72 h. Apoptosis was assessed using a Cell Death Detection Kit, and expressed as ELISA absorbance units. The *Bars* represent the mean ± SD ($n = 4$). $^{*}p < 0.05$, versus the control group; $^{\#}p < 0.05$, versus the preceding group. **b** Cells were exposed to vehicle or 25 or 100 μM ICT for 72 h and then stained with acridine orange/ethidium bromide. The representative graphs were shown. Viable cells had green fluorescent nuclei with organized structure. The early apoptotic cells had yellow chromatin in nuclei that were highly condensed or fragmented. Apoptotic cells also exhibited membrane blebbing. The late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented. The necrotic cells had bright orange chromatin in round nuclei. Arrows indicate apoptotic cells. Only cells with yellow, condensed or fragmented nuclei were counted as apoptotic cells in a blinded, nonbiased manner. For each sample, at least 500 cells/well and 4 wells/condition were counted, and the percentage of apoptotic cells was determined: percentage of apoptotic cells = total number of apoptotic cells/total number of cells counted. The Bars represent the mean ± SD ($n = 4$; $^{*}p < 0.05$, compared with the vehicle-treated group; $^{\#}p < 0.05$, vs. the 25 μM ICT-treated group)

The effects of icaritin on cyclin D1, CDK4, Bcl-2 and Bax protein expression

Possible effects of icaritin upon levels of cyclin D1, CDK4, Bcl-2 and Bax protein in human PSMCs were examined by Western blotting. At 10–100 μM for 72 h, icaritin induced a significant decreasing in the expression levels of cyclin D1 and CDK4 relative to the control cells (Fig. 4). After treatment with 10–100 μM icaritin for 72 h, the levels of Bcl-2 decreased in comparison with the control cells, and the levels of Bax significantly increased (Fig. 4). The Bax/Bcl-2 ratio in control cells was arbitrarily set at 1. Icaritin increased the Bax/Bcl-2 ratio, with a maximal increase to 35.8 with 100 μM.

Estrogen receptors are expressed in human PSMCs

As expected (Royuela et al. 2001; Huang et al. 2007b), Western blotting detected 66 kDa ER-α and 56 kDa ER-β proteins in human PSMC lysates (Fig. 5). There was no difference of ER-α or ER-β protein expression between two different PSMC cultures. This result shows that human PSMCs express ERs.

Icaritin stimulates sustained ERK1/2 activation in human PSMCs

We examined whether icaritin induced MAPK signaling in PSMCs. Icaritin (100 μM) has no effect on the activities of JNK and p38, and none of their phosphorylated forms were detected (Fig. 6a). However, icaritin treatment increased phosphorylated ERK1/2 levels within 10 min of incubation (Fig. 6a). Importantly, the icaritin-induced phosphorylation of ERK1/2 was strong and sustained and lasted for at least 8 h (Fig. 6a).

Figure 6b shows that the activation of ERK1/2 by icaritin was inhibited by the specific ERK inhibitor, PD98059. However, blocking of ER signaling pathway with ICI182780 had no effect on icaritin-induced phosphorylation of ERK1/2. These results indicate that icaritin induces a sustained ERK1/2 activation via ER-independent pathway(s) in human PSMCs.

Icaritin-induced growth inhibition and apoptosis with no association with estrogen receptors

Estrogens have been long presumed to play a role in the etiology of BPH (Prins and Korach 2008). Estrogens induce a preferential stimulation of prostate stromal cell proliferation (Prins and Korach 2008). Icaritin seems to

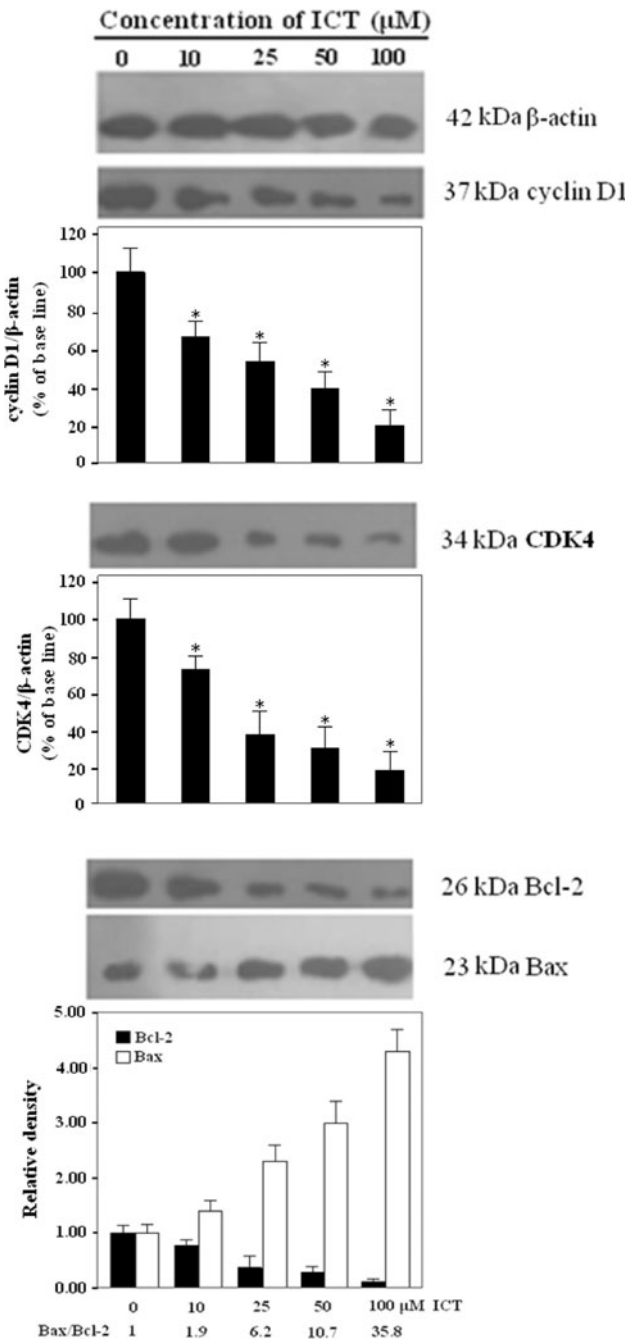

Fig. 4 Effects of different concentrations of icaritin (ICT) on cyclin D1, CDK4, Bcl-2 and Bax protein expression in human PSMCs. Cell lysates were subjected to Western blotting and incubated with cyclin D1, CDK4, Bcl-2, and Bax antibodies. The representative results were shown. Protein bands were quantitated by densitometric analysis and normalized for variations in β-actin protein levels. The relative mean ratio of Bax/Bcl-2 was determined. The bars represent the mean ± SD of three separate experiments. *$p < 0.05$ versus the control group

possess agonistic activity as well as somehow antagonistic activity at the ERs (Huang et al. 2007b; Wang et al. 2007; Ye and Lou 2005). In the present study, we clearly showed growth-inhibiting and pro-apoptotic effects of

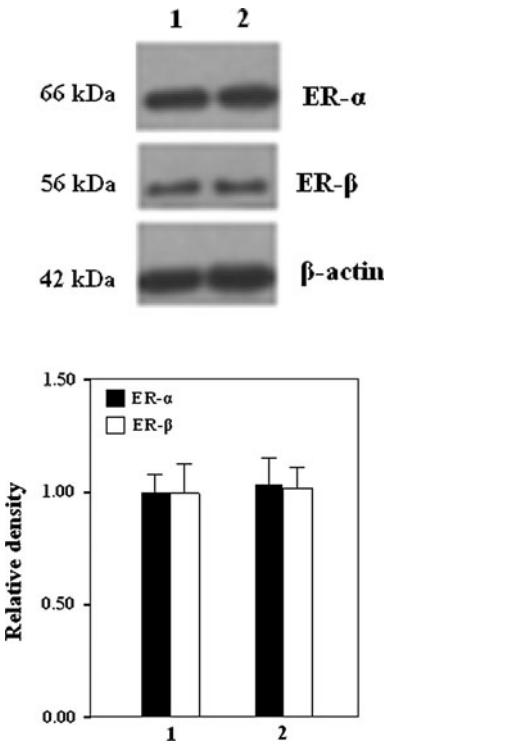

Fig. 5 Expression of estrogen receptors (ERs) in cultured human PSMCs as detected by Western blotting. Whole-cell lysates prepared from cultured human PSMCs were subjected to Western blotting using anti-ER-α, -ER-β, and -β-actin antibodies. 1 and 2 indicate two PSMC cultures isolated from two human prostatic tissue explants. The representative results were shown. Protein bands were quantitated by densitometric analysis and normalized for variations in β-actin protein levels. The ER-α/β-actin or ER-β/β-actin ratio in the PSMC culture 1 was arbitrarily set at 1. Data are means ± SD of three separate experiments

icaritin in human PSMCs (Figs. 1, 2, 3), implying an antagonist-type activity. Moreover, preincubation with 10 μM ICI182780, a specific high-affinity ER antagonist for 3 h caused negligible alteration to treatment of 100 μM icaritin for 72 h (Fig. 7). Thus, the cell growth inhibition and apoptosis related to icaritin treatment could not be associated with ERs.

ERK signaling pathway mediates the effects of icaritin on growth and apoptosis in human PSMCs

Since the icaritin treatment activated the ERK signaling pathway in human PSMCs, we next examined whether the icaritin-induced activation of the ERK signaling pathway plays a role in cell growth and apoptosis. Pretreatment of cell cultures with the ERK inhibitor PD98059 abolished the effects of icaritin on growth and apoptosis (Fig. 7). These data indicate that the growth-inhibiting and the pro-apoptotic effects of icaritin in human PSMCs are mediated by the ERK pathway.

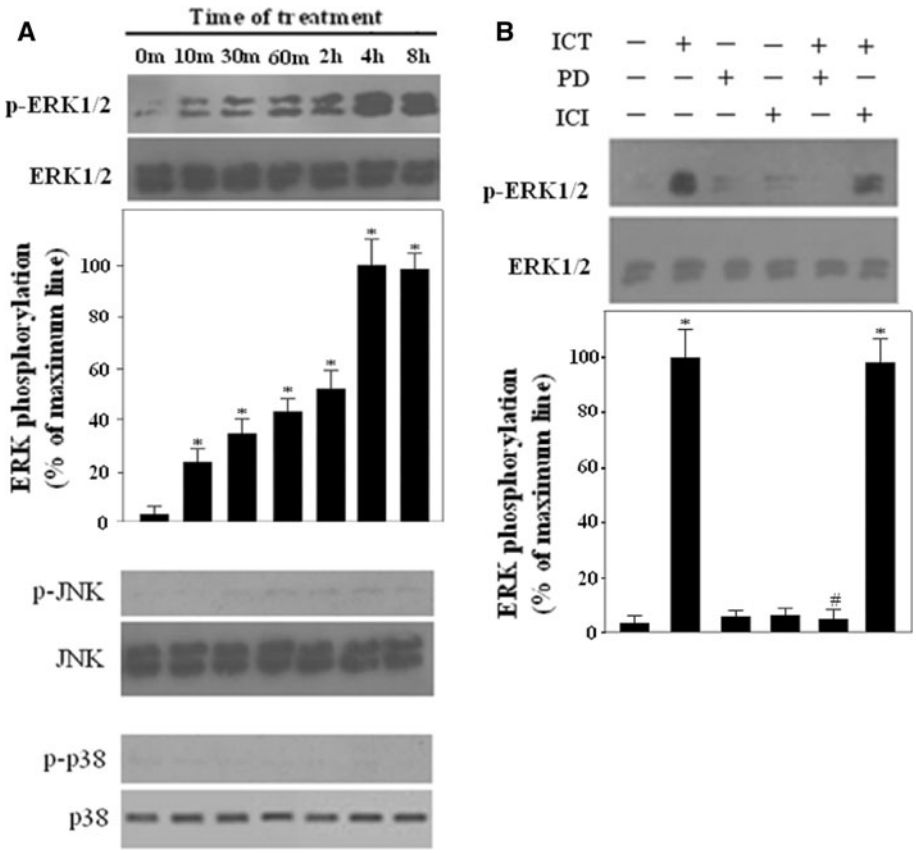

Fig. 6 Effects of icaritin (ICT) on MAPKs activation in human PSMCs. Cell lysates were subjected to Western blotting and incubated with p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, and p38 antibodies. The representative results were shown. p-ERK protein bands were quantitated by densitometric analysis and normalized for variations in ERK protein levels. The *bars* represent the mean ± SD of three separate experiments. **a** Cells were treated with 100 μM ICT for 10 min to 8 h. $^{*}p < 0.05$ versus the control group. **b** Cells were treated with 10 μM PD98059 (PD) or 10 μM ICI182780 (ICI) for 2 h prior to treatment with 100 μM ICT for 4 h. $^{*}p < 0.05$ versus the vehicle-treated group, $^{\#}p < 0.05$ versus the ICT-treated group

Discussion

The central and novel finding of the present study is the identification of anti-BPH efficacy of icaritin in human PSMCs. Icaritin was found to produce a cell growth inhibition and apoptosis via an ER-independent induction of sustained ERK activation.

It is well established that the cyclin-dependent protein kinase CDK4 interacts with cyclin D1 and drives cells from G1 to S phase (Malumbres and Barbacid 2005). The present study shows that icaritin inhibits cell growth by suppressing the expression of cyclin D1 and CDK4. Apoptosis is a tightly regulated physiological process, and the Bax/Bcl-2 ratio is correlated with the extent of apoptosis (Adams and Cory 1998). In our experiments, icaritin treatment resulted in an upregulation of Bax and a downregulation of Bcl-2, leading to an increased ratio of Bax/Bcl-2, which is accepted as a crucial factor in triggering apoptosis (Adams and Cory 1998).

In the present study, we detected ERα and ERβ protein expression in human PSMCs. This is consistent with several reports by other researchers (Konishi et al. 1993; Royuela et al. 2001), who demonstrated that ERs were expressed in the prostate stroma. Icaritin possesses estrogenic activity (Wang et al. 2007). In order to explore the possible role of ER signaling in icaritin-induced growth inhibition and apoptosis of human PSMCs, pure ER antagonist ICI182780 was employed, but pre-treatment with this high-affinity ER inhibitor could not block the effects of icaritin, indicating that they were not directly related to the classical, intracellular ERs.

Membrane-independent genomic signaling involves ligands binding to ER-α and ER-β (Heldring et al. 2007). Non-genomic estrogenic signaling is generally considered to be membrane-dependent and rapid. Membrane associated full-length ER-α exists that is at least in part responsible for such signaling (Razandi et al. 2004). Recent reports show that GPR30 acts as a membrane ER (Prossnitz et al. 2008a, b, introducing an additional receptor responsible for non-genomic estrogen signaling. GPR30 is a seven-transmembrane-domain G protein coupled receptor (GPCR) (Prossnitz et al. 2008a, b). Filardo et al. (2000) show that GPR30 mediates E_2 stimulation of growth factor-dependent responses in a breast cancer cell line lacking both ER-α and ER-β. GPR30 has subsequently been reported to signal via membrane, resulting in intracellular Ca^{2+} mobilization, Src activation, PI3 kinase activation, and ERK1/2 activation (Prossnit z et al. 2008a, b). Recent studies show that GPR30 is expressed in normal prostate stromal cells (Park et al. 2009; Zhang et al. 2008). Phytoestrogens have been reported as capable of binding to GPR30 and activating GPR30-mediated second messengers

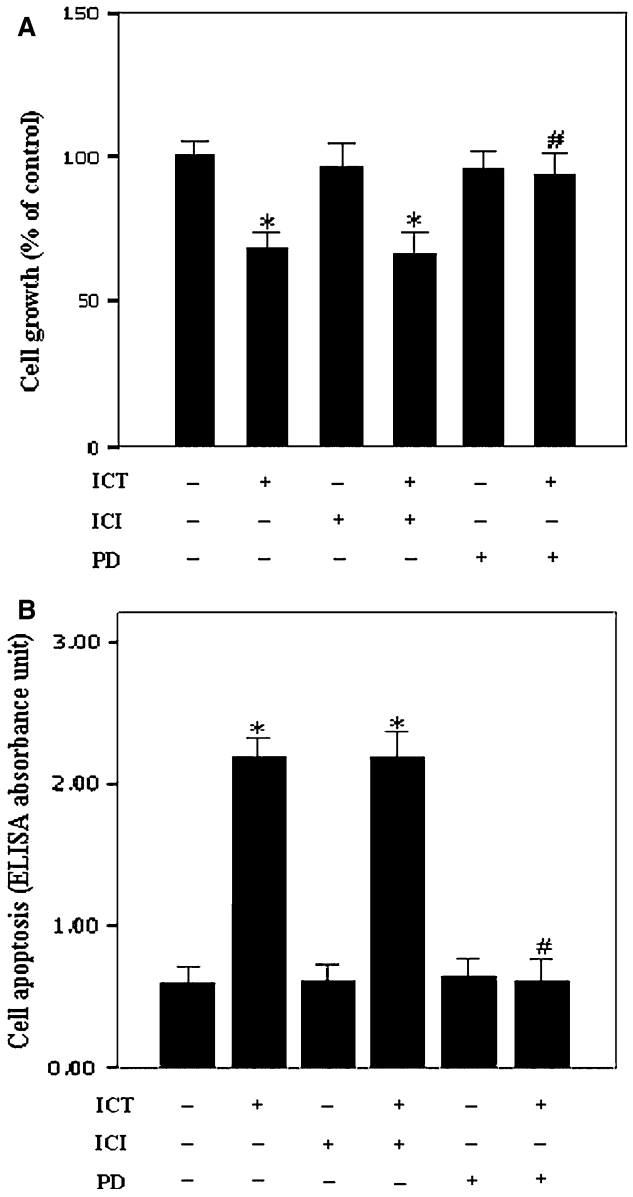

Fig. 7 ERK signaling pathway mediates icaritin-induced growth inhibition and apoptosis of human PSMCs. Cells were incubated with 10 μM ICI182780 (ICI) or 10 μM PD98059 (PD) for 3 h prior to treatment with 100 μM icaritin (ICT) for 72 h. Cell growth was determined by cell counting (**a**) and cell apoptosis was determined by Cell Death Detection Kit, and expressed as ELISA absorbance units (**b**). The *bars* represent the mean ± SD ($n = 4$; $^{*}p < 0.05$ versus the vehicle-treated group, $^{\#}p < 0.05$ versus the ICT-treated group)

in breast or thyroid cancer cells (Thomas and Dong 2006; Vivacqua et al. 2006).

Furthermore, cholesterol is an abundant component of the plasma membranes of eukaryotic cells and plays an essential role in maintaining membrane integrity and fluidity. Recent studies provide evidence that cholesterol plays an important role in signal transduction which is relevant to cell survival or apoptosis (López-Revuelta et al. 2006; Ma 2007; Zhuang et al. 2005). Dysregulation of cholesterol metabolism and accumulation of cholesterol in cancer cells may promote lipid raft synthesis and reduce apoptosis (Ma 2007; Zhuang et al. 2005). Inversely, depletion of cholesterol enhances apoptosis in epithelial, prostate, and cancer cells (Ma 2007; Zhuang et al. 2005). Phytosterols have been shown to promote apoptosis via extracellular signals (Moon et al. 2007). These steroids incorporate into the cell membranes and alter the membrane structure by lowering cholesterol in lipid rafts of the membranes, which may result in cell apoptosis (Awad et al. 2007; Hac-Wydro et al. 2007; Shin et al. 2007). Based on the present data, while there is no evidence for involvement of the classical ER in action of icaritin on human PSMCs, other plasma membrane signaling could be involved, e.g., due to GPR30 or perturbation of cholesterol, which needs to be investigated.

The MAPKs, including the JNK, ERK, and p38, are critically involved in cell growth, differentiation, and apoptosis (Chang and Karin 2001; Blüthgen and Legewie 2008). These enzymes are activated by phosphorylation, and in turn phosphorylate other intracellular enzymes and transcription factors (Chang and Karin 2001; Blüthgen and Legewie 2008). Several studies have shown that icaritin induces activation of ERK and p38 kinase in embryonic stem cells and neuronal cells (Wang et al. 2009b; Wo et al. 2008; Wang et al. 2007). Wang et al. (2009b) reported that icaritin enhanced co-expression of β-tubulin III and choline acetyltransferase in neuronal differentiation from mouse embryonic stem cells via a suppression of p38 phosphorylation and sustained ERK phosphorylation simultaneously, with no involvement of estrogen-like activities. Wo et al. (2008) demonstrated icaritin-induced cardiac differentiation of embryonic stem cells, connected to stimulation of intracellular ROS and followed by activation of ERK and p38 signaling cascades. Wang et al. (2007) showed that ERK pathway was involved in, and partly contributed to, the neuroprotective effects of icaritin against beta amyloid-induced neurotoxicity in primary cultured rat neuronal cells.

The present study indicated that icaritin activated ERK, but not JNK and p38 in human PSMCs. Since icaritin-induced sustained phosphorylation of ERK in PSMCs is not affected by ICI182780, icaritin should activate ERK via an ER-independent pathway. The present findings of activation of ERK by icaritin in human PSMCs and of the cell growth inhibition and apoptosis contrast the general view that ERK activation plays an important role in promoting cell growth signals (Ramos 2008). In general, ERK activation occurs rapidly, and if not protracted can enhance cell survival and proliferation (Fukunaga and Miyamoto 1998). However, a sustained ERK activation induces an apoptotic signal (Stanciu et al. 2000; Narasimhan et al. 2009). This study finds a sustained ERK activation by icaritin, leading to human PSMC growth inhibition and apoptosis. Sustained ERK

activation linked to induction of apoptosis was reported in a number of other studies. This includes the estrogen-induced apoptosis of mature osteoclasts (Chen et al. 2005), the vascular endothelial growth factor-induced apoptosis of cerebral endothelial cells (Narasimhan et al. 2009), and the calcimycin-induced apoptosis of lens epithelial cells (Li et al. 2005). However, the mechanisms underlying cell apoptosis linked to ERK signaling are still not well understood. The mechanisms of ERK activation associated with growth inhibition and apoptosis induction in icaritin-treated PSMCs also need to be clarified.

In the present study, high concentration range (10–100 μM) of icaritin was needed to generate growth inhibitory and pro-apoptotic effects in human PSMCs in vitro. In vivo, even though a recent paper reported that the maximal blood concentrations of icaritin around only 2 μM in rats fed with three doses of a standardized Epimedium (100, 300 and 600 mg/kg body weight) (Wong et al. 2009), its achievable blood concentrations in animal or human after administration with extracted pure icaritin remain unknown. Up to now, the in vivo studies of icaritin are very limited and fail to provide the detailed data concerning the optimal administration method, achievable concentrations, and biological metabolism of icaritin in animals or humans. In future, in vivo studies using animal models would provide more valuable information.

In conclusion, the present results provide experimental evidence that icaritin inhibited cell growth of human PSMCs associated with reductions in cyclin D1 and CDK4 protein expression and stimulated apoptosis of human PSMCs associated with up-regulation of Bax/Bcl-2 ratio. These icaritin growth-inhibitory and pro-apoptotic actions were mediated via the sustained ERK phosphorylation and independent of ER signaling pathway. Therefore, we expect that icaritin could be effective as a therapeutic agent against BPH.

Acknowledgments This work was supported by grants 30672089 from the China National Natural Scientific Foundation.

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