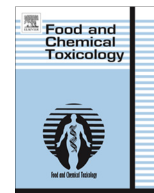
Food and Chemical Toxicology 60 (2013) 424–430

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox



Piperine inhibits the proliferation of human prostate cancer cells via induction of cell cycle arrest and autophagy

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A R T I C L E I N F O

Article history:
Received 7 April 2013
Accepted 4 August 2013
Available online 9 August 2013

Keywords:
Piperine
Prostate cancer cells
Cell cycle arrest
Chloroquine
Autophagy

A B S T R A C T

Piperine, an alkaloid from black and long peppers (*Piper nigrum* Linn & *Piper longum* Linn), has been reported to exhibit antitumor activities *in vitro* and *in vivo*. To further understand the antitumor mechanism of piperine, we investigated the growth inhibitory effects of piperine on human prostate cancer DU145, PC-3 and LNCaP cells. Piperine treatment resulted in a dose-dependent inhibition of the proliferation of these cell lines. Cell cycle arrest at G_0/G_1 was induced and cyclin D1 and cyclin A were downregulated upon piperine treatment. Notably, the level of $p21^{Cip1}$ and $p27^{Kip1}$ was increased dose-dependently by piperine treatment in both LNCaP and DU145 but not in PC-3 cells, in line with more robust cell cycle arrest in the former two cell lines than the latter one. Although piperine induced low levels of apoptosis, it promoted autophagy as evidenced by the increased level of LC3B-II and the formation of LC3B puncta in LNCaP and PC-3 cells. The piperine-induced autophagic flux was further confirmed by assaying LC3-II accumulation and LC3B puncta formation in the presence of chloroquine, a well-known autophagy inhibitor. Taken together, these results indicated that piperine exhibited anti-proliferative effect in human prostate cancer cells by inducing cell cycle arrest and autophagy.

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1. Introduction

Phytochemicals isolated from spices, including peppers, have been suggested to be a reservoir of potential antitumor agents (Lampe, 2003). Piperine is a major plant alkaloid present in black (*Piper nigrum* Linn) and long (*Piper longum* Linn) peppers, which is one of the most common spices used in food and in traditional Indian and Chinese medicine. It has been reported that piperine possesses a broad spectrum of pharmacological properties, including anticonvulsant (D'Hooge et al., 1996), antioxidant (Mittal and Gupta, 2000), anti-inflammatory (Mujumdar et al., 1990; Kim et al., 2012), hepatoprotective (Koul and Kapil, 1993), antimicrobial (Salie et al., 1996), immunomodulatory and anticancer activities (Sunila and Kuttan, 2004). The anticancer effect of piperine may be attributed to the inhibition of NF-κB, c-Fos, ATF-2 and CREB activities (Pradeep and Kuttan, 2004), suppression of angiogenesis by inhibiting Akt phosphorylation (Doucette et al., 2013), or blockade of the production of pro-inflammatory cytokines and the activity of matrix metalloproteinases that are likely to promote tumor growth and metastasis (Hwang et al., 2011; Pradeep and Kuttan, 2002). Piperine also inhibits P-glycoprotein-mediated transport and CYP3A4-mediated drug metabolism, thus increasing the efficacy of antitumor agents (Bhardwaj et al., 2002). For example, dietary piperine has been shown to increase the therapeutic effect of docetaxel against castration-resistant prostate cancer in xenograft animal models (Makhov et al., 2012). Despite this indirect effect of piperine, it remains to be identified whether other action of piperine is involved in its anti-proliferative effect on human prostate cancer cells.

Macroautophagy (hereafter referred to autophagy) is a celluar process that sequesters and engulfs long-lived proteins and damaged organelles into double-membrane autophagic vacuoles (autophagosomes) for degradation (Yorimitsu and Klionsky, 2005). The recycled amino acids are released into the cytosol to provide nutrients and energy for the cell to survive metabolic stresses such as hypoxia and starvation. In some circumstances, autophagy may become a cellular suicide pathway through which the essential intracellular proteins and structures are digested (Gozuacik and Kimchi, 2004). Whether autophagy benefits cancer progression or becomes a cell death mechanism other than apoptosis and necrosis seems to depend on the concrete cell types and stresses (Moretti et al., 2007). For example, autophagy is genetically impaired in DU145 cells due to the loss of wild-type ATG5 protein while it may take

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http://dx.doi.org/10.1016/j.fct.2013.08.007

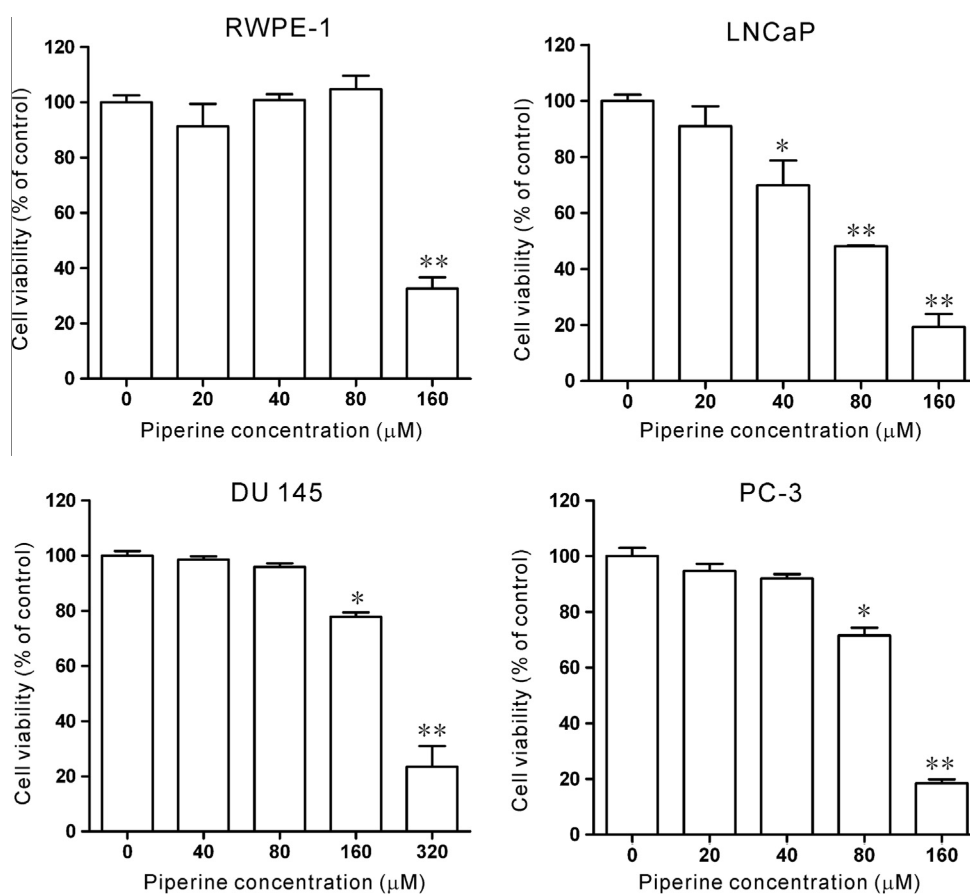

Fig. 1. Effect of piperine on the proliferation of human prostate cancer cells and normal prostate epithelial cells. Cells were treated with indicated concentrations of piperine for 48 h. Cell viability was measured by MTS assay. * $P < 0.05$, ** $P < 0.01$, compared to the control cells by Student's *t*-tests.

place in LNCaP and PC-3 cells (Ouyang et al., 2013). It is of interest whether piperine can induce autophagy in these prostate cancer cells. In view of the fact that LNCaP cells have wild-type p53, and DU145 cells harbor mutated p53 but PC-3 cells have null p53 (Carroll et al., 1993), it is also interesting to evaluate the effect of piperine on cell cycle progression in these human prostate cancer cell lines.

In this study, we investigated the effect of piperine on cell cycle progression and autophagy induction in human prostate cancer LNCaP, DU145 and PC-3 cells. Our results demonstrated that piperine treatment inhibited the proliferation of these cell lines by induction of cell cycle arrest at G_0/G_1 phase and autophagy.

2. Materials and methods

2.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO), propidium iodide (PI), Hoechst 33342, and chloroquine (CQ) were bought from Sigma–Aldrich (St. Louis, MO, USA). Piperine was obtained from Guangzhou Institute for Drug Control (Guangdong, China), dissolved in DMSO and stored at −20 °C. MTS (CellTiter 96 Aqueous ONE solution Cell Proliferation Assay) was a product of Promega (Madison, WI, USA). Antibodies against cyclin A, cycle B1, cyclin D1, cyclin E, $p21^{Cip1}$, $p27^{Kip1}$, androgen receptor (AR), LC3B, cleaved caspse-3 and β-tubulin were purchased from Cell Signaling Technology (Danvers, MA, USA). RNase A was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Cell lines and cell culture

Human prostate cancer cell lines LNCaP, PC-3 and DU145 and prostate epithelial cell RWPE-1 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). LNCaP cells were maintained in RPMI-1640 medium, DU145 cells were cultured in DMEM while PC-3 cells were maintained in DMEM/F12. These media were obtained from Invitrogen (Beijing China) supplemented with 10% fetal bovine serum (FBS) (Invitrogen; Australia), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Carlsbad, CA, USA). RWPE-1 cells were cultured in K-SFM medium containing bull pituitary extract and epithelial growth factor (Invitrogen). All cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

2.3. Cell viability assay

MTS assay was used to assess cell viability. Briefly, cells were seeded in 96-well plates at a density of 3300 cells/well. After 24 h, the cells were treated with indicated concentrations of piperine for 48 h. Then 20 μl of MTS solution was added to each well and further incubated for 1 h at 37 °C. The absorbance at 490 nm was measured by a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA) and the IC_{50} (50% inhibiting concentration) was determined from the growth curve.

2.4. Cell cycle analysis

Analysis of cell cycle and apoptosis (sub-G_1 peak) was performed as previously described (Ouyang et al., 2013). In brief, cells seeded in 6-well plates were incubated with various concentrations of piperine for 24 or 48 h. After treatment, the cells were collected, fixed and stained with phosphate-buffered saline (PBS) containing 50 μg/ml PI and 30 μg/ml of RNase A. DNA content data were acquired using CELLQuest software on a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA) and analyzed using ModFit LT (Verity, Topsham, ME).

2.5. Western blot analysis

Western blotting was performed essentially as described previously (Ouyang et al., 2013). Cells were washed with cooled PBS and lysed with 2× sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Thirty micrograms of total proteins was separated using SDS-PAGE, followed by electrotransfer to polyvinylidene difluoride (PVDF) membranes (Hybond-P; GE Healthcare Life Sciences, USA). The membranes were immunoblotted using primary antibodies. After incubation with horseradish peroxidase (HRP)-labeled secondary antibody, specific bands were visualized by enhanced chemiluminescence kit (BeyoECL Plus; Beyotime, Haimen, China) and recorded on X-ray films (Kodak; Xiamen, China). The densitometry of each band was quantified by FluorChem 8000 (AlphaInnotech; San Leandro, CA, USA).

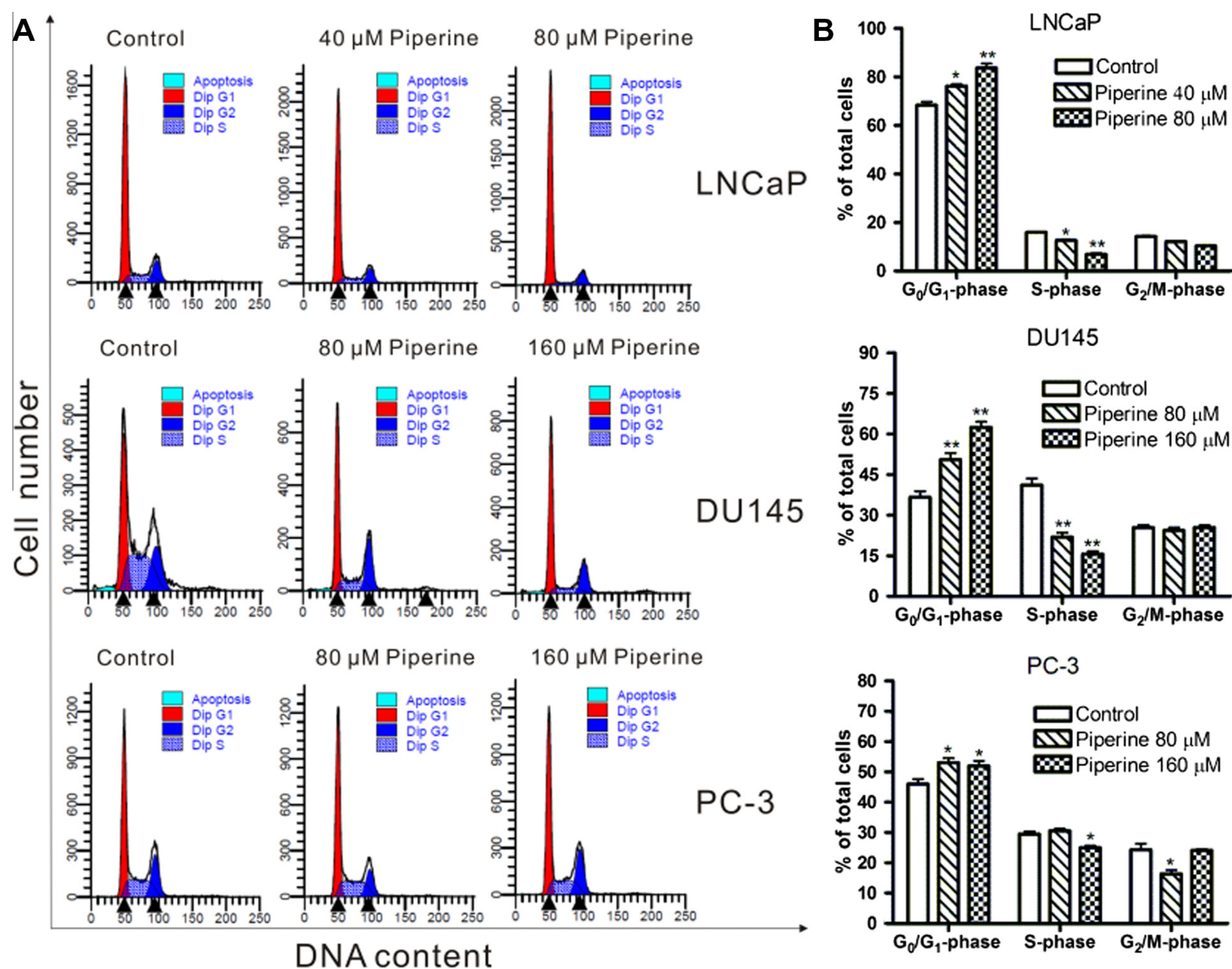

Fig. 2. Effect of piperine on cell cycle distribution of LNCaP, DU145 and PC-3 cells. Cells were treated with piperine for 24 h and the DNA contents were analyzed by flow cytometry. (A) Representative flow cytometric plots of three independent experiments. (B) Analysis of the cell cycle distribution using ModFit LT™ software. Data are presented as mean ± SD. Analysis of statistical significance is performed by comparing the data of piperine treatment groups with that of control group using Student's *t*-tests. * $P < 0.05$, ** $P < 0.01$.

2.6. Immunofluorescence staining

LNCaP or PC-3 cells were planted on glass bottom dishes and incubated at 37 °C overnight. After incubation with indicated reagents, cells were fixed in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS), permeabilized with ice-cold 100% methanol, and immunostained with rabbit anti-LC3B antibody, followed by CF568-conjugated goat–anti-rabbit IgG (Biotium, Hayward, CA, USA). Nuclei were revealed by Hoechst 33342 staining. Fluorescence images were observed and collected under a Leica DMIRB fluorescent microscope (Leica Microsystems, Wetzlar, Germany) armed with a Spinning Disk Confocal Microscopy system (UltraView cooled CCD; Perkin Elmer, Waltham, MA, USA).

2.7. Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA). One-way ANOVA followed by Newman–Keuls post test was used to compare groups, while Student's *t*-test was used to evaluate the differences between drug groups and control groups and a *P*-value < 0.05 was considered significant.

3. Results

3.1. Piperine dose-dependently inhibited the proliferation of prostate cancer cells

To determine the inhibitory effect of piperine on prostate cancer cells, LNCaP, DU145 and PC-3 cells were treated with various concentrations of piperine for 48 h. As shown in Fig. 1, piperine inhibited the growth of all the three cell lines in a dose-dependent manner. The IC_{50} of piperine for LNCaP, DU145 and PC-3 cells were 74.4 μM, 226.6 μM and 111.0 μM, respectively. Compared with androgen-independent DU145 and PC-3 cells, androgen-dependent LNCaP cells were more sensitive to piperine treatment. It was noticed that piperine had no cytotoxicity to normal prostate epithelial RWPE-1 cells at doses lower than 160 μM, suggesting that piperine exhibited certain specific inhibitory effect on prostate cancer cells.

3.2. Piperine induced cell cycle arrest at G_0/G_1-phase in LNCaP, DU145 and PC-3 cells

Next we sought to examine the effect of piperine on cell apoptosis and cell cycle progression by flow cytometry analysis of the DNA contents. The results showed that piperine treatment for 24 h resulted in a significant cell cycle arrest at G_0/G_1-phase ($P < 0.05$) in LNCaP, DU145 and PC-3 cells (Fig. 2A and B). The proportion of cells at G_0/G_1-phase was dose-dependently increased and that of S-phase cells were decreased for LNCaP and DU145 cells. As for PC-3 cells, piperine treatment also arrested the cells at G_0/G_1-phase, though the effect was lower than that of LNCaP and DU145 cells. However, piperine treatment for 24 h did not induce apoptosis (sub-G_0/G_1 phase) in these cell lines, and low proportions of sub-G_0/G_1-phase cells (<3%) were induced only when treated with high dose of piperine (160 μM) for 48 h (Fig. 3A and B). In line with this, only high dose of piperine induced a

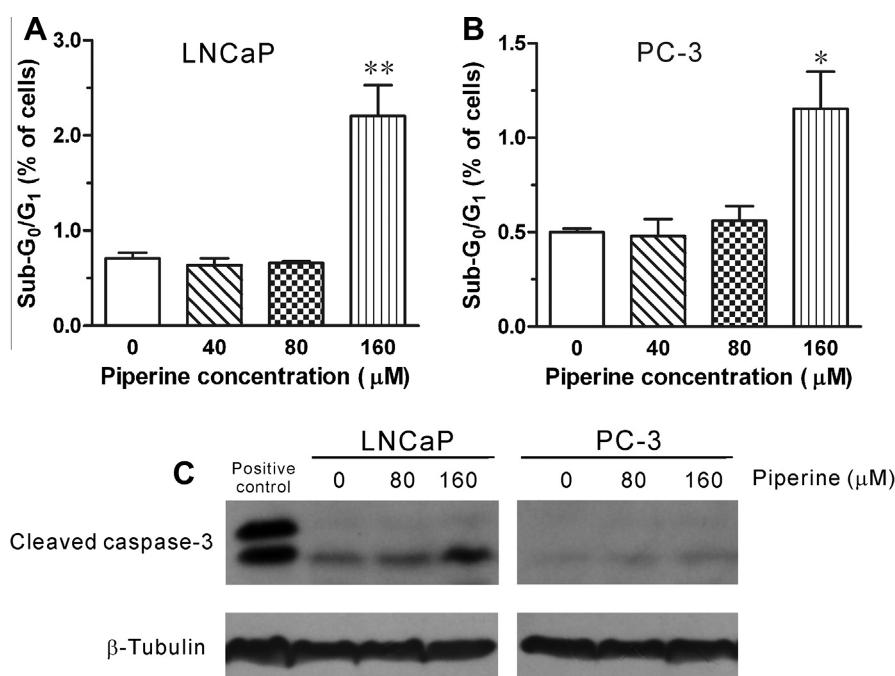

Fig. 3. Piperine induced low levels of apoptosis in prostate cancer cells. (A) LNCaP and PC-3 prostate cancer cells were treated with indicated concentrations of piperine for 48 h and the DNA contents were analyzed by flow cytometry. Sub-G_0/G_1 proportions were calculated by ModFit LT™ software as indicated above. (B) Cleaved caspase-3 was determined by Western blotting with a positive control sample of LNCaP cells, over 10% of which were apoptotic cells (Ren et al., 2012).

slightly-increased level of cleaved caspase-3, an executor of cell apoptosis, in both LNCaP and PC-3 cells (Fig. 3C) as compared with those bands of a positive control sample (Fig. 3C) of LNCaP cells with over 10% apoptosis (Ren et al., 2012). These results indicated that piperine treatment induced cell cycle arrest at G_0/G_1 phase in prostate cancer cells, but its apoptosis-inducing effect was low.

3.3. Piperine upregulated $p21^{Cip1}$ and $p27^{Kip1}$ expression and/or downregulated cyclin D1 and cyclin A in human prostate cancer cell lines

As LNCaP, DU145 and PC-3 are either androgen-dependent or -independent cell lines, we first determined whether piperine treatment influenced androgen receptor (AR) expression in these cells. As shown in Fig. 4A, AR protein was highly expressed in LNCaP cells but was inhibited by piperine at high dose (160 μM). As expected, AR was not expressed in DU145 and PC-3 cells, either in the presence or absence of piperine (Fig. 4A). Next, we evaluated the effect of piperine on cell cycle-related proteins. Cell cycle progression is tightly regulated by cyclin-dependent kinases (CDKs) and cyclins. For example, cyclin D1 regulates cell cycle at G_1 phase while cyclin A regulates at S phase. The CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ regulate the cell cycle progression from G_0 /G_1 phase into S phase, and induction of $p21^{Cip1}$ and $p27^{Kip1}$ may lead to blockade of G_1/S transition (Harper et al., 1993; Grimmler et al., 2007). To explore the mechanism of piperine-induced cell cycle arrest, we examined the expression of $p21^{Cip1}$, $p27^{Kip1}$ and cyclins by western blotting. As shown in Fig. 4, after piperine treatment, the expression of $p21^{Cip1}$ and $p27^{Kip1}$ was increased in LNCaP and DU145 cells, but was hardly detectable in PC-3 cells (Fig. 4B–D). Cyclin D1 was downregulated by piperine in both DU145 and PC-3 cells. While in LNCaP cells, cyclin D1 was upregulated by low dose (40 μM, 80 μM) but downregulated by high dose (160 μM) of piperine. Cyclin A was downregulated by piperine both in LNCaP and PC-3 cells. Cyclin B1 was upregulated by piperine in LNCaP cells while downregulated in PC-3 cells (Fig. 4B and E). These results suggested that piperine induced G_0/G_1-phase cell cycle arrest by increasing the expression of $p21^{Cip1}$ and $p27^{Kip1}$ while decreasing the expression of cyclin D1 and cyclin A.

3.4. Piperine induced autophagy in LNCaP and PC-3 cells

As piperine could only induce low level of apoptosis in the prostate cancer cells, we next examined whether it induced autophagy in LNCaP and PC-3 cells. The conversion of LC3 protein from LC3-I to LC3-II is widely considered as an autophagosomal marker for monitoring autophagy (Miszushima and Yoshimort, 2007; Rubinsztein et al., 2009). Western blot analysis showed that the LC3B-II level was increased by piperine treatment for 24 h in both LNCaP and PC-3 cells (Fig. 5A). No significant formation of LC3B-II could be observed in DU145 cells (data not shown), consistent with the lack of functional ATG5 expression in this cell line (Ouyang et al., 2013). To confirm whether piperine induced autophagic flux, we used lysosomal inhibitor chloroquine (CQ) to inhibit the autophagolysosome degradation during piperine treatment. As shown in Fig. 5A and B, CQ significantly increased the LC3B-II level which reflected the accumulation of autophagosomes due to the inhibition of autophagic flux. Treatment of LNCaP and PC-3 cells with both piperine and CQ further increased the accumulation of LC3B-II compared with piperine or CQ alone, indicating that piperine treatment did induce autophagic flux in these cells. This was further confirmed by the formation of LC3B puncta in LNCaP and PC-3 cells upon piperine treatment as revealed by immunofluorescence (Fig. 5C). With the treatment of piperine in the presence of CQ, the formation of LC3B puncta was significantly enhanced in both cells. Altogether, these data demonstrated that piperine induced autophagy in LNCaP and PC-3 cells.

Fig. 4. Effect of piperine on the expressions of androgen receptors (A) and cell cycle-related proteins (B). (A and B) Western blot analysis was used to determine the protein expression levels after cells were treated with 40, 80 and 160 μM piperine for 24 h. β-Tubulin was used as a loading control. (C–E) Analysis of the relative density of each p21^{Cip1} (C), p27^{Kip1} (D) or cyclin protein (E) bands to that of LNCaP control group (which was set as 1.0). Analysis of statistical significance is performed by comparing the density of piperine-treatment groups with the control group of each cell line by Student's *t*-tests. * $P < 0.05$, ** $P < 0.01$ (C–E).

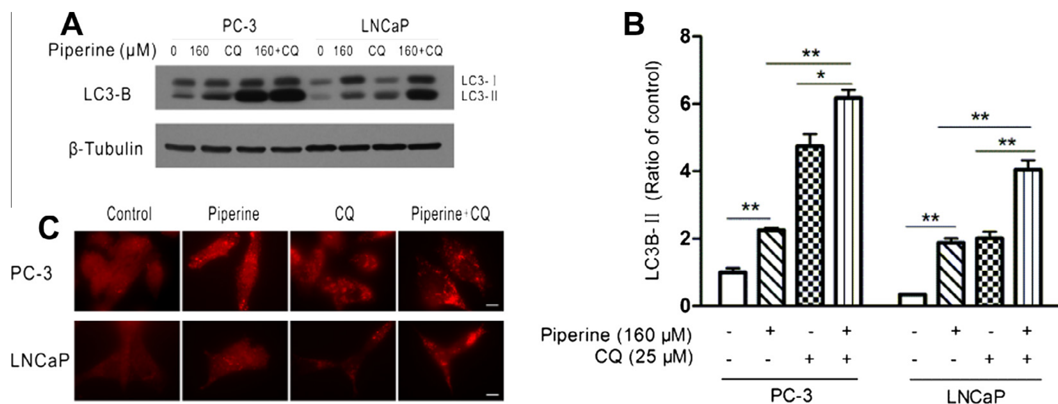

Fig. 5. Piperine induced autophagy in LNCaP and PC-3 cells. Cells were treated with piperine (160 μM), CQ (25 μM) or piperine plus chloroquine (CQ) for 24 h. (A) Western blot analysis of LC3B expression in PC-3 and LNCaP cells. (B) Analysis of the relative density of each LC3B-II band to that of PC-3 control group (which was set as 1.0). Significant differences were compared between treatment groups using Newman–Keuls post test. * $P < 0.05$ and ** $P < 0.01$. (C) Observation of piperine-induced LC3B puncta formation in PC-3 and LNCaP cells after immunofluorescence staining. Bars: 10 μm.

4. Discussion

Recently increasing evidence has indicated that piperine suppresses the growth of cancer cells (Yaffe et al., 2013; Lai et al., 2012). However, the anti-proliferative effect of piperine and its underlying molecular mechanism on human prostate cancer (PCA) cells have not been studied, to our best knowledge. Our results demonstrated that piperine inhibited the proliferation of PCA cells due to the induction of cell cycle arrest by upregulating $p21^{Cip1}$, $p27^{Kip1}$ and/or downregulating cyclin expression. Induction of autophagy by piperine might also contribute to its inhibitory effect on PCA cells.

We found that piperine was able to inhibit the proliferation of human PCA DU145, LNCaP and PC-3 cells in a dose-dependent manner, with the androgen-sensitive LNCaP cells more sensitive than the androgen-insensitive DU145 and PC-3 cells. Since induction of apoptosis and cell cycle could be important targets for cancer chemotherapy (Ahmad et al., 1997; Hartwell and Kastan, 1994), and piperine has been reported to induce apoptosis and cell-cycle arrest in other cancer cells (Lai et al., 2012; Yaffe et al., 2013), we investigated the effect of piperine on cell cycle distribution and apoptosis induction in these PCA cells. Our results demonstrated that piperine treatment induced a significant cell cycle arrest at G_0/G_1 phase in all the three cell types, but its apoptosis-inducing effect was low. This is consistent with previous observation that piperine possesses only weak cytotoxicity (Pradeep and Kuttan, 2002; Sunila and Kuttan, 2004; Bezerra et al., 2005) and inhibited the proliferation and G_1/S transition of human umbilical vein endothelial cells without causing cell death (Doucette et al., 2013). Collectively, these findings indicate that the inhibitory effect of piperine on the growth of human PCA cells may be mainly contributed by the induction of cell cycle arrest. Supporting our data, it has been recently reported that pipernonaline, a piperine derivate from *P. Longum* Linn., also induced G_0/G_1 phase arrest in PCA cells. But pipernonaline is more potential to induce apoptosis in PC-3 cells (Lee et al., 2013) as compared to piperine in this study. It would be interesting to study the structure-function relationship of piperine derivates with regarding their anti-PCA activities.

A critical difference among LNCaP, DU145 and PC-3 cells is that LNCaP cells express a wild-type p53/TP53, while DU145 cells express a mutant one and PC-3 cells are null of p53. It is believed that $p21^{Cip1}$ is an intermediate by which p53 acts as an inhibitor of cellular proliferation in response to DNA damage (El-Deiry et al., 1993). In addition, $p21^{Cip1}$ protein has been shown to inhibit the activity of CDKs and proliferating cell nuclear antigen (PCNA), resulting in cell cycle arrest (Harper et al., 1993; Liu et al., 2003). For example, $p21^{Cip1}$ blocks cell cycle progression at the G_1/S phase in both p53 wild-type (LNCaP), mutated (DU145) or deficient (PC-3) cells (Gotoh et al., 2003). Thus, it seems important to detect $p21^{Cip1}$ expression when comparing the cell cycle arrest in these prostate cancer cell lines. Our data demonstrated that piperine caused a significant increase of $p21^{Cip1}$ in LNCaP and DU145 cells, but not in PC-3 cells, which might account for why piperine treatment caused a significant cell cycle arrest in LNCaP and DU145 cells but only slightly arrested PC-3 cells in G_0/G_1 phase. Consistent with our previous data (Ren et al., 2012), it seems that $p21^{Cip1}$ expression could only be induced by more cytotoxic agents in PC-3 cells as compared to LNCaP and DU145 cells. Moreover, cyclin D1 (a G_1 phase cyclin) and cyclin A (an S phase cyclin) were all decreased by piperine treatment, at least at high doses, in all three human PCA cells. The changes of these cell cycle regulatory proteins were consistent with the results of cell cycle distribution by flow cytometer analysis. Thus, piperine-induced upregulation of $p21^{Cip1}$ protein and downregulation of the cyclins may be responsible for the induction of cell cycle arrest in PCA cells.

Autophagy is regarded as another cause of cell death, though this is still controversial. On the one hand, excessive self-digestion via autophagy may triggers cell death. On the other hand, it may protect the cells from undergoing apoptosis during nutrient stresses (Gozuacik and Kimchi, 2004; Boya et al., 2005). Previously we have demonstrated that autophagy was genetically impaired in DU145 cells (Ouyang et al., 2013). In this study, we showed that autophagy was induced by piperine both in LNCaP and PC-3 cells. Since piperine inhibited the proliferation of all the three PCA cell lines and LNCaP was more sensitive to piperine, our results suggested that induction of autophagy might not be the main cause of piperine that inhibited human PCA cells. However, one recent study suggested that autophagy might contribute to cell cycle arrest in Glioma cells upon resveratrol treatment (Filippi-Chiela et al., 2011). Thus we speculate that piperine-induced autophagy may indirectly inhibit the proliferation of human PCA cells via regulating cell cycle arrest, yet further works are needed to clarify this issue.

As LNCaP, DU145 and PC-3 are either androgen-dependent or -independent cell lines (van Bokhoven et al., 2003), the expression of androgen receptor (AR) may directly influence their growth under piperine treatment. In this study, we observed that AR was highly expressed in LNCaP cells but not in DU145 and PC-3 cells. Although piperine treatment did not change the status in these cells, it did downregulated AR expression in LNCaP cells. This suggests that AR downregulation may be involved in piperine-induced cell growth arrest in LNCaP cells, which warrants further investigation.

In summary, we demonstrated that piperine inhibited the proliferation of the human PCA cells by induction of cell cycle arrest at G_0/G_1 phase, which was likely due to upregulation of $p21^{Cip1}$, $p27^{Kip1}$ and/or downregulation of cyclin D1 and cyclin A as well as induction of autophagy. Our data provided experimental basis for further evaluation of piperine for co-therapy against human prostate cancer.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work is supported by Grants from the Specialized Research Program of "Twelfth Five-Year Plan" of China (No. 2011ZX09307-303-03), the National Natural Science Foundation of China (No. 81173604), and the Fundamental Research Funds for the Central Universities (No. 21612411).

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