Biochemical and Biophysical Research Communications 430 (2013) 406–412

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Pipernonaline from *Piper longum* Linn. induces ROS-mediated apoptosis in human prostate cancer PC-3 cells

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ARTICLE INFO

Article history:
Received 5 November 2012
Available online 15 November 2012

Keywords:
Pipernonaline
Prostate cancer
Cell cycle arrest
Reactive oxygen species
Mitochondrial membrane potential
Ca^{2+} flux

ABSTRACT

The antiproliferation effects of pipernonaline, a piperine derivative, were investigated on human prostate cancer PC-3 cells. It inhibited growth of androgen independent PC-3 and androgen dependent LNCaP prostate cells in a dose-dependent (30–90 μM) and time-dependent (24–48 h) manner. The growth inhibition of PC-3 cells was associated with sub-G_1 and G_0/G_1 accumulation, confirmed by the down-regulation of CDK2, CDK4, cyclin D1 and cyclin E, which are correlated with G_1 phase of cell cycle. Pipernonaline up-regulated cleavage of procaspase-3/PARP, but did not change expression of proapoptotic bax and antiapoptotic bcl-2 proteins. Its caspase-3 activation was confirmed by the caspase-3 assay kit. In addition, pipernonaline caused the production of reactive oxygen species (ROS), increase of intracellular Ca^{2+}, and mitochondrial membrane depolarization, which these phenomena were reversed by *N*-acetylcysteine, a ROS scavenger. The results suggest that pipernonaline exhibits apoptotic properties through ROS production, which causes disruption of mitochondrial function and Ca^{2+} homeostasis and leads to its downstream events including activation of caspase-3 and cleavage of PARP in PC-3 cells. This is the first report of pipernonaline toward the anticancer activity of prostate cancer cells, which provides a role for candidate agent as well as the molecular basis for human prostate cancer.

1. Introduction

The prostate cancer has been the critical health problem because of unacceptably increasing mortality in worldwide [1]. Geographical differences in the incidence of cancer indicate that agricultural, industrial and dietary factors play a critical role in carcinogenesis of a multistep process and a beneficial role in the prevention of certain cancer types [2]. At the time of clinical diagnosis, most prostate cancers represent a mixture of androgen-responsive and androgen-unresponsive cancer cells. Former cancer cells undergo rapid apoptosis on androgen ablation, whereas latter these generally evade apoptosis during androgen withdrawal. Thus it has proved difficult to identify agents that can eradicate these cells without incurring cytotoxicity in uninvolved normal cells [3].

One of the mechanisms involved in cancer cell death is apoptosis, which is a highly regulated process that involves the activation of a series of molecular events [4,5]. Understanding the basic mechanisms of apoptosis in these cancer cells is therefore critical for developing novel therapeutic strategies for prostate carcinoma [6]. Various extracellular stresses, including reactive oxygen species (ROS), ultraviolet radiation, and anticancer agents, have been known to induce apoptosis [7]. In particular, ROS are not only byproducts of mitochondrial respiration but also key signaling molecules that regulate mitochondrial dysfunction [8,9]. In addition, mitochondria manage apoptotic signals that include the following: change in electron transport, loss of mitochondrial membrane potential (MMP, $\Delta\Psi_m$), failure of Ca^{2+} flux homeostasis, generation of ROS, and release of caspase activators [10]. A breakdown in the MMP is an invariant feature of early apoptosis, which precedes DNA fragmentation regardless of the cell type and the apoptotic stimuli [11].

Chemotherapeutic drugs are not favorable for the treatment of malignancy due to their side effects and cytotoxicity in specific tissues. Therefore there is an urgent need to develop a next generation of drugs for prostate cancer, which are equally effective and less

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http://dx.doi.org/10.1016/j.bbrc.2012.11.030

toxic [12]. Medical herbs or medicinal plants may be alternative therapeutic strategies for anticancer agents because they possess a rich source of bioactive chemicals and have fewer side effects. Natural products or their structural derivatives still comprise about 50% of the drugs used in cancer chemotherapy, most of which were discovered through a routine examination of medicinal plants and herbs.

Peppers from the genus *Piper* (Piperaceae) are medicine plants traditionally used by many people of orient countries. Their chemical studies have revealed the occurrence of a variety of bioactive compounds [13]. Pipernonaline (Fig. 1A) is an alkaloid/amide component isolated from the fruit of *Piper* species exhibiting appreciable antihyperlipidemic activity *in vivo* [14], potent cell adhesion inhibition [15], platelet aggregation inhibition [16], acyl CoA:diacylglycerol acyltransferase inhibition [17], and mosquito larvicidal activity [18]. But, compared to some other piperine amide derivatives, pipernonaline has not been thoroughly studied and moreover its precise action mechanism of apoptosis has not been defined yet in prostate cancer cells.

The present study is to observe the effect of pipernonaline on proliferation of two human prostate PC-3 and LNCaP cancer cells and especially characterize the molecular mechanisms underlying its activity via the ROS-mediated apoptosis by observing a series of cellular apoptotic pathways including mitochondrial function and Ca^{2+} homeostasis in PC-3 cells.

2. Materials and methods

2.1. Chemicals

[3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT), *N*-acetylcysteine (NAC), propidium iodide (PI), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 3,3-dihexyloxacarbocyanine ($DiOC_6$) and Fluo-3/AM were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse anti-β-actin monoclonal antibody, rabbit anti-bax polyclonal antibody, mouse anti-bcl-2, and anti-poly (ADP-ribose) polymerase-1 (PARP-1) were also purchased from Sigma Chemical Co. Antibodies for caspase-3 and cyclin D1 were purchased from Cell Signaling (Danvers, MA, USA). Antibodies for CDK2 and cyclin E were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibody of CDK4 was from Stressgen Biotechnologies Co. (Victoria, Canada). Caspase-3 Colorimetric Assay Kit was purchased from R&D Systems Inc. (Minneapolis, MN, USA). The ECL Western blotting kit was purchased from Amersham (Arlington Heights, IL, USA). Pipernonaline was isolated in our laboratory [19] and diluted to the desired concentration in fresh medium just before use.

2.2. Cell line and cell culture

Human prostate cancer cell lines, androgen-independent PC-3 cells and androgen-dependent LNCaP cells, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Korean Cell Line Bank (KCLB, Seoul, Korea), respectively. These cells were maintained and cultured in Dulbecco's modified Eagle's medium (DMEM; WelGENE Inc., Daegu, Korea) and RPMI 1640 (WelGENE Inc.) supplemented with 10% fetal bovine serum (WelGENE Inc.), 100 units/ml of penicillin and 100 μg/ml of streptomycin (WelGENE Inc.), respectively. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were diluted in appropriate medium before each experiment.

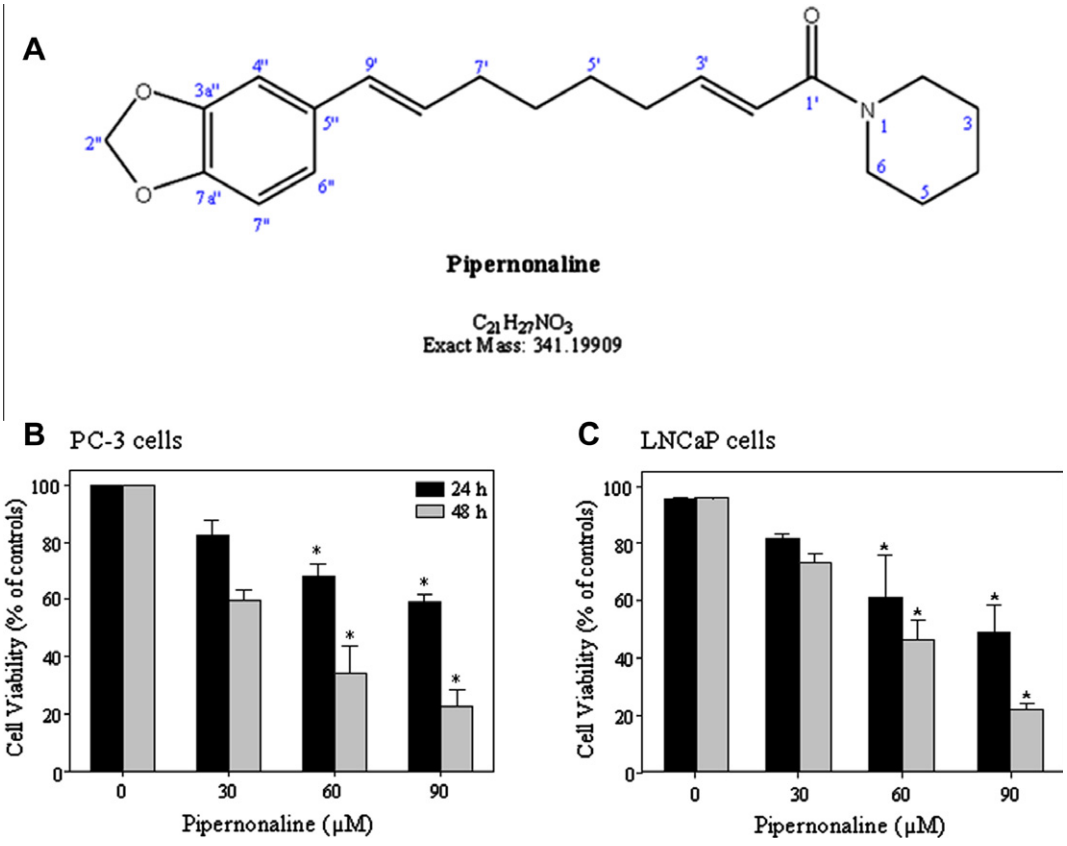

Fig. 1. Pipernonaline inhibits the proliferation and viability of prostate cancer cells. (A) Structure of pipernonaline. (B) PC-3 cells. (C) LNCaP cells. Cells (5×10^4 cells/ml) was treated with 30–90 μM pipernonaline for 24 or 48 h. Cell viability was determined by a MTT assay. Data are presented as mean ± SD ($n = 3$ in each group). $^*p < 0.05$ vs. the control group.

2.3. Cell viability assay

The cytotoxicity of pipernonaline on PC-3 cells and LNCaP cells was measured by a MTT assay [20]. The cells were inoculated at 1×10^4 cells/well in a 48-well plate. After 24 h incubation, the cells were treated to each well with pipernonaline of various concentrations. After the indicated incubation times, the medium was removed and 250 μl of the MTT solution (0.5 mg/ml in PBS) was added to each well for 4 h. Sequently, after the medium was aspirated, 250 μl of dimethyl sulfoxide was added to solubilize the MTT-formazan complex. The plate was analyzed on a $VERSA_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm to determine 50% inhibitory concentration on the growth of cancer cells (IC_{50} value).

2.4. Morphological analysis

Exponentially growing PC-3 cells were incubated at 1×10^5 - cells/well in 24-well plate [21]. After attachment, the cells were incubated in the absence or presence of pipernonaline at various concentrations for 24 h. For the observation by fluorescence microscopy after the medium was removed, the cells were rinsed with PBS, fixed with 4% formaldehyde, and then stained with 1 mg/ml of DAPI solution for 30 min. Stained nuclei were visualized under UV excitation and photographed using a Leica DMRB microscope (Leica Microsystems Holdings, GmbH, Germany).

2.5. Flow cytometric analysis

The cell cycle progression of PC-3 cells was analyzed by flow cytometry as reported previously [22]. The harvested cells were two times washed with PBS, fixed with ice-cold 70% ethanol and stored at 4 °C for overnight. The fixed cells were harvested, washed with PBS and incubated in 100 μl RNase A/PBS (200 μg/ml) at 37 °C for 30 min. Intracellular DNA was labeled with 100 μl PI (1 mg/ml) at 4 °C for 5 min and the stained cells were analyzed on a flow cytometer for relative DNA content, based on increased red fluorescence with a FACScalibur flow cytometer (Becton Dickinson Co., Franklin Lakes, NJ, USA). Data collection and analysis of cell cycle distribution were performed using CellQuest and Modfit software (Becton Dickinson Co.) and mean fluorescence intensity was obtained by histogram statistics.

2.6. Western blot analysis

Cells (1×10^6) were seeded in 6-well plate. After 24 h, the cells were incubated with vehicle or various concentrations of pipernonaline and incubated for 24 h, as described previously [23]. Cells were washed with ice-cold PBS and then lysed in lysis buffer, followed by centrifugation (15,000*g*, 30 min, 4 °C). Forty micrograms of protein were fractionated by 15% SDS–PAGE denaturing gels and then blotted onto PVDF membranes. The membranes were blocked in the 20 mM Tris-buffered saline-0.1% Tween-20 (TBST) buffer containing 5% skim milk at room temperature (RT) for 2 h and then probed with specific primary antibodies overnight at 4 °C. After washing with TBST for 40 min, the membranes were incubated with horse radish peroxidase-conjugated anti-rabbit/mouse IgG for 2 h at RT and then washed with TBST for 40 min. Finally, immunoblot signals were visualized using an ECL Western blotting detection reagent (Amersham).

2.7. In vitro caspase-3 activity

Caspase-3 activity was measured by using a commercial kit (R&D Systems Inc.) based on the cleavage of Asp-Glu-Val-Asp (DEVD)-*p*NA. Briefly, cells (2×10^6 cells/ml) treated with pipernonaline were lysed by incubation in cell lysis buffer on ice for 10 min and then centrifuged at 10,000*g* for 1 min. Enzyme reactions were carried out in a 96-well plate. Fifty microliter of cell lysate (200 μg of total protein) was added to each reaction mixture. The resultant fluorescence was measured at excitation and emission wavelengths of 400 and 505 nm respectively, on a microplate reader. The results were expressed as fold of the specific activity (UI/mg protein) of caspase-3.

2.8. Analysis of reactive oxygen species (ROS)

Intracellular ROS generation was measured using a DCFH-DA fluorescent dye. After treatment with an appropriate concentration of pipernonaline for 24 h, the cells were incubated with 10 μM DCFH-DA at 37 °C for 30 min and then washed twice with PBS. For each experiment, the cells were analyzed for fluorescence using a flow cytometer.

2.9. Analysis of the mitochondrial membrane potential (MMP)

MMPs ($\Delta\Psi_m$) were determined by the retention of the dye $DiOC_6$. Cells were collected 24 h after treatment with an appropriate concentration of pipernonaline and incubated with 100 nM $DiOC_6$ at 37 °C for 30 min. Cells were washed twice with PBS and analyzed with flow cytometry.

2.10. Analysis of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} levels were determined with the Ca^{2+}-sensitive fluorescence dye Fluo-3/AM. After exposure to pipernonaline at various concentrations for 24 h, cells were centrifuged and washed twice with PBS. And the cells were incubated with 5 μM Fluo-3/AM at 37 °C for 30 min. Then the cells were washed and subjected to flow cytometer.

2.11. Statistical analysis

Experiments were repeated at least 3 times with consistent results. Unless otherwise stated, data are expressed as the mean ± SD. ANOVA was used to compare the experimental groups with the control, whereas comparisons among multiple groups were performed using a Tukey's multiple comparison test. Results were statistically significant at $p < 0.05$.

3. Results

3.1. Pipernonaline inhibits proliferation of human prostate cancer cells

To determine the effect of pipernonaline on cell viability, we performed a MTT assay. The androgen-independent PC-3 cells and the androgen-dependent LNCaP cells were treated with various concentrations of pipernonaline at different time points. Pipernonaline induced a remarkable dose- and time-dependent reduction of cell viability (Fig. 1B and C). After 48 h exposure, viability underwent a clear decline at 30 and 90 μM ($p < 0.05$). Based on data collected from two independent experiments in duplicate, the IC_{50} value (50% growth inhibitory concentration) at 48 h could be estimated as 40 μM for PC-3 cells. We further investigated the pipernonaline-mediated antiproliferation mechanisms in hormone-independent PC-3 cells, which are resistant to chemotherapeutic treatment.

3.2. Pipernonaline induces cell cycle arrest in G_0/G_1 phase and accumulation in sub-G_1 phase in PC-3 cells

An effect of pipernonaline on cell cycle distribution was determined by using flow cytometry. Pipernonaline increased a significant cell population in G_0/G_1 phase at a concentration of 30 and 60 μM and a dramatic hypodiploid (sub-G_1) populations at even 90 μM for 24 h in PC-3 cells were shown in Fig. 2A, indicating that the arrested PC-3 cells enter into apoptosis. Our data thus implied that pipernonaline inhibits growth of PC-3 by blocking G_0/G_1 phase cell transition and inducing apoptosis. Therefore we further examined expression of target molecules related to cell cycle progression critical for G_1/S transition such as cyclins and cyclin dependent kinases (CDKs). After treatment with 30–90 μM pipernonaline for 24 h, their whole cell lysates were subjected to Western blot analysis (Fig. 2B and C). Compared to the β-actin control, pipernonaline decreased the levels of cyclin D1, cyclin E, CDK2 and CDK4, which are correlated with the cell cycle arrest of G_1/S phase transition. These observations suggested the roles for cyclins and CDKs in the mechanism underlying pipernonaline-mediated cell cycle arrest in G_0/G_1 phase.

3.3. Pipernonaline affects cytomorphology of PC-3 cells

In cytomorphological observation, pipernonaline caused the growth-inhibitory phenomena in PC-3 cells (Fig. 3A, upper panel). Untreated PC-3 cells showed a typical feature with adherent, homogeneous, and spanning morphology after 24 h culture under the phase contrast microscope. But the PC-3 cells treated with 60 and 90 μM pipernonaline for 24 h were changed to a non-adherent, detached, and round morphology in a dose-dependent manner. The further cytomorphological changes were examined by using fluorescence microscopy after staining with the fluorescent dye DAPI (Fig. 3A, lower panel). The nuclei of untreated PC-3 cells were round and homogeneous but the cells treated with pipernonaline displayed the representative apoptotic characteristics, indication cell shrinkage, vacuolization, and nuclei condensation. These morphological observations suggested that pipernonaline might reduce overall PC-3 cell numbers by inducing apoptosis and therefore be a powerful inducer of cell death in PC-3 cells.

3.4. Pipernonaline does not regulate the expression of bcl-2 family proteins in PC-3 cells

The bcl-2 family proteins play the key roles in regulation of apoptosis by functioning as either promoters (bax and bak) or inhibitors (bcl-2 and bcl-xL) of the cell death process. To determine whether the antiproliferative activity of pipernonaline is correlated with the expression of apoptotic proteins, levels of bcl-2 family protein was examined. As a result, 30–90 μM pipernonaline did not affect any changes in the expression of bcl-2 family proteins, bax protein and bcl-2 protein (Fig. 3B). These results suggest that pipernonaline induces cell death by other apoptotic protein different from bcl-2 family proteins.

3.5. Pipernonaline induces the cleavage of procaspase-3 proteins and poly-(ADP-ribose) polymerase (PARP) in PC-3 cells

In the cytosol, cytochrome c binds to Apaf-1, recruits and activates procaspase-9 in the apoptosome. Active caspase-9 cleaves

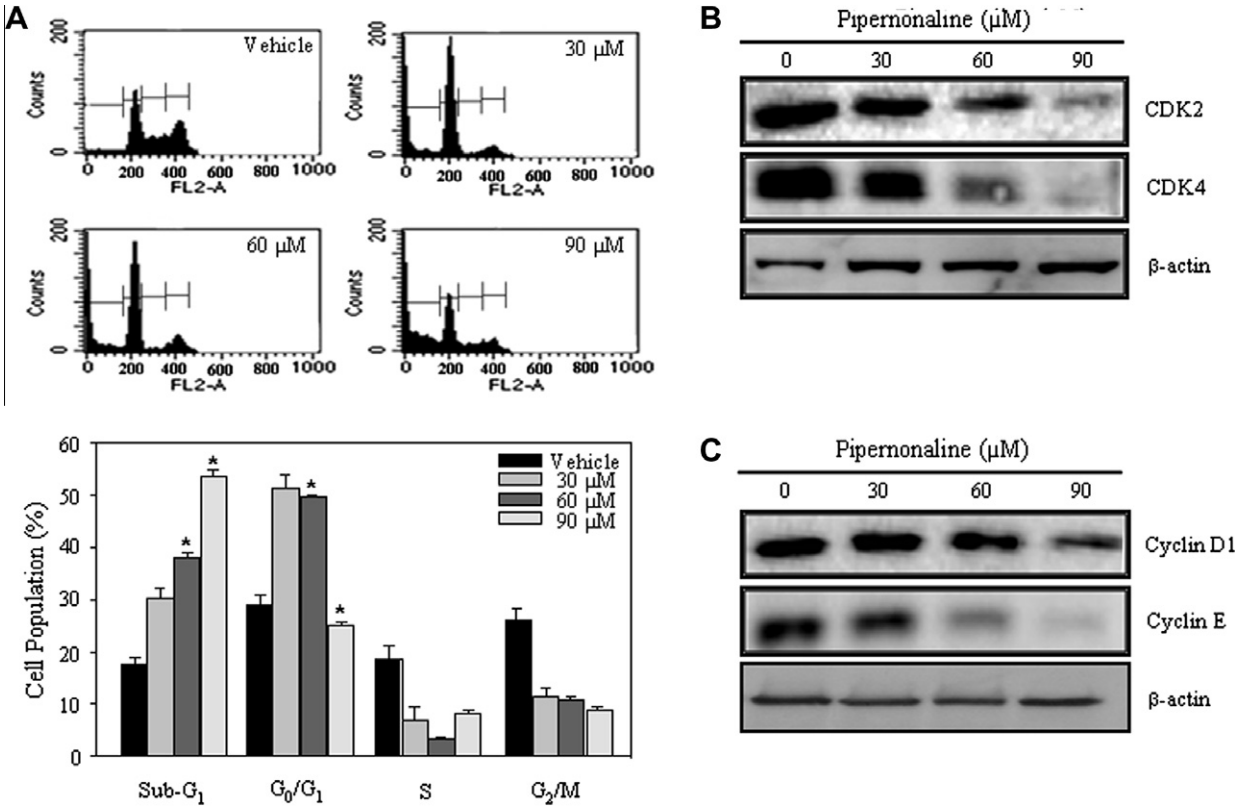

Fig. 2. Pipernonaline regulates cell cycle distribution in PC-3 cells. (A) Cell cycle arrest. PC-3 cells were seeded at 1×10^4 cells/well in 6-well plate and exposed to 30–90 μM pipernonaline for 24 h. Cells were harvested, washed, fixed with ice-cold 70% (v/v) ethanol, and stained with PI (1 mg/ml), followed by flow cytometry and cell cycle distribution was analyzed and graphed. Data are presented as mean ± SD ($n = 3$, each group). $^*p < 0.05$ vs. control group. (B) CDK2 and CDK4 expression. (C) Cyclin D1 and cyclin E expression. Cells were exposed to 30–90 μM pipernonaline for 24 h. Total cell lysates were electrophoretically separated on 15% polyacrylamide gel and immunoblotted with an antibody against each protein and β-actin was used as the internal control.

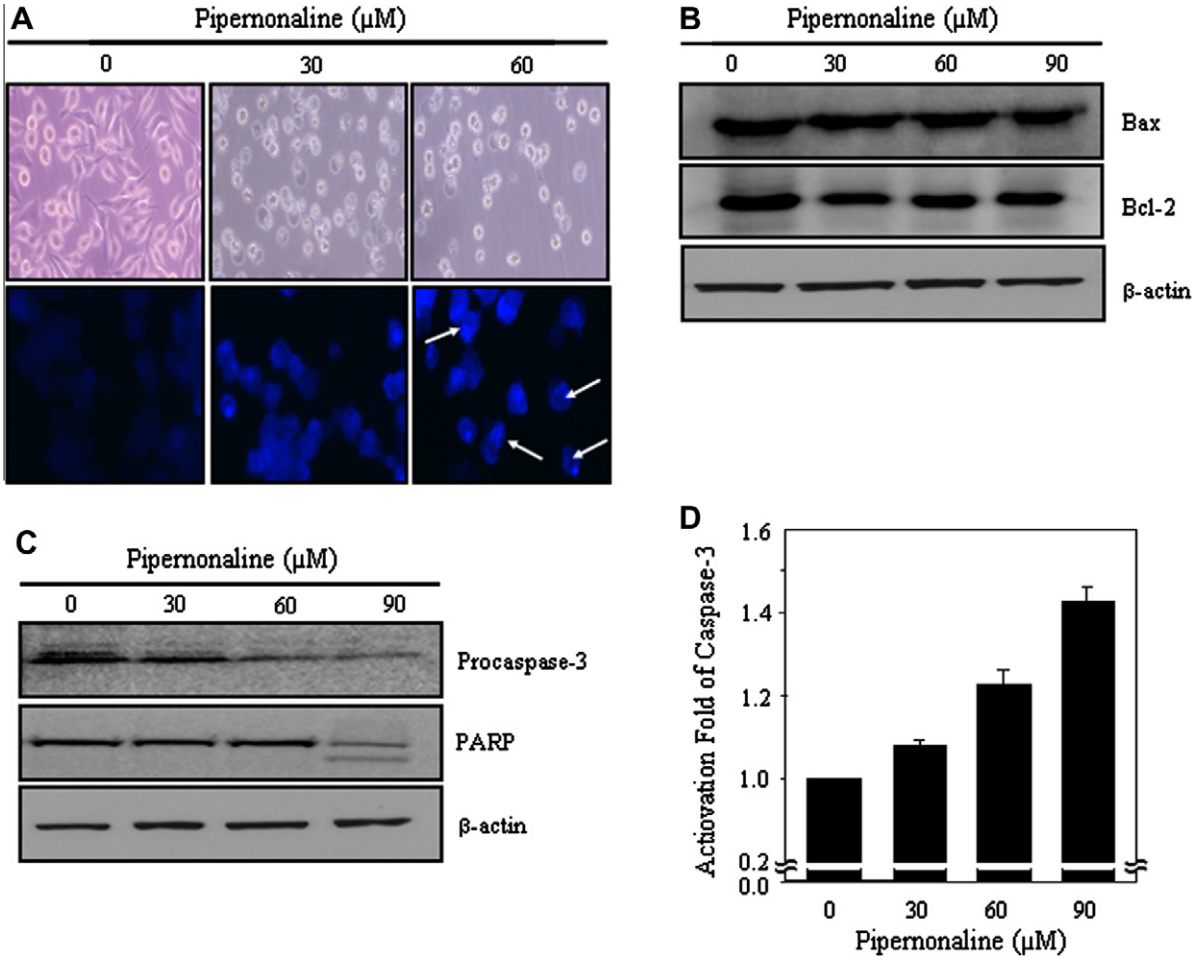

Fig. 3. Pipernonaline induces apoptosis in PC-3 cells. (A) Cytomorphological changes. After treatment with 60 and 90 μM pipernonaline for 24 h, apoptotic cells were detected by a fluorescence microscopy (400×), based on nuclear morphology change after DAPI staining at 37 °C for 30 min. Arrows show chromatin condensation and nuclear fragmentation by high concentrations of pipernonaline. (B) Bax and Bcl-2 expression. (C) Procaspase-3 and poly (ADP-ribose) polymerase (PARP) expression. Cells were exposed to 30–90 μM pipernonaline for 24 h. Total cell lysates were electrophoretically separated on 15% polyacrylamide gel and immunoblotted with an antibody against each protein and β-actin was used as the internal control. (D) Caspase-3 activity. The caspase-3 activity was determined by a colorimetric assay kit with a specific substrate (Ac-DEVD-pNA). Data are presented as mean ± SD ($n = 3$ in each group). $^{*}p < 0.05$ vs. the control group.

and activates executer caspases, including caspase-3, which in turn break a broad spectrum of cellular target proteins such as PARP, thus leading to cell death. Therefore, the effect of pipernonaline on the activation of caspase-3 and the cleavage of PARP, the well-characterized caspase-3 substrate, was observed. Treatment of PC-3 cells with 90 μM pipernonaline for 24 h resulted in a significant increase in the cleavage of procaspase-3 and PARP, compared to untreated cells (Fig. 3C). Also, caspase-3 activation induced by pipernonaline was confirmed by using a assay kit, showing a dose-dependent increase in the caspase-3 activity at 30–90 μM (Fig. 3D). Taken together, pipernonaline was deeply involved in apoptosis via eliciting a series of apoptotic cascade activation that are related to the apoptotic caspase-3 activation.

3.6. Pipernonaline induces production of reactive oxygen species (ROS) in PC-3 cells

In a variety of cell types, cancer chemotherapy induced tumor cell death in part by inducing the production of ROS [8]. To investigate whether the production of ROS is involved in pipernonaline-induced apoptosis of PC-3 cells, the state of ROS after pipernonaline treatment for 24 h was examined by using DCHF-DA fluorescence intensity. As shown in Fig. 4A, the production of ROS was increased in a dose-dependent manner. To determine the dependency of ROS in pipernonaline-induced apoptosis, we utilized *N*-acetylcysteine (NAC), a ROS scavenger. The application of 10 mM of NAC caused a dramatic recovery of ROS production by treatment with 30 and 90 μM pipernonaline, consistent with recovery of apoptotic cell death by 10 mM NAC (data not shown). The results indicated that ROS production plays an important role in the pipernonaline-induced apoptosis of PC-3 cells.

3.7. Pipernonaline affects mitochondrial membrane function and intracellular Ca^{2+} flux of ROS downstream in PC-3 cells

ROS is involved in aspects of mitochondrial dysfunctions such as the induction of permeability transition and disruption of intracellular calcium homeostasis is one of the characteristic events associated with mitochondrial membrane disruption and apoptosis [9]. To confirm whether pipernonaline-induced ROS production has an effect on its downstream events in PC-3 cells, mitochondrial membrane potential (MMP) and intracellular Ca^{2+} concentrations were determined. As shown in Fig. 4B, a decrease in MMP, depolarization, was detected in PC-3 cells treated with 30 and 90 μM by a flow cytometry analysis using $DiOC_6$ fluorescence dye staining. In addition, these occurrences were significantly blocked by pretreatment with 10 mM NAC (Fig. 4B). Also the fluorescence emitted from PC-3 cells treated with 30 and 90 μM, by a flow cytometry

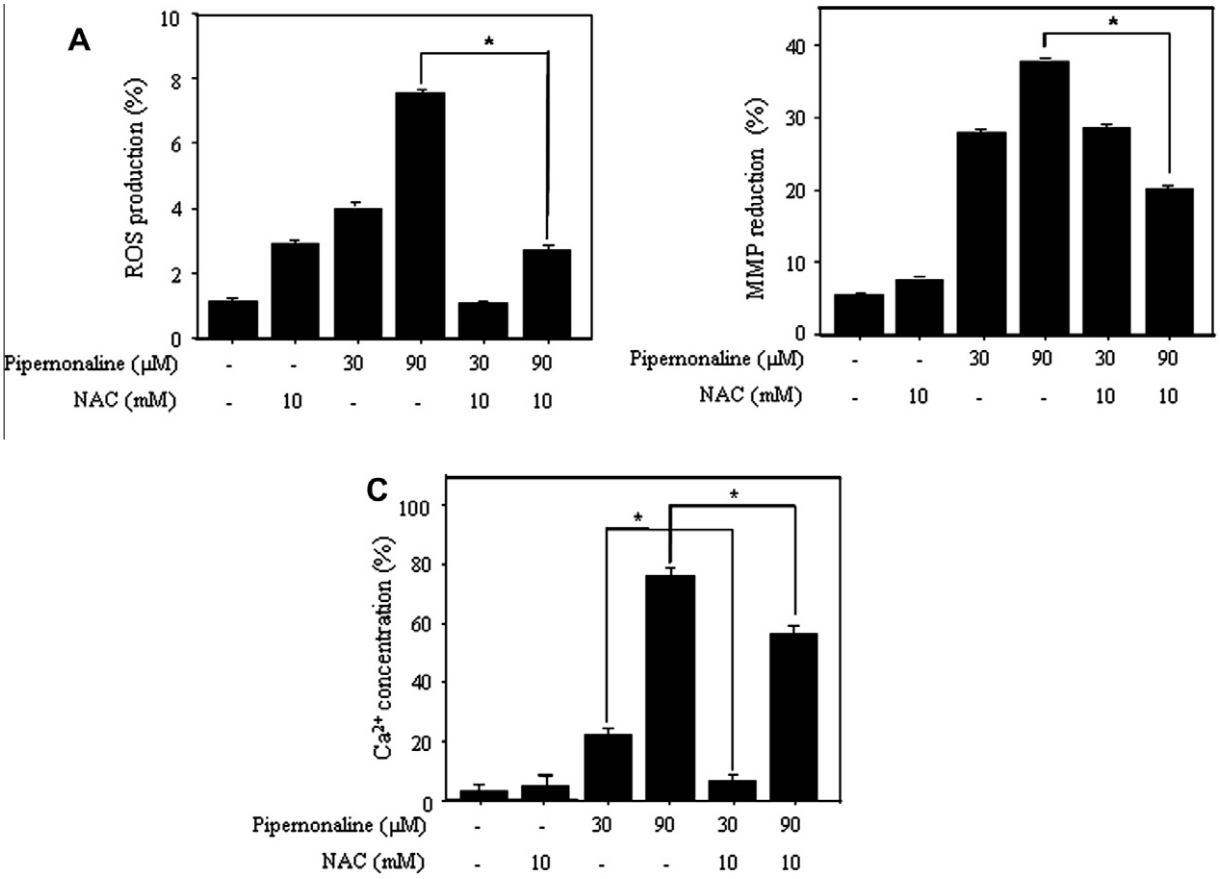

Fig. 4. Pipernonaline induces oxidative stresses to activate intracellular events in PC-3 cells. (A) Reactive oxygen species (ROS). (B) Mitochondrial membrane potential (MMP). (C) Intracellular Ca_i^{2+} concentration. Cells were treated with 30 and 90 μM pipernonaline for 24 h in the presence or absence of prior 1 h incubation with 10 mM *N*-acetylcysteine (NAC). The dichlorodihydrofluorescein (DCF), $DiOC_6$, Fluo-3/AM and fluorescence intensities were detected by flow cytometry analysis for ROS production, MMP changes, and Ca_i^{2+} concentration, respectively. Data are presented as mean ± SD ($n = 3$ in each group). $^*p < 0.05$ vs. the control group.

analysis using Fluo-3/AM fluorescence dye staining, was shifted to a higher intensity, indicating pipernonaline induced surge of the intracellular Ca_i^{2+} concentration (Fig. 4C). The rise in the Ca_i^{2+} concentration was blocked when the cells were pretreated with 10 mM NAC and this blockade was statistically significant (Fig. 4C). Finally these findings suggest that ROS production and the resulting oxidant stress as key player of apoptosis are involved in mitochondrial depolarization caused to mitochondrial membrane dysfunction and intracellular elevation to intracellular Ca_i^{2+} homeostasis disruption, leading to caspase-3 activation and consequently pipernonaline-induced apoptosis.

4. Discussion

Previously, pipernonaline showed antiproliferative effects against two prostate cancer cells, androgen-independent PC-3 and androgen-dependent LNCaP [19]. This information prompted us to further study the molecular mechanism behind pipernonaline activity in prostate cancer cells. In this study, we conducted an unprecedented investigation of how pipernonaline acts against human prostate cells, for the first time. Flow cytometric analysis showed cells arrest in the G_0/G_1 phase and subsequent cell accumulation in the sub-G_1 phase by pipernonaline. The arrest at a specific point (G_1 point) of the cell cycle means the formulation of a specific, programmed signaling response. Western blots revealed a dose-dependent decrease of cyclins/CDKs expression in the pipernonaline-treated PC-3 cells. These observations showed that pipernonaline arrests PC-3 cells at the G_0/G_1 phase by directly modulating cyclin/CDK complexes, which prevented them from entering synthesis and its growth inhibition is associated with sub-G_1 and G_0/G_1 cell accumulation.

Furthermore, it was investigated the major apoptotic molecules to understand the antiproliferative mechanism of pipernonaline at cellular and molecular levels. Western blot revealed a dose-dependent cleavage of procaspase-3 and PARP but no significant changes of bax and bcl-2 critical for apoptosis in pipernonaline-treated PC-3 cells. Based on the above phenomena, it was suggested that caspase-3 activation and its downstream PARP cleavage are involved in apoptosis process of PC-3 cells induced by pipernonaline, but not bax and bcl-2 level regulation.

Further researches were focused on the upstream factors and signaling pathway leading to caspase-3 activation and PARP cleavage. Some studies have reported that cancer chemopreventive agents induce apoptosis in part through ROS generation and disruption of redox homeostasis [8]. ROS act as secondary messengers because of their ability to influence the MMP and mitochondrial function, mediate the elevation of intracellular Ca^{2+}, and lead to the activation of the caspase cascade [9]. Pipernonaline induced the generation of ROS and in contrast antioxidant agent NAC significantly suppressed their production and recovered ROS-derived apoptosis in PC-3 cells. On the other hands, ROS production by pipernonaline caused to an effect on its downstream events, yielding to mitochondrial membrane depolarization and surge of intracellular Ca^{2+}, which these occurrences were significantly blocked and recovered by pretreatment with NAC. Therefore, pipernonaline-induced apoptosis might be mediated through ROS production, which subsequently commits the cell to death due to the decrease of MMP and Ca^{2+} flux.

In conclusion, the action mechanisms of pipernonaline-induced apoptosis were hypothesized to function through enhanced production of ROS in human prostate carcinoma PC-3 cells, which is located at upstream of mitochondrial dysfunction and Ca^{2+} event. Therefore these results present the molecular basis for potent leading antitumor agents to human prostate cancer and also pipernonaline may be a promising candidate as anticancer drug from *Piper* species medicinal herbs.

Acknowledgment

This study was supported by Medical Research Institute Grant (2008-17-00), Pusan National University.

References

[1] A. Jemal, F. Bray, M.M. Center, et al., Global cancer statistics, CA Cancer J. Clin. 61 (2011) 69–90.
[2] L. Reddy, B. Odhav, K.D. Bhoola, Natural products for cancer prevention: a global perspective, Pharmacol. Ther. 99 (2003) 1–13.
[3] M. Gleave, N. Bruchovsky, S.L. Goldenberg, et al., Intermittent androgen suppression for prostate cancer: rationale and clinical experience, Eur. Urol. 34 (Suppl. 3) (1998) 37–41.
[4] A. Degterev, J. Yuan, Expansion and evolution of cell death programmes, Nat. Rev. Mol. Cell Biol. 9 (2008) 378–390.
[5] K.M. Hajra, J.R. Liu, Apoptosome dysfunction in human cancer, Apoptosis 9 (2004) 691–704.
[6] X. Jing, N. Ueki, J. Cheng, et al., Induction of apoptosis in hepatocellular carcinoma cell lines by emodin, Jpn. J. Cancer Res. 93 (2002) 874–882.
[7] Z.B. Wang, Y.Q. Liu, Y. Zhang, et al., Reactive oxygen species, but not mitochondrial membrane potential, is associated with radiation-induced apoptosis of AHH-1 human lymphoblastoid cells, Cell Biol. Int. 31 (2007) 1353–1358.
[8] Y.H. Ling, L. Liebes, Y. Zou, et al., Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic response to Bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells, J. Biol. Chem. 278 (2003) 33714–33723.
[9] G. Paradies, G. Petrosillo, M. Pistolese, et al., Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage, Gene 286 (2002) 135–141.
[10] G. Kroemer, B. Dallaporta, M. Resche-Rigon, The mitochondrial death/life regulator in apoptosis and necrosis, Annu. Rev. Physiol. 60 (1998) 619–642.
[11] M.J. Liu, Z. Wang, H.X. Li, et al., Mitochondrial dysfunction as an early event in the process of apoptosis induced by woodfordin I in human leukemia K562 cells, Toxicol. Appl. Pharmacol. 194 (2004) 141–155.
[12] J. Mann, Natural products in cancer chemotherapy: past, present and future, Nat. Rev. Cancer 2 (2002) 143–148.
[13] S. Kumar, J. Kamboj, Suman, et al., Overview for various aspects of the health benefits of *Piper longum* linn fruit, J. Acupunct. Meridian Stud. 4 (2011) 134–140.
[14] Z. Jin, G. Borjihan, R. Zhao, et al., Antihyperlipidemic compounds from the fruit of *Piper longum* L, Phytother. Res. 23 (2009) 1194–1196.
[15] S.W. Lee, Y.K. Kim, K. Kim, et al., Alkamides from the fruits of *Piper longum* and *Piper nigrum* displaying potent cell adhesion inhibition, Bioorg. Med. Chem. Lett. 18 (2008) 4544–4546.
[16] B.S. Park, D.J. Son, Y.H. Park, et al., Antiplatelet effects of acidamides isolated from the fruits of *Piper longum* L, Phytomedicine 14 (2007) 853–855.
[17] S.W. Lee, M.C. Rho, H.R. Park, et al., Inhibition of diacylglycerol acyltransferase by alkamides isolated from the fruits of *Piper longum* and *Piper nigrum*, J. Agric. Food Chem. 54 (2006) 9759–9763.
[18] Y.C. Yang, S.G. Lee, H.K. Lee, et al., A piperidine amide extracted from *Piper longum* L fruit shows activity against Aedes aegypti mosquito larvae, J. Agric. Food Chem. 50 (2002) 3765–3767.
[19] K.Y. Kim, Y.J. Kim, W. Lee, et al., Purification and identification of apoptosis modulator pipernonaline from *Piper longum* Linn against prostate cancer cells, J. Life Sci. 15 (2009) 671–675.
[20] E.H. Kong, Y.J. Kim, Y.J. Kim, et al., Piplartine induces caspase-mediated apoptosis in PC-3 human prostate cancer cells, Oncol. Rep. 20 (2008) 785–792.
[21] C. Perez-Stable, 2-Methoxyestradiol and paclitaxel have similar effects on the cell cycle and induction of apoptosis in prostate cancer cells, Cancer Lett. 231 (2006) 49–64.
[22] G. Wang, H. Chen, M. Huang, et al., Methyl protodioscin induces G2/M cell cycle arrest and apoptosis in HepG2 liver cancer cells, Cancer Lett. 241 (2006) 102–109.
[23] K.Y. Kim, S.N. Yu, S.Y. Lee, et al., Salinomycin-induced apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization, Biochem. Biophys. Res. Commun. 413 (2011) 80–86.