行政院國家科學委員會專題研究計畫 成果報告

Labedipinedilol-A 對人類攝護腺細胞抑制增生及誘發細胞凋亡之機制探討

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行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

Labedipinedilol-A 對人類攝護腺細胞抑制增生及誘發細胞凋亡之機制探討

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Labedipinedilol-A 對人類攝護腺細胞抑制增生及誘發細胞凋亡之機制探討

Mechanisms of anti-proliferation and apoptosis induced by labedipinedilol-A in human prostate cell

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一、中文摘要

Labedipinedilol-A 是一新型的鈣離子阻斷劑並具有阻斷α1-腎上腺素受體的活性，先前實驗證明labedipinedilol-A 對於培養的老鼠血管平滑肌細胞有抑制細胞增生與細胞遷徙的作用。在本計畫中，我們進行一連串的離體實驗來評估攝護腺細胞對labedipinedilol-A 的反應，以了解此選擇性α_{1A}-腎上腺素受體阻斷劑抑制細胞增生與誘發細胞凋亡對於降低攝護腺細胞生長的影響。，我們分別培養人類正常攝護腺上皮細胞及人類攝護腺癌細胞並測試labedipinedilol-A 對細胞增生和誘發細胞凋亡的活性。結果發現 labedipinedilol-A可以有效地藉由G_0/G_1細胞週期停滯和減少DNA合成來抑制細胞增生。在細胞週期停滯方面，labedipinedilol-A可以降低cyclin D1/2、cyclin E、Cdk2、Cdk4、Cdk6 的表現，同時增加p21、p27的表現因此使細胞週期停滯於G_0/G_1。細胞凋亡的誘導方面，labedipinedilol-A造成細胞萎縮、染色質濃縮及增加Bax 的表現，降低Bcl-2的表現而啟動粒線體相關的細胞凋亡途徑，包括使cytochrome *c* 由粒線體釋放至細胞質、caspase-9 和caspase-3 的活化。本研究證明選擇性α1A-腎上腺素受體阻斷劑labedipinedilol-A在攝護腺癌細胞上具有抗增生與誘發細胞凋亡的作用。

Abstract

Labedipinedilol-A is a novel 1, 4-dihydropyridine-type calcium antagonist with α_1-receptor blocking activity. We have been demonstrated that labedipinedilol-A inhibited cell proliferation, and suppressed cell migration on cultured rat vascular smooth muscle cells. In this research, we conducted an in vitro analysis to evaluate the prostate cell response to labedipinedilol-A in order to determine the effect of this selective α_{1A}-adrenoceptor antagonist to suppress prostate cell growth by affecting cell proliferation and apoptosis. Human prostate cancer LNCaP (androgen-sensitive) cells and PC-3 (androgen-insensitive) cells were used for the measurement of anti-carcinoma activities of 0–75 μM labedipinedilol-A. Normal human prostatic epithelial cells (PZ-HPV-7) used as cell growth selective control. The growth-inhibitory effects were analyzed using XTT assay, 5-bromo-2’-deoxyuridine (BrdU) incorporation, flow cytometry, and immunoblotting were employed to illustrate the possible mechanisms. When treated with labedipinedilol-A for 24 to 48 h, cell growth was strongly inhibited. Labedipinedilol-A-induced growth inhibition was associated with G_0/G_1 arrest, and G2-M arrest depending upon doses. Consistently with G_0/G_1 arrest, labedipinedilol-A

increased protein expressions of cyclin D1/2, cyclin E, Cdk2, Cdk4, and Cdk6 p21 and p27. In addition, ellipticine also induced apoptosis in PC-3 cells, as determined by using both Hoechst 33342 staining (chromatin condensation) and Annexin-V staining assay. Labedipinedilol-A increased the expression of Bax, but decreased the level of Bcl-2, and subsequently triggered the mitochondrial apoptotic pathway (release of cytochrome *c*, and activation of caspase-9 and -3). In this study, we found that the α_{1A}-adrenoceptor inhibitor labedipinedilol-A shows not only cell-proliferation inhibition, but also induction of apoptosis in PC-3.

Keywords: α_{1A}-adrenoceptor antagonist; prostate cancer; anti-proliferation, cell cycle, apoptosis

Introduction

Prostate cancer is one of the leading causes of cancer related deaths in men worldwide (1). In the United States, one of nine men over the age of 65 years is diagnosed with prostate cancer (1, 2). Molecular mechanism underlying onset and progression of prostate cancer is not fully understood, but age, race, diet, and androgen secretion and metabolism are the identifiable risk factors for this malignancy (3, 4). Therapeutic options exist for localized disease, including surgery, radiation therapy, and hormonal therapy. The major cause of the mortality associated with this disease is the metastasis of cancer cells that fail to respond to hormone ablation therapy (5, 6). As surgery and current chemotherapeutic options seem to be inadequate in curing or controlling prostate cancer, there is a pressing need for the identification of alternative chemopreventive and chemotherapeutic strategies.

Benign prostatic hyperplasia (BPH) is attributable to proliferation of the epithelial and stroma smooth muscle components of the prostate gland, resulting in glandular enlargement. The exact cause of BPH is not fully understood but is believed to involve interactions between epithelial and stromal components under androgenic influence. The prevailing theory is that the combined effects of static (mechanical pressure on the urethra exerted by increased stromal smooth mass) and dynamic (α_1-adrenoceptor-mediated increase of smooth muscle tone) components cause bladder outlet obstruction. Therefore, disruption of the molecular mechanisms that regulate cells proliferation and cell death (apoptosis) may underlie the abnormal postatic cell growth leading to BPH. Labedipinedilol-A is a novel 1, 4-dihydropyridine-type calcium antagonist with α1-receptor blocking activity. We have previously demonstrated that labedipinedilol-A inhibited cell proliferation, and suppressed cell migration on cultured rat vascular smooth muscle cells. In this research, we will conduct an in vitro analysis to evaluate the prostate cell response to labedipinedilol-A in order to determine the effect of this selective α_{1A}-adrenoceptor antagonist to suppress prostate cell growth by affecting cell proliferation and apoptosis.

Materials and methods

Reagents and materials

Fetal bovine serum (FBS), penicillin G, streptomycin, amphotericin B and RPMI-1640 were obtained from GIBCO BRL (Gaithersburg,MD). Dimethylsulfoxide (DMSO), RNase, propidium iodide (PI) and the antibodies to p27, CDK4, CDK6 and β-actin were purchased from Sigma (St Louis, Missouri, USA). XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis [4-methoxy-6-nitro] benzene sulfonic acid hydrate) was obtained from Roche Diagnostics (Mannheim, Germany). Caspase-3 and -9 activity assay kits were purchased from Calbiochem (Cambridge, Massachusetts, USA). The antibodies to cyclin D1/2, cyclin E, p21, Bcl-2 and Bax were obtained from Upstate Biotechnology (NY, USA). The antibodies to cytochrome C was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Cell culture

Human prostate cancer LNCaP (androgen-sensitive) cells and PC-3 (androgen-insensitive) cells were obtained from American Type Culture Collection (Manassas, Virginia, USA). They were

grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml of penicillin G, 100 μg/ml streptomycin and 0.25 mg/ml amphotericin B at 37℃ and 5% CO_2. All studies were performed with exponentially growing cells. LNCaP and PC-3 cells were seeded in cell culture plates or dishes. After 24 h, cells were treated with various concentrations of labedipinedilol-A and incubated for the indicated times. Cells were harvested by trypsinization for flow cytometry analysis and scraped for other experiments.

Determination of Cell Number

The tetrazolium salt, XTT, is especially useful in quantifying viable cells. This assay is designed for the spectrophotometric quantification of cell growth and viability without the use of radioactive isotopes and is based on the cleavage of yellow tetrazolium salt, XTT, to form an orange formazan dye by metabolically active cells. LNCaP and PC-3 cells were seeded into 96-well plates at a density of 1×10^3 cells/well. Incubation with labedipinedilol-A was started 24 hours after cells were adherent. After 24 and 48 hours, 50 mL of XTT labeling mixture per well was added and the microtiter plate was incubated for 4 hours. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a scanning multiwell spectrophotometer enzyme-linked immunosorbent assay (ELISA) reader at 450 nm. The reference wavelength was 690 nm.

Determination of DNA Synthesis

DNA synthesis as assayed by the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into nuclei was measured to investigate the effects of labedipinedilol-A on cell growth. Briefly, cells were plated in 96-well culture plates (1×10^3cells/well). After 24 h incubation, the cells were treated with vehicle control and labedipinedilol-A (0, 1, 10, 25, 50 and 75 μM) for 24 and 48 h and then, for 4 hours in the same medium containing BrdU (10μM). Cells were harvested for the detection of DNA synthesis by incorporation of BrdU using a cell proliferation ELISA (Roche Molecular Biochemicals, Germany).

Cell cycle analysis

To determine cell cycle distribution analysis, 10^5 cells were plated in a 60-mm dish for 24 h and then treated with various concentrations of labedipinedilol-A (0, 1, 10, 25, 50 and 75 μM) for 24 h. After treatment,, the cells were trypsinized, centrifuged at 1,250 ×g for 5 minutes, washed with PBS, and then cleared of RNA with RNase A (10 mg/mL). The DNA was stained with propidium iodide (50 mg/mL) for 30 minutes at 37℃, and analyzed by a Coulter Epics XL-MCL (Beckman Coulter, USA).

Apoptosis assay

Labedipinedilol-A-induced apoptosis in human prostate cancer cells and normal prostate epithelial cells was determined by flow cytometry using the Annexin V–conjugated Alexa Fluor 488 (Alexa488) Apoptosis Detection Kit following the instructions of the manufacturer and as previously described (7). The apoptotic and necrotic cells from the same samples were quantified using quantitative FACS analysis. This method utilizes the binding of FITC-labeled annexin V to phosphatidylserine in the cell membrane that surfaces only during the early phase of apoptosis, indicating the loss of cell membrane phospholipid asymmetry. However, these apoptotic cells with intact cell membranes do not stain with the propidium iodide. By utilizing the morphological changes that occur in both apoptotic and necrotic cells, the samples were stained simultaneously with annexin–FITC and propidium iodide. The samples were then subjected to flow cytometric analyses to detect the percentage of apoptotic (FITC-stained cells) and necrotic cells (PI-stained cells) in a given population. A minimum of 10,000 cells were maintained for all the samples. The samples were analyzed by a Coulter Epics XL-MCL (Beckman Coulter, USA).

Hoechst 33342 staining

The PC-3 cells were plated in 12-well

culture plates (1×10^5 cells/well). After 24 h incubation, the cells were treated with vehicle control and labedipinedilol-A (0, 1, 10, 25, 50 and 75 μM) for 48 h and then stained with 10 μg/ml Hoechst 33342 for 60 min. The nuclear morphology of the cells was visualized using a fluorescence microscope (Zeiss Axioskop 2 plus, Japan).

Assay for caspase activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrates of caspase-9 (LEHD-pNA) and caspase-3 (Ac-DEVD-pNA). Cell lysates were incubated with peptide substrate in assay buffer (100mM NaCl, 50mM HEPES, 10mM dithiothreitol, 1mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) for 2 h at 37℃. The release of p-nitroaniline was monitored at 405 nm. Results are represented as the percent change of the activity compared to the untreated control.

Western Blot Analysis

Cells were treated with various concentrations of labedipinedilol-A for the indicated times. Mitochondrial and cytoplasmic fractions were separated using the cytochrome *c* releasing apoptosis assay kit (BioVision, Mountain View, CA). Reactions were terminated by washing twice with cold PBS and then cells were harvested. Total cell extracts were prepared in lysis buffer (20 mM Tris-HCl (pH 7.5), 1mM dithiothreitol (DTT), 5 mM EGTA, 2 mM EDTA, 0.5 mM PMSF, 20 μM leupeptin, and 20 μM aprotinin. The cell lysate was centrifuged at 15,000 ×g for 30 min, and the supernatant fraction was collected for western blot. Equivalent amount of protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (10-14%) and transferred to polyvinylidene difluoride membranes. After blocking for 1 h in 5% non-fat dry milk in Tris-buffered saline, the membrane was incubated with the desired primart antibody for 2 h. The membrane was then treated with appropriate horseradish peroxidase (HRP) conjugated secondary antibody (Chemicon), and the immunoreactive bands were detected by chemiluminescence (ECL) reagents (Amersham, USA).

Statistics

The results are expressed as mean 6 SEM. Statistical significance was estimated by one-way analysis of variance (ANOVA) followed by Newman-Keuls test. P , 0.05 was considered significantly significant. Analysis of the data and plotting of the figures were done with SigmaStat and SigmaPlot software (Version 5.0, San Rafael, CA) run on an IBM-compatible computer (IBM, White Plains, NY).

Results

Labedipinedilol-A inhibits LNCaP and PC-3 cells proliferation

In the first series of experiments, an XTT test was used to study labedipinedilol-A ability to alter proliferation of LNCaP and PC-3 cells. As shown in Figure 1A, labedipinedilol-A inhibited cell proliferation in both cancer cell lines in a concentration-dependent manner.

Labedipinedilol-A inhibits LNCaP and PC-3 cells DNA Synthesis

Additional experiments were done to determine the DNA synthesis of labedipinedilol-A by the incorporation of 5-bromo-2'-deoxyuridine (BrdU). Both cancer cell lines were incubated with different concentrations of labedipinedilol-A. After short incubation periods of 24 and 48 hours, the mitotic indices in the BrdU-ELISAwere dose-dependently reduced (Fig. 1B).

Labedipinedilol-A inhibits PC-3 cell growth

We examined the effect of labedipinedilol-A on the growth of human prostate cancer PC-3 cells (Fig. 2). The PC-3 cells were grown with different concentrations of labedipinedilol-A. After 48 hours of incubation, high concentrations of labedipinedilol-A (25, 50 and 75 μM) decreased the cell density.

Labedipinedilol-A induces cell cycle arrest

To examine the mechanism responsible for labedipinedilol-A-mediated cell proliferation

inhibition, we evaluated the effect of labedipinedilol-A on the cell cycle progression (Fig. 3). The ability of labedipinedilol-A to inhibit cell cycle progression was determined by a combination of propidium iodide staining and the flow cytometric analysis. These findings indicated that labedipinedilol-A-treated cells showed a concentration-dependent block of cell-cycle progression. Treatment of the PC-3 cells with 25 μM labedipinedilol-A for 24 h resulted in an accumulation of cells in the G0/G1 phase (74.0 %) alone with a decrease in the number of cells in the S phase.

Labedipinedilol-A alters the expression of cell cycle-related proteins

Since our studies have shown that labedipinedilol-A treatment of LNCaP and PC-3 cells results in G0/G1 phase cell cycle arrest, we examine the effects of labedipinedilol-A on cell cycle-regulatory proteins, including cyclin D1/2, cyclin E, Cdk2, Cdk4, and Cdk6. Figure 4 showed that labedipinedilol-A treatment of the cells resulted in a concentration-dependent decrease in the protein expression of cyclin D1/2, cyclin E, Cdk2, Cdk4, and Cdk6 in both cancer cell lines. Numerous proteins, including the cell-dependent kinase inhibitors p21 and p27, control the cell cycle progression. We next evaluated the effect of labedipinedilol-A on the protein levels of p21 and p27. As shown in Figure 4, the treatment with labedipinedilol-A increased the expression of p21 and p27. These data clearly demonstrated that labedipinedilol-A inhibits cell proliferation by inducing a G_0/G_1 cell cycle arrest through p21-mediated and Cdk inhibition pathway.

Labedipinedilol-A induces apoptosis in PC-3 cells

To investigate whether labedipinedilol-A induces apoptosis in PC-3 cells, we examined the apoptotic morphological changes in the PC-3 cells with labedipinedilol-A. The PC-3 cells were treated with 75 μM labedipinedilol-A for 48 h and then stained with Hoechst 33342 (Fig. 5). Labedipinedilol-A induced chromatin condensation, which is characteristic of apoptotic cells. To confirm this result, we performed annexin V binding and propidium iodide staining. The annexin V is one of the high-affinity-binding proteins that specifically bind to phosphotidylserine residues. The annexin V-conjugated FITC specifically binds to phosphotidylserine residues of apoptotic cells. Labedipinedilol-A (75 μM) significantly ($P < 0.05$) increases apoptosis, resulting in a 12-fold increase in annexin V-positive cells when compared with control (Fig. 6).

Labedipinedilol-A induce s apoptosis through the activation of the mitochondrial pathway

To investigate the mitochondrial apoptotic events involved in labedipinedilol-A-induced apoptosis, we evaluated the effect of labedipinedilol-A on the protein levels of several apoptosis-related molecules (Fig. 7). Labedipinedilol-A increased the pro-apoptotic protein Bax. In contrast, the anti-apoptotic protein Bcl-2 decreased. The cytosolic fraction from untreated prostate cancer cells contained no detectable amounts of cytochrome c, whereas it did become detectable after 25 and 10 μM labedipinedilol-A treatment in LNCaP and PC-3 cells, respectively. Next, we investigated the implication of initiator caspases and effector caspases in labedipinedilol-A-induced apoptosis. The activities of upstream caspase-9 were significant increased, indicating that treatment with labedipinedilol-A increased caspase-9 activity in PC-3 cells, consistent with the release cytochrome c into the cytosol (Fig. 8). Furthermore, Labedipinedilol-A subsequently increased executor caspase-3 activity (Fig. 8). Taken together, these results indicated that labedipinedilol-A induced apoptosis in PC-3 cells, and that labedipinedilol-A-induced apoptosis is dependent on the caspase activation.

Discussion

Control of cell cycle progression in cancer cells is considered to be a potentially effective strategy for the control of tumor

growth (8, 9) as the molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (10, 11). Our in vitro data indicated that treatment of both androgen-sensitive (LNCaP) and androgen-insensitive (PC-3) cells with labedipinedilol-A resulted in significant G_0/G_1 phase arrest of cell cycle progression, which indicates that one of the mechanisms by which labedipinedilol-A may act to inhibit the proliferation (Fig. 1) of cancer cells is inhibition of cell cycle progression.

Cell cycle progression is also regulated by the relative balance between the cellular concentration of cyclin-dependent kinase (CDK) inhibitors (CKIs), such as members of the CDK-interacting protein/CDK-inhibitory protein (CIP/KIP) and inhibitor of cyclin-dependent kinase (INK) families, and that of cyclin–CDK complexes. The Cip/Kip family, including CIP/p21 and KIP/p27, binds to cyclin–CDK complexes and prevents kinase activation, andsubsequently blocks the progression of cell cycle at the G0/G1 or G2/M phase (12, 13). Our finding of a significant decrease in cyclins D1/D2, and E and Cdk2, Cdk4, and Cdk6 in PC-3 cells on labedipinedilol-A suggests the disruption of the uncontrolled cell cycle progression of these cells (Fig. 4) and that the labedipinedilol-A induced G0/G1 arrest is mediated through the up-regulation of Cip1/p21 and Kip1/p27 proteins, which enhances the formation of heterotrimeric complexes with the G1-S Cdks and cyclins thereby inhibiting their activity (Fig. 3). Based on the data (Figs. 3 and 4), it seems that cyclin D1/D2 and Cdk4 are responsible for most of the cell cycle arrest observed in response to labedipinedilol-A because these regulators are effectively inhibited at the lowest concentration of labedipinedilol-A (10 μM). In addition, ellipticine treatment also increases the expression of Cip1/p21 and KIP1/p27. Thus, it is reasonable to postulate that labedipinedilol-A treatment may cause cell cycle arrest.

G1-phase arrest of cell cycle progression provides an opportunity for cells to either undergo repair mechanisms or follow the apoptotic pathway. We therefore determined the effect of labedipinedilol-A on the induction of apoptosis PC-3 cells. Our flow cytometry data indicate that treatment of PC-3 cells with labedipinedilol-A resulted in significant induction of apoptosis (Fig. 6)

Apoptosis is tightly regulated by antiapoptotic and proapoptotic effector molecules, including proteins of the Bcl-2 family, and can be mediated by several different pathways. The proteins of the Bcl-2 family either promote cell survival (e.g., Bcl-2 and Bcl-xL) or induce programmed cell death (e.g., Bax) (14, 15). The ratio of Bax/Bcl-2 is critical for the induction of apoptosis and this ratio determines whether cells will undergo apoptosis (5, 16). An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome c from mitochondria into the cytosol). The cytosolic cytochrome c then binds to Apaf-1, leading to the activation of caspase-3 and poly (ADP-ribose) polymerase (17, 18). Therefore, we investigated the contribution of Bcl-2 family proteins to labedipinedilol-A-induced apoptosis of PC-3 cells. We found that labedipinedilol-A treatment was not only associated with an increase in the pro-apoptotic Bax protein level, but also a decrease in the anti-apoptotic Bcl-2 amount (Fig. 7), and subsequently also mediated the release of cytochrome *c*, finally activating the caspase cascade (caspase-9 and -3) (Fig. 8).

In conclusion, the results of the present study indicate that labedipinedilol-A inhibits proliferation and induces G_0/G_1 phase arrest and apoptosis in PC-3 cells. we provide mechanistic evidence that labedipinedilol-A-induced apoptosis in PC-3 cells not only alters the balance between proapoptotic Bax protein and anti-apoptotic Bcl-2, toward cell death, but also induces the release of cytochrome *c*, and activation of caspase-9 and -3, resulting in apoptosis.

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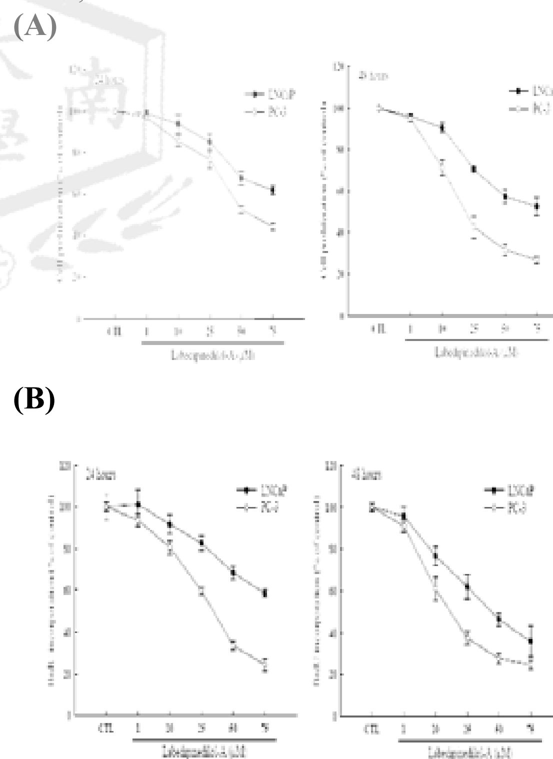


Figure 1. Effects of labedipinedilol-A on cell proliferation and BrdU incorporation. **(A)** After 24 and 48 h exposure, total cells were treated with MTT for 4 h and cell numbers were harvested as absorbance value in two prostate cancer cell lines. **(B)** After only 24 hours incubation, the DNA synthesis rate decreased. Application of labedipinedilol-A

for 48 hours induced a marked inhibition of proliferation (*n*=3, each experiment with eight replicates). All data are expressed as percentage of proliferation of control cultures and given as the mean ± SEM.

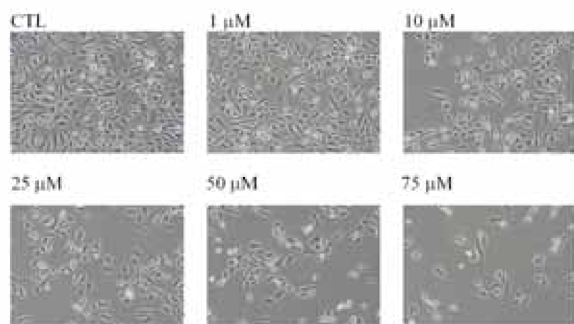


Figure 2. Inhibitory effects of labedipinedilol-A on PC-3 cells growth. Cell morphology in vehicle and different concentrations of labedipinedilol-A treated PC-3 cells (magnification 100×).

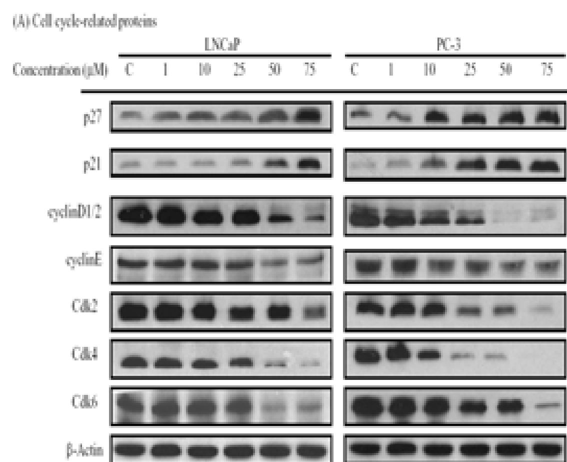


Figure 4. The effects of labedipinedilol-A on cell cycle-related proteins. The various protein levels of labedipinedilol-A-treated cancer cells were determined by immunoblot. Similar results were obtained in three other experiments.

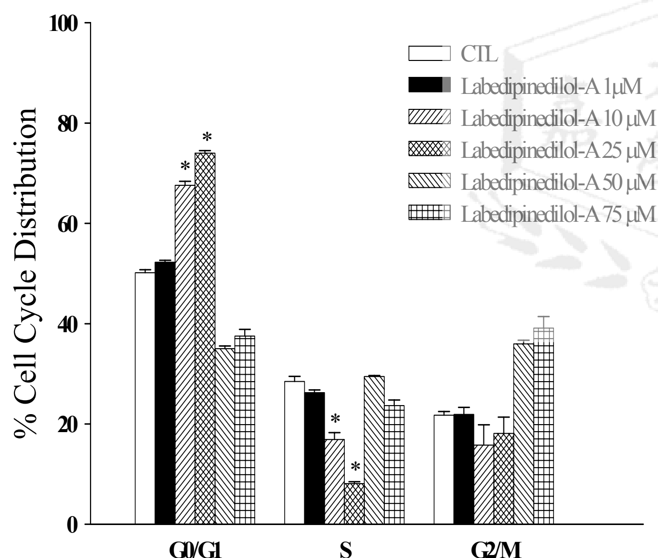


Figure3. The effects of labedipinedilol-A on the progression of cell cycle in PC-3 cells. Cells were treated with vehicle and labedipinedilol-A for 24 hours, and cell cycle distribution was assessed by flow cytometry. Results are expressed as the mean ± SEM form three separate experiments, with duplicate determinations in each experiment. **P* < 0.05 versus control.

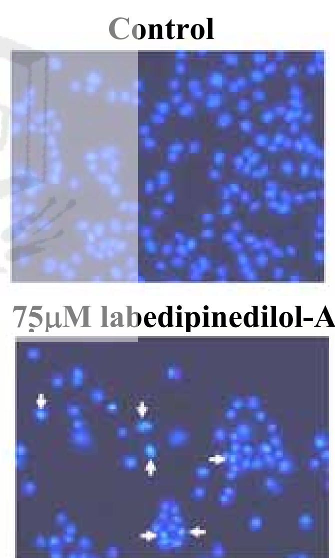


Figure 5. Induction of apoptosis by labedipinedilol-A in PC-3 cells. Chromatin condensation by labedipinedilol-A treatment. PC-3 cells were cultured with 50μM labedipinedilol-A for 48 h, and then the cells were stained with Hoechst 33342. The arrows indicate condensed chromatins.

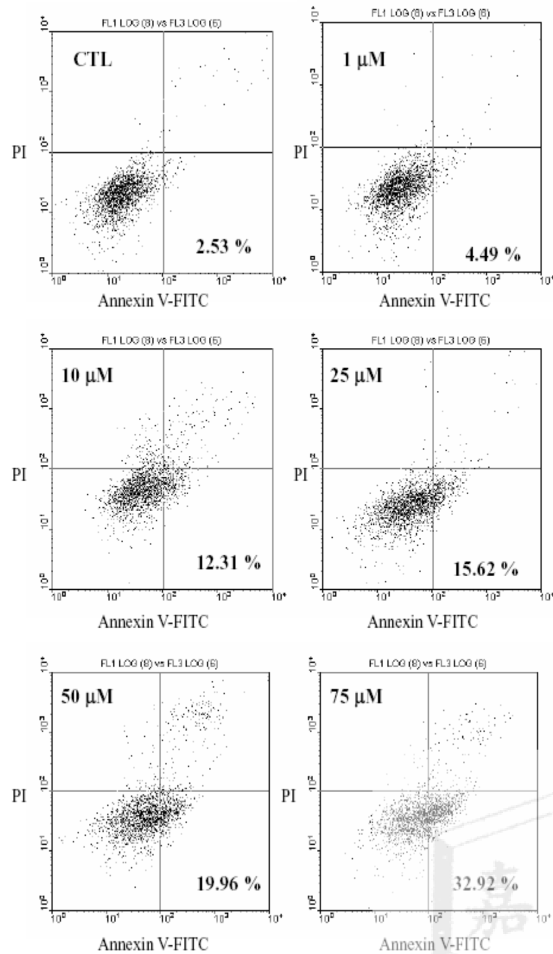


Figure 6. Labedipinedilol-A induces apoptosis in human prostate carcinoma PC-3 cells. Cells were treated with varying concentrations of Labedipinedilol-A (0, 1, 10, 25, 50 and 75 μM) for 48 h, then harvested for analysis of apoptosis using the Annexin V-Alexa Fluor 488 (Alexa488) Apoptosis Vybrant Assay Kit as detailed in Materials and Methods. Lower right (LR) quadrant, percentage of early apoptotic cells (Alexa488-stained cells); upper right (UR) quadrant, percentage of late apoptotic cells (Alexa488 + propidium iodide–stained cells). One representative experiment out of three is shown.

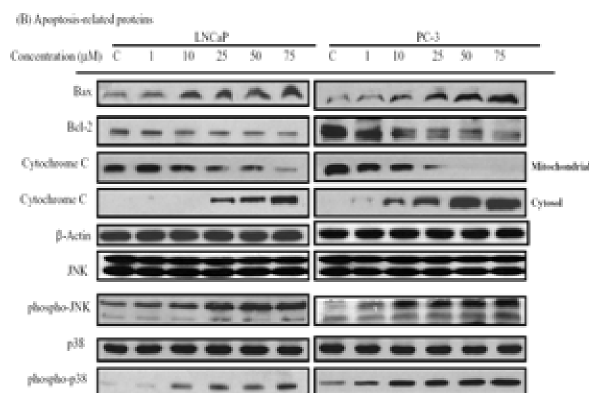
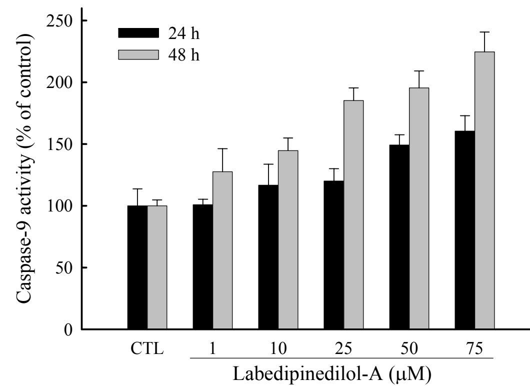


Figure 7. The effects of labedipinedilol-A on apoptosis-related molecules. PC-3 cells were cultured with the indicated concentrations of labedipinedilol-A for 48h. The protein extracts were applied to Western blot analysis. Similar results were obtained in three other experiments.



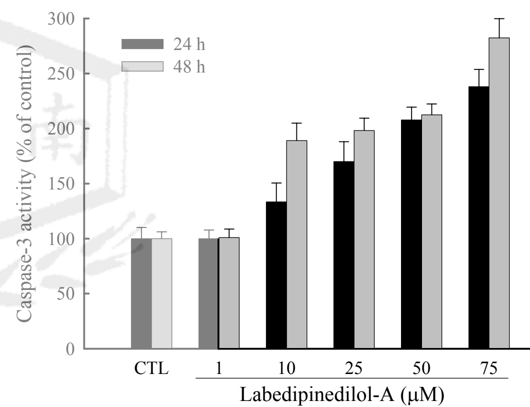


Figure 8. Labedipinedilol-A-induced apoptosis through caspase-dependent pathway. The activation of caspase-9 and caspase-3 in labedipinedilol-A treated PC-3 cells. The activity of caspase-9 and caspase-3 was assessed by caspase-9 and caspase-3 activity assay kit. Results are expressed as the mean ± SEM form three separate experiments, with duplicate determinations in each experiment. *$P < 0.05$ versus control.