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# Antirestenosis Effect of Butein in the Neointima Formation Progression

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**ABSTRACT:** The development of restenosis involves migration and hyperproliferation of vascular smooth muscle cells (VSMCs). Platelet-derived growth factor (PDGF) is one of the major factors. Butein modulates inflammatory pathways and affects the proliferation and invasion of the tumor. We investigated the hypothesis that butein might prevent the restenosis process via a similar pathway. Our results demonstrated that butein inhibited PDGF-induced VSMC proliferation and migration as determined by BrdU proliferation and two-dimensional migration scratch assay. Butein also concentration-dependently repressed PDGF-induced phosphorylation of PDGF-receptor $\beta$, mitogen-activated protein kinases, phosphoinositide 3-kinase/Akt, and phopholipase C$\gamma$/c-Src in VSMCs. In addition, in vivo results showed that butein attenuated neointima formation in balloon-injured rat carotid arteries. These results indicate that butein may inhibit PDGF-induced VSMC proliferation and migration, resulting in attenuation of neointima formation after percutaneous transluminal coronary angioplasty. Our study demonstrates for the first time that systemic administration of butein is able to reduce neointima formation after vascular injury.

**KEYWORDS:** *butein, smooth muscle cell, PDGF, restenosis, migration*

## INTRODUCTION

Restenosis may be an uncontrolled and rapid response to tissue injury characterized by marked intimal growth and vascular smooth muscle cell (VSMC) proliferation and migration. The pathophysiology of restenosis involves accumulation of new tissue within the arterial wall, and the process can be divided into several stages. Platelets, fibrin, and neutrophils accumulate around the damaged site by stenting and immediately adhere to launching the coagulation cascade in the first stage. The coagulation effect triggers fibrin accumulation in the vessel surface in the second stage. Various cytokines, chemoattractants, and growth factors will increase the adhesion of inflammatory cells on the thrombus.[1] In addition, the accumulation of chronic inflammatory cells also sustains a release of platelet-derived growth factor (PDGF) to lead to the phenotypic modification of medial smooth muscle cells (SMCs) followed by their migration and subsequent proliferation in the intima.[2] The chronic inflammation and fibrin deposition continue in the third stage. The persistent inflammation close to the stent struts and restenosis formation are completed in the last stage. In recent studies, positive regulator expression, including that of growth factor receptors, matrix metalloproteinases, and integrins, was reported to be correlated with increased angiogenesis, and these regulators are under investigation as therapeutic targets.

Percutaneous transluminal coronary angioplasty (PTCA) was the most important method of revascularization in the treatment of coronary artery disease during the mid-1990s. Bypass surgery and balloon angioplasty with stent implantation were used to restore blood flow in the vessels.[3] The dilated segment after PTCA can be reoccluded, causing restenosis.[4] After stent placement over 1−6 months, about 30% of patients have been observed to have the dilated segment reoccluded in the clinic.[5]

VSMCs in the arterial media are fully differentiated to play their physiological roles, such as regulators of vascular wall tension. Hyperproliferation and migration of VSMCs in response to arterial injury play a crucial role in the pathogenesis of vascular disease, such as atherosclerosis, hypertension, and restenosis.[6] After the endothelial denudation, the activated platelets release chemoattractants to provide mitogenic stimuli for smooth muscle cell growth.[7] PDGF is a potent chemoattractant and is a member of the highly hydrophilic cationic glycoprotein family (28−35 kDa) produced by platelets, monocytes/macrophages, endothelial cells, and VSMCs when a vascular lesion is activated.[8] Upon injury, the expression of PDGF and its receptor gradually increases. In the cardiovascular system, PDGF-BB expression can induce rapid down-regulation of SMCs that infiltrate the artery in response to pathological stimuli[9] and stimulate SMC proliferation and migration in arterial injury models.[10] PDGF is released from degranulating platelets and present in the wound fluid to play an important role in each stage of wound healing.[11] An alternative approach in preventing the proliferation and migration of VSMCs may be achieved by blocking the downstream intracellular signaling events responsible for transduction of the signals from the various growth factor receptors.[12]

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3,4,2′,4′-Tetrahydroxychalcone (butein) is a type of chalcone derivative which is a flavonoid.[13] Flavonoid is an agent of polyphenol from numerous plant tissues, including the stem bark of cashews (*Semecarpus anacardium*), the heartwood of *Dalbergia odorifera*, and *Caragana jubata* and *Rhus verniciflua* stokes[14] in traditional Chinese and Tibetan medicinal herbs. The extract of native butein predominantly contains the potent chalcone with reported benefits, including antioxidant activity, anti-inflammatory activity, elicitation of endothelium-dependent vasodilation, protein kinase inhibition, and antitumor activity in a variety of human tumor cells.[14,15] Several flavonoids have been reported to block injury-induced neointimal hyperplasia via inhibiting VSMC proliferation and migration.[16] Thus, we hypothesized that butein has a similar inhibition effect in injury-induced neointimal hyperplasia in VSMCs. In the present study, we aimed to gain more understanding about the mechanism of the antirestenosis activities of butein.

## MATERIALS AND METHODS

**Chemicals.** Butein was purchased from Extrasynthese Corp. (Genay, France). Recombinant Rat PDGF-BB was purchased from R&D Systems (Minneapolis, MN). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic−antimycotic, and trypsin−EDTA were purchased from Gibco (Grand Island, NY). Propidium iodide (PI), toluidine blue O, RNase A, and 3-(4,5-dimethylthiazol-2-y1)-2,4-diphenytetrazolium bromide (MTT) were purchased from Sigma Chemical Corp. (St. Louis, MO). Lactate dehydrogenase (LDH) assay reagents were purchased from Promega Corp. (Madison, WI).

**Primary Aortic Smooth Muscle Cell Culture.** Rat primary VSMCs were isolated from the normal aorta of Wistar rats by the PBS and dithiothreitol (DTT) explant method. The aortic ring was cut into thin pieces and incubated in six-well plates with DMEM, supplied 10% FBS, 100 U/mL antibiotics, 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 1 mM sodium pyruvate at 37 °C in a 5% $CO_2$ atmosphere. After the cells proliferated from the rings for about 14 days, they were treated and cut with PBS and 0.05% trypsin−EDTA in a 10 cm dish. The cells were serially passaged and used between passages 3 and 8.

**MTT Assay.** VSMCs ($1.5 \times 10^4$ cells/well) were subcultured onto 24-well plates and starved with serum-free DMEM for 48 h. Then VSMCs were grown in DMEM, including 10% FBS or PDGF (20 ng/mL), in the absence or presence of various concentrations of butein (0.1, 1, 10, and 100 $\mu$M) for 48 h. The cells were incubated with MTT (0.5 mg/mL) for 2−4 h. Formazan crystal (purple) was lysed by dimethyl sulfoxide (DMSO), and the absorbance was measured at 550 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Thermo, Waltham, MA).

**Lactate Dehydrogenase Assay.** VSMCs were seeded onto 48-well plates at a density of $2 \times 10^4$ cells/well, starved for 48 h with serum-free DMEM, and incubated with or without vehicle and the indicated concentrations of butein (0.1, 1, and 10 $\mu$M) in DMEM with 10% FBS. The percentage of LDH release was calculated from the ratio of LDH activity in the medium to LDH activity in the cell lysate.

**BrdU Incorporation Assay.** VSMCs were cultured in 96-well plates ($1 \times 10^4$ cells/well) and starved with serum-free DMEM for 48 h. The culture was treated with or without vehicle and the indicated concentrations of butein (0.1, 1, and 10 $\mu$M) for 48 h. Cell proliferation was measured by a BrdU Cell Proliferation Assay Kit (Chemicon, Temecula, CA) following the manufacturer's protocol. The absorbance was read at 450 nm.

**Migration Assay.** VSMC migration was measured using a 12-well modified Boyden chamber hosting a gelatin-treated polycarbonate filter with 8 $\mu$m pores (Transwell apparatus, 8 $\mu$m pores size, Costar) as described after cultivation with serum-free DMEM for 48 h. The upper chambers were reversed to coat with 0.2% gelatin. VSMCs were suspended in serum-free DMEM with or without pretreatment for 30 min with the indicated concentrations of butein (0.1, 1, and 10 $\mu$M) and seeded on the upper compartment of the Transwell. The lower chambers were filled with 0.6 mL of serum-free DMEM in the absence or presence of PDGF (20 ng/mL) as a chemotaxis. The chamber was incubated at 37 °C in 5% $CO_2$. After incubation for 16 h, the cells were fixed with 4% paraformaldehyde at room temperature, and nonmigrated cells on the upper face of the filters were removed by wiping with a cotton swab. The migrated cells were stained with 1% toluidine blue in 4% paraformaldehyde. Cell migration was quantified by counting the number of stained cells in three random fields (100×) with an inverted contrast phase microscope (Nikon, Japan) and photographed.

**Two-Dimensional Migration Scratch Assay.** Subcultured VSMCs were seeded into six-well plates. VSMCs were starved with serum-free DMEM for 48 h. Part of the dish was denuded by scratching along a straight line with a 200 $\mu$L pipet tip and incubated with or without vehicle and the indicated concentrations of butein (0.1, 1, and 10 $\mu$M) in DMEM with 10% FBS. After 24 h of incubation at 37 °C, images of the wounded area were captured immediately by a microscope (Nikon, Japan).

**Western Blotting.** VSMCs were subcultured onto a six-well plate and starved with serum-free DMEM for 48 h. After the cells were pretreated with or without various concentrations of butein (0.1, 1, and 10 $\mu$M) and vehicle in serum-free DMEM for 30 min, PDGF (20 ng/mL) was added as a basal control for 30 min. The cells were harvested and lysed in a lysis buffer containing 15 mM Tris−HCl, 50 mM NaCl, 5 mM EGTA (ethyleneglycoltetraacetic acid), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM NaF, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM $Na_3VO_4$, 10 $\mu$g/mL aprotinin, and 10 $\mu$g/mL leupeptin, pH 7.4. The protein samples separated on 10% SDS−polyacrylamide gel electrophoresis (PAGE) were then transferred to PVDF membranes by electroblotting. The membranes were blocked with 0.25% (w/v) gelatin at room temperature for 1 h. Afterward, immune detection for phosphorylated (activated) platelet-derived growth factor receptor $\beta$ (PDGFR$\beta$), phosphorylated Akt, phosphorylated phosphoinositide 3-kinase (PI3K), phosphorylated c-Src, phosphorylated extracellular-signal-regulated kinase (ERK), and $\beta$-actin was accomplished with antibody in 0.25% (w/v) gelatin. The blots were washed for at least 30 min in wash buffer (Tris/phosphate/saline/Tween). After incubation with each antibody, the immunoreactive band was detected by an enhanced chemiluminiscence (ECL) Western blotting detection system.

**Balloon-Injury Rat Carotid Artery Model.** We used the established rat carotid artery (CA) model of balloon angioplasty to examine the in vivo arterial response to injury.[17] Briefly, male Wistar rats weighing 300−350 g were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and the right CA was exposed. A Fogarty 2F embolectomy balloon catheter was inserted into the right external carotid artery through arteriotomy incision and advanced to the aortic arch. The balloon was inflated and withdrawn three times with rotation at the same pressure. Followed by removal of the catheter, the external carotid artery was ligated and the wound closed. Animals were given standard rat chow and tap water ad libitum for 2 weeks, at which time they were sacrificed, and the sections from both the right and left (collateral control group) CA were harvested to fix with 4% paraformaldehyde in PBS overnight for study. All procedures involving animal experiments were approved by the Institutional Animal Care and Use Committee at the College of Medicine, Tzu-Chi University, approval number 99015.

**Histological Examination.** Tissues were processed by standard procedures and paraffin-embedded. Sections (5 $\mu$m) were cut using a rotary microtome and placed on pretreated slides. Histological sections were stained with hematoxylin and eosin. Morphometric analysis of cross-sectional areas was performed on arterial sections by using computer-assisted image analysis (NIH Image, National Institutes of Health, Bethesda, MD).

**Statistical Analysis.** Results are expressed as the mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA). $P$ values less than 0.05 ($P < 0.05$) were considered to indicate a significant difference.

## RESULTS

**Effects of Butein on VSMC Cytotoxicity, Viability, and Apoptosis.** To investigate whether the effect of butein was due to its cytotoxicity to VSMCs, we performed an LDH assay. Our data indicate that butein at 10 $\mu$M did not cause VSMC cytotoxicity (Figure 1A) or affect viability (Figure 1B). However,

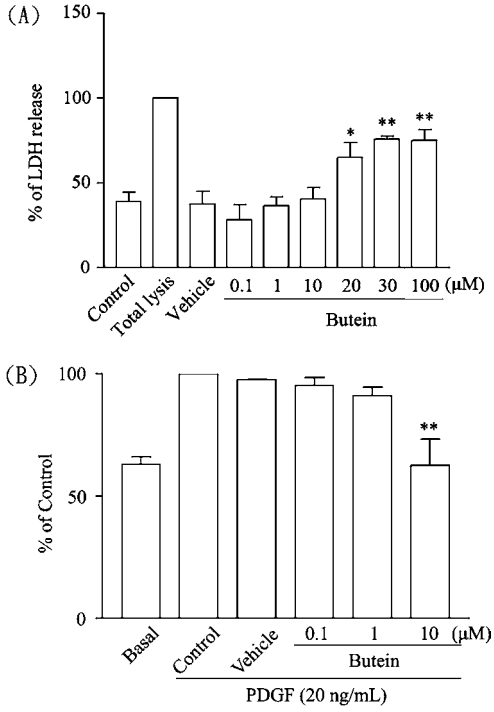


**Figure 1.** Effects of butein on VSMC cytotoxicity and viability. VSMCs were seeded onto plates for 24 h and starved for another 48 h with serum-free DMEM. Quiescent cells were cultivated with DMEM containing 10% FBS in the absence or presence of various concentrations of butein for 48 h. (A) Medium was detected via the manufacturer's protocol, and finally the absorbance was recorded at 490 nm. The control was taken as the basal condition of LDH release. (B) With 48 h of treatment, VSMCs were added to MTT agent to detect the effect of butein on VSMC viability. The results are presented as percentages of the control (mean ± SEM, $n$ = 3). All $P$ values were obtained from comparisons between the control and indicated concentration-treated cells. Two asterisks indicate $P < 0.01$.

treatment with higher concentrations of butein (20−100 $\mu$M) caused a significant LDH release. Therefore, we used the range of butein between 0.1 and 10 $\mu$M for further experiments in VSMCs.

**Effects of Butein on PDGF-BB- and Serum-Stimulated VSMC Proliferation.** After 48 h of incubation, VSMC growth was significantly increased after treatment with 20 ng/mL PDGF-BB (about 30%) or 10% FBS (about 57%) compared to that of the serum-free group. A 10 $\mu$M concentration of butein inhibited PDGF-BB (Figure 2A) and serum-induced (Figure 2B) cell proliferation. The BrdU incorporation assay revealed a result similar to that of the MTT assay on VSMC viability (Figure 1B).

**Butein Inhibition of PDGF-BB-Stimulated Migration and Serum-Stimulated Cell Motility.** Migration plays a major role in restenosis when VSMCs are stimulated by growth factor.[18] The Boyden (microchemotaxis) chamber assay showed comparable results (Figure 3A). Pretreatment with various concentrations of butein (0.1, 1, and 10 $\mu$M) in

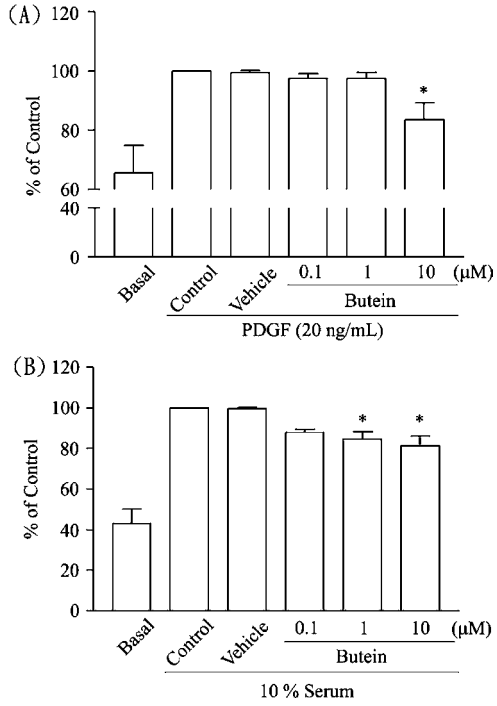


**Figure 2.** Effect of butein on VSMC proliferation. VSMCs were seeded onto plates for 24 h and starved for another 48 h with serum-free DMEM. Quiescent cells were cultivated with DMEM containing 10% FBS in the absence or presence of various concentrations of butein for 48 h. Proliferation was assayed by incorporation of BrdU. (A) PDGF-BB stimulation. (B) Serum stimulation. The data are shown as the mean ± SEM, $n$ = 3 per group. An asterisk indicates $P < 0.05$ (versus the control untreated cells).

the upper well significantly inhibited VSMC migration induced by PDGF-BB. Furthermore, we produced a scrape wound on a monolayer of VSMCs to observe the continuous movement of VSMCs in closing the gap that was denuded.[19] After 24 h of continuous treatment, butein (0.1, 1, and 10 $\mu$M) inhibited the serum-stimulated cell motility of VSMCs (Figure 3B). Both migration responses in the Boyden chamber and scrape models were significantly reduced in a concentration-dependent manner.

**Butein Suppression of Neointima Formation after Balloon Injury in Rat Carotid Artery in Vivo.** We further determined the inhibitory effect of butein on neointima formation after carotid artery injury. The neointima formation was attenuated in the butein-treated group compared with the control group. As shown in Figure 4A, the sham (noninjuried) group was free of intimal thickening. After balloon catheter injury of the artery of the control group, there was evident neointima thickening (Figure 4B) 14 days after the operation. On the other hand, the vessels after butein treatment in various doses (Figure 4D−F) exhibited reduction of neointimal formation as compared with the control. We found that butein significantly inhibited the N/M ratio (neointima/media) as it was administered intraperitoneally (ip) at 1.37 (mg/kg)/day (Figure 4G).

**No Butein Arrest of Serum-Stimulated Cells in the G1 Phase.** The cell cycle progression of quiescent cells induced by 10% FBS and the effect of butein (1−10 $\mu$M) are illustrated in Figure 5A. Butein did not induce an apparent increase in the G0/G1 phase of the cell cycle in VSMCs with a slight decrease in the population of cells in the S phase.


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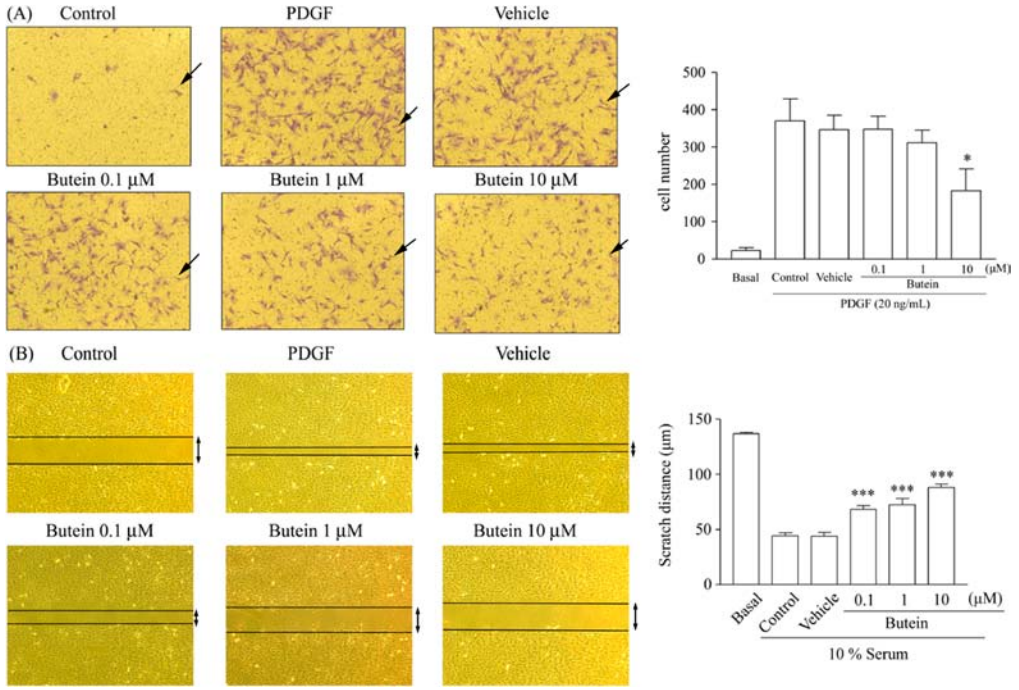


**Figure 3.** Effect of butein on PDGF-induced VSMC migration and serum-stimulated cell motility. (A) VSMCs ($4 \times 10^4$) cultured in serum-free DMEM were pretreated with or without butein for 30 min and placed in the upper chamber of a Transwell containing a 0.2% gelatin-coated filter membrane. Serum-free DMEM (basal) or 20 ng/mL PDGF was added to the lower chamber. After removal of nonmigrated cells and fixation, cells that migrated to the underside of the filter membrane were stained and quantified by phase-contrast light microscopy under a high-power field (HPF; magnification 100×). Three fields per filter were counted at 100× magnification. (B) Cells were subjected to injury by scratching with a plastic pipet tip (200 $\mu$L). The cells were then treated for 24 h with or without serum or butein. Data are presented as percentages of the control (mean ± SEM, $n = 3$). One asterisk indicates $P < 0.05$ and three asterisks indicate $P < 0.001$ versus the control group.

However, the cycle progression was not significantly modulated by butein.

**Inhibitory Effect of Butein on PDGF-Induced Kinase Activation.** The inhibitory effect of butein on the activation of signaling pathways induced by PDGF-BB was investigated. The levels of phospho-PDGFR$\beta$, ERK1/2, PI3K, and Akt stimulated by PDGF-BB in VSMCs were inhibited by 10 $\mu$M butein (Figure 6). In addition, the PLC$\gamma$- and c-Src/FAK-mediated signaling cascade also plays a role in VSMC migration and mitogenic responses.[20] We also assessed the effect of butein on PLC$\gamma$ and c-Src/FAK phosphorylation. We found the levels of phospho-PLC$\gamma$ and c-Src/FAK induced by PDGF-BB were inhibited by butein at 10 $\mu$M (Figure 7). Since the downstream signals of PDGFR$\beta$ involve PI3K/Akt, ERK1/2, and PLC$\gamma$ activation, the inhibitory effect of butein on the activation of phospho-PI3K/Akt, ERK1/2, PLC$\gamma$, and c-Src/FAK may also be contributed by its activation of PDGFR$\beta$.

## DISCUSSION

Growth factors are key modulators of many biological processes during pathophysiological events, such as cardiovascular diseases, including atherosclerosis and restenosis.[21] Subsequent to vascular injury, various inflammatory cytokines and growth factors derived from either the vessel itself or platelet-induced VSMC dedifferentiation are released. The migration and proliferation of VSMCs are the key points in atherosclerosis and restenosis.[12,22] In this study, we found that butein has the ability to inhibit the PDGF-induced VSMC proliferation and migration (Figures 1B, 2, and 3). Since the experimental concentration of butein (10 $\mu$M) did not affectthe VSMC survival, this antiproliferation and antimigration effect of butein on VSMCs was not due to its cytotoxicity (Figure 1A). Cell proliferation is usually associated with cell cycle progression. Our findings also showed that, in the presence of serum stimulation in VSMCs, there is only a slight sub-G1 phase accumulation after butein treatment (Figure 5). It was reported that the G2/M phase inhibition effect observed at 30 $\mu$M butein was approximately 58% and the effect of the control/20 $\mu$M butein treatment condition was approximately 20% in cancer cells.[23] Their results demonstrate that butein inhibits the proliferation of cancer cells by inducing G2/M transition at a higher dose (30 $\mu$M) only. As compared to the Moon et al. studies,[23] this present study has similar results in that a low dose of butein (10 $\mu$M) may not be associated with cell cycle arrest. This result may be interpreted in that butein blocked injury-induced neointimal hyperplasia mainly via the inhibition of VSMC migration, without inducing apoptosis or cell cycle arrest.[16]

The activation of surface tyrosine kinase receptors that remodel the surrounding tissue will increase the efficiency of VSMC migration and modulate the migration mode, enabling the switch to pathological cell migration as in restenosis.[24] In the Transwell assay and linear wound healing assay, PDGF-induced (Figure 3A) or serum-induced (Figure 3B) migration was reduced in a concentration-dependent manner. c-Src kinase activity was reported to be essential for cellular activities such as migration,[25] and PDGFR$\beta$-mediated PLC-$\gamma$ activation stimulates cell motility.[26] We found that butein significantly inhibited PDGF-induced c-Src phosphorylation in a concentration-dependent manner in VSMCs (Figure 7A), indicating that butein inhibited PDGF-induced VSMC migration in part


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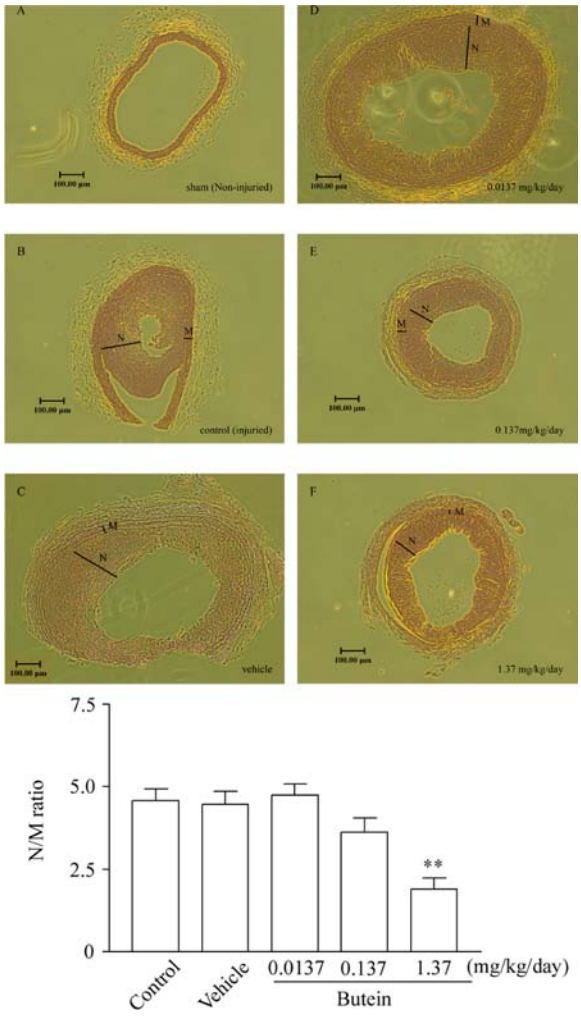


**Figure 4.** Effect of butein on neointimal formation after arotid artery balloon injury in rats in vivo. Rats were subjected to butein treatment by ip injection beginning 3 days before injury and lasting until 14 days after the injury. (A) Sham (noninjuried). (B) Control (injuried). (C) Vehicle. (D–F) Butein-treated (0.0137, 0.137, and 1.37 (mg/kg)/day). "N" indicates neointima, and "M" indicates media. Data are presented as percentages of the control (mean ± SEM, $n = 7–8$). Two asterisks indicate $P < 0.01$ versus the control group.

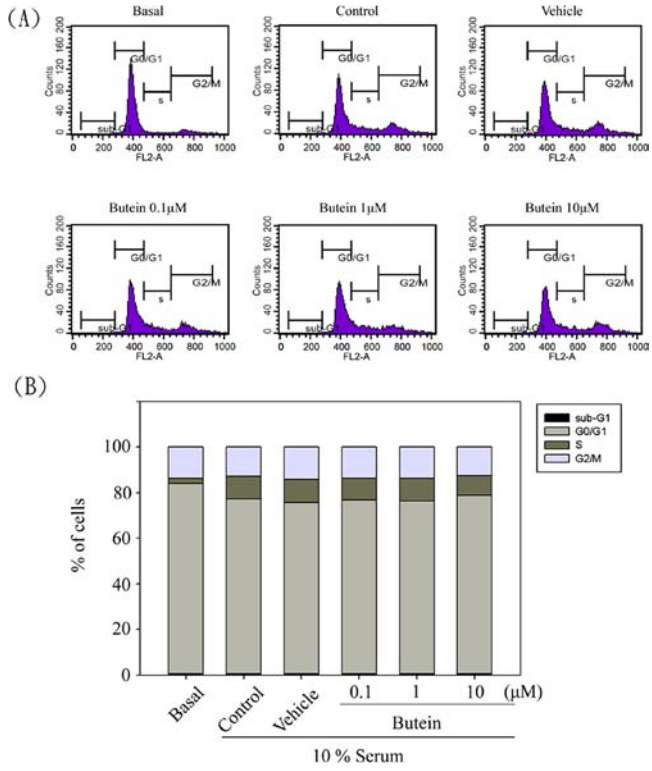


**Figure 5.** Effect of butein on serum-induced VSMC cell cycle progression. VSMCs treated with the indicated concentrations of butein in the presence of 10% FBS stimulation for 48 h were analyzed by flow cytometry. The results are expressed as percentages of the total cells in the sub-G1, G0/G1, S, and G2/M phases of the cell cycle. Values are presented as the mean ± SEM, $n = 3$.

through affecting c-Src activation. Butein also inhibited PDGF-stimulated PDGFR$\beta$ and PLC$\gamma$/c-Src phosphorylation, thus providing evidence that the inhibitory effects on butein in VSMC migration and proliferation may be through the inhibition on PDGFR$\beta$ and its downstream signal pathways. Although butein was shown to be a specific protein tyrosine kinase inhibitor, there are very few studies on its receptor tyrosine kinase inhibition activity. Yang et al. showed that the inhibition of EGF-induced EGFR tyrosine phosphorylation by butein was observed in human hepatocellular carcinoma HepG2 cells, and its $IC_{50}$ was 8 $\mu$M.[27] Our study is the first to demonstrate that butein also has the ability to inhibit another receptor tyrosine kinase, PDGFR$\beta$.

Recent studies have demonstrated that the MAPK, PI3K, and PLC$\gamma$ pathways play a crucial role in VSMC proliferation following PDGF treatment.[28] It has also been shown that Akt, which is downstream of PI3K, is a major mediator for growth factors in cell survival.[29] On the other hand, PI3K is required downstream of PDGF signaling for activating proteins involved in cell migration, PDGF-BB-induced proliferation, and cell cycle progression.[30] LY-294002, a PI3K inhibitor, showed a similar inhibitory effect on PDGF-induced proliferation and migration of VSMCs.[31] Thus, the inhibitory effects of butein on the proliferation and migration of PDGF-BB-stimulated RASMCs may be through inhibition of the MAPK, PI3K, and PLC$\gamma$ signaling pathways.

Our data also demonstrate that the ip administration of butein, at a dose of 1.37 mg/kg, is able to reduce by approximately 60% the neointimal formation after balloon injury (Figure 4). Although the antiproliferation effect of butein has been studied by many researchers, there were very few in vivo studies conducted before. In fact, our study demonstrates for the first time that systemic administration of butein is able to reduce the neointima formation after vascular injury. Butein has also been reported to affect inflammatory responses and tumor cell survival, which are associated with inhibition in NF-$\kappa$B activation and down-regulation of MMP-9.[32] However, the detailed effects of butein on PDGF-stimulated upstream and downstream signaling pathways of restenosis need further investigation.

In summary, the present study provides evidence that butein inhibits balloon injury-induced neointimal formation and inhibits VSMC migration/proliferation. Our data suggest that the suppression of PDGFR$\beta$, PI3K/Akt, PLC$\gamma$, c-Src/FAK, and MAPK activity may be responsible for the inhibition of PDGF-increased VSMC migration and proliferation in response to butein. Butein may provide valuable information regarding drug development for the prevention and treatment of vascular diseases such as vascular restenosis.

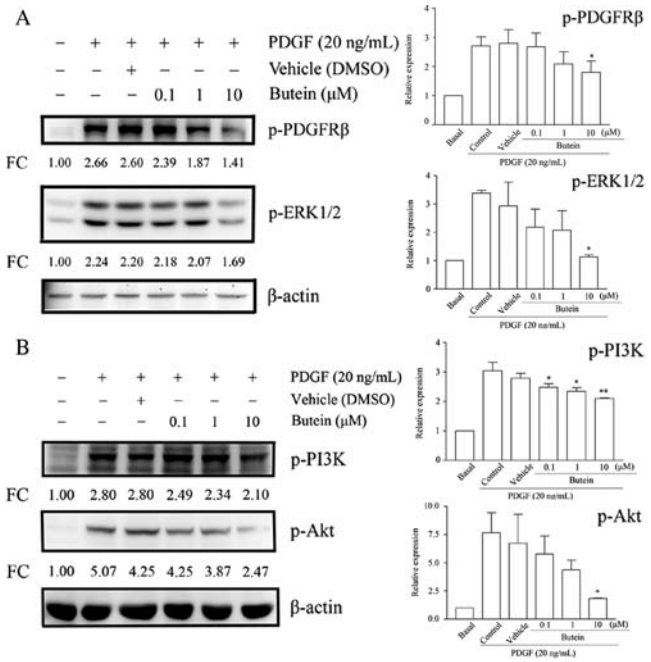


**Figure 6.** Effect of butein on PDGF-induced PDGFR$\beta$/ERK/Akt/PI3K phosphorylation. The cells were cultured in six-well plates until confluence, and the medium was replaced with serum-free medium in the presence or absence of butein for 30 min. After pretreated butein, the VSMCs were stimulated with PDGF (20 ng/mL) for 30 min. The cell lysates were analyzed by Western blot with antibodies as indicated. (A) PDGFR$\beta$ and ERK phosphorylation. (B) Akt and PI3K phosphorylation. Bar charts of the statistics are shown on the right. "FC" indicates fold change. Data are presented as percentages of the control (mean ± SEM, collected by several independent experiments, $n$ = 7−8). One asterisk indicates $P < 0.05$ and two asterisks indicate $P < 0.01$ versus the control group.

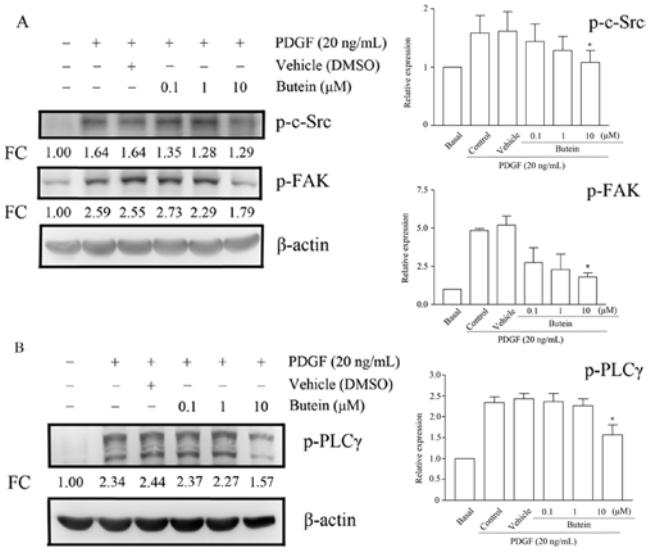


**Figure 7.** Effect of butein on PDGF-induced c-Src/FAK/PLC-$\gamma$ phosphorylation. The cells were cultured in six-well plates until confluence, and the medium was replaced with serum-free medium in the presence or absence of butein for 30 min. After pretreated butein, the VSMCs were stimulated with PDGF (20 ng/mL) for 30 min. The cell lysates were analyzed by Western blot with antibodies as indicated. (A) c-Src and FAK phosphorylation. (B) PLC$\gamma$ phosphorylation. Bar charts of the statistics are shown on the right. "FC" indicates fold change. Data are presented as percentages of the control (mean ± SEM, collected by several independent experiments, $n$ = 7−8). One asterisk indicates $P < 0.05$ and two asterisks indicate $P < 0.01$ versus the control group.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

BrdU, 5-bromodeoxyuridine; butein, 3,4,2′,4′-tetrahydroxychalcone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ECL, enhanced chemiluminiscence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ERK 1/2, extracellular-signal-regulated kinase 1/2; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PDGFR$\beta$, platelet-derived growth factor receptor $\beta$; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PTCA, percutaneous transluminal coronary angioplasty; VSMCs, vascular smooth muscle cells

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