Full Paper

Clitocybin A, a Novel Isoindolinone, From the Mushroom *Clitocybe aurantiaca*, Inhibits Cell Proliferation Through G1 Phase Arrest by Regulating the PI3K/Akt Cascade in Vascular Smooth Muscle CellsKyu-Dong Yoo^{1,†}, Eun-Seok Park^{1,†}, Yong Lim², Shin-Il Kang¹, Su-Hyang Yoo¹, Ha-Hee Won¹, Young-Hee Kim³, Ick-Dong Yoo³, Hwan-Soo Yoo¹, Jin Tae Hong¹, and Yeo-Pyo Yun^{1,*}¹College of Pharmacy, Research Center for Bioresource and Health, Chungbuk National University, Cheongju 361-763, Korea²Department of Clinical Laboratory Science, Dong-eui University, Busan, Korea³Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea

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Abstract. Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays an essential role in the pathogenesis of vascular diseases, such as atherosclerosis, hypertension, and restenosis. Clitocybin A, a novel isoindolinone, isolated from the culture broth of mushroom *Clitocybe aurantiaca* has been reported to possess free radical scavenging activity. However, the antiproliferative effects of clitocybin A on VSMCs are unknown. In the present study, we investigated the effect of clitocybin A on platelet-derived growth factor (PDGF)-BB-induced proliferation of VSMCs and examined the molecular basis of the underlying mechanism. Clitocybin A inhibited DNA synthesis and cell proliferation. In accordance with these findings, clitocybin A blocked the PDGF-BB-inducible progression through G0/G1 to S phase of the cell cycle in synchronized cells and decreased the expression of cyclin-dependent kinase (CDK) 2, CDK4, cyclin D1, cyclin E, and proliferative cell nuclear antigen. In addition, clitocybin A inhibited the PDGF-BB-induced phosphorylation of phosphatidylinositol 3 kinase (PI3K) / Akt kinase. However, clitocybin A did not change the expression levels of extracellular signal-related kinase (ERK) 1/2, phospholipase C- γ 1, and PDGF-R β phosphorylation. These results indicate that clitocybin A may inhibit VSMCs proliferation through G1 phase arrest by regulating the PI3K/Akt pathway.

Keywords: clitocybin A, vascular smooth muscle cell, platelet-derived growth factor (PDGF)-BB, PI3K/Akt pathway

Introduction

Vascular smooth muscle cells (VSMCs) proliferation within the intimal layer of the vessel wall is a key step in atherosclerotic plaque development (1). Vascular injury can induce cytokines such as interferon- γ and interleukin 1 and growth factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). These factors stimulate VSMC proliferation by regulation of downstream signal transduction such as the mito-

gen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3 kinase (PI3K) / Akt pathway (2). PDGF-BB-stimulated VSMCs synthesize and excrete extracellular matrix components and collagen, which lead to neointimal thickening and restenosis.

PDGF-BB-induced proliferation and mitogenesis of VSMCs are prerequisites of intimal thickening, which is invariably observed after angioplasty (3). The binding of PDGF-BB to the PDGF receptor (PDGF-R) can activate three major signal transduction pathways including PI3K/Akt, phospholipase C (PLC)- γ 1, and extracellular regulated kinase 1/2 (ERK1/2) (4, 5). Once PDGF binds to PDGF-R in VSMCs, PI3K is then activated, followed by the stimulation of Akt. The phosphorylation level of PI3K/Akt is increased in cell proliferation and survival

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of VSMCs from thoracic aortas of male Sprague–Dawley rats and human VSMCs (6, 7).

Cell proliferation is regulated primarily by the cell cycle, which consists of G0/G1, S, G2, and M phases. The G1 to S transition in cell cycle progression is controlled by the action of cyclin-dependent kinases (CDKs) and their activating subunits, the cyclins (8, 9). Complexes of cyclin D1–CDK4 and cyclin E–CDK2 predominantly act in sequence during the G1/S transition (10).

Recently, clitocybin A, novel isoindolinone, was isolated from the mushroom *Clitocybe aurantiaca* (11), and this compound inhibited reactive oxygen species (ROS)-induced cell death by reducing the activation of caspase 3 and 9, cytochrome *c* release from mitochondria, and NF- κ B activation in IMR-90 lung fibroblast cells (12). It suggests that clitocybins are novel ROS scavengers that can protect cells from apoptosis.

However, the effect of clitocybin A on VSMCs proliferation has not been clearly demonstrated. We attempted to elucidate the antiproliferative activity and machinery targets of clitocybin A in the PDGF-BB–induced signaling pathway. These results provide evidence that clitocybin A can inhibit proliferation and cell cycle progression via cell cycle–related proteins by regulating the PI3K/Akt pathway.

Materials and Methods

Materials

Clitocybin A was synthesized and characterized as previously described (12) (Fig. 1A). It was dissolved in dimethylsulfoxide (DMSO) and added to Dulbecco's modified Eagle's medium (DMEM) with a maximum final DMSO concentration of 0.05%. The cell culture materials were obtained from Gibco-BRL (Rockville, MD, USA), and other chemical reagents were from Sigma Chemical Co. (St. Louis, MO, USA). [3 H]Thymidine was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). PDGF-BB was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Polyclonal antibodies specific for phospho-ERK1/2, phospho-Akt, phospho-PLC- γ 1, phospho-PDGFR- β , ERK1/2, Akt, PLC- γ 1, and PDGF-R β were supplied by Cell Signaling Technology Inc. (Beverly, MA, USA). PCNA, cyclin D1, cyclin E, CDK2, and CDK4 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were of analytical grade.

Cell culture

Rat aortic VSMCs were isolated by enzymatic dispersion using a slight modification of the method reported

Fig. 1. Inhibitory effect of clitocybin A on PDGF-BB–stimulated cell proliferation. A: Chemical structure of clitocybin A. B: VSMCs were cultured in serum-free medium for 24 h; treated with clitocybin A at 0, 5, 10, and 20 μ M for 24 h; and then stimulated by 25 ng/mL PDGF-BB for 24 h. The cells were trypsinized and counted using a hemocytometer. Data represent the mean $\pm$ S.E.M. from 4 different sets of experiments. ** $P < 0.01$ vs. VSMCs stimulated with PDGF-BB alone.

by Chamley (13). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 g/mL streptomycin, 8 mM HEPES, 2 mM L-glutamine at 37°C in an incubator with a humidified atmosphere of 95% air and 5% CO₂. The purity of rat VSMCs culture was confirmed by western blotting of α -smooth-muscle actin. The passage of VSMCs used in this experiment was 5–8.

Cell proliferation and DNA synthesis

The cell proliferation and DNA synthesis of VSMCs were measured as described previously (14). In brief, VSMCs were seeded into 12-well culture plates at 4×10^4 cells/mL with DMEM containing 10% FBS. Cells were incubated at 37°C for 24 h. The medium was then replaced by serum-free medium in the presence or absence of clitocybin A. The cells were stimulated with 25 ng/mL PDGF-BB and then trypsinized with trypsin-EDTA. Cells were counted using a hemocytometer under a microscope. For the [3 H]thymidine incorporation experiments, VSMCs were seeded and cultured for 20 h in 24-well culture plates under the same conditions as the ones in the cell proliferation assay. [3 H]Thymidine (1

$\mu\text{Ci/mL}$) was then added to the medium and the cells were incubated further for 4 h. The cultures were then placed on ice and reactions were terminated by aspiration of the medium, followed by sequential washes with PBS containing 10% trichloroacetic acid and ethanol/ether [1:1, v/v]. Acid-insoluble [^3H]thymidine was lysed with 300 μL of 0.5N NaOH, and the lysate was mixed with 3 mL scintillation cocktail (Ultimagold; Packard Bioscience, Meriden, CT, USA) and quantified using a liquid scintillation counter (LS3801; Beckman, Düsseldorf, Germany).

Cell cycle progression analysis

To estimate the proportion of cells in the various phases of the cell cycle, cellular DNA contents were measured by flow cytometry. Cells were harvested, fixed in 70% ethanol, and stored at -20°C . The cells were then washed twice with ice-cold PBS and incubated with RNase and propidium iodide (PI). Cell cycle phase analysis was performed using the FACSCalibur (BD Biosciences, San Jose, CA, USA) and the proportion of cells within the G1, S, and G2/M phases was analyzed with ModFit LT V2.0 (Verity Software House, Topsham, ME, USA).

Western blot analysis

The immunoblotting of VSMCs was performed as previously described (14). Briefly, VSMCs were seeded into 6-well culture plates at a density of 1×10^5 cells/mL and then cultured in DMEM containing 10% FBS at 37°C for 24 h. When cells reached up to 70% confluence, the medium was replaced with serum-free medium, and the cells were incubated for 24 h. The medium was then changed to serum-free medium containing various concentrations of clitocybin A, and the cells were incubated for 24 h. VSMCs were stimulated with 25 ng/mL PDGF-BB for 1 min for PDGF-R β , 5 min for ERK 1/2 and PLC- γ 1, 15 min for PI3K and Akt phosphorylation assays. For the assay of cyclin D1, cyclin E, CDK2, CDK4, and PCNA expressions, VSMCs were stimulated by PDGF-BB (25 ng/mL) for 24 h. The cell pellets were lysed, and the proteins were separated on 10%–15% SDS-PAGE. The proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech). The membranes were then incubated with a 1:1000 dilution of the following antibodies: phospho-PDGF-R β , phospho-ERK1/2, phospho-Akt, phospho-PI3K, phospho-PLC- γ 1, PDGF-R β , ERK1/2, Akt, PI3K, PLC- γ 1, PCNA, CDK2, CDK4, cyclin D1, and cyclin E. The blots were washed with TBS/T and then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated IgG secondary antibody (New England Biolabs, Beverly, MA, USA). The proteins were detected using an ECL plus kit (Amersham

Pharmacia Biotech), followed by exposing the membranes to Hyperfilm ECL (Amersham Pharmacia Biotech). The band intensities were quantified using Scion-Image Software.

Statistical analysis

The experimental results are expressed as the mean $\pm$ S.E.M. One-way analysis of variance (ANOVA) was used for multiple comparison followed by Dunnett's test. P -values < 0.05 and < 0.01 were considered significant.

Results

Effect of clitocybin A on VSMCs proliferation

Direct cell counting was performed to determine whether clitocybin A inhibited PDGF-BB-stimulated VSMCs proliferation. Treatment of VSMCs with 25 ng/mL PDGF-BB for 24 h increased the number of cells from 33.0×10^4 to 67.5×10^4 cells/well. Treatment with both PDGF-BB and 5, 10, or 20 μM clitocybin A for 24 h decreased the number of cells to 45.5×10^4 , 38.0×10^4 , or 33.5×10^4 cells/well, in a concentration-dependent manner, compared to treatment with PDGF-BB alone (Fig. 1B).

Effects of clitocybin A on DNA synthesis and cell cycle progression

To investigate the effects of clitocybin A on DNA synthesis in VSMCs, we performed a [^3H]thymidine incorporation assay. The incorporation of [^3H]thymidine into DNA was found to be significantly increased by adding PDGF-BB to VSMCs, and clitocybin A significantly inhibited [^3H]thymidine incorporation into DNA in a concentration-dependent manner (Fig. 2A). Inhibition percentages were 49.5%, 72.2%, and 84.0% at concentrations of 5, 10, and 20 μM , respectively.

As shown in Fig. 2B, the serum-deprivation of VSMCs for 24 h resulted in an approximately 92.36% synchronization of the cell cycle at the G0 phase, and PDGF-BB treatment of the VSMCs increased the percentage of cells in the S phase from 2.18% to 15.98%. Clitocybin A diminished the PDGF-BB-stimulated cell cycle progression in a concentration-dependent manner: approximately 15.98% of the S phase shown in PDGF-BB-treated cells reduced gradually to the levels of 11.7%, 7.5%, and 5.0% in the presence of 5, 10, and 20 μM clitocybin A, respectively. Additionally, clitocybin A inhibited the phosphorylation of PCNA, which regulates the G1 to S phase transition in the cell cycle (Fig. 2C). This result suggests that clitocybin A is effective against DNA synthesis during the early events of the cell cycle.

Fig. 2. Effects of clitocybin A on [3 H]thymidine incorporation, cell cycle progression, and the expression or activation of cell cycle regulatory proteins in PDGF-stimulated VSMCs. VSMCs cultured in serum-starved medium were stimulated with 25 ng/mL PDGF-BB, and the effect of various concentrations of clitocybin A (5–20 μ M) on DNA synthesis by the addition of 1 μ Ci/mL [3 H]thymidine (A), cell cycle progression (B), and PCNA protein by SDS–PAGE followed by immunoblot using antibodies for PCNA (C) were measured. Data represent the mean $\pm$ S.E.M. from 4 different sets of experiments. * P < 0.05 and ** P < 0.01 vs. VSMCs stimulated with PDGF-BB alone.

Effects of clitocybin A on the expression of cell cycle regulatory proteins

To clarify whether this inhibition involves the regulation of cell cycle-related proteins, we assessed G1-checkpoint proteins in clitocybin A-treated cell cycle arrest. As shown in Fig. 3, clitocybin A significantly decreased the level of CDK2, cyclin E, CDK4, and cyclin D1. Therefore, these results indicate that clitocybin A regulates the activity of G1 and S phase-related G1-checkpoint proteins in association with its cell cycle arrest effects in VSMCs.

Effect of clitocybin A on PDGF-R β , PI3K/Akt, ERK1/2, and PLC- γ 1 activation

To investigate the inhibitory mechanism of clitocybin A on VSMCs proliferation, we examined whether clitocybin A is involved in the activation of PDGF-R β , PI3K/Akt, ERK1/2, and PLC- γ 1 signaling. In our previous studies, we determined the maximum phosphorylation in 1 min for PDGF-R β , 5 min for ERK1/2 and PLC- γ 1, and 15 min for Akt by PDGF-BB (25 ng/mL) stimulation of VSMCs. As shown in Fig. 4, clitocybin A did not activate PDGF-R β , ERK1/2, or PLC- γ 1. However, clitocybin A inhibited PI3K and Akt phosphorylation induced by PDGF-BB in a concentration-dependent manner, with inhibition of 58.7% (P < 0.01, n = 3), 67.8% (P < 0.01, n = 3), and 71.4% (P < 0.01, n = 3) for PI3K phosphorylation and 55.0% (P < 0.01, n = 3), 83.3% (P < 0.01, n = 3), and 89.2% (P < 0.01, n = 3) for Akt phosphorylation at 5, 10, and 20 μ M, respectively (Fig. 4). These results indicate that the PI3K/Akt-mediated signaling pathway is involved in the antiproliferative action of clitocybin A.

Effect of clitocybin A on PI3K/Akt pathway

To confirm whether inhibiting PI3K/Akt pathways can reach a synergistic inhibition on VSMCs proliferation, we examined the synergistic effects of LY294002 (a PI3K/Akt pathway inhibitor) and clitocybin A when they were treated in combination. LY294002 (1 μ M) or clitocybin A (2 μ M), partially inhibited DNA synthesis; however, when they were treated in combination, synergistic inhibitory effects were shown (Fig. 5). Therefore, these results indicate that the inhibitory effect of clitocybin A on PI3K/Akt activation may be involved in the clitocybin A-induced inhibition of cell cycle progression and VSMCs proliferation.

Discussion

We reported here that clitocybin A, a ROS scavenger, had an inhibitory effect on VSMC proliferation, which was associated with inhibition of the PI3K/Akt signaling

Fig. 3. Effects of clitocybin A on PDGF-BB-stimulated CDK2, CDK4, cyclin D1, and cyclin E. Quiescent VSMCs were stimulated with 25 ng/mL PDGF-BB in the presence or absence of clitocybin A at 5, 10, and 20 μ M for 24 h. The cells were then lysed, and proteins were analyzed using 12% SDS-PAGE. A: Western blot analysis was performed with antibodies specific for CDK2, CDK4, cyclin D1, and cyclin E. α -Actin was used for normalization. Representative data from 3 different experiments are presented. B: The data were quantified with densitometry and are expressed as the mean $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ vs. VSMCs stimulated with PDGF-BB alone.

pathways, and the inhibitory effect of clitocybin A on PI3K/Akt signaling pathways induced the suppression of DNA synthesis via G0/G1 arrest by regulating cell cycle-mediated proteins such as CDK2/4 and cyclin D1/E. For the first time, we showed the antiproliferative action of clitocybin A in VSMCs, suggesting that it may be a candidate agent for the treatment of vascular restenosis and atherosclerosis.

In this study, we found that clitocybin A inhibited PDGF-BB-induced VSMCs proliferation. The activations of PI3K/Akt, ERK1/2, and PLC- γ 1 are enhanced by binding of PDGF to its tyrosine kinase and then stimulating signaling pathways (4, 15). Clitocybin A inhibited PDGF-BB-induced phosphorylation of PI3K/Akt, but not that of PDGF-R β , ERK1/2, and PLC- γ 1 (Fig. 4). PI3K positively regulates cell survival. Akt, a serine/threonine protein kinase, is activated through the PI3K pathway (16). These kinases have each been implicated in VSMC proliferation, cell cycle progression, and cell

survival (16, 17). The treatment of LY294002 with clitocybin A in VSMCs caused significant suppression of DNA synthesis (Fig. 5), indicating that this may be a synergic effect of VSMC proliferation. These findings indicated that the PI3K/Akt pathway might be a potential target for clitocybin A in VSMCs.

In the cell cycle of mammalian cells, cell division and growth are tightly controlled by regulators that act at sequential points throughout the cell cycle. The CDK2 and CDK4 are key mediators during the G1 to S phase transition of the cell cycle by forming complexes with cyclins E and D (18). These complexes phosphorylated a large number of proteins, resulting in hyperphosphorylation of Rb, which then releases transcription factors that promote DNA synthesis (19, 20). The effects of clitocybin A on expressions of CDK2, cyclin E, CDK4, and cyclin D1 were investigated. Clitocybin A inhibited CDK2, cyclin E, CDK4, and cyclin D1 expressions (Fig. 3), indicating that cell cycle arrest in the G1-phase might

Fig. 4. Effects of cliticocin A on the PDGF-induced activation of PDGF-R β , PI3K, Akt, ERK1/2, and PLC- γ 1. VSMCs cultured in serum-free medium were stimulated with 25 ng/mL PDGF-BB, and the ability of various concentration of cliticocin A (5 – 20 μ M) to reduce the PDGF-induced phosphorylation of PDGF-R β , ERK1/2, and PLC- γ 1 (A); PI3K (B); and Akt (C) were measured by SDS-PAGE and immunoblotting using primary antibodies for PDGF-R β , phospho-PDGF-R β , Akt, phospho-Akt, ERK1/2, phospho-ERK1/2, PI3K, phospho-PI3K, PLC- γ 1, and phospho-PLC- γ 1. Total amounts of PDGF-R β , Akt, ERK1/2, PI3K, or PLC- γ 1 were used for normalization and immunoblots were analyzed by densitometry; the values are given as a percentage of the control. Data were quantified with densitometry and are expressed as the mean $\pm$ S.E.M. (n = 3). ** P < 0.01 vs. VSMCs stimulated with PDGF-BB alone.

Fig. 5. Synergistic effect of PI3K inhibitor on suppression of cell proliferation by cliticocin A. Confluent cells were cultured in serum-free medium with 1 μ M LY294002 plus 2 μ M cliticocin A for 24 h. The cells were then treated with 25 ng/mL PDGF-BB for 20 h before 1 μ Ci/mL [³H]thymidine was added. Four hours later, the labeling reaction was terminated and quantified using a liquid scintillation counter. Data represent the mean $\pm$ S.E.M. from 4 different sets of experiments. * P < 0.05 and ** P < 0.01 vs. VSMCs stimulated with PDGF-BB alone.

be due to the downregulation of CDKs/cyclins complex expression (8). Therefore, the inhibition of CDK2, cyclin E, CDK4, and cyclin D1 expressions may be necessary for cell cycle progression arrest in the early G0/G1 and S phases of the cell cycle. To further evaluate the effects of cliticocin A on the regulation of VSMCs at the nuclear level, we examined PCNA expression level. PCNA is synthesized in the early G1- and S-phases of the cell cycle and is required for transition from the G1 to the S-phase (21). Therefore, PCNA can be employed as a marker for proliferating cells. Cliticocin A suppressed PCNA expression (Fig. 2C), suggesting that cliticocin A inhibited VSMCs proliferation by blocking G1 to S progression.

ROS have been reported to be critical signaling molecules in cell proliferation, which is important in the development of intimal thickening in atherosclerosis and restenosis (22, 23). Antioxidants such as *N*-acetylcysteine inhibited PDGF-BB-induced ROS production by scavenging ROS (22). In addition, a previous report indicated that an antioxidant, ROS scavenger, inhibits Akt activation in VSMCs (24) in agreement with this finding.

At the present stage, the molecular mechanism by which the cliticocin A exerts its influence on VSMCs proliferation has not been fully clarified. However, these results indicate that inhibition of the PI3K/Akt pathway induced by PDGF-BB may be involved in the cliticocin A-induced inhibition of cell proliferation and the ROS

scavenging effect of clitocybin A may partially contribute to the inhibition of PDGF signal transduction.

Taken together, these findings provide evidence that clitocybin A inhibits PDGF-BB-induced VSMCs proliferation by inhibiting PI3K- and Akt-mediated signaling pathways. In addition, the expressions of cyclin D, cyclin E, CDK2, and CDK4 are subsequently downregulated, and PCNA phosphorylation is inhibited, leading to G0/G1 cell cycle arrest. Thus, clitocybin A may be a candidate antiproliferative agent for the treatment of vascular restenosis and stent implantation.

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