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Double blockade of cell cycle progression by coptisine in vascular smooth muscle cells

H. Tanabe, H. Suzuki, H. Mizukami, M. Inoue *

Laboratory of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan

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Abstract

Coptisine, an isoquinoline alkaloid isolated from rhizome of *Coptis japonica*, inhibits proliferation of vascular smooth muscle cells (VSMCs). The aim of this study was to evaluate the action of coptisine, along with berberine (a structurally similar isoquinoline alkaloid), on progression of the cell cycle in VSMCs. Coptisine displayed antiproliferative action against VSMCs by blocking the cell cycle at G_1 and G_2/M phases. The G_1 block was shown by inhibition of [^{3}H]thymidine incorporation into VSMCs at coptisine concentrations higher than 15 μM. The mechanism underlying the G_1 arrest involved a decrease in cyclin D1 protein, although cyclin E, A, and B were not affected by coptisine treatment. The selective reduction in cyclin D1 protein was mainly attributable to accelerated proteolysis via proteasome-dependent pathway, since it was inhibited by a proteasome inhibitor, *N*-carbobenzoxy-L-leucinyl-L-leucinyl-L-norleucinal (MG132) and further the mRNA level of cyclin D1, protein synthesis, and mitogen-activated protein kinase (MAPK) activity remained unaltered. The mechanism underlying the G_2/M arrest involved partial inhibition of tubulin polymerization, which was apparent at coptisine concentration of 3 μM. Berberine arrested the cell cycle at G_1 phase via a mechanism identical with coptisine, but did not cause block at G_2/M phase. The results demonstrate that a small difference in the structure between isoquinoline alkaloids produces a big difference in activity, and that coptisine has a unique double action in arresting the cell cycle of VSMCs.

Keywords: Coptisine; Berberine; Isoquinoline alkaloid; Vascular smooth muscle cell; Cell cycle; Selectivity

1. Introduction

Vascular smooth muscle cell (VSMC) proliferation has long been considered a critical event in the pathology of coronary artery atherosclerosis, restenosis following angioplasty, and hypertension. VSMC proliferation and differentiation are intricately regulated by a complex array of local environmental influences including growth factors, contractile agonists, inflammatory stimuli, and mechanical stresses [1]. Although medial VSMCs are normally highly specialized for contraction, they retain the ability to modulate their phenotype to a more proliferative synthetic state. Retention of this plasticity allows vessel repair after injury, but may also increase susceptibility to atherogenic risk factors [2]. In atherosclerosis, the change in medial VSMCs from the contractile to the synthetic state is at present the most widely accepted mechanism of neointimal formation. Inhibition of excessive proliferation of VSMCs might therefore provide protection from vascular disorders, and might also dedifferentiate VSMCs from the synthetic to the contractile state and induce apoptosis.

Recently, a new strategy to treat vascular diseases has been proposed. It involves the use of antineoplastic agents such as doxorubicin, vincristine, colchicine, and paclitaxel, which have a strong dose-dependent antiproliferative effect on VSMCs in vitro [3–5]. Of these, paclitaxel has been shown to act on microtubules, and to cause mitotic arrest leading to apoptosis in certain tumor cell lines [6].

Abbreviations: bFGF, basic fibroblast growth factor; CDK, cyclin dependent kinase; DEPC, diethyl pyrocarbonate; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP, mitogen-activated protein; PDGF, platelet-derived growth factor; PVDF, polyvinylidene dufluoride; RNase, ribonuclease; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; VSMC, vascular smooth muscle cell

* Corresponding author. Tel.: +81 52 836 3415; fax: +81 52 836 3415.
E-mail address: inoue@phar.nagoya-cu.ac.jp (M. Inoue).

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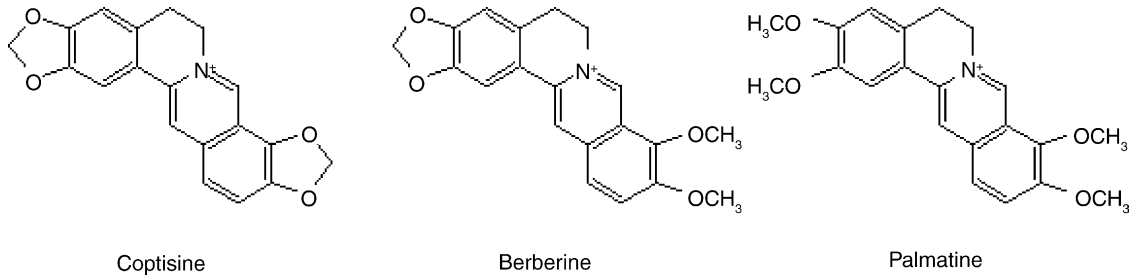


Fig. 1. Structure of coptisine and the related isoquinoline alkaloids.

In VSMCs, paclitaxel induces postmitotic cell cycle arrest rather than cell death [5]. Paclitaxel also reduces neointimal hyperplasia after angioplasty, when administered orally [7] or by implantation of a paclitaxel-eluting stent [8]. However, the usefulness of drugs of this type is likely to be limited by their toxicity and oral bioavailability.

Although some compounds derived from medicinal plants have been found to suppress VSMC proliferation [9–11], none have been reported with antiproliferative activity specific to VSMC. In searching for such agents, we found that coptisine, an ingredient of coptis rhizome (rhizome of *Coptis japonica*), inhibits proliferation in VSMCs at a lower concentration ($GI_{50} = 3.3$ μM) than in other cells (e.g. 3Y1 fibroblast, dRLh-84 hepatoma, B16 melanoma, and HeLa cells, $GI_{50} = 35$ to >140 μM) [12]. Coptisine is an isoquinoline alkaloid with a structure very similar to berberine and palmatine (Fig. 1). However, berberine is much less potent than coptisine in suppressing VSMC proliferation (GI_{50} about one-thirtieth), while palmatine failed to show any inhibitory activity [12]. Thus, these three alkaloids possess differing potency in their antiproliferative activities, despite their similarity in structure.

The aim of this study was to examine the mechanisms underlying the inhibitory action of coptisine and berberine on VSMC proliferation, with a focus on their effects on the regulation of cell cycle progression.

2. Materials and methods

2.1. Chemicals and reagents

Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) with high glucose were obtained from Irvine Scientific Co. Antibiotics (penicillin and streptomycin) were from Life Technologies Inc. [^{3}H]thymidine and [3,4-^{3}H]leucine were purchased from Amersham Biosciences Corp. Other reagents and their sources were: basic fibroblast growth factor (bFGF), BD Biosciences; platelet-derived growth factor (PDGF)-BB, AUSTRAL Biologicals; *N*-carbobenzoxy-L-leucinyl-L-leucinyl-L-norleucinal (MG132), Sigma; GTP, Sigma; vincristine, Nippon Kayaku Co., Ltd.; DMEM without leucine, Cell Science & Technology Institute Inc.

2.2. Cell culture

Aortic VSMCs were isolated from the thoracic and abdominal aortas of 10-week-old male Sprague–Dawley rats (Nippon Crea Co., Ltd.) using the enzymatic method described previously [12]. VSMCs were maintained in DMEM supplemented with 10% heat-inactivated FCS (Irvine Scientific Co.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). VSMCs were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The identity of VSMCs was confirmed by morphological examination and by RT-PCR analysis of l-caldesmon, h-caldesmon, and smooth muscle myosin heavy chain-1. Passages between 5 and 15 were used for the following experiments.

2.3. Cell cycle analysis

To evaluate the effect of alkaloids on cell cycle progression, VSMCs were growth-arrested at G_0/G_1 phase as follows. VSMCs were seeded at a concentration of 6×10^3 cells/cm^2 and cultured for 24 h in DMEM supplemented with 10% FCS. After washing the cells with PBS, VSMCs were cultured in DMEM with high glucose in the absence of FCS, for 96 h, to synchronize the cells at G_0/G_1 phase. The cells were then incubated in medium supplemented with 10% FCS in the presence of various concentrations of alkaloids. At the indicated time points after release from G_0/G_1 phase, cells were harvested by trypsinization. The cells were fixed with ice-cold 70% methanol for 30 min, washed with PBS, and then treated with 100 μL of 1 mg/mL RNase A in 1 mM Tris–HCl and 1.5 mM NaCl, pH 7.4, at 37°C for 30 min. Cells were then harvested by centrifugation at $400 \times g$ for 5 min, and stained with 250 μL of nuclear staining solution (10 mg propidium iodide, 0.1 mg trisodium citrate, and 0.03 mL Triton X-100 dissolved in 100 mL H_2O) at room temperature for 1 h in the dark. After adding 750 μL PBS, the DNA content in each cell cycle phase was determined using a flow cytometer (FACSort, Becton Dickinson) and analytic software (ModFit, Becton Dickinson). To examine the effect of alkaloids on cell cycle progression at S or G_2/M phase, VSMCs were serum-starved for 96 h to arrest the cells at G_0/G_1 phase, and then stimulated with 10% FCS. At 24 h after release from G_0/G_1 phase, coptisine or

berberine was added to the culture at the indicated concentration. Cells were harvested 21 h after the addition of the alkaloid and the progression of cell cycle was determined by flow cytometry as described above.

2.4. Measurement of DNA synthesis

VSMCs were seeded at a concentration of 3.0×10^4 cells/well into 24-well plates and cultured for 24 h. The cells were then cultured in serum-free medium for 96 h to arrest them at G_0/G_1 phase, and exposed to 1% FCS, 10 ng/ml PDGF, or 10 ng/ml bFGF for 21 h before adding 1 μCi/well [^{3}H]thymidine to the medium. Twenty-four hours later, the medium was removed and the cell monolayer was washed sequentially with ice-cold PBS (three times), 0.5 mL of ice-cold 10% trichloroacetic acid for 3 min, and ice-cold ethanol. Acid-insoluble [^{3}H]thymidine was then extracted with 0.5 mL of 1N NaOH. The extract (0.4 mL) was neutralized, added to 10 mL scintillation cocktail (Clear-sol 2, Nacalai Tesque), and analyzed with a liquid scintillation counter.

2.5. Western blot analysis

After the different treatments, VSMCs were harvested and lysed by sonication (5 s, five times) in 50 μL of lysis buffer containing 50 mM HEPES, pH 7.9, 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 40 mM Na_3VO_4, 0.4 mM NaF, 0.1% NP-40, 10% glycerol, and protease inhibitor cocktail tablets (Roche Diagnostics). The lysates were pelleted ($1000 \times g$, 10 min) at 4°C, and the supernatant was assayed for protein concentration (Bradford method, Bio-Rad Laboratories). Lysates (protein 20 μg) were then separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, 0.02% SDS, 20% methanol). The membrane was blocked for 2 h in a solution of 5% powdered skim milk in Tris-buffered saline (TBS) and then incubated with the appropriate primary antibody (dilution 1:200) overnight at 4°C. The antibodies used were cyclin A (C-19), cyclin B1 (H-433), Cdc2 p34 (C-19), cyclin D1 (H-295), and cyclin E (M-20), all from Santa Cruz Biotechnology. The blot was washed in four changes of wash buffer (0.05% Tween-20 in TBS) and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG antibody (Cell Signaling) in 1% powdered skim milk and 0.05% Tween-20 in TBS for 1 h at room temperature. The blot was thoroughly washed in five changes of wash buffer, and proteins were detected using CDP-Star™ (Roche Diagnostics) as the substrate for alkaline phosphatase.

2.6. Semiquantitative RT-PCR

Total RNA from VSMCs was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The extracted RNA was treated with DNaseI (Invitrogen) to degrade contaminating DNA. The RNA was dissolved in diethyl pyrocarbonate-treated water and quantified by GeneQuant II (Amersham Pharmacia Biotech). To prepare first strand cDNA, 500 ng of total RNA was reverse-transcribed using Revertra Ace-α (Toyobo Co.) according to the manufacturer's instructions. The resulting cDNA was subjected to PCR amplification with Taq polymerase (Roche Diagnostics) using the following specific PCR primers: for cyclin D1—sense primer, CTG CAT GTT CGT GGC CTC TAA G and antisense primer, AGA AAG TGC GTT GTG CGG TAG C; for GAPDH—sense primer, GTG ACA AAG TGG ACA TTG TTG and antisense primer, ATG AGC CCT TCC ACG ATG C. A thermal cycle of 30 s at 94°C, 30 s at 56°C, and 75 s at 72°C was applied for 30 cycles (cyclin D1) or 20 cycles (GAPDH), following denaturation for 120 s at 94°C, using a Program Temp Control System PC-707 (Astec). PCR products were analyzed on 1% agarose gels stained with ethidium bromide. The density of each DNA band was measured, and the value was expressed as a ratio to the value for GAPDH amplified from an aliquot of the same RT reaction.

2.7. Measurement of protein synthesis

VSMCs were seeded at a concentration of 3.0×10^4 cells/well into 24-well plates, and cultured for 24 h. After washing with PBS, the cells were cultured in the medium supplemented by DMEM without FCS for 96 h to arrest the cell cycle at G_0/G_1 phase. To examine the effect of drugs, VSMCs synchronized at G_0/G_1 phase were stimulated with 10% FCS in leucine-free DMEM in the presence of various concentrations of coptisine. The cells were pulse-labeled with [3,4-^{3}H]leucine (1.0 μCi/well) for 4 h every 4 h. The medium was removed, and the cell monolayer was washed sequentially with cold PBS (three times), 0.5 mL of ice-cold 10% trichloroacetic acid for 3 min, and ice-cold ethanol. The cells were then solubilized with 0.5 mL of 1N NaOH. The solution (0.4 mL) was neutralized, added to 10 mL of Clear-sol 2 (Nacalai Tesque), and then analyzed by liquid scintillation counter.

2.8. Effect of a proteasome inhibitor on cyclin D1 protein reduction

To assess the effect of a proteasome inhibitor, MG132, on the decrease in cyclin D1 protein, VSMCs (6×10^3 cells/cm^2) synchronized at G_0/G_1 phase were stimulated with 10% FCS in the presence of 30 μM coptisine or berberine. At 15 h after release from G_0/G_1 phase, 10 μM MG132 was added to the cultures and further incubated for another 9 h. The cells were harvested and subjected to Western blot analysis to determine cyclin D1 and cyclin E protein as described above.

2.9. Tubulin polymerization assay

Tubulin polymerization was carried out using tubulin polymerization assay kit (Cytoskeleton Inc.) according to the manufacturer's instructions. Briefly, polymerization was conducted in a 96-well microtiter plate with 2 mg/mL tubulin, 1 mM GTP, and 3 μM coptisine in a buffer containing 80 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]sequisodium salt (pH 6.9), 2 mM magnesium chloride, 0.5 mM ethylene glycol-bis(b-amino-ethyl ether)*N*,*N*,*N*′,*N*′-tetra-acetic acid, 10% (v/v) glycerol, 8 μM fluorescent reporter, and was monitored by an increase in fluorescence emission at 450 nm over a 30 min period at 37°C (excitation wavelength 345 nm; model MTP-650, Corona Electric Co.). Paclitaxel and vincristine were used as control compounds at a concentration of 3 μM.

2.10. Statistical analysis

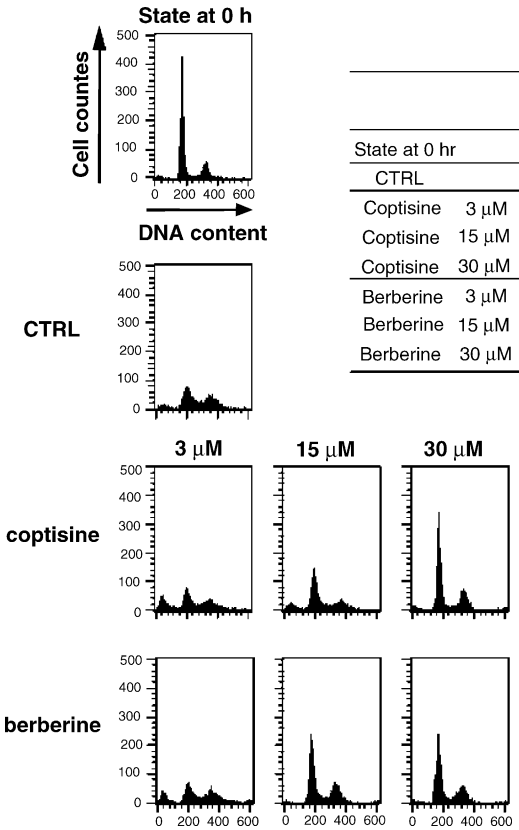
The data were represented as means ± S.D. Statistical differences between different groups were determined by ANOVA followed by Bonferroni-type multiple *t*-test.

3. Results

3.1. Effect of coptisine and berberine on cell cycle progression

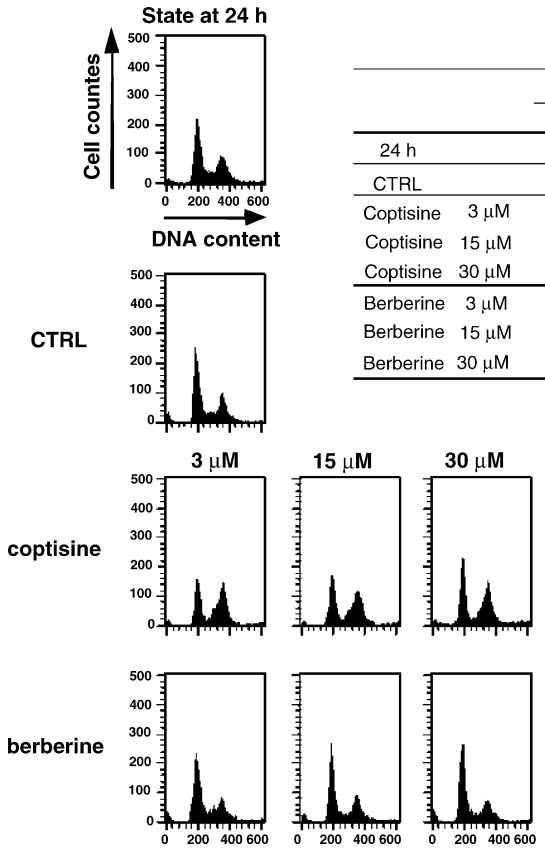
To compare coptisine and berberine in their cytostatic activity against VSMCs, we evaluated the effects of the two alkaloids on the progression of the cell cycle. For these experiments we used VSMCs synchronized at G_0/G_1 phase by serum deprivation for 96 h. In control VSMCs, the progression of the ell cycle from G_0/G_1 phase to S and G_2/M phases was observed at 27 h after the addition of the medium containing 10% FCS. In contrast, the addition of coptisine or berberine to the cultures blocked the serum-stimulated progression of cell cycle from G_0/G_1 phase at concentrations higher than 15 μM (Fig. 2). However, although the GI_{50} (the 50% growth inhibitory concentration) value of coptisine against VSMCs was 3.3 μM [12], inhibitory activity was not seen at a coptisine concentration of 3 μM.

After VSMCs progressed to S phase with stimulation by FCS, coptisine or berberine was added to the cultures to evaluate the effect on the progression of the cell cycle after release from G_0/G_1 phase (Fig. 3). Berberine did not arrest



		phase (%)		
		G0/G1	S	G2/M
State at 0 hr		74.74	9.98	15.31
CTRL		40.63	26.94	32.43
Coptisine	3 μM	43.32	25.11	31.17
Coptisine	15 μM	60.50	18.03	21.47
Coptisine	30 μM	66.50	9.81	23.69
Berberine	3 μM	39.70	24.38	35.92
Berberine	15 μM	59.47	14.00	26.53
Berberine	30 μM	64.54	15.45	20.01

Fig. 2. Effects of coptisine and berberine on cell cycle progression in VSMCs synchronized at G_0/G_1 phase by serum deprivation. VSMCs at a density of 6×10^3 cells/cm^2 were maintained in DMEM in the absence of FCS for 96 h to arrest the cells at G_0/G_1 phase. The cells were then stimulated with 10% FCS in the presence of coptisine or berberine at the indicated concentrations. At 27 h after release from G_0/G_1 phase, cells were harvested and the progression of the cell cycle was determined by flow cytometry after staining the cells with propidium iodide.



		phase (%)		
		G0/G1	S	G2/M
24 h		50.63	18.82	30.55
CTRL		54.12	16.60	29.29
Coptisine	3 μM	35.57	17.12	47.31
Coptisine	15 μM	42.05	17.24	40.70
Coptisine	30 μM	43.70	14.79	41.52
Berberine	3 μM	55.66	18.90	25.44
Berberine	15 μM	53.43	16.64	29.93
Berberine	30 μM	61.30	15.52	23.18

Fig. 3. Effects of coptisine and berberine on the cell cycle progression of VSMCs in S and G_2/M phases. VSMCs were serum-starved for 96 h to arrest the cells at G_0/G_1 phase and then stimulated with 10% FCS. At 24 h after release from G_0/G_1 phase, coptisine and berberine were added to the cultures at the indicated concentration. Cells were harvested 21 h after addition of the alkaloids and progression of the cell cycle was determined by flow cytometry, after staining the cells with propidium iodide.

the cell cycle at S or G_2/M phase at any concentration, but coptisine caused the cells to accumulate at G_2/M phase at a concentration of 3 μM.

3.2. Effect of coptisine on DNA synthesis

The incorporation of [^{3}H]thymidine into VSMCs synchronized at G_0/G_1 phase was measured following stimulation with FCS, bFGF, and PDGF. Coptisine treatment markedly suppressed FCS- or PDGF-induced DNA synthesis at concentrations higher than 15 μM, and moderately inhibited bFGF-induced DNA synthesis at equivalent concentrations (Fig. 4). However, coptisine did not suppress [^{3}H]thymidine incorporation at 3 μM, a concentration at which progression of the cell cycle from G_2/M to G_1 phase was blocked.

3.3. Effect of coptisine and berberine on G_1 cyclin

To clarify the mechanism underlying the block of cell cycle progression at G_0/G_1 phase by coptisine or berberine, G_1 cyclin protein was quantified by Western blot analysis (Fig. 5A). In control VSMCs, cyclins D1 and E protein levels increased 12, 18, and 24 h after stimulation with FCS. Treatment with coptisine or berberine significantly inhibited the induction of cyclin D1, but not cyclin E. However, when mRNA levels were assessed by RT-PCR at 5, 10, and 15 h after stimulation with FCS, treatment with coptisine or berberine was not associated with reduced cyclin D1 mRNA (Fig. 5B). Treatment of VSMCs with coptisine and berberine did not affect the activity of mitogen-activated protein kinase (MAP), extracellular signal-regulated kinase (ERK) or the mRNA induction of *c-fos* and *c-jun*.

3.4. Effect of coptisine on protein synthesis and degradation

To further examine the mechanism underlying the decrease in cyclin D1 protein, the effect of coptisine on protein synthesis in VSMCs was evaluated by measuring the [^{3}H]leucine incorporation into cellular proteins. Treatment with coptisine for 4 h did not show a strong inhibition of [^{3}H]leucine incorporation, but a slight dose-dependent reduction was observed 8 and 12 h after the treatment (Fig. 6). Cyclin D1 is also known to be regulated by proteasome-mediated protein degradation [13]. We therefore evaluated whether a proteasome inhibitor abrogated coptisine-caused decrease in cyclin D1 protein or not. In control VSMCs, cyclin D1 was induced 24 h after release

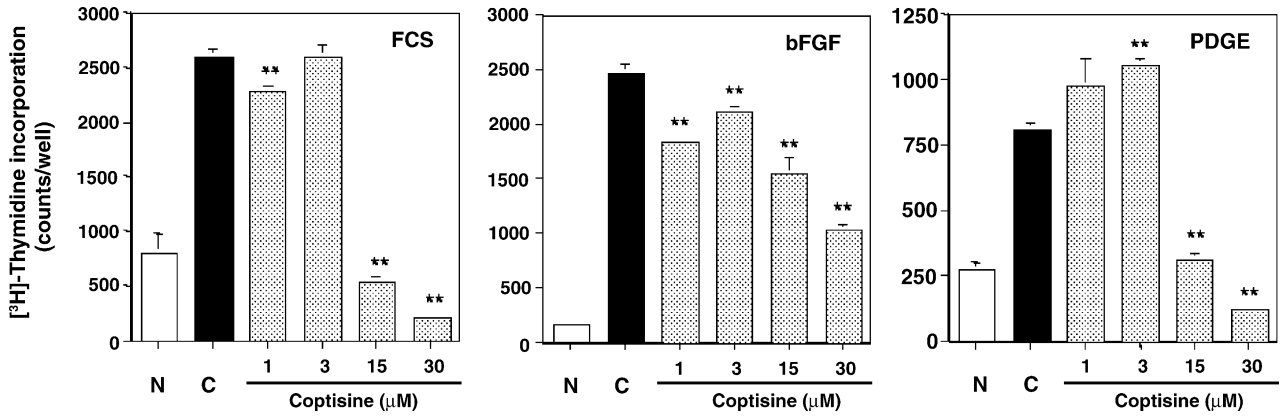


Fig. 4. Effect of coptisine on the incorporation of [^{3}H]thymidine into VSMCs. VSMCs were serum-starved for 96 h to arrest the cells at G_0/G_1 phase and then were stimulated with 1% FCS, 10 ng/ml PDGF, or 10 ng/ml bFGF in the presence of coptisine at the indicated concentrations. After 21 h incubation with serum or growth factors, the cells were incubated with [^{3}H]thymidine for a further 24 h. The incorporation of [^{3}H]thymidine was determined by liquid scintillation counter. Data are representative of three independent experiments with similar results and are expressed as means ± S.D. ($n = 3$).

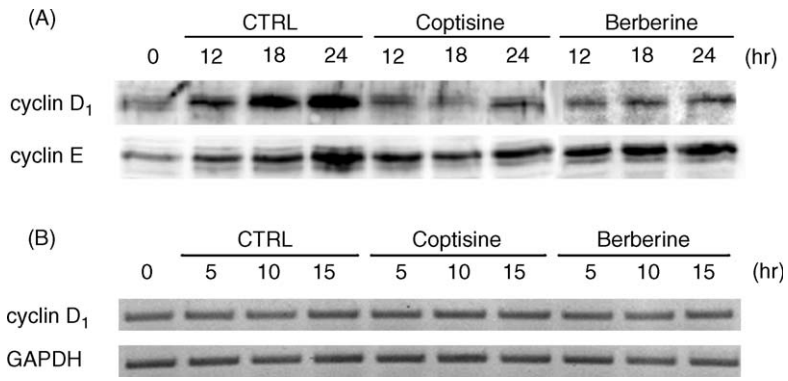


Fig. 5. Effects of coptisine and berberine on the protein (A) or mRNA (B) expressions of cyclins D1 and E. VSMCs were serum-starved for 96 h to arrest the cells at G_0/G_1 phase and then stimulated with 10% FCS in the presence of 30 μM coptisine or berberine. At the indicated time after release from G_0/G_1 phase, (A) cells were lysed and 20 μg protein was analyzed with SDS-PAGE; (B) cells were harvested and mRNA levels were detected by RT-PCR as described in Section 2. The data are representative of three independent experiments with similar results.

from G_0/G_1 phase and the addition of MG132 further increased cyclin D1 protein. On the other hand, coptisine or berberine treatment decreased cyclin D1 expression and MG132 abolished the reduction (Fig. 7). Although data were not shown, MG132 had no effect on cyclin E expression in VSMCs as well as coptisine or berberine treatment.

3.5. Effect of coptisine on tubulin polymerization

To elucidate difference between the inhibitory effects of coptisine and berberine on VSMC proliferation, the proteins of cyclin A, cyclin B, and cdc2, which are involved in the progression at S and G_2/M phases, were quantified by Western blot analysis. No effect of treatment with coptisine was detected (data not shown).

Tubulin polymerization is a central event in mitosis, and is a target for some alkaloids, that show antiproliferative activity against cancer cells. In an in vitro assay, tubulin polymerization is stimulated by paclitaxel and inhibited by vincristine [13,14]. In the present study, coptisine inhibited

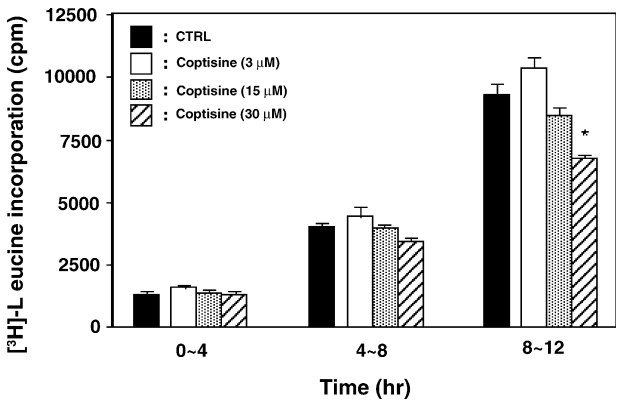


Fig. 6. Effect of coptisine on the incorporation of [^{3}H]leucine into VSMCs. VSMCs were serum-starved for 96 h to arrest the cells at G_0/G_1 phase and then incubated in the leucine-free DMEM supplemented with 10% FCS in the presence of coptisine at the indicated concentrations. For every 4 h after release from G_0/G_1 phase, cells were incubated with [^{3}H]leucine and the incorporation of [^{3}H]leucine was determined by liquid scintillation counter. Data are representative of three independent experiments with similar results and are expressed as means ± S.D. ($n = 3$).

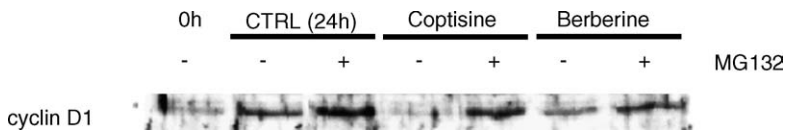


Fig. 7. Effect of a proteasome inhibitor, MG132, on decrease in cyclin D1 following coptisine or berberine treatment. VSMCs synchronized at G_0/G_1 phase were stimulated with 10% FCS in the presence of 30 μM coptisine or berberine. At 15 h after release from G_0/G_1 phase, 10 μM MG132 was added to the cultures and further incubated for another 9 h. The cells were harvested and subjected to Western blot analysis as described in Section 2.

tubulin polymerization, although the maximum inhibition was not more than approximately 50% at a concentration of 3 μM (Fig. 8).

4. Discussion

We previously reported that coptisine has a much more potent antiproliferative effect against VSMC than berberine (30-fold difference in GI_{50}), and that the effect of coptisine is selective for VSMC [12]. In the present study, we found that both coptisine and berberine completely abrogated the induction of cyclin D1 protein in serum-stimulated VSMCs by accelerating proteasome-mediated proteolysis, leading to the cell-cycle arrest at G_0/G_1 phase. In addition, coptisine, but not berberine, prevented the progression of the cell cycle at G_2/M phase at a concentration lower than that effective for G_0/G_1 block, probably by inhibiting tubulin polymerization.

In the arterial media, VSMCs are normally quiescent and remain in the G_0/G_1 phase of the cell cycle. In response to atherogenic stimuli or vessel injury, VSMCs migrate into the intimal layer of the arterial wall, where they leave

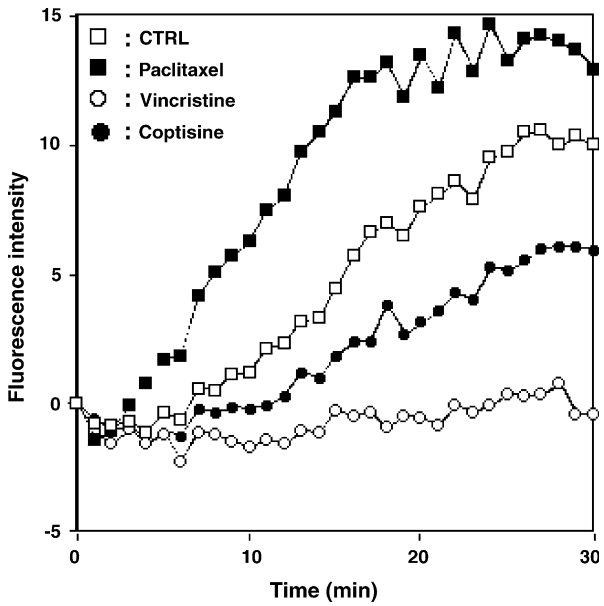


Fig. 8. Inhibitory effect of coptisine on tubulin polymerization. Tubulin polymerization was carried out using a tubulin polymerization assay kit as described in Section 2. The effect of coptisine was determined at a concentration of 3 μM, and paclitaxel and vincristine were used as control compounds at the same concentration. This result is representative of three independent experiments with similar results.

their quiescent state and reenter the cell cycle. In many cells, the transit from G_1 to S phase requires induction and activation of cyclin and cyclin-dependent kinase (CDK) complexes, predominantly cyclin D1/CDK4,6 and cyclin E/CDK2, which phosphorylate the retinoblastoma protein, releasing factors needed for the progeression into S phase. Cyclin D1 is expressed in low abundance in quiescent cells, but quickly accumulates upon the stimulation with serum or mitogens [15,16]. MAPK cascades are involved in the induction of cyclin D1 and especially ERK pathway is known to up-regulate the expression of cyclin D1 by activating *c-fos* and *c-jun*-mediated transcription [17–19]. When cells enter the S phase, cyclin D1 is rapidly degraded by proteasome–ubiquitin-mediated proteolysis [20]. In the present study, we examined the effect of coptisine and berberine on the activity of ERK1/2 and the mRNA expression of *c-fos* and *c-jun*. Neither alkaloids had any effect on ERK1/2 activity in VSMCs stimulated by serum or PDGF (data not shown). In addition, coptisine treatment did not influence the induction of mRNA for transcription factors *c-fos* or *c-jun*. Taken together, these results indicate that coptisine could not inhibit the signal transduction pathway upstream of cyclin D1 induction. In addition, coptisine selectively reduced the protein levels of cyclin D1 without affecting cyclin E, A, and B, and without affecting cyclin D1 mRNA. This discounts the possibility that down-regulation of cyclin D1 protein is mediated by inducing the CDK inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ or p53 [19].

There are several mechanisms that could explain the reduction of cyclin D1 protein by coptisine or berberine treatment. One possibility is that the translation of cyclin D1 mRNA was inhibited. However, total protein synthesis was not significantly reduced by coptisine, and a mechanism that could specifically suppress translation for cyclin D1 remains to be elucidated. Another possibility is that cyclin D1 is rapidly degraded following coptisine or berberine treatment. The evidence that a proteasome inhibitor abrogated coptisine- or berberine-induced reduction in cyclin D1 protein strongly suggests that coptisine and berberine activate proteasome-mediated proteolysis, finally leading to G_1 arrest. There are some antitumor compounds that down-regulate cyclin D1. GL331, a novel podophyllotoxin-derived compound down-regulates cyclin D1 expression in human lung adenocarcinoma cells without the effects on the expression of cyclins E, A, and B1, but reduces cellular cyclin D1 mRNA level down to 45% of control [21]. Tetrandrine induces early G_1-phase arrest

against human colon carcinoma cells by down-regulating several key G_1 regulatory proteins, such as CDK4, CDK6, cyclin D1, pRB, and E2F1 [22]. Retinoic acid triggers proteasome-dependent degradation of cyclin D1 as well as cyclin E in human bronchial epithelial cells [23]. Taking into consideration that coptisine shows antiproliferative effect specific to VSMCs and activates proteasome-mediated proteolysis specific to cyclin D1, coptisine appears to be a novel cell cycle regulatory compound, compared with these compounds. Recently, peroxisome proliferator-activated receptor-γ agonist troglitazone has been shown to down-regulate cyclin D1 expression as part of the mechanism for causing cell-cycle arrest and growth inhibition in breast cancer cells [24]. Moreover, the ablative effect is specific to cyclin D1 and the mRNA level of cyclin D1 remains unaltered in drug-treated cells. These results are in reasonably good agreement with our data, except the cells used in the study, suggesting the possibility that coptisine might act as a PPARγ agonist.

The difference in antiproliferative activity between coptisine and berberine is that coptisine causes G_2/M blolck via a partial inhibition of tubulin polymerization. We found that coptisine inhibited tubulin polymerization at a concentration of 0.1 μM (data not shown), but did not completely prevent tubulin polymerization even at 3 μM. Given that 3 μM coptisine accumulated VSMCs at G_2/M phase, the maximum inhibition of approximately 50% appeared sufficient to block cell cycle progression. However, the relationship between the potency of G_2/M block and the structure of the alkaloids remains to be investigated in detail.

Tubulin inhibitors such as vinca alkaloids, taxols and colchicines are used clinically as antineoplastic drugs [24]. A number of other tubulin inhibitors have been isolated from fungi, sponges, and marine mollusks [25–27]. These agents bind tubulin at distinct sites, and by inhibiting or stabilizing tubulin polymerization, lead to G_2/M arrest. Tubulin inhibitors have mostly been evaluated for antiproliferative or cytotoxic activity against tumor cell lines. However, taxol has been shown to interfere with VSMC migration and proliferation in vitro, and to inhibit neointimal formation in a rat carotid injury model [28]. Unfortunately, high systemic toxicity and low bioavailability prevent its use for vascular disorders.

VSMCs cause cardiovascular disease, and synovial cells cause arthritis, when they are abnormally activated by pathogenic stimuli or when they acquire the ability to proliferate excessively. The regulation of the cell proliferation should therefore be an important target for development of therapeutic agents for cardiovascular disease and arthritis, in addition to cancer. A specific inhibitor of VSMC proliferation, or of tubulin polymerization in VSMCs would be particularly valuable.

In conclusion, our findings show that coptisine blocks the cell cycle progression of VSMCs at G_0/G_1 phase by accelerating proteasome-mediated degradation of cyclin D1, and at G_2/M phase by inhibiting tubulin polymerization. Berberine, a structurally related isoquinoline alkaloid, instead exhibited block at G_0/G_1 but not at G_2/M. The difference between coptisine and berberine in antiproliferative activity against VSMCs was therefore dependent on the ability of coptisine to arrest the progression of the cell cycle at G_2/M phase. In addition, we have shown here for the first time the existence of a selective inhibitor with a cytostatic effect on VSMCs. These results may provide critical insights for the development of novel therapeutic agents against progressive atherosclerotic diseases, including restenosis.

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