Original Article

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Binding of phenochalasin A, an inhibitor of lipid droplet formation in mouse macrophages, on G-actin

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SUMMARY Phenochalasin A, a unique phenol-containing cytochalasin produced by the marine-derived fungus *Phomopsis* sp. FT-0211, was originally discovered in a cell morphological assay of observing the inhibition of lipid droplet formation in mouse peritoneal macrophages. To investigate the mode of action and binding proteins, phenochalasin A was radio-labeled by ^{125}I. Iodinated phenochalasin A exhibited the same biological activity as phenochalasin A. [^{125}I]Phenochalasin A was found to be associated with an approximately 40 kDa protein, which was identified as G-actin. Furthermore, detail analyses of F-actin formation in Chinese hamster ovary cells (CHO-K1 cells) indicated that phenochalasin A (2 μM) caused elimination of F-actin formation on the apical site of the cells, suggesting that actin-oriented specific function(s) in cytoskeletal processes are affected by phenochalasin A.

Keywords Phenochalasin, cytochalasin, actin, lipid droplet, macrophages

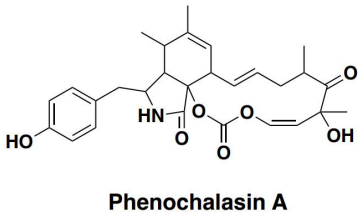
1. Introduction

Marine-derived microorganisms are a promising natural resource for new drug discovery because they produce a wide range of structurally diverse compounds. Phenochalasins A and B, produced by the marine-derived fungus *Phomopsis* sp. FT-0211 which was isolated from seawater collected near Ponape Island (*1,2*), were originally discovered in a cell morphological assay of observing the inhibition of lipid droplet formation in mouse peritoneal macrophages (*3*). These compounds belong to the well-known cytochalasan family (*4,5*). Intriguingly, phenochalasin A (Figure 1) possesses a unique phenol moiety in the structure, and this type of cytochalasans has not been reported to date. Thus, the marine-derived *Phomopsis* sp. FT-0211 is valuable for phenochalasin A production. Furthermore, cytochalasans are generally considered as mycotoxins showing cytotoxic activity to mammalian cells by binding to the actin filaments (*6*). Importantly, phenochalasin A inhibited lipid droplet formation in mouse macrophages without cytotoxic effect (*3*). These findings prompted us to investigate the mechanism of action of phenochalasin A on lipid droplet formation in mouse macrophages. In this study, we examined the action of phenochalasin A compared to cytotoxic cytochalasin on actin filament formation.

2. Materials and Methods

2.1. Materials

Phenochalasin A was produced by fermentation of the marine-derived fungus *Phomopsis* sp. FT-0211 in the production medium (seawater concentration was changed from 50% to 25%) at 27°C for 4 days according to a previously reported method (*1*). From the 10 L culture, pure phenochalasin A (15 mg) was obtained as white powder. Cytochalasin E, phosphatidylcholine, phosphatidylserine, dicetylphosphate, cholesterol, G-actin (derived from rabbit muscle), Ham's F-12 medium, and bovine serum albumin (BSA) (fatty acid free, fraction V) were purchased from Sigma-Aldrich (now Millipore Sigma, St. Louis, MO, USA). Iodination Beads were purchased from Pierce (now Thermo Fisher Scientific, Waltham, MA, USA). [^{125}I]Sodium iodinate and [^{14}C]oleic acid were purchased from PerkinElmer (Waltham, MA, USA). GIT medium was purchased from Nippon Seiyaku Co. (Tokyo, Japan). Penicillin (10,000 units/mL) and streptomycin (10,000 mg/mL) were purchased from Invitrogen (now Thermo Fisher Scientific, Waltham, MA, USA).



Phenochalasin A

Figure 1. The structure of phenochalasin A.

2.2. Preparation of [^{125}I]phenochalasin A

Preparation of [^{125}I]phenochalasin A was performed according to the instruction manual for Iodination Beads(*7-9*) with a slight modification. In brief, 100 µg of phenochalasin A (0.20 µmol) was dissolved in 500 µL of 20% methanol/phosphate-buffered saline (PBS)(-), and then 5.0 µL of [^{125}I]sodium iodinate (20.0 MBq) was added. After 30-min incubation at room temperature, the supernatant was collected and the beads were washed with 100 µL PBS(-). The aqueous solutions were two times extracted with 500 µL of ethyl acetate. The organic layer was collected and ethyl acetate was evaporated under nitrogen atmosphere. [^{125}I]Phenochalasin A solution was prepared at a concentration of 1 mg/mL using acetonitrile and stored at -20°C until use.

2.3. Cell culture

CHO-K1 cells (a generous gift from Dr. Kentaro Hanada, National Institute of Infectious Diseases, Tokyo, Japan) were maintained at 37°C in 5% CO_2 in medium A containing Ham's F-12 medium supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL) and streptomycin (100 mg/mL) using a method described previously (*10*).

2.4. Assay for [^{14}C]neutral lipid synthesis in mouse macrophages

The assay for the synthesis of neutral lipid from [^{14}C] oleic acid was conducted according to a previously described method (*11*). In brief, primary mouse peritoneal macrophages (5×10^5 cells/250 µL of GIT medium) in each well of a 48-well plastic plate (Corning Co., Corning, NY, USA) were incubated at 37°C in 5% CO_2 for 2 h. The medium was then replaced with 250 µL of DMEM containing 8% (v/v) lipoprotein-deficient serum (LPDS), penicillin (100 units/mL) and streptomycin (100 mg/mL) (hereafter referred to as medium B). After another 2-h preincubation, a test compound (2.5 µL in acetonitrile solution) and liposomes (10.0 µL, 1.0 µmol phosphatidylcholine, 1.0 µmol phosphatidylserine, 0.20 µmol dicetylphosphate and 1.5 µmol cholesterol, suspended in 1.0 mL of 0.3 M glucose) together with [^{14}C]oleic acid (5 µL (1.85 kBq) in 10% ethanol/PBS(-) solution) were added to each well. Following 14-h incubation, the medium was removed and the cells in each well were washed three times with PBS(-). The cells were lysed with 250 µL of 10 mM Tris·HCl/PBS(-) containing 0.1% (w/v) sodium dodecyl sulfate (SDS), and the cellular lipids were extracted by the method of Bligh and Dyer (*12*). After concentrating the organic solvent, the total lipids were separated on a TLC plate (silica gel F254, 0.5 mm thick, Merck, Darmstadt, Germany) and analyzed with a BAS-2000 (Fuji Film, Tokyo, Japan).

2.5. Binding of [^{125}I]phenochalasin A to macrophage cell lysate and G-actin

2.5.1. Preparation of lysate of mouse peritoneal macrophages

The microsomes of mouse peritoneal macrophages were prepared according to a previously described method (*13,14*). Briefly, mouse peritoneal macrophages (2×10^8 cells) were homogenized in 3.0 mL of cold Tris·HCl (pH 7.8) containing protease inhibitor cocktail (Roche) in a Teflon homogenizer. The supernatant was collected by centrifugation at 10,000 rpm for 15 min at 4°C. The lysate was prepared at a concentration of 5 mg of protein/mL and stored at -80°C until use.

2.5.2. Binding assay using [^{125}I]phenochalasin A

The macrophage lysate or G-actin (derived from rabbit, 40 µg or 10 µg in 80 µL of Tris·HCl (pH 7.8), respectively) was incubated with cytochalasin E (10 µL, 0-400 µmol) at 4°C for 15 min, then [^{125}I]phenochalasin A (370 kBq, 4 µmol, 2 µL) was added, and the samples were incubated at 4°C for 15 min. The binding assay was stopped by transferring an equal amount of 2× sample buffer containing 2.0% of 2-mercaptoethanol, which was boiled for 5 min at 95°C and centrifuged. A portion of supernatant was separated by SDS-PAGE at a constant current of 20 mA for 2 h. [^{125}I]Phenochalasin A binding proteins were detected by radioactivity using image analyzer BAS-2000.

2.6. Analysis of actin filaments in CHO-K1 cells by confocal microscopy

CHO-K1 cells (3×10^3 cells in 200 µL of medium A) in each well of an 8-well of glass chamber slide (Nalge Nunc International K.K., Rochester, NY, U.S.A.) were incubated at 37°C in 5% CO_2. Following the overnight recovery, a sample (2.0 µL in acetonitrile solution) was added to each well. After 1 and 6 h of incubation, cells were washed three times with 200 µL of PBS(-), and then fixed by soaking in 100 µL of 3.7% formalin for 20 min at room temperature. After washing three

times with PBS(-), actin filaments were stained with Alexa Fluor 594-phalloidin (0.66 µM, 100 µL in 3% BSA/PBS(-)) for 1 h at room temperature under shading condition. After washing three times with PBS(-), the cells were mounted with Prolong Antifade reagent (Thermo Fischer Scientific) according to the manufacturer's instructions. The formation of actin filaments was examined by confocal laser scanning microscopy (LSM-510 META, Carl Zeiss, Oberkochen, Germany).

2.7. MALDI-TOF-MS analysis of phenochalasin A treated-G-actin

A complex of a test sample (phenochalasin A and cytochalasin E) and G-actin was prepared by incubation of 2.1 µM of actin with 20 µM of a test sample in milliQ for 3 h on ice. The molecular weight of the actin incubated with or without a test sample was determined by a MALDI-TOF/MS spectrometer (AXIMA-CFR, SHIMADSU, Kyoto, Japan). Sinapinic acid (for proteome research, Wako) was used as the matrix for the measurement.

3. Results

3.1. Inhibition of macrophage-derived lipid droplet formation by iodinated phenochalasin A

As demonstrated in the previous study, phenochalasin A blocked the lipid droplet formation by selectively inhibiting cholesteryl ester (CE) synthesis (IC_{50}, 0.61 µM) without any cytotoxic effect (even at the highest dose of 20 µM) in mouse peritoneal macrophages. Furthermore, the target site of phenochalasin A is limited to within the prelysosomal stages of cholesterol metabolism in the lipid droplet synthetic process (*3*). To investigate the target molecule of phenochalasin A in this process, radiolabeled phenochalasin A was prepared by taking advantage of having a phenol residue in the structure to produce [^{125}I]phenochalasin A. During the setup conditions for the iodination reaction, monoiodinated ([I]) and diiodinated ([I_2])phenochalasin A were prepared. We confirmed that [I]- and [I_2] phenochalasin A exhibited inhibitory activity of lipid droplet formation and CE synthesis (Figure 2) in mouse macrophages analogous to phenochalasin A. Indeed, their IC_{50} values of CE synthesis were the range of 0.1 to 1.0 µM (Figure 2). Finally, under 30 min reaction time for the iodination, [^{125}I]phenochalasin A (including [^{125}I] and [$^{125}I_2$]phenochalasin A) was prepared and used in the subsequent experiments.

3.2. Analysis of [^{125}I]phenochalasin A-binding proteins in macrophage lysate

After the macrophage lysate (40 µg protein) was incubated with [^{125}I]phenochalasin A (370 kBq, 4 µmol), proteins in the lysate were separated by SDS-PAGE and radiolabeled proteins were detected by autoradiography. As shown in Figure 3 (right 3 lanes), an approximately 40 kDa protein was mainly radiolabeled. Preincubation of the lysate with excess of cytochalasin E (40 and 400 µmol) caused elimination of the radioactivity from the protein, suggesting that [^{125}I]phenochalasin A is strongly bound to actin filaments in the lysate. To validate this, pure G-actin (10 µg) was similarly treated with [^{125}I]phenochalasin A and analyzed (Figure 3 left 3

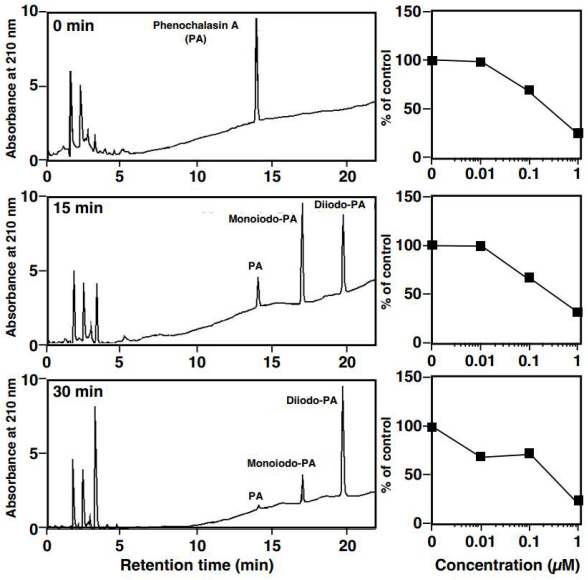


Figure 2. Iodination of phenochalasin A and its inhibitory effect on CE synthesis. Left lane shows HPLC chromatogram of iodination at indicated times. HPLC conditions were as follows; column, YMC-Pack D-ODS-5 (4.6 × 250 mm); mobile phase, 30-70% acetonitrile/H_2O-0.1% H_3PO_4 20-min linear gradient; flow rate, 1.0 mL/min; detection, UV at 210 nm. Right lane shows the inhibitory activity of CE synthesis of mixture of iodinated phenochalasin A at indicated reaction times.

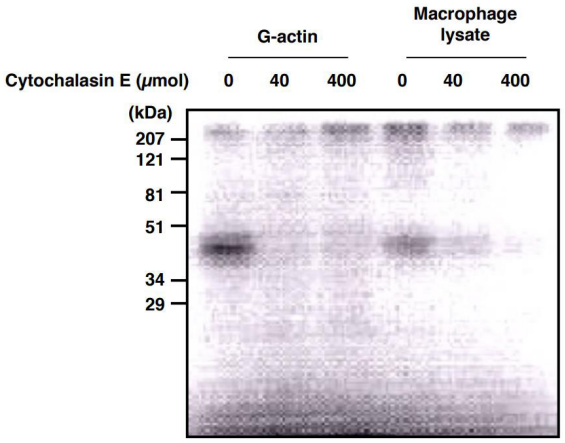


Figure 3. Binding of [^{125}I]phenochalasin A on G-actin and macrophage lysate. G-actin or macrophage lysate was incubated with [^{125}I]Phenochalasin A (4 µmol, 370 kBq) after the preincubation with cytochalasin E (0, 40, 400 µmol). Bound proteins were analyzed using SDS-PAGE and radio autography.

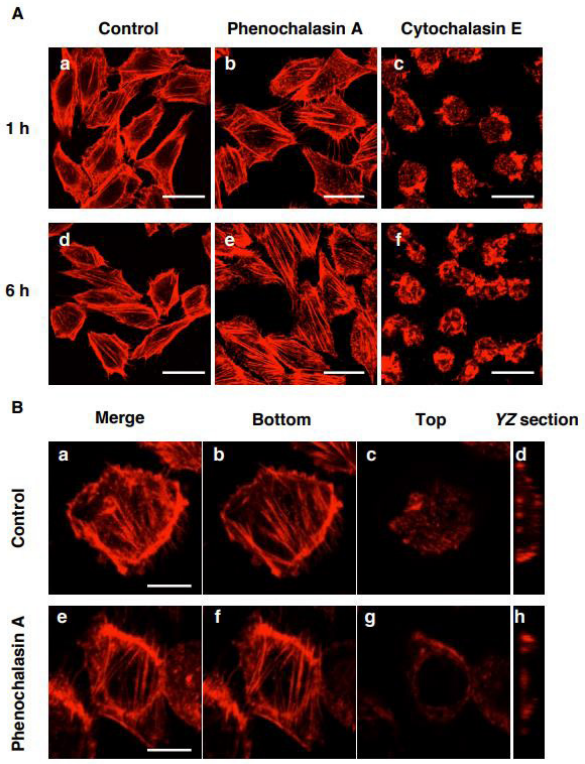


Figure 4. Effect of phenochalasin A on F-actin in CHO-K1 cells. **(A)** CHO-K1 cells were treated with phenochalasin A (2 μM) or cytochalasin E (2 μM). Following 1-h and 6-h incubation, cells were stained with Alexa Fluor 594-phalloidin. **a**, **d**: control cells; **b**, **e**: phenochalasin A-treated cells; **c**, **f**: cytochalasin E-treated cells. Bars, 20 μm. **(B)** CHO-K1 cells were treated with (**e-h**) or without phenochalasin A (2 μM) (**a-d**). Following 6-h incubation, cells were stained with Alexa Fluor 594-phalloidin. **a**, **e**; whole scanning images, **b**, **f**; bottom scanning images, **c**, **g**; top scanning images, **d**, **h**; sectioning images. Bars, 10 μm.

lanes). G-actin was found to be radiolabeled with [^{125}I] phenochalasin A, but not radiolabeled by preincubation with cytochalasin E. Thus, it was concluded that phenochalasin A is exclusively bound to actin filaments of mouse macrophages.

3.3. Effect of phenochalasin A on actin filament formation in CHO-K1 cells.

Since it is difficult to observe actin filaments in intact mouse peritoneal macrophages, CHO-K1 cells were used to investigate the effect of phenochalasin A on actin filament formation. Confocal microscopical observation (Figure 4A) indicated that ostensibly similar F-actin filaments were formed in control cells and phenochalasin A-treated cells, whereas F-actin filaments were fragmented/broken (at 1 h) and aggregated (at 6 h) in cytochalasin E-treated cells. To search for the difference between control and phenochalasin A-treated cells, F-actin formation was compared at the bottom (basal) and top (apical) sites between the cells (Figures 4B-g and -h). Intriguingly, F-actins disappeared at the top site of phenochalasin A-treated cells, suggesting that the specific function of actin is affected by phenochalasin A.

4. Discussion

In the previous study (*3,15*), we reported that 7 cytochalasans tested (two phenochalasins, three cytochalasins and two aspochalasins) inhibited macrophage-derived lipid droplet formation. Six cytochalasans except phenochalasin A caused morphological abnormality in macrophages at the doses corresponding to the IC_{50} values (0.20-3.0 μM) for CE synthesis. Only phenochalasin A did not induced such morphological abnormality even at a much higher dose (19 μM) than its IC_{50} value (0.61 μM) for CE synthesis. Regarding cytotoxic effects on macrophages measured by the MTT assay, cytochalasins D and E induced 10-fold higher IC_{50} values than the doses causing morphological abnormality. These findings indicate that most cytochalasans cause both morphological abnormality and plasma membrane damage on macrophages at similar doses, and certain ones like cytochalasins D and E induce morphological abnormality without plasma membrane damage at lower doses (*3,16*). Intriguingly, among cytochalasans, phenochalasin A has a very unique characteristic of lack ability to cause morphological abnormality and plasma membrane damage even at higher doses. Indeed, phenochalasin A did not show any cytotoxic effects on measurements by the alamar blue (detectable cell viability) and LDH assays (detectable cell membrane damage) in macrophages, and did not inhibit growth of CHO-K1 cells (Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=118*). Note that, to date, phenochalasin A is a rare cytochalasan with a phenol moiety in the structure only produced by the marine-derived fungus *Phomopsis* sp. FT-0211.

Cytochalasans are well known to disrupt the actin cytoskeleton (*4-6*) by binding to the barbed end of actin filaments (*17*), causing morphological abnormality and cytotoxic effect on mammalian cells. Therefore, we initially considered that the target molecule of phenochalasin A is not actin. However, phenochalasin A was found to be also bound to G-actin in macrophage lysates and purified G-actin using [^{125}I]phenochalasin A (Figure 3), which showed the same biological activity as phenochalasin A on CE synthesis on macrophages (Figure 2). Furthermore, the binding site of [^{125}I] phenochalasin A in G-actin was the same as that of cytochalasin E (Figure 3), and one phenochalasin A that appeared bound to one G-actin by MS analysis (Figure S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=118*). Accordingly, the biological characteristics observed by phenochalasin A appear very mysterious among cytochalasan family. To approach this question, actin filaments in CHO-K1 cells were observed after 1 (or 6)-h treatment of the

drug (phenochalasin A or cytochalasin E) (Figure 4A). Researchers have utilized CHO-K1 cells to investigate actin filament formation because clear actin filaments are observed in the cells. The dose of 2 μM phenochalasin A showed no effects on CHO-K1 cells by MTT assay, whereas 2 μM cytochalasin E caused 50% damage to the cells. The effects on actin filament formation in CHO-K1 cells are in good agreement with morphological changes and cytotoxicity observed in mouse macrophages, suggesting that phenochalasin A ostensibly caused almost no effects on actin filament formation in CHO-K1 cells even though it was bound to the actin. Although actin-cytochalasan complex structures have not been extensively studied, Nair *et al*. reported that cytochalasin D binds the hydrophobic cleft between subdomains 1 and 3 of G-actin based on the crystal structures (*17*). They showed 6 hydrogen bonds between the protein and the ligand, 5 of which were mediated by the isoindolone core and its adjacent cyclohexynol ring. The benzene ring in cytochalasin D structure did not appear to have any interaction with surrounding amino acids nor face outside from actin. According to the 3D structure, it might be that the hydroxy moiety of the phenol in phenochalasin A may be involved in a hydrogen bonding to an amino acid in actin or outside proteins to provide a different function of actin. Intriguingly, detailed analysis of actin filament formation using a confocal microscopy revealed that F-actins disappeared at the apical site of phenochalasin A-treated cells (Figures 4B-g and -H). Actin cytoskeleton interacts with a number of proteins such as actin related proteins (Arp2/3 *etc*) and actin accessory proteins (thymosin, profilin, formin and so on), to have diverse functions and interaction with plasma membranes (*18-20*). Therefore, it is plausible that phenochalasin A binding to G-actin affects a specific function of actin cytoskeleton, leading to blockade of lipid droplet formation in mouse macrophages. Further studies are required to clarify the mechanism of phenochalasin A.

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