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Effect of Fungal Metabolites Cytochalasans on Lipid Droplet Formation in Mouse Macrophages

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Effects of seven cytochalasans including cytochalasins B, D and E and novel phenochalasins A and B were tested on cytosolic lipid droplet formation and neutral lipid synthesis in mouse peritoneal macrophages. Phenochalasin A inhibited lipid droplet formation in a dose-dependent manner at least up to 20 μM without any morphological changes in macrophages. Cytochalasins D and E also inhibited lipid droplet formation only in a narrow range of concentrations, around 1 and 0.1 μM, respectively. At higher concentrations they gave morphological changes in macrophages. The other four cytochalasans only showed severe morphological changes in macrophages. Phenochalasin A and cytochalasins D and E inhibited cholesteryl ester (CE) synthesis specifically with IC_{50} values of 0.61, 2.4 and 0.20 μM, respectively, while the other cytochalasans inhibited both CE and triacylglycerol syntheses. Thus, among the cytochalasans tested, phenochalasin A showed very specific inhibition of CE synthesis and gave the lowest morphological changes in macrophages, resulting in the best inhibitor of lipid droplet formation in macrophages.

In the early stages of atherosclerogenesis, macrophages penetrate into the intima, efficiently take up modified low density lipoprotein (LDL), store cholesterol and fatty acids as neutral lipids in cytosolic lipid droplets, and are converted into foam cells, leading to the development of atherosclerosis in the arterial wall. The main components of neutral lipids in the lipid droplets are cholesteryl ester (CE) and triacylglycerol (TG). Therefore, inhibitors of macrophage-derived foam cell formation would be expected to retard the progression of atherosclerosis[1~4].

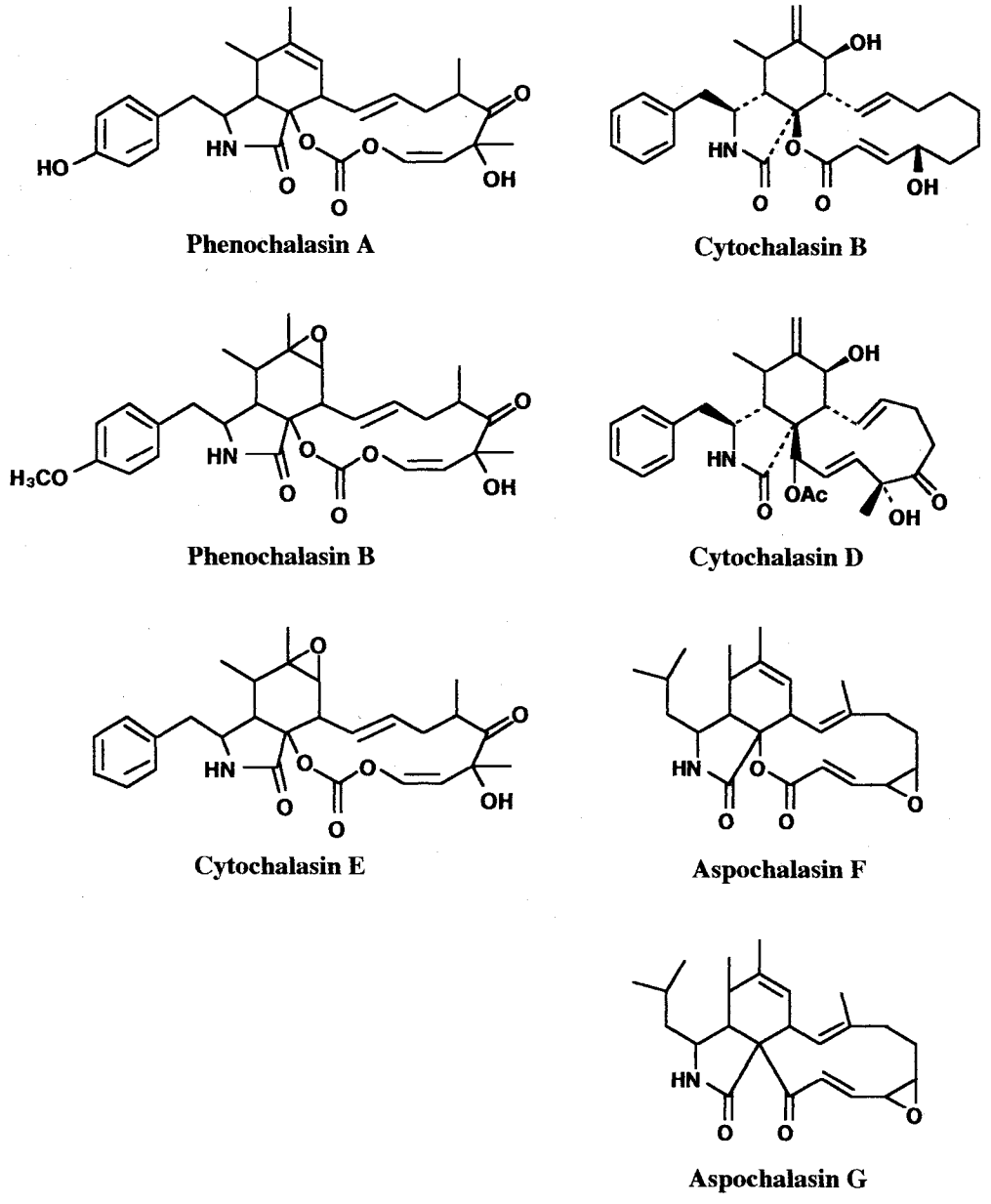
During our screening program for microbial inhibitors of mouse macrophage-derived foam cell formation, novel cytochalasans named phenochalasins A and B were isolated from the culture broth of *Phomopsis* sp. FT-0211[5,6]. TABAS *et al.* reported that cytochalasin D, a typical member of the cytochalasan family, inhibits lipid droplet formation by specifically blocking CE synthesis in mouse macrophages, suggesting that actin cytoskeleton is responsible for CE synthesis in the process of macrophage-derived foam cell formation[7]. As described in the previous paper[5], phenochalasin A showed inhibition of lipid droplet formation without any morphological changes in macrophages, while phenochalasin B caused severe morphological changes. These findings prompted us to compare the effects of seven different cytochalasans (Fig. 1) on lipid droplet formation, neutral lipid synthesis in mouse peritoneal macrophages and toxicity to macrophages. This paper shows that phenochalasin A is the most specific inhibitor of mouse macrophage-derived foam cell formation among the cytochalasans tested.

Materials and Methods

Materials

Phenochalasins A and B were purified from the culture broth of *Phomopsis* sp. FT-0211 as reported previously[5]. Aspochalasins F and G were purified from the culture broth of *Aspergillus* sp. FO-4282[8]. [1-^{14}C]Oleic acid (50 mCi/mmol) was purchased from DuPont NEN. Dulbecco's modified Eagle's medium (DMEM) and Hank's buffered salt solution (HBSS) were purchased from Nissui Pharma-

Fig. 1. Structures of cytochalasans.



ceutical Co., Ltd., GIT medium was from Nippon Seiyaku Co., Ltd., and penicillin (10,000 units/ml), streptomycin (10,000 μg/ml), and glutamine (200 mM) solutions were from GIBCO. Phosphatidylcholine, phosphatidylserine, dicetylphosphate, cholesterol, 3β-hydroxy-5-pregnen-20-one (pregnenolone), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and cytochalasins B, D and E were purchased from Sigma. CL-283,546, an ACAT inhibitor[9], was a generous gift from Dr. HANS-JURGEN HESS, Pfizer, Groton, CT. Other reagents used were of commercially available analytical grade.

Mouse peritoneal macrophages were prepared from female ICR mice (25~30 g, Japan SLC Inc.) as described previously[11].

Assay for Lipid Droplet Formation by Macrophages

Assay for lipid droplet formation in mouse peritoneal macrophages was carried out by the method described previously[11]. In brief, primary mouse peritoneal macrophage (2.5×10^5 cells in GIT medium) in each well of a 96-well plastic microplate (Corning Co.) were incubated in a humidified CO_2 (5% v/v) atmosphere at 37°C for 2

hours. The medium was then replaced with 0.125 ml DMEM containing 8% (v/v) lipoprotein-deficient serum (LPDS), penicillin (100 units/ml) and streptomycin (100 μg/ml) (hereafter referred to as medium A). After another 2-hour preincubation, 1.25 μl of a sample (in MeOH), and 5.0 μl of liposomes (phosphatidylcholine 1.0 μmol, phosphatidylserine 1.0 μmol, dicetylphosphate 0.20 μmol and cholesterol 1.5 μmol suspended in 1.0 ml of 0.3 M glucose) were added to each well. After a 14-hour incubation, the cells were washed with PBS and then fixed by soaking in 10% formalin. After staining with hematoxylin and oil red O, nuclei, intracellular lipid droplet formation and morphology of macrophages were examined under a light microscope (Vanox-S model, Olympus).

Assay for Cell Viability

Mouse macrophage viability after a 14-hour incubation under conditions of lipid droplet formation was measured using MTT by the established method[10].

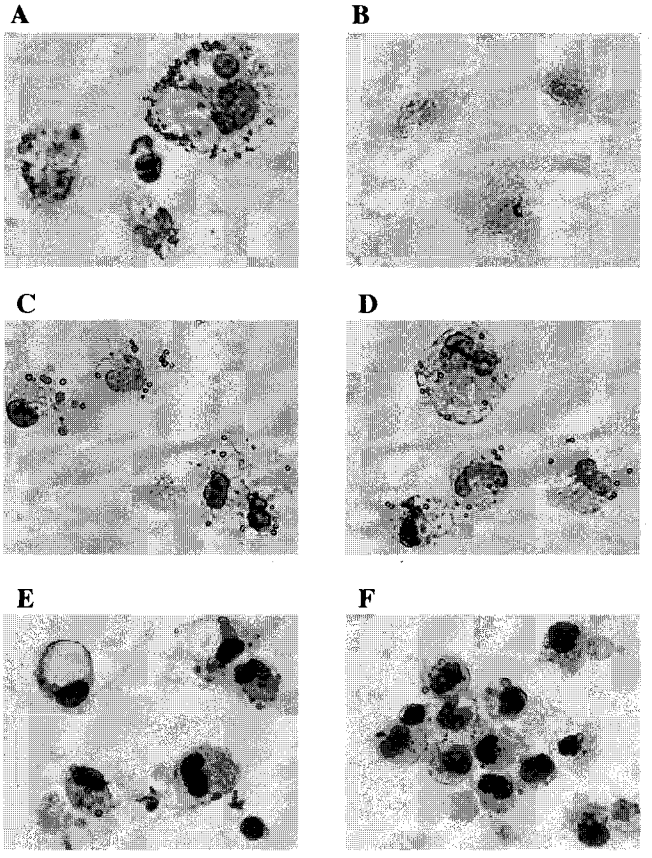
Assay for [^{14}C]Neutral Lipid Synthesis by Macrophages

Assay for [^{14}C]CE and [^{14}C]TG syntheses from [^{14}C]oleic acid in macrophages was carried out by the method described previously[11]. In brief, macrophages (5×10^5 cells/0.25 ml medium A) were cultured in each well of a 48-well plastic microplate (CORNING), and then 2.5 μl of a sample (MeOH solution) and 10 μl of liposomes together with 5 μl of [^{14}C]oleic acid (1 nmol, 0.05 μCi, 10% EtOH/PBS solution) were added to each culture. Following a 14-hour incubation, the medium was removed, and the cells in each well were washed three times with HBSS. The cells were lysed by adding 0.25 ml of PBS containing 0.1% (w/v) sodium dodecyl sulfate, 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 F_{254}, 0.5 mm thick, Merck) and analyzed with a radioscanner (AMBIS Systems, Inc.) as described previously[11].

Assay for Lysosomal [^{14}C]Cholesterol Metabolism in Macrophages

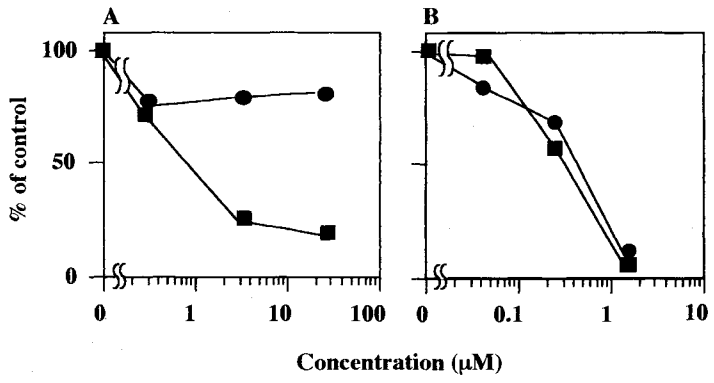
The metabolism of lysosomal [^{14}C]cholesterol in mouse macrophages was measured by the method reported by FURUCHI *et al.*[13]. In brief, macrophages (5×10^5 cells) in each well of a 48-well plastic microplate were cultured in 0.25 ml of medium A containing [^{14}C]cholesterol (3.7 nmol, 200 nCi)-supplemented liposomes (10 μl) and pregnenolone (in 2.5 μl methanol at a final concentration of 10 μM). After a 12-hour incubation, [^{14}C]cholesterol were accumulated in lysosomes. The metabolism of lysosomal [^{14}C]cholesterol was restarted by washing the cells twice with buffer B (150 mM NaCl and 50 mM Tris-HCl, pH 7.4) containing BSA (2 mg/ml) and once with buffer B, and then by incubating the cells in 0.25 ml of medium A in the presence or absence of phenochalasin A or CL-283,546 (in 2.5 μl MeOH). After a 5-hour incubation, cellular lipids were extracted with hexane - 2-propanol (3 : 2, 1 ml×2), and separated on a TLC plate to analyze the distribution of [^{14}C]CE and [^{14}C]cholesterol by the same method described above.

Fig. 2. Effect of cytochalasans on lipid droplet formation in mouse peritoneal macrophages and morphology to macrophages.



Macrophage monolayers (4.0×10^5 cells in 0.2 ml medium) grown in 96-well plastic microplate were incubated for 16 hours with liposomes (8 μl) (A), without liposomes (B), or with liposome (8 μl) in the presence of 2 μM phenochalasin A (C), 20 μM phenochalasin A (D), 10 μM cytochalasin D (E), or 1 μM cytochalasin E (F). Original magnification, ×200.

Fig. 3. Effect of phenochalasins on [^{14}C]neutral lipid synthesis from [^{14}C]oleic acid by mouse macrophages.



Macrophage monolayers obtained from 5×10^5 cells/well in 48-well plastic microplate were incubated in 0.25 ml of medium A with a liposome and [^{14}C]oleic acid in the absence or presence of indicated amounts of phenochalasin A (A) or phenochalasin B (B). After a 14-hour incubation, cholesteryl [^{14}C]oleate (■) and [^{14}C]triacylglycerol (●) were separated on a TLC and determined with a radioscanner as described in the "Materials and Methods". The results are plotted as % of control (without a drug).

Results

Effect of Cytochalasans on Lipid Droplet Formation in Macrophages

When mouse peritoneal macrophages were incubated with liposomes, a number of lipid droplets were observed in cytosols of macrophages (Fig. 2A). Phenochalasin A inhibited lipid droplet formation in a dose-dependent manner at least up to 20 μM in that the size and the number of lipid droplets decreased (Fig. 2C and D). Cytochalasins D and E showed moderate inhibition of the lipid droplet formation at 1.0 and 0.1 μM (data not shown), but caused morphological changes in macrophages at 10 (Fig. 2E) and 1.0 μM (Fig. 2F), respectively, where about 50% of macrophages became smaller in size and round in shape. The other cytochalasans showed only severe morphological changes in almost all macrophages at concentrations tested (0.1~10 μM, Table 1). These findings indicate that phenochalasin A inhibits lipid droplet formation without any morphological changes in mouse macrophages up to 20 μM.

Effect of Cytochalasans on the Synthesis of [^{14}C]Neutral Lipids from [^{14}C]Oleic Acid in Macrophages

Under conditions of lipid droplet formation in the presence of [^{14}C]oleic acid, [^{14}C]oleic acid is incorporated into CE and TG fractions[14]. Therefore, the effect of cytochalasans on [^{14}C]CE and [^{14}C]TG syntheses from [^{14}C]oleic acid was investigated. As shown in Fig. 3, phenochalasin A inhibited [^{14}C]CE synthesis in a dose-dependent manner with an IC_{50} value of 0.61 μM, but showed almost no effect on [^{14}C]TG synthesis. However, phenochalasin B inhibited both [^{14}C]CE and [^{14}C]TG syntheses to similar extents. The IC_{50} values of cytochalasans for CE or TG synthesis are summarized in Table 1. As well as phenochalasin A, cytochalasin E and D inhibited CE synthesis specifically with IC_{50} values of 0.61, 0.20 and 2.4 μM, respectively. Cytochalasin B was rather specific in CE inhibition (IC_{50}; 2.9 *vs.* 11 μM), but the other three inhibited both CE and TG syntheses.

Effect of Cytochalasans on Macrophage Viability and Morphology

Cytotoxic effect of cytochalasans on mouse macrophages was evaluated by the MTT assay[10]. As shown in Table 1, phenochalasin B, cytochalasin E and aspochalasins F and G caused cytotoxic effect with IC_{50} values of 0.5, 2.0, 2.0 and 1.0 μM, respectively. Cytochalasin B showed moderate cytotoxicity. However, cytochalasin D gave very weak cytotoxicity to macrophages with 77% viability at 20 μM, and phenochalasin A caused no cytotoxic effect even at 20 μM. From observation of macrophage morphology under conditions of lipid droplet formation, only phenochalasin A

Table 1. Effects of cytochalasans on cholesteryl ester and triacylglycerol syntheses, morphology and toxicity in mouse macrophages.

Compound	IC_{50}[a]		Morphology [b]	Cytotoxicity [c]	Cytotoxicity / IC_{50} of CE
	CE	TG			
	(μM)				
Phenochalasin A	**0.61**	**>19**	**>19**	**>20**	**>33**
Phenochalasin B	**0.23**	**0.38**	**0.18**	**0.50**	**2.2**
Cytochalasin B	**2.9**	**11**	**1.8**	**10**	**3.4**
Cytochalasin D	**2.4**	**>19**	**1.8**	**>20**	**>8.3**
Cytochalasin E	**0.20**	**>19**	**0.19**	**2.0**	**10**
Aspochalasin F	**3.0**	**5.0**	**2.3**	**2.0**	**0.67**
Aspochalasin G	**1.0**	**2.0**	**2.4**	**1.0**	**1.0**

a: Concentration of a compound which inhibits [^{14}C]CE or [^{14}C]TG synthesis from [^{14}C] oleic acid by 50% in macrophages.

b: Minimal concentration of a compound which shows morphological changes in macrophages.

c: Concentration of a compound which causes 50% viability of macrophages by MTT assay.

caused no effect even at 19 μM, while the others including cytochalasin D produced morphological changes in macrophages at 0.18~2.4 μM (Table 1). As a result, phenochalasin A was found to give the lowest effect on mouse macrophages in both MTT and morphological analyses among the seven cytochalasans.

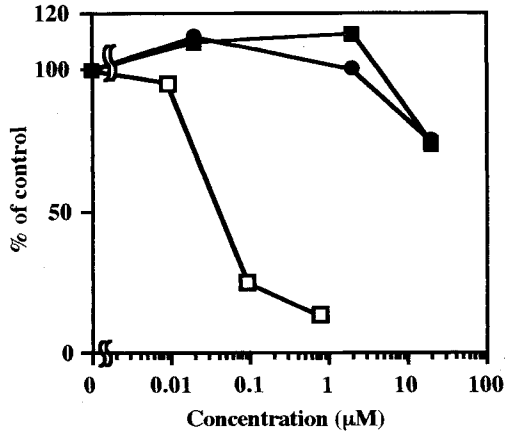
Effect of Phenochalasin A on the Metabolism of Lysosomal [^{14}C]Cholesterol

When macrophages are incubated with [^{14}C]cholesterol-supplemented liposomes in the presence of pregnenolone, unesterified [^{14}C]cholesterol is accumulated in lysosomes of macrophages. The lysosomal [^{14}C]cholesterol can be metabolized by washing the cells to remove pregnenolone and then by incubating again to yield [^{14}C]CE in cytosolic lipid droplets. With this experiment, it can be speculated whether the inhibition site of a drug lies in a prelysosomal or postlysosomal stage[13]. As shown in Fig. 4, phenochalasin A and cytochalasin D showed almost no effect on [^{14}C]CE synthesis at 2.0 μM and only a slight inhibition at 20 μM. However, CL-283,546, an ACAT inhibitor, inhibited [^{14}C]CE synthesis in a dose-dependent fashion with an IC_{50} value of 40 nM, which is almost the same as that of CL-283,546 for [^{14}C]CE synthesis from [^{14}C]oleic acid synthesis (data not shown). These results indicated that phenochalasin A blocks the prelysosomal stages of the cholesterol metabolism as well as cytochalasin D.

Discussion

Fungal metabolites cytochalasans such as cytochalasins B, D and E have been reported to disrupt the actin cytoskeleton[15]. Phenochalasins, recently discovered as inhibitors of lipid droplet formation in mouse macrophages, are members of the cytochalasan family. TABAS *et al.* reported that cytochalasin D inhibits lipid droplet formation by blocking the cholesterol esterification in macrophages, leading a conclusion that actin cytoskeleton plays an important role in CE synthesis[7]. In order to speculate on the relationship between synthesis of lipid droplets and function of actin cytoskeleton, effects of seven cytochalasans including phenochalasins and cytochalasin D were compared on lipid droplet formation, neutral lipid synthesis in macrophages and macrophage viability. From microscopical observations, phenochalasin A showed a dose-dependent inhibition of lipid droplet formation

Fig. 4. Effect of phenochalasin A on the metabolism of lysosomal [^{14}C]cholesterol.



Macrophages (5×10^5 cells in 0.25 ml medium) grown in a 48-well plastic microplate were incubated with 10 ml of [^{14}C]cholesterol (3.7 nmol, 200 nCi)-supplemented liposomes for 12 hours in the presence of 10 μM pregnenolone. After incubation, the medium was removed, and the cells in each well were washed twice with buffer B containing BSA (2 mg/ml) and once with buffer B, and then incubated in 0.25 ml of medium A containing phenochalasin A (■) or cytochalasin D (●) or CL-283,546 (□). After a 5-hour incubation, [^{14}C]CE was separated by TLC, and measured with a radioscanner as described in "Materials and Methods". The results are plotted as % of control (without a drug).

without morphological changes in macrophages up to 20 μM (Fig. 2C and 2D). Cytochalasins D and E also inhibited lipid droplet formation in a narrow range of drug concentrations, but caused morphological changes at higher concentrations (Table 1). The other cytochalasans showed severe morphological changes. From biochemical analyses, phenochalasin A and cytochalasins D and E inhibited CE synthesis specifically, but the others inhibited both CE and TG syntheses (Table 1). Thus, it is demonstrated that phenochalasin A and cytochalasins D and E inhibit CE synthesis specifically, leading to an inhibition of lipid droplet formation in macrophages. On the other hand, the other cytochalasans cause cytotoxic effect and morphological changes on macrophages, resulting in inhibition of both CE and TG syntheses. Interestingly, except for phenochalasin A, a good correlation was shown between the IC_{50} values for CE synthesis and minimal concentrations causing morphological changes (Table 1).

Taken together, phenochalasin A is the best inhibitor of lipid droplet formation in mouse macrophages due to the specific inhibition of CE synthesis and the lowest cytotoxic effect among cytochalasans tested. Although phenochalasin A blocked prelysosomal stages of the cholesterol metabolism as well as cytochalasin D (Fig. 3), the inhibition site of phenochalasin A seems different from that of cytochalasin D because of different effects on macrophage morphology. Further studies on the effect of phenochalasin A on actin cytoskeleton and the inhibition site in lipid droplet formation remain to be investigated.

Acknowledgments

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