Molecular Target of Piperine in the Inhibition of Lipid Droplet Accumulation in Macrophages

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Received February 23, 2008; accepted March 20, 2008; published online April 2, 2008

An alkaloid piperine isolated from the *Piper Nigrum* was found to inhibit lipid droplet accumulation in mouse macrophages, and especially inhibited cholesteryl ester (CE) synthesis (IC₅₀: 25 μ M). The metabolism of cholesterol from lysosome to lipid droplet was inhibited with a similar IC₅₀ (18 μ M), indicating that the site of inhibition is one of the steps between the lysosomes and the endoplasmic reticulum. Therefore, effects of piperine on acyl-CoA:cholesterol acyltransferase (ACAT) activity in the microsomes prepared from mouse macrophage and liver were studied, to show that the compounds inhibited the activity in both cases (IC₅₀: 9.1, 7.0 μ M, respectively). Furthermore, piperine was found to inhibit both ACAT1 and ACAT2 isozymes to a similar extent (IC₅₀: 16, 18 μ M, respectively) in cell-based assays using ACAT1- or ACAT2-expressing cells. Thus, it was suggested that piperine inhibited macrophage ACAT to decrease CE synthesis, leading to a reduction of lipid droplets.

Key words lipid droplet accumulation; atherosclerosis; acyl-CoA:cholesterol acyltransferase; black pepper; piperine; functional food

In the early stage of atherosclerosis, macrophages penetrate into the intima, efficiently take up modified low density lipoprotein (LDL), store cholesterol and fatty acid as the respective forms of cholesteryl ester (CE) and triacylglycerol (TG) in cytosolic lipid droplets, and are converted to foam cells, leading to the development of atherosclerosis in the arterial wall. Therefore, inhibitors of lipid droplet accumulation in macrophages would be expected to retard the progression of atherosclerosis.^{1–4)}

We have established a cell-based assay for lipid droplet synthesis using mouse macrophage.⁵⁾ During the course of our screening program, microbial metabolites of beauveric acid, phenochalasin,^{6–9)} spylidone,^{10,11)} isobisvertinol,¹²⁾ and K97-0239¹⁴⁾ were discovered as inhibitors of lipid droplet accumulation in macrophages. In further screening studies, a known alkaloid, piperine¹⁵⁾ (Fig. 1), was found to show the activity. Piperine is one of the major components of long peppers and black peppers (*Piper longum* and *Piper nigrum*), which have been used as spices, medicines, preservatives and perfumes.¹⁶⁾

Here, we show that piperine inhibits lipid droplet accumulation by blocking acyl-CoA:cholesterol acyltransferase (ACAT) activity in macrophages. We further show that the compound inhibits both ACAT1 and ACAT2 isozymes to a similar extent.

MATERIALS AND METHODS

Materials Piperine was purchased from the National

Fig. 1. Structure of Piperine

Pharmaceutical Engineering Center (NPEC) for Solid Preparation in Chinese Herbal Medicine (Nanchang, China). [1-¹⁴C]Oleic acid (1.85 GBq/mmol) and [4-¹⁴C]cholesterol (1.85 GBq/mmol) were purchased from PerkinElmer Life & Analytical Sciences. [1-¹⁴C]Oleoyl-coenzyme A (CoA) (1.85 GBq/mmol) was purchased from Amersham Biosciences. Dulbecco's modified Eagle medium (DMEM) and Hank's buffered salt solution (HBSS) were purchased from Nissui Pharmaceutical Co., GIT medium was from Nippon Seiyaku Co., and penicillin (10000 units/ml), streptomycin (10000 mg/ml), and glutamine (200 mM) solution were from GIBCO. Phosphatidylcholine, phosphatidylserine, dicetylphosphate, cholesterol and 3 β -hydroxy-5-pregnen-20-one (pregnenolone) were purchased from Sigma.

Assay for Lipid Droplet Formation in Macrophages

The assay for lipid droplet formation in mouse peritoneal macrophages was carried out according to the method described previously.¹⁵⁾ In brief, primary mouse peritoneal macrophages (2.5×10^5 cells in GIT medium) in each well of a 96-well plastic microplate (Corning Co.) were incubated in a humidified CO₂ (5% v/v) atmosphere at 37°C for 2 h. The medium was then replaced with 0.2 ml of DMEM containing 8% (v/v) lipoprotein-deficient serum (LPDS), penicillin (100 units/ml) and streptomycin (100 mg/ml) (hereafter referred to as medium A). After another 2-h preincubation, 2.0 μ l of a sample in methanol, and 8.0 μ l of liposomes (phosphatidylcholine 1.0 μ mol, phosphatidylserine 1.0 μ mol, dicetylphosphate 0.20 μ mol and cholesterol 1.5 μ mol, suspended in 1.0 ml of 0.3 M glucose) were added to each well. After a 14-h incubation, the cells were washed with PBS and then fixed by soaking in 10% formalin. Nuclei and intracellular neutral lipid droplets were then stained with hematoxylin and oil red O, respectively. The lipid droplet formation and morphological changes in macrophages were examined by confocal-laser scanning microscopy (LSM-510 META, Carl Zeiss).

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Cytotoxicity of Piperine to Macrophages The cytotoxicity of piperine to mouse macrophages after a 14-h incubation was measured using alamar blue reagent (Biosource) according to the established method.⁹⁾

Assay for Neutral Lipid Synthesis in Macrophages The assay for the synthesis of [¹⁴C]CE and [¹⁴C]TG from [¹⁴C]oleic acid in mouse macrophages was carried out according to the method described previously.⁵⁾ In brief, mouse peritoneal macrophages (5×10^5 cells/0.25 ml medium A) were cultured in each well of a 48-well plastic microplate (Corning Co.), and then 2.5 μ l of a test compound (MeOH solution) and 10 μ l of liposomes together with 5 μ l (1.85 kBq) of [¹⁴C]oleic acid (10% EtOH/PBS solution) were added to each culture. Following a 14-h incubation, the medium was removed, and the cells in each well were washed three times with PBS. 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.¹⁷⁾ After concentrating the organic solvent, the total lipids were separated on a TLC plate (silica gel F254, 0.5 mm thick, Merck) and analyzed with a BAS2000 (Fuji Film).

Assay for Lysosomal Cholesterol Metabolism in Macrophages The metabolism of lysosomal [¹⁴C]cholesterol in mouse macrophages was measured as described previously.¹⁸⁾ Briefly, macrophages (5×10^5 cells) were incubated for 2 h in a 48-well plastic microplate, washed with HBSS, and placed in 0.25 ml of medium A containing 10 μ l of liposomes supplemented with [¹⁴C]cholesterol (7.4 kBq) and pregnenolone (added as 2.5 μ l of a methanol solution to make a final concentration of 10 μ M). After incubation for 12 h, the medium was removed, and the cells were washed twice with buffer B (150 mM NaCl and 50 mM Tris-HCl, pH 7.4) containing BSA (2 mg/ml), then with buffer B without BSA, and subsequently incubated in 0.25 ml of medium A containing a test compound (added as 2.5 μ l of a methanol solution) for 5 h. The cells were washed three times with PBS, and then 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.¹⁷⁾ After reducing the organic solvent by evaporation, the total lipids were separated on a TLC plate and the radioactivity of [¹⁴C]CE was measured according to the same method described above.

Assay for ACAT Activity in Microsomes Prepared from Mouse Macrophages and Livers Mouse peritoneal macrophages (2×10^8 cells) and mouse livers (0.5 g) were homogenized in 3.0 ml of cold buffered sucrose solution (pH 7.2) containing 100 mM sucrose, 50 mM KCl, 40 mM KH₂PO₄ and 30 mM EDTA (hereafter referred to as Buffer C) in a Teflon homogenizer. The microsomes were pelleted by centrifugation at $100000 \times g$ for 1 h at 4 °C, resuspended in the same buffer at a concentration of 5 mg protein/ml and stored at -80 °C until use.

ACAT activity was determined using the isotope method described previously¹⁹⁾ with minor modifications. Briefly, an assay mixture containing 2.5 mg/ml BSA in Buffer C and [¹⁴C]oleoyl-CoA (18.5 kBq) together with a test compound (added as 10 μ l of a methanol solution to make a final concentration of 0–150 μ M), and the microsomal fraction (100 μ g of protein) in a total volume of 200 μ l were incu-

bated at 37 °C for 5 min. The reaction was stopped by adding 1.2 ml of CHCl₃:MeOH (2:1), and the product [¹⁴C]CE was extracted by the method of Bligh and Dyer.¹⁷⁾ After removing the organic solvent by evaporation, the lipids were separated on a TLC plate and the radioactivity of [¹⁴C]CE was measured as described above.

Assay for ACAT Isozyme Activity ACAT1-CHO and ACAT2-CHO cells were kindly provided by Prof Rudel (Wake Forest University) and were cultured by the method described previously.²⁰⁾ In brief, both cell lines were maintained at 37 °C in 5% CO₂ in Ham's F-12 medium supplemented with MEM vitamins, geneticin (G418 sulfate) (300 μ g/ml) and 10% heat inactivated fetal bovine serum (FBS) (hereafter referred to as medium B). ACAT1- or ACAT2-CHO cells (1.25×10^5 cells/0.25 ml in medium B) were cultured in a 48-well plastic microplate and allowed to recover overnight at 37 °C in 5% CO₂. The assays were done with cells that were at least 80% confluent. Following the overnight recovery, 2.5 μ l of a sample (methanol solution) and 5 μ l of [1-¹⁴C]oleic acid (1 nmol, 1.85 kBq, 10% ethanol/PBS solution) were added to each culture at 37 °C in 5% CO₂. After a 6-h incubation, the medium was removed, and the cells in each well were washed twice with PBS. The cells were lysed by adding 0.25 ml of 10 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) sodium dodecyl sulfate, and [¹⁴C]CE were analyzed by the same method described above. In this assay, [¹⁴C]CE was produced by the reaction of ACAT1 or ACAT2. ACAT inhibitory activity (%) is defined as $(1 - [\text{¹⁴C]CE-drug} / [\text{¹⁴C]CE-control}) \times 100$. The IC₅₀ value is defined as the drug concentration causing 50% inhibition of an enzyme (or biological) activity.

RESULTS

Inhibition of Lipid Droplet Accumulation in Macrophages by Piperine In this macrophage assay, significant amounts of lipid droplets accumulated in the cytosol, as observed microscopically after oil red O staining, when the macrophages were incubated with liposomes. Under these conditions, piperine was found to dose-dependently inhibit lipid droplet accumulation: significant inhibition at 19 μ M (Fig. 2A). However, piperine showed no observable morphological changes or cytotoxic effects in macrophages at these concentrations.

Selective Inhibition of Cholesteryl Ester Synthesis in Macrophages by Piperine Under the conditions of lipid droplet accumulation in mouse macrophages, about 40% of exogenously added [¹⁴C]oleic acid was incorporated into the neutral lipids [¹⁴C]CE and [¹⁴C]TG, which are the main constituents of lipid droplets in the cytosol.⁵⁾ As shown in Fig. 2B, piperine selectively inhibited [¹⁴C]CE synthesis with an IC₅₀ value of 25 μ M. But a slight inhibition of [¹⁴C]TG synthesis was observed at 200 μ M. No cytotoxic effects were observed even at the highest dose of the drug. These data are consistent with the results found in the assay of lipid droplet accumulation (Fig. 2A).

Inhibition of Postlysosomal Cholesterol Metabolism in Macrophages by Piperine In order to gain insight regarding the target molecule of piperine in the inhibition of lipid droplet accumulation in macrophages, the effects of piperine on the postlysosomal process of cholesterol metabolism were

Fig. 2. Inhibition of Lipid Droplet Accumulation in Macrophages by Piperine

(A) Macrophage monolayers (5×10^5 cells in 0.2 ml of medium) grown in tissue culture chambers (LAB-TEK 8-chamber, Nunc) were incubated with 8 μ l of liposomes for 16 h in the absence or presence of 0.19, 1.9, or 19 μ M piperine. Fixation and staining with oil red O and hematoxylin were performed. Bar=20 μ m. (B) Effect of piperine on synthesis of [14 C]neutral lipid from [14 C]oleic acid in macrophages. Macrophage monolayers obtained from 5×10^5 cells/well in a microplate were incubated in 0.25 ml of medium with phospholipid/cholesterol liposomes composed of phosphatidylcholine, phosphatidylserine, dicetylphosphate, and cholesterol at a molar ratio of 10:10:2:15 and [14 C]oleic acid in the absence or presence of the indicated amounts of piperine. After a 14-h incubation, cholesteryl [14 C]oleate (■) and [14 C]triacylglycerol (●) were separated by TLC, and the intensities of their signals were determined with a bioimage analyzer as described in Materials and Methods and plotted as % of control (without drugs). Macrophage monolayers obtained from 5×10^5 cells/well in a microplate were incubated in 0.25 ml of medium with alamar BlueTM. After a 3-h incubation, viability (▲) was determined by measurement of the amount of alamar BlueTM as described in Materials and Methods and plotted as % of control (without drugs). Values represent means $\pm$ S.D. of more than two duplicate experiments.

studied. When macrophages were incubated with [14 C]cholesterol-supplemented liposomes in the presence of 10 μ M pregnenolone, [14 C]CE formation was almost completely suppressed, and unesterified [14 C]cholesterol accumulated in the lysosomes of the macrophages.¹⁸ After pregnenolone was removed by washing the cells with buffer, the metabolism of lysosomal [14 C]cholesterol to [14 C]CE was restarted in the presence or absence of piperine. As shown in Fig. 3, piperine inhibited [14 C]CE synthesis in a dose-dependent fashion with an IC_{50} value of 18 μ M, which is similar to that for the synthesis of [14 C]CE from [14 C]oleic acid (Fig. 2B). These data indicate that piperine blocks the postlysosomal process of cholesterol metabolism.

Inhibition of ACAT Activity by Piperine The experiment described above using pregnenolone indicated that the site of inhibition of piperine is one of the steps between the exiting of cholesterol from the lysosomes and the step of cholesterol esterification in the ER. Therefore, the effects of piperine on ACAT activity were evaluated. For this, the microsomal fractions prepared from mouse macrophages and livers were used as enzyme source. Piperine was found to inhibit the ACAT activity in mouse macrophage and liver microsomal fractions with an IC_{50} value of 9.1 and 7.0 μ M, respectively (Fig. 4A). It is well known that macrophages ex-

Fig. 3. Inhibition of the Metabolism of Lysosomal [14 C]Cholesterol by Piperine in Macrophages

Macrophages (5×10^5 cells in 0.25 ml of medium) grown in a 48-well plastic microplate were incubated with 10 μ l of [14 C]cholesterol-supplemented liposomes for 12 h in the presence of 10 μ M pregnenolone. After incubation, the medium was removed, and the cells in each well were washed twice with buffer B containing BSA and once with buffer B, and then incubated in 0.25 ml of medium A containing piperine. After a 5-h incubation period, [14 C]CE was separated by TLC, and radioactivity was measured as described in Materials and Methods. The results were plotted as % of control (without drugs). Values represent means $\pm$ S.D. of more than two duplicate experiments.

Fig. 4. Inhibition of ACAT Activity by Piperine

(A) Mouse macrophages (2×10^8 cells) and livers (0.5 g) were suspended in 3 ml of cold buffered sucrose solution (pH 7.2) containing 100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4 and 30 mM EDTA (hereafter referred to as buffer C) in a Teflon homogenizer. The microsomal fractions of macrophages (●) and livers (■), prepared as described in Materials and Methods, were used as the enzyme source. ACAT activity was assayed in an assay mixture containing 2.5 mg/ml BSA in buffer C and 20 μ M [14 C]oleoyl-coenzyme A, together with piperine (0–100 μ M) and the microsomal fraction (100 μ g of protein). After a 5-min incubation at 37 $^{\circ}$ C, [14 C]CE was separated by TLC, and radioactivity was measured as described in Materials and Methods. The results were plotted as % of control (without drugs). Values represent means $\pm$ S.D. of more than two duplicate experiments. (B) Effect of piperine on synthesis of [14 C]neutral lipid from [14 C]oleic acid in ACAT1- or ACAT2-CHO cells. ACAT1- (●) or ACAT2-CHO (■) cell monolayers obtained from 1.25×10^5 cells/well in a microplate were incubated in 0.25 ml of medium in the absence or presence of the indicated amounts of piperine. After a 6-h incubation, cholesteryl [14 C]oleate and [14 C]triacylglycerol were separated by TLC, and the intensities of their signals were determined with a bioimage analyzer as described in Materials and Methods and CE signals were plotted as % of control (without drugs). Values represent means $\pm$ S.D. of more than two duplicate experiments.

press ACAT1, while livers express ACAT2, suggesting that piperine is a dual inhibitor of ACAT1 and ACAT2.

Selectivity in Inhibition of ACAT Isozyme by Piperine As reported previously, we established a cell-based assay method to determine the selectivity of inhibitors toward ACAT1 or ACAT2 isozyme using CHO cell clones that were

selectively expressing ACAT1 or ACAT2 in ACAT-deficient AC29 cells.²⁰⁾ To confirm whether piperine was a dual inhibitor, we studied the selectivity by using these cells. As shown in Fig. 4B, piperine inhibited both ACAT1 and ACAT2 to a similar extent with IC_{50} values (16, 18 μ M, respectively) similar to those calculated from Fig. 4A. Thus, we concluded that piperine is a dual inhibitor of ACAT1 and ACAT2.

DISCUSSION

Piperine, a main component of peppers was found to inhibit the lipid droplet accumulation in mouse macrophages (Fig. 2A). The inhibition mechanism was investigated in this study. Piperine inhibited CE synthesis rather selectively in macrophages (Fig. 2B), and the site of inhibition was between the exiting of cholesterol from lysosomes and cholesterol esterification in the ER. Furthermore, we found that piperine inhibited ACAT activity in microsomes prepared from macrophages. The IC_{50} values (25, 18 μ M) are almost consistent in both cell-based assays shown in Figs. 2 and 3, but that of ACAT assay using macrophage-derived microsome (IC_{50} ; 9.1 μ M, Fig. 4A) is slightly lower. This discrepancy of the IC_{50} values in the cell-based assays and microsomal assay might be due to the permeability of piperine to intact cell membranes.⁹⁾ A simple interpretation of these data is that piperine inhibits lipid droplet accumulation by blocking the ACAT activity in mouse macrophages.

ACAT has been focused as an ideal target of inhibition for the development of a new type of anti-atherosclerotic drugs. Extensive studies on ACAT inhibitors have been done²¹⁾ except ACAT2 selective inhibitor. Thus, the side effect of the inhibition of ACAT2 remains unclear. But the data from the knockout mice²²⁾ indicated that inhibition of ACAT2 would not induce wrong side effects. Daiichi Sankyo Pharmaceutical Company and Pfizer tried to develop the dual ACAT inhibitor, pactimibe²³⁾ and avasimibe,²⁴⁾ respectively. However, their development as a pharmaceutical drug has failed recently. These still appear to be a number of debates about ACAT inhibitors. Chang *et al.* emphasized that dual ACAT inhibitors are still promising.²⁵⁾ Nissen *et al.* concluded that ACAT inhibition was not an effective strategy for limiting atherosclerosis because it increased the level of free cholesterol in macrophages, which caused a cytotoxic effect on macrophages.²³⁾ In this study, piperine, a major component of pepper, showed moderate dual ACAT inhibition. Pepper has been used as a spice for a long time, so black pepper is expected to be used as a functional food for atherosclerosis.

Acknowledgments This work was supported by JSPS Japan-China Medical Exchange Program and by a Grant-in-

Aid for Scientific Research on Scientific Research (B) 18390008 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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