Magnolol Stimulates Lipolysis in Lipid-Laden RAW 264.7 Macrophages

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Abstract This study investigated the effect of magnolol, a compound isolated from Magnolia officinalis, on lipolysis in lipid-laden RAW 264.7 macrophages. Treatment of macrophages with magnolol led to dissolution of lipid droplets. This phenomenon was accompanied by a dose-dependent release of glycerol and cholesterol and a concomitant reduction in intracellular levels of glycerol and cholesterol. Furthermore, adipose differentiation-related protein (ADRP), a lipid droplet-associated protein, was down-regulated by magnolol in a dose- and time-dependent manner by Western blot analysis. Immunofluorescence studies also showed that ADRP became detached from the surface of lipid droplets after magnolol treatment. The lipolytic effect of magnolol was not mediated through the cAMP-protein kinase A (PKA) system, an authentic lipolytic pathway for macrophages, since magnolol did not induce an increase of intracellular cAMP levels, and pretreatment with either of PKA inhibitors, PKI and KT5720, did not abrogate the lipolytic response to magnolol. We conclude that magnolol induces lipolysis of lipid-laden macrophages by down-regulation of ADRP expression and detachment of ADRP from the lipid droplet surface by a cAMP-independent mechanism. Lipolysis of lipid-laden macrophages may occur when the amount of ADRP on the surface of lipid droplets is not enough to stabilize the lipid droplets. J. Cell. Biochem. 94: 1028–1037, 2005. © 2004 Wiley-Liss, Inc.

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The formation of macrophage foam cells in vivo is initiated by the oxidation of low-density lipoprotein (LDL) to oxidized LDL, which is taken up by endocytotic pathway into subendothelial macrophages, in which the cholesterol is stored in lipid droplet structure [Kruth, 2001]. Accumulation of macrophage foam cells and T lymphocytes in the innermost layer of the arterial wall results in “fatty streak,” the earliest recognizable lesion of atherosclerosis [Diaz et al., 1997]. Adipose differentiation-related protein (ADRP), a lipid droplet-associated protein, is expressed in several types of lipid-containing cells [Heid et al., 1996]. In human atherosclerotic lesions, expression of ADRP mRNA is localized in a subset of lipid-rich macrophages [Wang et al., 1999]. Using an in vitro model of foam cells, we have previously demonstrated that the lipid droplets within sterol ester-laden RAW 264.7 macrophages are coated with ADRP, that up- or down-regulation of ADRP is accompanied, respectively, by accumulation or dissolution of lipid droplets, and that this is regulated by protein kinase C (PKC) [Chen et al., 2001a, 2002a]. Magnolol is one of the active compounds isolated from Magnolia officinalis. Its most interesting effect is its antioxidant activity, which is about 1,000 times greater than that of...
α-tocopherol (vitamin E) [Lo et al., 1994]. More recently, magnolol has been shown to attenuate the intimal thickening in cholesterol-fed rabbits by reducing the expression of macrophage chemotactic protein-1 and vascular cell adhesion molecule-1 [Chen et al., 2001, 2002]. Treatment of cholesterol-fed rabbits with magnolol significantly inhibits the Cu²⁺-induced formation of oxidized LDL [Chen et al., 2001b], which is a known chemotactic factor for monocytes. However, this effect of magnolol cannot be attributed solely to its antioxidant activity, since prevention of LDL oxidation is not sufficient to preserve endothelial function [Keaney et al., 1994] and further studies are therefore required to elucidate the underlying mechanism of action of magnolol on intimal structure. Previous results from our laboratory showed that magnolol stimulates lipolysis in a cAMP-independent manner by decreasing the total surface area of lipid droplets in each cell and by detaching P160, a lipid droplet surface protein, from the lipid droplet surface in rat adrenal cells [Wang et al., 2000]. The ability of magnolol treatment to attenuate intimal hyperplasia led us to propose that it might stimulate lipolysis in macrophage foam cells, thus accounting for reduced intimal hyperplasia. Oleic acid in combination with albumin and cholesterol has been widely used to induced the formation of intracellular lipid droplets in J774.2 macrophages [Jepson et al., 1996] and adipocyte precursors [Gao and Serrero, 1999]. Over 60% of the oleic acid will be formed in the neutral lipid pool and 30% in the phospholipids [Gao and Serrero, 1999]. In this study, we used sterol ester to induce the accumulation of lipid droplets in RAW 264.7 macrophages as a foam cell model to test the function of magnolol.

Hormonally regulated lipolysis in adipocytes and adrenocortical cells is stimulated by cAMP and the subsequent activation of cAMP-dependent PKA, which phosphorylates hormone-sensitive lipase (HSL), a rate-limiting enzyme in lipolysis [Vahouny et al., 1984]. A similar activation mechanism for neutral cholesterol ester hydrolase activity has been demonstrated in macrophages [Kho et al., 1981, 1993; Goldberg and Kho, 1990]. A study by Namatame et al. [1999] reported that triacylglycerol synthesis, as well as cholesterol synthesis, is responsible for foam cell formation, based on the observation that triacsin C, an inhibitor of long chain acyl-CoA synthetase, is effective in preventing foam cell formation. Whether triacylglycerol hydrolase is involved in magnolol-mediated action in foam cells is an interesting issue.

The findings that stable expression of ADRP in COS-7 cells transfected with ADRP cDNA stimulates long-chain fatty acid uptake and that ADRP expression is activated by long-chain fatty acids in 1,246 cells, support the function of ADRP in lipid droplet formation [Gao and Serrero, 1999; Gao et al., 2000]. This function is further confirmed by the observation that ADRP protein aggregates appear prior to lipid accumulation in 3T3-L1 preadipocytes during sterol ester-induced lipogenesis [Wang et al., 2003]. Since ADRP is located at the lipid droplet surface of lipid-loaded RAW 264.7 macrophages, it remains to be determined whether ADRP is involved in the regulation of lipolysis in macrophage foam cells. Thus, efforts were made to study the effects of magnolol on the distribution and expression of ADRP, and the possible mechanisms mediating ADRP expression. Elucidation of the function of ADRP is therefore important in understanding the mechanism of foam cell formation.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

RAW 264.7 macrophages (ATCC TIB-71) were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco’s modified Eagle medium (Gibco, Long Island, NY) supplemented with 10% CPSR-1 (a low-lipid serum replacement) (Sigma, St. Louis, MO), 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Macrophages were loaded for 16 h with 100 µg/ml of oleic acid (Sigma) by the method of Jepson et al. [1996], then changed to the fresh culture medium and treated with various agents for the indicated times. All inhibitors were added 30 min prior to the addition of magnolol. Triacylglycerol hydrolase inhibitor E600 (diethyl-p-nitrophenyl phosphate; Hilaire et al., 1995), KT5720 and PKI (PKA inhibitors), RHC-80267 (diacylglycerol kinase lipase inhibitor), lipopolysaccaride (lipoprotein lipase inhibitor; Hill et al., 1995), and U73122 (phospholipase C inhibitor) were purchased from Calbiochem (La Jolla, CA) or Biomol Research Laboratory, Inc. (Plymouth Meeting, PA). Magnolol, isolated from the barks of Magnolia officinalis, was purchased from the Pharmaceutical Industry Technology and Development...
Center (Taipei, Taiwan). According to the manufacturer’s instruction, magnolol is purified to >99%, as judged by two HPLC column chromatography (Cosmosil 5C 18-AR and Nucleosil 10 μm NH₂). For sequential microscopic observations, lipid-laden cells were vitally stained with Nile red (Sigma) and photographed before and after treatment for 6 h with 40 μM magnolol in DMSO.

Rat Peritoneal Macrophages and THP-1 Macrophages

Four milliliters of 0.34M sucrose was injected into the peritoneal cavity of the rat. The peritoneal fluids were collected and peritoneal macrophages obtained by centrifugation for 10 min at 400g. Rat primary macrophages were cultured in the same medium as described above. Human monocyte cell line THP-1 cells were purchased form the American Type Culture Collection and cultured in 10% FBS and 90% of RPMI (Gibco) containing 20 μM of β-mercaptoethanol.

Measurement of Cytotoxicity

Lipid-laden RAW macrophages grown in 24-well plates were treated with different concentrations of magnolol (15–60 μM) for 6 h or and then subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Mitochondrial dehydrogenase activity was measured using the MTT assay described by Welder [1992]. After washes with PBS, 500 μl of MTT medium (0.5 mg/ml MTT in culture medium) was added to each well and reacted for 4 h at 37°C in a CO₂ incubator. After removal of the MTT medium, 500 μl DMSO was added to each well for 5 min to dissolve the reaction products. The absorbance at 590 nm of each well was measured and recorded.

Immunofluorescence Microscopy

After treatments, RAW 264.7 macrophages on coverslips were fixed in 10% formalin, 0.15% Triton X-100 in phosphate buffered saline (PBS) for 10 min at room temperature, washed, and incubated with 1:200 diluted rabbit anti-ADRP antibody, a kind gift from Dr. Thomas Keenan [Heid et al., 1996]. After PBS washes, the cells were then reacted with 1:50 diluted FITC-conjugated anti-rabbit IgG (Sigma), washed, mounted, and photographed using a Zeiss epifluorescence microscope (Carl Zeiss0, Oberkuecheu, Germany).

Measurement of cAMP Levels

The cAMP assay has been described previously [Wang et al., 2000]. Briefly, cells in 35 mm dishes were treated with 40 μM magnolol or DMSO for 30 min in the presence of 500 μM 3-isobutyl-1-methylxanthine (Sigma) to inhibit phosphodiesterase activity. After aspirating off the culture medium, the cells were washed with PBS, then treated overnight with 0.5 ml of 0.01N HCl to extract the cAMP. The supernatant was collected by aspiration, neutralized with 5 μl of 1N NaOH, and centrifuged at 13,000g for 5 min. Intracellular cAMP levels were measured using the Amersham Pharmacy (³H)-cAMP assay system (Amersham, Arlington Heights, IL).

Measurement of Glycerol and Cholesterol Release

After incubation of cells in 35 mm culture dishes with various agents for 6 h, the culture medium (0.5 ml) was collected and the cells resuspended in 200 μl of PBS and sonicated. Intracellular and extracellular levels of glycerol and cholesterol were determined using colorimetric assays for glycerol (GPO-trinder, Sigma) and cholesterol (cholesterol assay kit, Sigma). The protein concentration in the samples was determined using a Biorad protein assay kit (BioRad laboratories, Richmond, CA). The data were expressed as micromole of glycerol or cholesterol per milligram of protein.

Nile Red Staining

To observe lipid droplets, macrophages were fixed in 10% formalin in PBS for 10 min at room temperature, then stained with Nile red (Sigma) at a final concentration of 10 μg/ml. Following brief washes with PBS, the cells were mounted and examined. For time course study, live cells were incubated in the culture medium in the presence of Nile red at a final concentration of 10 μg/ml, and photographed at 0, 2.5, and 4 h, respectively, after the application of magnolol or DMSO.

Western Blot Analysis

After various treatments, RAW 264.7 macrophages were collected from culture dishes, homogenized in lysis buffer (10 mM EGTA, 2 mM MgCl₂, 0.15% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 μg/ml of leupeptin and pepstatin, 60 mM PIPES, 25 mM
HEPES, pH 6.9) by sonication, and the protein concentrations of the homogenates determined (BioRad protein assay kit). Cell homogenates (50 μg/lane) were electrophoresed on a 10% SDS–polyacrylamide gel [Fritz et al., 1989], then the proteins were transferred onto a nitrocellulose membrane. Membrane strips were blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris base, pH 8.2), then incubated overnight at 4°C with rabbit anti-ADRP (Dr. Heid) or mouse anti-β actin (Sigma), diluted in PBS. After washes with TBS-0.1% Tween, the strips were reacted with alkaline phosphatase-conjugated secondary antibodies (1:7,500 dilution, Sigma), and bound antibody visualized using a substrate solution (3.3 mg/ml nitro blue tetrazolium and 1.65 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM MgCl₂, 100 mM Tris base, pH 9.5). The density of the bands on the nitrocellulose membrane was quantified by densitometric scanning using an Image Master (Pharmacia Biotech, Hong Kong) and expressed as a percentage of that in the controls.

Statistical Analysis

All results are expressed as the mean ± SD. Statistical differences between means were assessed using Student’s t-test, a P value less than 0.05 being considered significant.

RESULTS

Lipolysis Induced by Magnolol

The effect of magnolol on lipolysis was evaluated by morphological and biochemical methods. Loading with oleic acid for 16 h was enough to induce accumulation of lipid droplets, which were varied in sizes and could be visualized by differential interference contrast microscopy. Figure 1 shows that extracellular levels of both glycerol and cholesterol were increased by magnolol for 6 h treatment in a dose-dependent manner. The increase in cholesterol was much less than that of glycerol (Fig. 1B). Exposure of lipid-loaded macrophages to increasing concentrations of the magnolol from 15 to 60 mM for 6 h did not affect the cell viability, as judged by MTT test (data not shown). To ensure that magnolol stimulated lipolysis by inducing dissolution of lipid droplets and the subsequent release of glycerol or cholesterol, Nile red staining was used to visualize the changes of lipid droplets in live cells. Figure 2 shows Nile red vitally stained cells which were photographed at 0, 2.5, and 4 h, respectively, after magnolol treatment. The results show the complete disappearance of Nile red-stained lipid droplets in the cytoplasm after 6 h of magnolol treatment (Fig. 2). The effective reaction time depended on the number of lipid droplets originally present in the cell and ranged from 6 to 16 h.

Down-Regulation of ADRP Induced by Magnolol

Since ADRP is associated with lipid droplets in RAW 264.7 macrophages and its up-regulation and down-regulation are, respectively, correlated with lipid droplet accumulation and dissolution [Chen et al., 2001], we next exam-
ined the distribution and expression of this protein after magnolol treatment. Immunofluorescence studies in these lipid-laden macrophage showed that ADRP was exclusively distributed around the surface of lipid droplets, large droplets displaying particularly clear bright rings of ADRP staining (Fig. 3A,B). After 3 h treatment with 40 μM magnolol, the intensity of ADRP labeling was reduced in some lipid droplets, some being entirely devoid of ADRP staining (Fig. 3C,D). After 6 h treatment with 40 μM magnolol, almost all cells were negative for ADRP immunostaining, this effect being concomitant with the disappearance of intracellular lipid droplets (Fig. 3E,F). We carried out similar examinations on primary rat peritoneal macrophage culture, and demonstrated that magnolol significantly decreased the number of cellular lipid droplets (Fig. 4B, control 4A), concomitantly with the disappearance of ADRP from the lipid droplet surface (Fig. 4D, control 4C). However, magnolol did not exert such lipolytic effect on human THP-1 cell lines, as revealed by the presence of intact ADRP ring staining at the lipid droplet surface and many large lipid droplets in the cytoplasm (data not shown). Western blot analysis confirmed that magnolol caused a decrease in ADRP protein levels in a dose- and time-dependent manner (Fig. 5). A duplicate membrane was immunoblotted with monoclonal anti-β-actin antibody in order to confirm the same loading amount in each lane (Fig. 5).

Mechanism of Magnolol-Induced Lipolysis and -ADRP Down-Regulation

In order to determine whether the signaling mechanism of magnolol-induced lipolysis involves PKA activation, a traditional signaling pathway responsible for lipolysis in macrophages [Goldberg and Khoo, 1990], we measured intracellular cAMP levels in lipid-laden RAW macrophages after magnolol treatment. This treatment did not alter intracellular cAMP levels (Fig. 6A). Consistent with this observation, pretreatment with PKA inhibitor PKI did not prevent magnolol-induced lipolysis (Fig. 6B). Similar results were obtained with another PKA inhibitor KT5720 (data not shown). In addition, triacylglycerol hydrolase inhibitor E600 (2–4 μM) failed to block the effect of magnolol on lipolysis (data not shown). Oleate has been demonstrated to be incorporated into diacylglycerol and phospholipids [Hilaire et al., 1995]. Therefore, diacylglycerol lipase and phospholipase may be possibly involved in the production of extracellular glycerol. Lipoprotein lipase, catalyzing the hydrolysis of triacylglycerol component of chylomicrons and very low density lipoprotein, is also synthesized and secreted by macrophages [Mead et al., 1999]. Efforts have been made to examine the roles of these lipases by using diacylglycerol kinase inhibitor RHC-80267, lipoprotein lipase inhibitor lipopolysaccharide, and phospholipase C inhibitor U73122. None of them were able to...
abolish the lipolytic effect of magnolol (data not shown).

**DISCUSSION**

In the present study, we have provided evidence that magnolol can induce lipid droplet dissolution and lipolysis by the release of both glycerol and cholesterol from lipid-laden macrophages. Oleic acids are reported to incorporate into polar lipids (60%–80%) and triacylglycerol (20%–40%) in human fibroblasts [Hilaire et al., 1995]. In oleic acid-laden 3T3-L1 preadipocytes, newly formed lipid droplets mainly consist of triacylglycerol (>99%), as analyzed by quantitative thin layer chromatography assay [Braasamle et al., 2000]. Our results also showed that magnolol induced much more release of glycerol than cholesterol from the lipid droplets in RAW macrophages. This lipolytic effect is accompanied by down-regulation of ADRP and detachment of ADRP from the surface of various sizes (B), which are decorated by ADRP immunostaining (A). After 3 or 6 h treatment with magnolol (40 μM) (C–F), the ADRP staining becomes weaker (arrows) or is even absent (arrowheads) on the surface of some lipid droplets. Scale bar, 15 μm.

**Fig. 3.** Distribution of adipose differentiation-related protein (ADRP) in magnolol-treated lipid-laden RAW macrophages. A, C, and E: Immunostained with anti-ADRP antibody; B, D, and F, corresponding differential interference contrast images. Macrophages incubated with oleic acid accumulate lipid droplets of various sizes (B), which are decorated by ADRP immunostaining (A). After 3 or 6 h treatment with magnolol (40 μM) (C–F), the ADRP staining becomes weaker (arrows) or is even absent (arrowheads) on the surface of some lipid droplets. Scale bar, 15 μm.
lipid droplets. In contrast to the PKA-mediated lipolytic mechanism in macrophages, adipocytes and steroidogenic cells, the lipolytic action of magnolol is PKA-independent. We also explored the potential application of magnolol in a human monocyte/macrophage THP-1 cell line. Unexpectedly, we did not observe any sign of lipolysis. It is noted that magnolol treatment did not...
not cause the detachment of ADRP protein from the lipid droplet surface in this cell line, suggesting the presence of different regulation mechanisms of ADRP expression by magnolol between macrophages of different origin.

After magnolol treatment of lipid-laden macrophages, ADRP immunostaining at the surface of lipid droplets decreased, and ADRP protein levels were reduced to ~15% of the control levels, coincident with the increased lipolysis. These data support that a reduction in ADRP expression is one of the mechanisms by which magnolol increased macrophage lipolysis. The previous findings that overexpression of ADRP stimulates lipid accumulation and lipid droplet formation without induction of other adipocyte-specific genes in murine fibroblasts [Imamura et al., 2002], and tiny ADRP aggregates appear prior to the lipid accumulation in 3T3-L1 preadipocytes during sterol ester-induced lipogenesis [Wang et al., 2003] support the role of ADRP in the assembly of nascent lipid droplets. In addition to its role in lipid formation, being a barrier to prevent lipid droplet hydrolysis from lipase activity, ADRP at the lipid droplet surface may also stabilize the lipid droplet structure. Thus, down-regulation of ADRP by magnolol may prevent the accumulation of lipid droplets in the cytoplasm, and facilitates the lipolytic process. Currently, it is not known whether the downregulation of ADRP is mediated at translational or transcriptional levels, or by accelerating the degradation of ADRP.

Since the accumulation of lipid droplets is known to be due to an equilibrium between the formation and hydrolysis of sterol ester, the effect of magnolol on sterol ester formation should also be taken into consideration. Magnolol has been demonstrated to inhibit acyl-CoA: cholesterol acyltransferase activity [Kwon et al., 1997]. Since acyl-CoA: cholesterol acyltransferase is responsible for the formation of cholesteryl ester, the main component of lipid droplets, it is plausible that magnolol-stimulated lipolysis may result from impeded esterification of cholesterol, thus blocking the formation of lipid droplets. It is also possible that magnolol up-regulates some fatty acid transporters to promote efflux of fatty acids, cholesterol or phospholipids to extracellular acceptors, though it needs to be elucidated.

Magnolol has been shown to attenuate neointimal thickening and reduce the expression of monocyte chemotactic protein-1 and vascular cell adhesion molecule-1, which recruits monocytes to the subendothelial space, in endothelial cells [Chen et al., 2001b, 2002b]; however, the effect of magnolol on lipolysis of macrophage foam cells was not investigated in their studies. The present finding that magnolol stimulates lipolysis in lipid-laden macrophages may provide another viewpoint to explore the direct effect of magnolol. We suggest that the magnolol-induced attenuation of neointimal thickening may be due, at least in part, to its action on lipolysis and the clearance of lipid droplets in foam cells and that this may prevent the subendothelial formation of fatty streak.

Fig. 6. Relation between protein kinase A (PKA) and magnolol-induced lipolysis. A: Effect of magnolol on intracellular cAMP level. Dibutyryl cAMP (Bt2cAMP) serves as a positive control. B: Effect of PKA inhibitor PKI on magnolol-induced lipolysis. RAW macrophages were loaded for 16 h with oleic acid, then treated with DMSO, 1 μM PKI, 40 μM magnolol (Mag), or magnolol plus PKI (Mag + PKI) for 6 h. The data shown are the mean ± SD for one typical triplicate determination. n = 3. *, P < 0.05, as compared to DMSO (vehicle) control.
When macrophages were loaded with oleate, cholesteryleoleate should be formed according to the chase and pulse study by Hakamata et al. [1994]. HSL is the enzyme hydrolyzing tri-, di-, and mono-acylglycerol and cholesterol ester [Yeaman et al., 1994], and this enzyme is responsible for the majority of neutral cholesterol esterase activity in several types of macrophages, including RAW 264.7 macrophages [Small et al., 1989; Khoo et al., 1993; Escary et al., 1998]. HSL activity in macrophages has been shown to be stimulated by cAMP [Khoo et al., 1981; Small et al., 1989, 1991; Goldberg and Khoo, 1990]. This study failed to support the involvement of triacylglycerol hydrolyase in magnolol-induced lipolysis by using the inhibitor E600. E600 has been reported to inhibit degradation of short/medium-chain triacylglycerols (C4–C10) in human fibroblasts [Hilaire et al., 1995] and to effectively inhibit the hydrolysis of triacylglycerols in 3T3-L1 preadipocytes [Brasaemle et al., 2000]. These results are consistent with previous findings that magnolol-induced lipolysis is cAMP-independent in rat adrenal cells [Wang et al., 2000] and in sterol ester-loaded 3T3-L1 preadipocytes [Huang et al., 2004].

In conclusion, our results show that magnolol induces lipolysis of lipid-laden macrophages, that this process is accompanied with the down-regulation of ADRP and detachment of ADRP from the lipid droplet surface. Based on the fact that magnolol treatment stimulates lipolysis in foam cells (present study), we suggest that it may be a possible candidate for treatment of atherosclerosis.

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REFERENCES


